Effects of dexamethasone and transforming growth factor- β 2 on group II phospholipase A₂ mRNA and activity levels in interleukin 1 β - and forskolin-stimulated mesangial cells

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The expression of 14 kDa group II phospholipase A₂ [also referred to as secretory PLA₂ (sPLA₂)] is induced in rat glomerular mesangial cells by exposure to inflammatory cytokines and forskolin, a cAMP elevating agent. Previously we have shown that dexamethasone and transforming growth factor- β 2 (TGF- β 2) suppress sPLA₂ protein synthesis and enzyme activity induced by cytokines and forskolin. The regulation of sPLA₂ by pro-inflammatory cytokines suggests that the enzyme may play a role in glomerular inflammatory reactions. In order to understand the regulation of sPLA₂ in more detail, we investigated whether dexamethasone and TGF- β 2 also suppress sPLA₂ mRNA after its induction by either interleukin-1 β (IL-1 β) or forskolin. We found that IL-1 β -induced sPLA₂ mRNA in rat mesangial cells is not down-regulated by pretreatment of the cells with dexamethasone, even at a concentration of 10 μ M, which dramatically decreases sPLA₂ protein levels and activity. Metabolic labelling

experiments indicated that the decreased sPLA₂ levels under these conditions can be explained by inhibition of the rate of sPLA₂ synthesis from the elevated mRNA levels. In contrast, the forskolin-induced elevation of sPLA₂ mRNA is inhibited by dexamethasone in a concentration-dependent manner. Likewise, TGF- β 2 inhibits the elevation of sPLA₂ mRNAs induced by either IL-1 β or forskolin. The decrease in sPLA₂ mRNA caused by TGF- β 2 corresponds with the decrease in sPLA₂ enzyme levels and activity. These data suggest that cytokine- and forskolin-induced sPLA₂ expression is tightly controlled via both transcriptional and post-transcriptional mechanisms. Furthermore, we show that pretreatment of mesangial cells with epidermal growth factor prior to stimulation with IL-1 β or forskolin had no suppressing effect on sPLA₂ levels or enzyme activity, as has been reported previously for osteoblasts.

INTRODUCTION

Phospholipases A₂ (PLA₂s) release the fatty acid at the *sn*-2 position of glycerophospholipids. PLA₂ is the dominant factor in most cells in the regulation of arachidonic acid liberation, assumed to be the rate-limiting step in eicosanoid production [1]. PLA₂s play a role in different pathophysiological events [2,3], including inflammatory processes. During inflammation several cells express high amounts of 14 kDa group II PLA₂ [also referred to as secretory PLA₂ (sPLA₂)] in response to various cytokines. Interleukin-1 β (IL-1 β) and tumour necrosis factor (TNF) are potent inflammatory cytokines which play an important role in the mediation of inflammatory processes. These two cytokines have been reported to share a number of biological effects in various tissues, although they are distinct proteins and use distinct receptors [4]. Rat glomerular mesangial cells are being increasingly recognized as important target and effector cells in the pathogenesis of acute and chronic forms of renal diseases [5] and are one of the target cells for the induction of PLA₂s by cytokines.

In rat mesangial cells both a sPLA₂ and a high-molecular-mass 85 kDa or cytosolic PLA₂ (cPLA₂) have been described [6,7]. The role of the sPLA₂ in inflammation has been discussed extensively by Vadas et al. [8]. IL-1 β and TNF can induce the synthesis and

secretion of sPLA₂ in parallel to enhanced prostaglandin E₂ (PGE₂) formation in many cell types, including mesangial cells [6,9,10], rat vascular smooth muscle cells [11], rabbit chondrocytes [12,13] and HepG2 cells [14]. This effect is inhibitable by the RNA and protein synthesis inhibitors actinomycin D and cycloheximide respectively, indicating that *de novo* RNA and protein synthesis is involved. IL-1 β and TNF were also found to increase sPLA₂ mRNA levels dramatically in a wide variety of tissues [10–13,15–17]. It has been shown that the IL-1 β -induced increases in sPLA₂ activity and mRNA are coordinate and follow the same time course as cytokine-stimulated PGE₂ synthesis in rat mesangial cells [18]. In rat mesangial cells [6] and vascular smooth muscle cells [11] forskolin, a cAMP elevating agent, can also induce sPLA₂ protein expression and the release of sPLA₂ enzyme synergistically with IL-1 β and TNF.

Pretreatment of the cells with the anti-inflammatory agent dexamethasone prevents the cytokine- and forskolin-induced synthesis and secretion of sPLA₂ and PGE₂ in a dose-dependent manner [19,20]. Initially it was believed that glucocorticoids exert their effect by the induction of a PLA₂ inhibitory protein, termed annexin. Later it was found that the inhibition of sPLA₂ by annexin *in vitro* could be overcome by increasing the substrate concentration, and the inhibitory action was ascribed to substrate sequestration [21,22]. Recently we showed that, in rat mesangial

Abbreviations used: EGF, epidermal growth factor; IL-1 β , interleukin-1 β ; NF, nuclear factor; PDGF, platelet-derived growth factor; PGE₂, prostaglandin E₂; PLA₂, phospholipase A₂; sPLA₂, secretory PLA₂ (14 kDa group II PLA₂); cPLA₂, cytosolic PLA₂; TGF- β 2, transforming growth factor- β 2; TNF, tumour necrosis factor.

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cells, dexamethasone induces neither annexin I protein synthesis nor translocation of annexin I from the cytosol to the membrane, which is required for sequestering the substrates for the enzyme [23]. We concluded from these experiments that the dexamethasone-induced decrease in sPLA₂ activity does not result from enzyme inhibition and is caused solely by direct inhibition of sPLA₂ protein expression [19,23].

Several growth factors can markedly down-regulate sPLA₂ expression. The presence of large quantities of transforming growth factor- β (TGF- β) in chronic inflammatory conditions has been demonstrated, and the presence of high-affinity receptors on mesangial cells has been reported [24]. Pretreatment of mesangial cells with TGF- β 2 prior to stimulation with IL-1 β or IL-1 β +forskolin can completely suppress the induced synthesis and secretion of sPLA₂ in these cells [25]. This suppression leads to an inhibition of the induced PGE₂ formation but, in contrast to dexamethasone, TGF- β 2 does not completely block PGE₂ synthesis. This can be explained by the observation that TGF- β 2 on the one hand suppresses sPLA₂ and on the other hand increases the activity of cPLA₂, which in turn can generate arachidonic acid for the production of PGE₂ [26]. Besides TGF [27], it has been shown that platelet-derived growth factor (PDGF) can attenuate sPLA₂ gene expression [15], and Pruzanski et al. reported that epidermal growth factor (EGF) also down-regulates sPLA₂ activity in osteoblasts [28]. Several reports have suggested that EGF affects prostaglandin production by cells in culture [29–31]. EGF is produced in large quantities by the kidney [32], and the effect of EGF on prostaglandin production may have particular physiological relevance in this organ, since many renal functions are controlled by prostaglandins. Furthermore, EGF-binding sites have been identified on cultured rat renal mesangial cells [33].

In view of the above we studied the effect of EGF in rat glomerular mesangial cells on IL-1 β - and forskolin-induced sPLA₂ activity. We also wanted to know whether dexamethasone and TGF- β 2 exert their suppressing effects on IL-1 β - and forskolin-induced sPLA₂ gene expression at the transcriptional or post-transcriptional level. In this paper we show that the suppression of IL-1 β -induced sPLA₂ synthesis by dexamethasone is mainly at a post-transcriptional level, whereas the down-regulation of forskolin-induced sPLA₂ synthesis appears to be caused by inhibition of mRNA synthesis. In IL-1 β - as well as forskolin-stimulated cells the suppression of sPLA₂ synthesis by TGF- β 2 is due to a decrease in sPLA₂ mRNA levels. Pretreatment of the cells with EGF prior to stimulation with IL-1 β or forskolin did not attenuate sPLA₂ activity or protein synthesis in rat mesangial cells.

MATERIALS AND METHODS

Cell culture and incubation

Cultivation and characterization of rat mesangial cells were performed as described previously [34]. Briefly, the cells were grown in RPMI 1640 supplemented with 15% (v/v) fetal calf serum, penicillin (100 units/ml), streptomycin (100 μ g/ml), and bovine insulin (0.66 unit/ml). For the experiments passages 8–12 were used. Confluent mesangial cells cultured in 75 cm² flasks were rinsed once with PBS and incubated with 10 ml of serum-free medium containing 0.1 mg/ml fatty acid-free BSA (Sigma). The cells were preincubated with or without agents for 5.5 h and then stimulated with IL-1 β or forskolin for 18 h. Thereafter, the medium was withdrawn and assayed for PLA₂ activity and the cells were washed with ice-cold PBS. Subsequently total mesangial cell RNA was isolated as described below or the cells were scraped with a rubber policeman into homogenization

buffer containing 50 mM Hepes, 0.25 M sucrose, 1 mM EDTA, 1 mM EGTA, 10 μ M leupeptin, 1 μ M pepstatin, 50 mM NaF, 250 μ M Na₃VO₄ and freshly added 1 mM PMSF. Thereafter the cells were sonicated on ice (3 $\times$ 5 s) using a Branson sonifier and assayed for PLA₂ activity. The protein content was determined by the method of Bradford [35] with BSA as standard.

Northern-blot analysis

Total mesangial cell RNA was extracted using the guanidinium isothiocyanate/phenol/chloroform method [36]. The RNA was precipitated with isopropyl alcohol and the pellets were washed with 75% ethanol. Samples of 20 μ g of RNA were separated on 0.8% agarose gels containing 6.6% formaldehyde and transferred on to a Gene Screen membrane by a standard method [37]. Equal loading of RNA was verified by visual inspection of ethidium bromide-stained rRNA. The filters were baked at 80 °C for 2 h and prehybridized for about 4 h at 42 °C in 50% (v/v) formamide, 10% (w/v) dextran sulphate, 1% (w/v) SDS, 1 M NaCl and 100 μ g/ml denatured herring sperm DNA. Thereafter the filters were hybridized at 42 °C overnight with rat sPLA₂ cDNA [38] labelled with [α -³²P]dCTP according to the random primed labelling technique described by Feinberg and Vogelstein [39]. Finally, the filters were washed twice in 2 $\times$ SSC for 30 min and several times at 65 °C in 0.2 $\times$ SSC/1% SDS. The signal was detected by autoradiography. To correct for variations in RNA amount, the sPLA₂ probe was stripped and the blots were rehybridized to the ³²P-labelled *Bam*HI-*Sal*I cDNA insert from clone pEX6 coding for β -actin. For quantification, autoradiograms of the filters were scanned densitometrically. The signal density of each RNA sample hybridized to the sPLA₂ probe was divided by that of the same sample hybridized to the β -actin probe. The corrected density is expressed as a percentage decrease from that recorded with IL-1 β or forskolin stimulation.

PLA₂ assay

PLA₂ activity was assayed using 0.2 mM *sn*-2-labelled [1-¹⁴C]linoleoylphosphatidylethanolamine (specific radioactivity 3000 d.p.m./nmol) in 0.2 ml of 0.1 M Tris/HCl, pH 8.5, in the presence of 10 mM CaCl₂. Reactions were stopped after 30 min and the liberated ¹⁴C-labelled fatty acids were extracted by a modified Dole extraction procedure [40]. The substrate 1-acyl-2-[1-¹⁴C]linoleoylphosphatidylethanolamine was prepared as described previously [41].

Immunoblotting

SDS/PAGE using a 15% polyacrylamide gel was done according to Laemmli [42] at a constant voltage of 200 V. For immunoblotting, the proteins were transferred to nitrocellulose during 1.5 h at 2 mA/cm² [43]. Non-specific binding sites were blocked with 0.1% (w/v) milk powder in PBS for 45 min at room temperature followed by incubation with primary antibody, i.e. a 1:60 dilution of each of the hybridoma supernatants 2E7 and 2B9 [44] in 0.01% milk powder. After the primary antibody incubation, the blot was incubated with alkaline phosphatase-conjugated goat anti-mouse IgG. After washing, alkaline phosphatase activity was detected by developing the blots in Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate [6].

Cell labelling and immunoprecipitation

Cells were grown in 60 mm-diam. culture dishes and incubated with agents as described above. Thereafter the medium was

withdrawn and the cells were preincubated for 20 min with methionine- and cysteine-free medium containing 0.1 mg/ml BSA, with or without agents as present during the original incubation. Thereafter, the cells were pulsed for different time periods with the same medium containing L-[³⁵S]methionine/L-[³⁵S]cysteine (200 μ Ci per dish; sp. radioactivity 1000 Ci/mmol). Following aspiration of the media, the cells were washed twice with ice-cold PBS and scraped into 500 μ l of cold radio-immunoprecipitation assay buffer (RIPA; 20 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1mM PMSF, 5 mM benzamide, 50 mM NaF, 250 μ M Na₃VO₄ and 10 mM Na₂HPO₄). The lysates were centrifuged at 14000 *g* for 45 min at 4 °C to remove unsolubilized material and the supernatants were transferred to another tube and used for immunoprecipitation. The samples were incubated at 4 °C overnight with a specific monoclonal antibody [44] against rat sPLA₂ in the presence of 0.5 M KCl. The sPLA₂-antibody complex was removed by a 1 h incubation with 6 mg of Protein A-Sepharose. The immunoprecipitate was washed twice with RIPA/1 M KCl (1:1, v/v) and twice with RIPA/water (1:1, v/v). The samples were boiled for 5 min in 30 μ l of Laemmli sample buffer and applied to SDS/PAGE (15% gel). Gels were then soaked in Amplify (Amersham) for 20 min, dried and exposed to a film at -70 °C. For quantitative determinations appropriate gel areas were cut out and radioactivity was counted in a scintillation counter.

Chemicals

Nylon membranes (Gene Screen) were purchased from New England Nuclear, Boston, MA, U.S.A. [α -³²P]dCTP (3000 Ci/mmol) and [1-¹⁴C]linoleic acid were obtained from Amersham International. The Random Primed Labelling Kit was from Boehringer Mannheim, Mannheim, Germany. Recombinant IL-1 β was prepared in the Biotechnology Department of Ciba-Geigy Ltd., Basel, Switzerland, and forskolin was obtained from Calbiochem, Luzern, Switzerland. Recombinant human TGF- β 2 was kindly donated by Drs. N Cerletti, D. Cox and G. McMaster of Ciba-Geigy Ltd. The cDNA clone pEX6, coding for human β -actin, was a gift from Dr. U. Aebi, Basel, Switzerland. EGF (receptor grade) was a product of Biomedical Technologies, Stoughton, MA, U.S.A. Nitrocellulose membranes were from Schleicher and Schull, Dassel, Germany. Alkaline phosphatase-conjugated goat anti-mouse IgG was a product of Bio-Rad. Nitro Blue Tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate and dexamethasone 21-acetate were obtained from Sigma, St. Louis, MO, U.S.A.

RESULTS

Dexamethasone fails to inhibit sPLA₂ mRNA induction by IL-1 β

Our previous results have shown that treatment of rat mesangial cells with IL-1 β or IL-1 β + forskolin can induce the synthesis and secretion of sPLA₂ in parallel with the formation of PGE₂ [6,9]. Pretreatment of the cells with dexamethasone prior to stimulation caused an inhibition of sPLA₂ activity in the cells and culture medium [19]. Western blot analysis indicated that this was not due to an inhibition of enzyme activity, but that it was caused by the suppression of the cytokine- and forskolin-induced synthesis of sPLA₂. We then analysed whether the suppression of induced sPLA₂ synthesis by dexamethasone is accompanied by a decrease in the amount of sPLA₂ mRNA. Previous experiments have shown that combined stimulation of mesangial cells with IL-1 β + forskolin resulted in increased mRNA levels which were only partially suppressed by dexamethasone under conditions

where sPLA₂ protein synthesis was totally inhibited [27]. The use of an oligonucleotide for sPLA₂ mRNA detection at the time allowed only experiments involving maximal stimulation of the cells by IL-1 β + forskolin. The use of the recently cloned cDNA for rat sPLA₂ [38] now provided a sufficiently sensitive detection method to differentiate between the effects of dexamethasone in cells stimulated with either IL-1 β or forskolin alone. The results show that IL-1 β alone is able to induce sPLA₂ mRNA synthesis (Figure 1B). At the dexamethasone concentrations used this IL-1 β -induced mRNA accumulation was not suppressed (Figure 1B), whereas the sPLA₂ activity in the culture medium was decreased to near the control level (Figure 1A), indicative of the complete inhibition of sPLA₂ protein synthesis [19,25].

Since the sPLA₂ activity levels in the experiments in Figure 1(A) were assessed after 18 h of IL-1 β treatment, the decreased levels of sPLA₂ in dexamethasone-pretreated cells could theoretically be explained by either a decreased rate of sPLA₂ synthesis or an increased rate of sPLA₂ degradation. To differentiate between these two possibilities, we measured sPLA₂ synthesis from radioactive amino acids after IL-1 β stimulation of cells pretreated or not with dexamethasone. As can be seen in Figure 2, the rate of sPLA₂ synthesis clearly decreased with increasing dexamethasone concentrations. The synthesis of sPLA₂ was linear with time over the 2 h period. No evidence for an initially identical synthesis rate followed by increased degradation in dexamethasone-treated cells was observed. We conclude from the combined data in Figures 1 and 2 that the decreased levels of sPLA₂ in the dexamethasone-pretreated cells, which still contain high levels of sPLA₂ mRNA, result from inhibition of sPLA₂ protein synthesis.

Forskolin-induced sPLA₂ mRNA production is completely blocked by dexamethasone

Experiments in which rat mesangial cells were stimulated with forskolin alone showed that this agent can also induce pronounced sPLA₂ activity and mRNA production (Figures 3A and 3B). Forskolin-induced sPLA₂ mRNA production was almost completely blocked at dexamethasone concentrations greater than 10 nM. This decrease in mRNA production parallels the decrease in sPLA₂ activity in the culture medium. From the results in Figures 1–3 we can conclude that dexamethasone can inhibit induced sPLA₂ gene expression at both the transcriptional and the post-transcriptional level, depending on the stimulus used.

TGF- β 2 suppresses both cytokine- and forskolin-induced sPLA₂ mRNA expression in a concentration-dependent manner

Previously it has been reported that pretreatment of rat mesangial cells with TGF- β 2 suppresses IL-1 β - and forskolin-induced sPLA₂ protein synthesis and secretion [25]. In addition, inhibition of cytokine-evoked PGE₂ synthesis was observed. Again the effect of TGF- β 2 on sPLA₂ could only be studied after maximal stimulation of the cells in the simultaneous presence of both IL-1 β and forskolin. In these experiments TGF- β 2 lowered the sPLA₂ mRNA levels to 30–40% of those found in the IL-1 β + forskolin-stimulated cells, thus leaving the possibility open that either the IL-1 β - or forskolin-induced elevation of sPLA₂ mRNA was insensitive to TGF- β 2. In order to investigate the effect of TGF- β 2 on sPLA₂ mRNA induced by either of the stimulating agents, we carried out Northern blots of IL-1 β - or forskolin-stimulated mesangial cells which had been pretreated with different concentrations of TGF- β 2. The sPLA₂ activity in the culture medium of IL-1 β -stimulated cells was nearly completely abolished at a TGF- β 2 concentration of 5 ng/ml (Figure

Figure 1 Effect of dexamethasone on sPLA₂ mRNA levels and enzyme secretion in IL-1 β -stimulated mesangial cells

Rat mesangial cells were treated with dexamethasone (DEX) at various concentrations for 5.5 h, and thereafter stimulated with IL-1 β (1 nM) for 18 h. The culture supernatants were analysed for PLA₂ activity (**A**). Cellular RNA (20 μ g) was analysed by Northern blotting as described in the Materials and methods section (**B**). Values in (**A**) are means $\pm$ S.D. of four independent experiments. A representative blot out of a series of four repeats showing identical results is given in (**B**). Corrected density as percentage of that on IL-1 β stimulation: lane 1, 0%; lane 2, 100%; lane 3, 84%; lane 4, 99%; lane 5, 86%; lane 6, 103%.

Figure 2 Rate of sPLA₂ synthesis in IL-1 β - and dexamethasone-treated mesangial cells

Rat mesangial cells were pretreated with two concentrations of dexamethasone for 5.5 h and thereafter stimulated with IL-1 β for 18 h. Subsequently, the cells were pulse-labelled for 0, 0.25, 0.5, 1.0 or 2.0 h with 200 μ Ci of [³⁵S]methionine/[³⁵S]cysteine. At the end of each pulse period cell lysates were prepared, and equal volumes of lysates were immunoprecipitated with an anti-sPLA₂ antibody. Immunoprecipitated protein was electrophoresed on an SDS/PAGE gel and exposed to X-ray film. The autoradiographs are shown. For quantification, bands corresponding to sPLA₂ were excised from the gel and the radioactivity was determined. A radioactivity (d.p.m.) versus pulse-time (h) plot is shown; dexamethasone: none, $\circ$; 10 nM, $\triangle$; 10 μ M, $\blacktriangle$; C, control.

4; [25]). In addition the Northern blot showed that the IL-1 β -stimulated increase in sPLA₂ mRNA was also almost completely blocked at this concentration (Figure 4).

Figure 5 shows the effect of TGF- β 2 on sPLA₂ mRNA and activity in forskolin-stimulated cells. The results demonstrate enhanced sPLA₂ activity in the culture medium of forskolin-treated cells which decreased to near control levels after pre-treatment of the cells with TGF- β 2. The inhibitory effect of TGF- β 2 on sPLA₂ activity was closely paralleled by a decrease in sPLA₂ mRNA levels (Figure 5). Collectively we can conclude from these results that TGF- β 2 prevents both IL-1 β - and

forskolin-induced sPLA₂ synthesis, primarily at the transcriptional level, in a concentration-dependent manner.

EGF does not inhibit induced sPLA₂ activity or synthesis

In addition to TGF- β 2, the isoforms TGF- β 1 and TGF- β 3 [27], and PDGF-BB, but not PDGF-AA [15], attenuate the IL-1 β +forskolin-induced elevation of sPLA₂ mRNA and protein synthesis in mesangial cells. A third class of growth factors, namely EGF, has also been reported to attenuate IL-1 β -induced sPLA₂ activity in osteoblasts [28]. On the other hand, it has recently been claimed that, in embryonic mouse cells, prolonged treatment with EGF did not affect sPLA₂ synthesis, accumulation of its transcript or the transcription rate of the gene [45]. In view of this discrepancy we investigated the effect of EGF on the induction of sPLA₂ by IL-1 β or forskolin in mesangial cells. As for the experiments described above for TGF- β 2, the cells were pretreated for 5.5 h with different concentrations of EGF prior to the addition of IL-1 β or forskolin. Thereafter the cells were harvested and cell homogenates and culture medium were assayed for PLA₂ activity. The results presented in Table 1 clearly show that EGF did not suppress the IL-1 β - or forskolin-induced increase in PLA₂ activity in cells or medium, except possibly the IL-1 β -induced increases at very high EGF concentrations (100 ng/ml). Rather, at lower EGF concentrations a modest increase in PLA₂ activity was found, especially in IL-1 β -stimulated cells. The fact that EGF alone or in combination with IL-1 β can induce enhanced cPLA₂ expression and activity in mesangial cells [46]; C. G. Schalkwijk, unpublished work) could be partially responsible for the increased PLA₂ activity in the cells on EGF treatment, but cannot explain the increase in the medium. Western blots confirmed that the increase in PLA₂ activity in EGF-pretreated cells (Table 1) is, if anything, accompanied by a slight increase in the sPLA₂ protein level (Figure 6), and certainly not by a decrease.

DISCUSSION

IL-1 β and forskolin have been shown to induce sPLA₂ protein synthesis and secretion in rat mesangial cells [6,9] that is accompanied by an increase in sPLA₂ mRNA [15,18,27]. It was

Figure 3 Effect of dexamethasone on sPLA₂ mRNA levels and enzyme secretion in forskolin-stimulated mesangial cells

Rat mesangial cells were treated with various concentrations of dexamethasone (DEX) for 5.5 h, and thereafter stimulated with forskolin (10 μM) for 18 h. The culture supernatants were analysed for PLA₂ activity (**A**). Cellular RNA (20 μg) was analysed by Northern blotting as described in the Materials and methods section (**B**). Data presentation is as in Figure 1. Corrected density as a percentage of that on forskolin stimulation: lane 1, 10%; lane 2, 100%; lane 3, 53%; lane 4, 44%; lane 5, 17%; lane 6, 12%.

Figure 4 Dose-dependence of the effect of TGF-β2 pretreatment on sPLA₂ mRNA levels and enzyme secretion in IL-1β-stimulated mesangial cells

Confluent monolayers of mesangial cells were incubated for 5.5 h with the indicated concentrations of TGF-β2 and subsequently stimulated for 18 h with IL-1β (1 nM). The culture supernatants were analysed for PLA₂ activity (bars), and cellular RNA (20 μg) was analysed by Northern blotting. Values represented by the bars are means ± S.D. of three independent experiments. A representative blot out of a series of three repeats is depicted. Corrected density as percentage of that on IL-1β stimulation: lane 1, 5%; lane 2, 100%; lane 3, 96%; lane 4, 6%; lane 5, 10%; lane 6, 3%.

also demonstrated that the IL-1β-induced increase in steady-state sPLA₂ mRNA abundance results from a moderate increase in the sPLA₂ transcription rate that is amplified by the prolonged existence of the transcript [18]. Using nuclear run-on assays we have also tried to measure the transcription rate of the inducible sPLA₂ gene. However, a sufficient signal for nascent sPLA₂ nuclear mRNA in run-on assays could not readily be detected. It is possible that the regulation of the sPLA₂ gene occurs predominantly at the post-transcriptional level and to a much lesser extent at the transcriptional level.

We and others have shown that the anti-inflammatory glucocorticosteroid dexamethasone inhibits the cytokine- and forskolin-induced synthesis and secretion of sPLA₂ [19,20]. For unknown reasons the induction of sPLA₂ by forskolin, as well as the suppression of the IL-1-induced increase in sPLA₂ activity by dexamethasone, were not found by Konieczkowski and Sedor [18]. We have recently shown that the decrease in sPLA₂ protein

Figure 5 Dose-dependence of the effect of TGF-β2 pretreatment on sPLA₂ mRNA levels and secretion in forskolin-stimulated mesangial cells

Confluent monolayers of mesangial cells were incubated for 5.5 h with the indicated concentrations of TGF-β2 and subsequently stimulated for 18 h with forskolin (FK; 10 μM). The culture supernatants were analysed for PLA₂ activity (bars), and cellular RNA (20 μg) was analysed by Northern blotting. Data presentation is as in Figure 4. Corrected density as a percentage of that on forskolin stimulation: lane 1, 14%; lane 2, 100%; lane 3, 74%; lane 4, 28%; lane 5, 19%.

in mesangial cells is solely responsible for the decrease in sPLA₂ activity and that this decrease is not mediated by the putative sPLA₂ inhibitory protein annexin I [23]. In the present study we demonstrate that dexamethasone inhibits the forskolin-induced elevation of sPLA₂ mRNA (Figure 3) but does not decrease sPLA₂ mRNA induced by IL-1β (Figure 1), not even at concentrations which dramatically affect sPLA₂ activity. Nakano et al. [20] reported similar inhibition of TNF-induced sPLA₂ release in rat smooth muscle cells by glucocorticosteroids without a decrease in sPLA₂ mRNA, whereas the forskolin-induced elevation of sPLA₂ mRNA was completely blocked. It now appears that similar events to those observed for TNF in smooth muscle cells are also involved in the suppression of IL-1β-induced sPLA₂ synthesis by dexamethasone in rat mesangial cells.

Glucocorticoids act by binding to a specific receptor, a ligand-activated transcription factor, to induce target gene transcription.

Table 1 Effect of EGF on IL-1 β - or forskolin-induced sPLA₂ synthesis and secretion by rat mesangial cells

Confluent mesangial cells were preincubated with the indicated concentrations of EGF for 5.5 h and then stimulated with IL-1 β (1 nM) or forskolin (10 μ M). Thereafter, the medium was withdrawn and a cell homogenate was prepared. PLA₂ activity was determined as described in the Materials and methods section. Results of two independent experiments are given.

Addition	PLA ₂ activity	
	Cells (nmol $\cdot$ min ⁻¹ $\cdot$ mg ⁻¹)	Medium (nmol $\cdot$ min ⁻¹ $\cdot$ ml ⁻¹)
Control	0.33; 0.22	0.19; 0.27
IL-1 β	2.11; 1.80	2.68; 1.78
IL-1 β + EGF (5 ng/ml)	4.12; 2.68	3.00; 2.72
IL-1 β + EGF (15 ng/ml)	2.86; 2.41	3.66; 2.11
IL-1 β + EGF (25 ng/ml)	2.07; 3.31	3.15; 2.48
IL-1 β + EGF (50 ng/ml)	2.37; 2.66	3.62; 2.55
IL-1 β + EGF (100 ng/ml)	1.63; 1.86	1.80; 1.56
Control	0.02; 0.10	0.10; 0.14
Forskolin	0.72; 0.75	0.66; 0.79
Forskolin + EGF (5 ng/ml)	0.82; 0.59	0.80; 0.90
Forskolin + EGF (15 ng/ml)	1.01; 0.87	0.78; 0.84
Forskolin + EGF (25 ng/ml)	1.00; 1.04	0.77; 0.63
Forskolin + EGF (50 ng/ml)	0.57; 0.67	0.60; 0.73
Forskolin + EGF (100 ng/ml)	0.74; 0.79	0.71; 0.60

Figure 6 Effect of EGF on IL-1 β - or forskolin-induced sPLA₂ levels in rat mesangial cells

Confluent monolayers of mesangial cells were incubated for 5.5 h with increasing concentrations of EGF and subsequently stimulated for 18 h with 1 nM IL-1 β (A) or with 10 μ M forskolin (B). Cellular protein (20 μ g) was analysed by Western blotting. Lanes from left to right: control cells; stimulated cells; stimulated cells pretreated with 5, 15, 25, 50, or 100 ng/ml EGF respectively.

The same receptor can also negatively regulate expression. Two mechanisms have been proposed for these down-regulations. The activated glucocorticoid receptor can bind to DNA sequences that overlap cAMP-responsive element sites [47] or can interfere with the AP-1 transcription factor through protein-protein interactions [48]. Recently it has been shown that the promoter region of the human sPLA₂ gene contains a sequence homologous to the consensus sequence of the cAMP-responsive element [49]. To our knowledge the presence of a glucocorticoid response element in the sPLA₂ promoter/enhancer region has not yet been reported. Possibly, the suppression of the forskolin-induced sPLA₂ mRNA elevation by dexamethasone (Figure 3) can be explained by inhibition of the cAMP-responsive element-binding protein transcription factor through interaction with the activated glucocorticoid receptor, by analogy to what has been observed for AP-1 [48].

The IL-1 β -induction of sPLA₂ gene expression in mesangial cells involves nuclear factor (NF)- κ B activation [50]. This activation generally involves degradation of the cytoplasmic inhibitor I κ B α and translocation of the released NF- κ B to the

nucleus. It has recently been shown that dexamethasone induces the transcription of the I κ B α , resulting in enhanced levels of I κ B α protein which quickly re-associates with the released NF- κ B to prevent its activation and target gene transcription [51]. However, this mechanism does not appear to be operative in the suppression of sPLA₂ gene expression in mesangial cells, since dexamethasone did not inhibit the IL-1 β -induced elevation of sPLA₂ mRNA (Figure 1).

The mechanism underlying the post-transcriptional regulation of sPLA₂ expression by dexamethasone is as yet unknown. We have shown that the decrease in sPLA₂ levels, despite the continued high levels of sPLA₂ mRNA, is caused by inhibition of sPLA₂ protein synthesis in dexamethasone-pretreated cells (Figure 2). For prostaglandin H synthase it has been reported [52] that glucocorticosteroids regulate translational control by converting the mRNA into a cryptic form, perhaps by binding of a glucocorticoid-induced or -activated protein to the 3'-untranslated region. It remains to be seen whether this is a valid explanation and whether it also applies to sPLA₂ mRNA from mesangial cells.

Previously we showed that pretreatment of rat mesangial cells with TGF- β 2 prior to stimulation with IL-1 β + forskolin inhibited the induced release of PGE₂ [25]. At the same time the synthesis and secretion of sPLA₂ were inhibited. Here we show that TGF- β 2 suppresses both IL-1 β - and forskolin-induced sPLA₂ mRNA elevation and enzyme activity in a dose-dependent manner (Figures 4 and 5). These findings extend other studies which demonstrated that three human isoforms of TGF- β displayed an equally potent inhibitory effect on sPLA₂ mRNA and activity after its induction by IL-1 β + forskolin in rat mesangial cells [27]. However, at the highest concentration used (30 ng/ml) all three isoforms of TGF- β were not able to suppress the mRNA levels and activity completely. We now demonstrate that TGF- β 2 at a concentration of 15 ng/ml can suppress sPLA₂ mRNA levels and activity completely when induced by one of the agents alone. That this result was not found after stimulation by both agents together may be due to the fact that these agents act synergistically and that much higher concentrations of TGF- β would be required to completely inhibit sPLA₂ under these circumstances. We conclude that the regulation of sPLA₂ expression by TGF- β 2 occurs entirely at the transcriptional level, as has also been found for regulation by PDGF [15].

Stimulation of arachidonic acid release by prolonged treatment with EGF has been found in many cell types expressing the EGF receptor, including rat mesangial cells [33]. In these cells EGF induced increased cPLA₂ activity, but the effect on sPLA₂ was not studied [46]. Recently it has been claimed that prolonged treatment of embryonic mouse cells with EGF stimulated the expression of cPLA₂ mRNA while leaving sPLA₂ synthesis and accumulation of its transcript unaffected [45]. Although no data at the sPLA₂ protein level were shown, it was concluded that neither EGF nor TGF- β 1 affected the synthesis of proteins immunoprecipitated with antibodies to sPLA₂. It is not clear why no suppression of sPLA₂ synthesis by TGF- β 1 was found in these cells, in contrast to our findings clearly indicating such suppression after IL-1 β or forskolin stimulation [25,27]. The authors also concluded that neither EGF nor TGF- β 1 treatment affected the steady-state levels of a 2.2 kb mRNA for the low-molecular-mass sPLA₂, in apparent contrast to what we found for TGF- β 2 in mesangial cells (Figures 4 and 5). It should be noted, however, that the conclusions for the effects of both growth factors were based on changes, or rather lack thereof, in the intensity of a 2.2 kb transcript, whereas the transcript of sPLA₂ is generally reported to have a size of 0.8–1.0 kb [18,38,53]. At variance with the lack of EGF effects on sPLA₂ expression, it

has also been reported [28] that in osteoblasts EGF caused suppression of sPLA₂ induction, similar to what was found for other growth factors such as TGF- β and PDGF. Our results clearly demonstrate that this notion is not valid for mesangial cells. In contrast to TGF- β 2 (Figures 4 and 5) and PGDF [15], EGF was not able to markedly decrease IL-1 β - or forskolin-induced sPLA₂ activity in rat mesangial cells (Table 1). At lower EGF concentrations, in fact, a slight increase in PLA₂ activity was measured and this was accompanied by a slight increase in sPLA₂ protein levels (Figure 6).

In conclusion, our data demonstrate that in rat mesangial cells dexamethasone can modulate sPLA₂ expression via two distinct mechanisms depending on the stimulus used. Whereas forskolin-induced sPLA₂ synthesis is suppressed at the transcriptional level, that induced by IL-1 β is modulated post-transcriptionally. TGF- β 2, like PDGF, attenuates sPLA₂ gene expression, independent of whether enzyme synthesis is induced by IL-1 β or by forskolin, at the transcriptional level. EGF does not mimic these effects of TGF- β 2 or PDGF on induced sPLA₂ protein expression in rat mesangial cells.

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