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Suppression by cyclosporin A of interleukin 1β-induced expression of group II phospholipase A_2 in rat renal mesangial cells

*Gaby Walker, *Dieter Kunz, †Werner Pignat, ‡Henk van den Bosch & *,§,[1]Josef Pfeilschifter

*Department of Pharmacology, Biozentrum, University of Basel, Klingelbergstr. 70, CH-4056 Basel, Switzerland, †Research Department, Pharmaceuticals Division, Ciba-Geigy Ltd., CH-4002 Basel, Switzerland, ‡Centre for Biomembranes and Lipid Enzymology, Padualaan 8, 3584 CH Utrecht, The Netherlands and §Zentrum der Pharmakologie, Klinikum der Johann Wolfgang Goethe-Universität, Theodor-Stern-Kai-7, D-60590 Frankfurt, Germany

1 We investigated whether cyclosporin A, a potent immunosuppressive drug, affects group II phospholipase A_2 (PLA_2; EC 3.1.1.4) induction in rat renal mesangial cells.

2 Previously we showed that the expression of group II PLA_2 in rat renal mesangial cells is triggered by exposure of the cells to inflammatory cytokines such as interleukin 1β (IL-1β) or tumour necrosis factor α and agents that elevate cellular levels of cyclic AMP. Treatment of mesangial cells with IL-1β for 24 h induced PLA_2 activity secreted into cell culture supernatants by about 16 fold. Incubation of mesangial cells with cyclosporin A inhibited IL-1β-induced PLA_2 section in a dose-dependent fashion, with an IC_{50} value of 4.3 μM. Cyclosporin A did not directly inhibit enzymatic activity of PLA_2.

3 Immunoprecipitation of radioactively labelled PLA_2 protein from mesangial cell supernatants revealed that the inhibition of PLA_2 activity is due to a suppression of PLA_2 protein levels. This effect was preceded by a reduction of PLA_2 mRNA steady state levels, as demonstrated by Northern blot analyses of total cellular RNA isolated from stimulated mesangial cells.

4 In order to evaluate whether cyclosporin A would affect the transcriptional activity of the PLA_2 gene, we performed nuclear run on transcription experiments and provided evidence that the transcription rate of the PLA_2 gene is reduced by cyclosporin A.

5 Previously we found that the nuclear transcription factor κB (NFκB) is an essential component of the IL-1β-dependent upregulation of PLA_2 gene transcription. By electrophoretic mobility shift analysis, we demonstrated that cyclosporin A diminishes the formation of NFκB DNA-binding complexes, thus suggesting that this transcription factor is a target for cyclosporin A-mediated repression of PLA_2 gene transcription.

6 The data presented in this study strongly suggest that the cellular mechanism involved in the IL1β -dependent transcriptional upregulation of the PLA_2 gene in mesangial cells is a target for the action of cyclosporin A.

Keywords: Group II phospholipase A_2; cyclosporin A; interleukin-1β; nuclear factor κB; renal mesangial cells

Introduction

Phospholipase A_2 (PLA_2) 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 (Van den Bosch, 1980; Glaser *et al*., 1993). Arachidonic acid and its numerous metabolites act as intracellular and intercellular messengers contributing to normal cellular physiology by modifying the activity of intracellular enzymes and ion channels. Moreover, PLA_2 and its products function as substrates for the generation of inflammatory lipid mediators that play an important role in the pathogenesis of inflammatory diseases. In most tissues and cells the synthesis of eicosanoids is limited by the availability of intracellular free arachidonic acid. The major mechanism considered to control the level of intracellular free arachidonic acid is the activation of PLA_2 and the direct release of the fatty acid from membrane glycerophospholipids.

In recent years four distinct types of phospholipases have been identified: (1) secretory PLA_2 ($sPLA_2$), a disulphide cross-linked, low molecular weight protein of 13–15 kDa which is secreted by cells and requires mM Ca^{2+} for optimal activity; (2) cytoplasmic PLA_2 ($cPLA_2$), a protein with a molecular weight of 70–80 kDa with an increased sensitivity to Ca^{2+} (μM rather than mM Ca^{2+}); (3) intracellular Ca^{2+}-independent PLA_2 ($iPLA_2$) with a molecular weight of 80 kDa; (4) platelet-activating factory acetylhydrolase (PAF-AH or PAF-PLA_2), a group of several isozymes with molecular weights ranging from 29 kDa to 45 kDa which specifically release short chains, most prominently acetyl groups (for review see Roberts, 1996).

Based on their primary structure, the $sPLA_2$s are further classified into two groups (Heinrikson *et al*., 1977). Mammalian group I PLA_2 comprises the pancreatic type of PLA_2 and mammalian group II PLA_2 is synthesized and secreted from many cell types and is believed to play a role in the initiation and propagation of inflammatory processes (for review see Pruzanski & Vadas, 1991).

Two potent proinflammatory cytokines, interleukin-1β (IL-1β) and tumour necrosis factor-α (TNFα), have been shown to induce the synthesis and secretion of group II PLA_2 in mesangial cells (Pfeilschifter *et al*., 1989b; Schalkwijk *et al*., 1991) and in a variety of other cell types (for review see Pfeilschifter, 1995). 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-1β and TNFα increase the level of mRNA for group II PLA_2 in these cells (Mühl *et al*., 1991; Nakazato *et al*., 1991). We also demonstrated an increased secretion of group II PLA_2 in rat vascular smooth muscle cells (Pfeilschifter *et al*., 1989a) and rat mesangial cells (Pfeilschifter *et al*., 1990; 1991) in response to adenosine 3′:5′-cyclic monophosphate (cyclic AMP)-elevating agents. Furthermore, forskolin, an activator of adenylate cyclase, synergistically in-

[1]Author for correspondence at: Zentrum der Pharmakologie, Klinikum der Johann Wolfgang Goethe-Universität, Theodor-Stern-Kai-7, D-60590 Frankfurt, Germany.

teracts with IL-1β to increase group II PLA_2 mRNA levels in mesangial cells (Mühl *et al*., 1992). Cyclosporin A and FK 506 are potent immunosuppressive drugs clinically used for the prevention of graft rejection following organ transplantation. These drugs bind to intracellular receptor proteins, collectively termed immunophillins, that have peptidylprolyl *cis-trans* isomerase activity. The complex between drug and its cognate immunophilin inhibits the intracellular Ca^{2+}-dependent protein phosphatase calcineurin. The activity of calcineurin is required for the activation and nuclear translocation of a subunit of the transcription factor NF-AT, known to be important in the transcriptional regulation of T-cell specific genes. Thereby, the drugs suppresss Ca^{2+}-dependent T-cell responses, most importantly the T-cell receptor-mediated transcription of interleukin 2 (for review see Schreiber & Crabtree, 1992). However, the therapeutic application of these drugs is limited by considerable side-effects, notably nephrotoxicity and neurotoxicity (Mason, 1989). Recent evidence shows that nephrotoxicity may be linked to immunophilin-mediated inhibition of calcineurin (Dumont *et al*., 1992) as well as inhibition of gene transcription (Morris *et al*., 1992). The transcription factors which are potential targets for cyclosporin A and FK 506 in nonimmune cells are likely to be different from those in T cells.

We now show that cyclosporin A suppresses IL1-β-induced increase in group II PLA_2 activity secreted into cell culture supernatants, thus confirming previous observations in our laboratory (Pfeilschifter *et al*., 1989c). This is preceded by a reduction of group II PLA_2 protein and mRNA steady state levels. Furthermore, by nuclear run on experiments and electrophoretic mobility shift analyses, we have identified the transcriptional machinery regulating the IL-1β-dependent transcription rate of the group II PLA_2 gene, most notably nuclear factor κB (NFκB) to be a target of cyclosporin A action. The data presented here may help to improve the understanding of the basic mechanisms which are involved in the modulation of cytokine-induced group II PLA_2 activity in renal mesangial cells by immunosuppressive drugs.

Methods

Cell culture

Rat mesangial cells were cultured as described previously (Pfeilschifter & Vosbeck, 1991). In a second step, single cells were cloned by limited dilution in 96-micro- well plates. Clones with apparent mesangial cell morphology were used for further processing. The cells exhibited the typical stellate morphology. Moreover, there was positive staining for the intermediate filaments desmin and vimentin, which are considered to be specific for myogenic cells, positive staining for Thy 1.1 antigen, negative staining for the factor VIII-related antigen and cytokeratin, excluding endothelial and epithelial contamination, respectively. The generation of inositol trisphosphate upon activation of the angiotensin II AT_1 receptor was used as a functional criterion for characterizing the cloned cell line. The cells were grown in RPMI 1640 supplemented with 10% foetal calf serum, penicillin (100 units ml^{-1}), streptomycin (100 μg ml^{-1}) and bovine insulin 0.66 units ml^{-1} (Sigma). For the experiments passages 6–16 of mesangial cells were used. We measured lactate dehydrogenase (LDH) release as a measure of cell injury and could not detect significant activities even with 30 μM cyclosporin A. However, with a more sensitive dye uptake test (MTT test), a tendency for a decrease in cell viability was observed, but this did not reach statistical significance.

Phospholipase A_2 assay

Phospholipase A_2 activity was determined with [1-^{14}C]-oleate-labelled *Escherichia coli* as substrate as described previously (Märki & Franson, 1986). The substrate was prepared by growing *E. coli* in the presence of [1-^{14}C]-oleate, followed by autoclaving to inactivate endogenous phospholipases. Over 95% of the label incorporated by *E. coli* was in phospholipid and, as demonstrated by hydrolysis with snake venom (*Crotalus adamanteus*), more than 95% of the [1-^{14}C]-oleate was in the sn-2 position of the phospholipids. Assay mixtures (1.0 ml) contained 100 mM Tris/HCl buffer (pH 7.0), 1 mM $CaCl_2$, 2.5×10^8 [1-^{14}C]-oleate-labelled *E. coli* (5 nmol of phospholipids, 5000–8000 c.p.m.) and the enzyme to be tested at a dilution producing 5% substrate hydrolysis. 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 of propane-2-ol/n-heptane/1 M-H_2SO_4 (40 : 10 : 1, by vol.) followed by 2 ml of heptane and 3 ml of water. After vigorous shaking and phase separation, a portion (2.5 ml) of the upper phase was passed over a column of silicic acid (3.5 cm × 0.5 cm). Free [1-^{14}C]-oleic acid was quantitatively eluted with 1 ml of ethylacetate. Radioactivity was determined in a scintillation counter. Phospholipase A_2 activity is expressed as [1-^{14}C]-oleate radioactivity released by 100 μl of cell culture supernatant.

Metabolic labelling and immunoprecipitation

Newly synthesized proteins were labelled by addition of 80 μCi of L-[^{35}S]-methionine (1175 Ci $mmol^{-1}$; DuPont NEN) to 2 ml methionine-free culture medium for 6 to 8 h. Subsequently, immunoprecipitation was performed by using 2 ml of culture supernatant containing proteinase inhibitors at a final concentration of 1 mM phenylmethylsulphonyl fluoride, 10 μM leupeptin and 20 μM pepstatin. One hundred microlitres of a 1 : 1 slurry of a specific monoclonal anti-sPLA$_2$ antibody (De Jong *et al*., 1987; Aarsman *et al*., 1989) coupled to protein G - Sepharose were added and incubated overnight at 4°C. The proteins were solubilized from the beads by heating for 5 min at 95°C in 0.1 ml of sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS/PAGE) sample buffer (3.6%, w/v) SDS, 15% (w/v) glycerol, 120 mM Tris/HCl, pH 6.8, 0.125 M dithiothreitol) and subjected to SDS/PAGE (15% (w/v) acrylamide gel).

Northern blot analysis

Confluent mesangial cells were washed twice with PBS and incubated in DMEM, supplemented with 0.1 mg ml^{-1} fatty acid-free bovine serum albumin, with or without agents for the indicated time periods. Cells were washed twice with PBS and harvested with a rubber policeman. Total cellular RNA was extracted from the cell pellets by the guanidinium thiocyanate/cesiumchloride method (Sambrock *et al*., 1989). Samples of 20 μg RNA were separated on 1% agarose gels containing 0.66 M formaldehyde before the transfer to gene screen membranes (New England Nuclear). After u.v.-crosslinking and prehybridization for 2 h, the filters were hybridized for 16–18 h to a ^{32}P-labelled EcoRI/HindIII- cDNA insert from p139-1 coding for rat group II phospholipase A_2 (Van Schaik *et al*., 1993). To correct for variation in RNA amount, the PLA_2 probe was stripped with boiling 0.1 × SSC/01%SDS and the blots were rehybridized to the ^{32}P-labelled Bam H I/Sal I cDNA insert from clone pEX 6 coding for human β-actin. DNA-probes ($0.5–1 \times 10^6$ d.p.m. ml^{-1}) were radioactively labelled with [^{32}P]-dATP by random priming (Boehringer Mannheim). Hybridization reactions were performed in 50% (v/v) formamide, 5 × SSC, 5 × Denhardt's solution, 1% (w/v) SDS, 10% (w/v) dextran sulphate and 100 μg ml^{-1} salmon sperm DNA. Filters were washed 3 times in 2 × SSC/0.1% SDS at room temperature for 15 min, and then twice in 0.2 × SSC/1%SDS at 65°C for 30 min. Filters were exposed for 24–48 h to Kodak X-Omat XAR-film with intensifying screens.

Nuclear run-on transcription

For the nuclear run-on transcription assay, a nuclei suspension was prepared and mixed with 0.2 ml of 2 × reaction buffer

(100 mM HEPES, pH 8.0, 10 mM $MgCl_2$, 300 mM KCl, 200 u RNasin (Boehringer Mannheim) ml^{-1}, 1 mM each ATP, GTP, and CTP, 150 μCi (1 μCi = 37 kBq) of [^{32}P]-UTP (3000 Ci $mmol^{-1}$; Amersham, Dübendorf, Switzerland)) and incubated for 30 min at 30°C. Transcription was stopped by adding 20 μg of DNase I, followed by 80 μg of proteinase K. The ^{32}P-labelled RNA was purified by extraction with phenol/chloroform and two sequential precipitations with ammonium acetate. Equal amounts of ^{32}P-labelled RNA were hybridized in 50% formamide, 5 × SSC, 5 × Denhardt's solution and 1% SDS (1 × SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0) at 42°C for 72 h. Filters contained 10 μg each of linearized plasmids immobilized on GeneScreen membranes (DuPont de Nemours International, Regensdorf, Switzerland) after blotting in 12 × SSPE with a dot-blot apparatus. After hybridization filters were rinsed for 30 min in 2 × SSC at 60°C, for 5 min in 2 × SSC containing 10 μg of RNase A ml^{-1} at 37°C and, finally for 1 h in 2 × SSC at 37°C. Filters were air dried and were kept at −70°C for 2 to 4 days. Densitometrical analyses were performed and the amount of sample hybridizing to β-actin was used for normalization. The corrected density is presented as fold induction when compared to unstimulated mesangial cells.

Electrophoretic mobility shift assay

The sequence of the double stranded oligonucleotides used to detect the DNA binding activities of the nuclear factor NFκB was 5′-GTATGAGGGCTTTTCCGCCCT-3′. This sequence motif is identical to the published sequence of the promoter of the rat group II PLA_2 gene (Ohara *et al*., 1990; Komada *et al*., 1990). The complementary DNA-strands were radioactively labelled by T4 polynucleotide kinase with [γ-^{32}P]-ATP (3000 Ci $mmol^{-1}$; Amersham, Dübendorf, Switzerland). Nuclear extracts from cultured cells were isolated as described by Dignam *et al*. (1993) with the modification that buffer D was supplemented with 0.1% NP-40. Binding reaction were performed for 20 min on ice with 5 μg of total protein in 20 μl of a solution containing 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 10% glycerol, 1 μg acetylated bovine serum albumin, 2 μg poly (dI-dC), 1 mM dithiothreitol, 1 mM phenylmethylsulphonylfluoride and 20 000 d.p.m. of ^{32}P-labelled oligonucleotides. DNA-protein complexes were separated from unbound DNA probe on native 4.0% polyacrylamide gels at 20 mA in 34 mM Tris-HCl (pH 7.5), 17 mM sodium acetate and 0.5 mM EDTA (pH 8.0). Gels were vacuum dried and exposured to Kodak X-Omat XAR film for 12–24 h at −80°C. For supershift experiments, binding reactions were incubated in the presence of 10 μl of anti-p50, anti-p65 and anti-c-Rel antisera (Santa Cruz Biotechnology, Basel, Switzerland), for 8 h at 4°C, before the addition of the radioactively labelled oligonucleotide. For competition experiments, unlabelled double stranded oligonucleotides containing the NFκB binding site from the rat inducible nitric oxide synthase (iNOS) promoter (5′-CTACTGGGGACTCTCCCTTTG-3′) (Eberhardt *et al*., 1996) was used in a 10 fold excess.

Materials

Recombinant human IL-1β was generously supplied by Dr Christiane Rordorf, Ciba-Geigy Ltd. (Basel, Switzerland). The cDNA-clone pEX 6, coding for human β-actin was a gift from Dr U. Aebi (Basel, Switzerland). [1-^{14}C]-oleic acid and [^{32}P]-dATP (specific activity 3000 Ci $mmol^{-1}$) were from Amersham (Dübendorf, Switzerland). Nylon membranes (Gene Screen) were purchased from DuPont de Nemours International (Regensdorf, Switzerland). Cyclosporin A was kindly provided by Sandoz Ltd. (Basel, Switzerland) and was dissolved in dimethylsulphoxide (DMSO). Cell culture media and nutrients were from Gibco BRL (Basel, Switzerland) and other chemicals used were either from Merck (Darmstadt, Germany) or Fluka (Basel, Switzerland).

Statistics

Statistical analysis was done by Student's t test and $P < 0.05$ was used as the criterion for statistical significance.

Results

Stimulation of mesangial cells for 24 h with IL-1β (2 nM) increased PLA_2 activity measured in culture supernatants by approximately 16 fold when compared to unstimulated cells (Table 1). Coincubation of the cells with IL-1β (2 nM) and different amounts of cyclosporin A ranging from 0.1 μM to 30 μM dose-dependently inhibited cytokine-stimulated secretion of PLA_2 activity into cell-culture supernatants with an IC_{50} of 4.3 μM.

In order to determine whether the inhibition of PLA_2 activity is due to changes in PLA_2 protein levels, we performed immunoprecipitation of radioactively labelled PLA_2 protein from mesangial cell-culture supernatants. The cells were incubated with IL-1β (2 nM) for 12 h in the presence of [^{35}S]-methionine to label metabolically newly synthesized proteins. Subsequently, cell-culture supernatants were used for immunoprecipitation with a monoclonal anti-PLA_2 antibody. As shown in Figure 1, IL-1β induced the formation of PLA_2 protein secreted from mesangial cells into the cell culture supernatant. Coincubation of the cells with IL-1β (2 nM) and different amounts of cyclosporin A dose-dependently inhibited cytokine-stimulated secretion of PLA_2 protein. With 30 μM cyclosporin A virtually no PLA_2 protein was detectable.

We further intended to evaluate whether cyclosporin A would affect PLA_2 mRNA levels. Northern blot analyses were performed from mesangial cells that were incubated either with vehicle (Con), IL-1β (2 nM) or IL-1β (2 nM) plus cyclosporin A (0.1 μM up to 30 μM) for 24 h. Total cellular RNA was isolated and Northern blots were probed with a radioactively labelled cDNA coding for group II PLA_2. Whereas no PLA_2 mRNA was detectable in unstimulated cells, IL-1β strongly induced the formation of PLA_2 mRNA, as shown in Figure 2. Cyclosporin A inhibited the formation of PLA_2 mRNA steady state levels upon induction by IL-1β in a concentration-dependent fashion. Complete inhibition was observed at a concentration of 30 μM cyclosporin A. Nuclear run on experiments were performed in order to evaluate whether cyclosporin A affects the transcription rate of the PLA_2 gene induced by IL-1β. Mesangial cells were incubated for 6 h with vehicle, IL-1β (2 nM) or a combination of IL-1β (2 nM) plus cyclosporin A (10 μM) and subsequently nuclei were isolated and used for *in vitro* transcription reaction. As shown in

Table 1 Effects of cyclosporin A on IL-1β-stimulated PLA_2 activity secreted from mesangial cells

Addition	*PLA_2 secretion (c.p.m./100 µl)*	*Fold induction*
Control	411 ± 32***	1
IL-1β (2 nM)	7105 ± 252	16.1
IL-1β (2 nM) + cyclosporin A (0.1 μM)	7228 ± 470	16.4
IL-1β (2 nM) + cyclosporin A (1 μM)	6663 ± 708	15.1
IL-1β (2 nM) + cyclosporin A (10 μM)	2113 ± 83***	4.8
IL-1β (2 nM) + cyclosporin A (30 μM)	514 ± 49***	1.2

Confluent mesangial cells were incubated with indicated concentrations for 24 h. Thereafter, the medium was withdrawn and PLA_2 activity determined as described in Methods. Results are means ± s.e.mean ($n = 4$). ***$P < 0.001$ versus IL-1β alone (Student's t test).

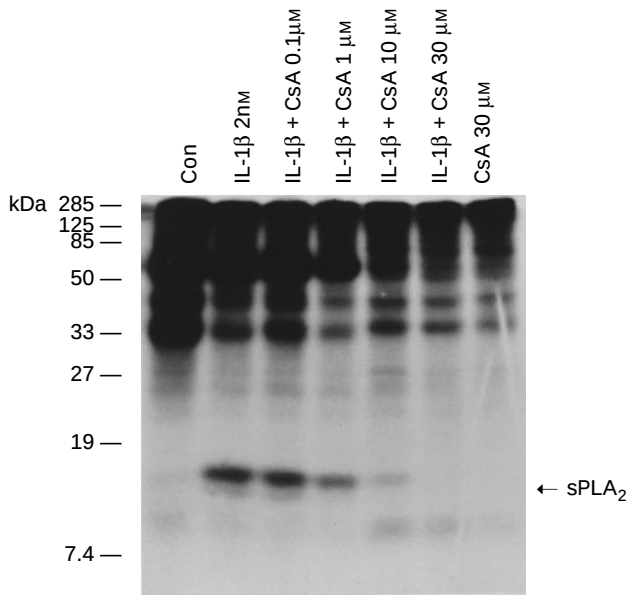

Figure 1 Inhibition of IL-1β-stimulated PLA_2 protein expression by cyclosporin A (CsA). Mesangial cells were incubated for 12 h with vehicle (Con), IL-1β (2 nM) or IL-1β (2 nM) plus the indicated concentrations of cyclosporin A in the presence of [^{35}S]-methionine. Thereafter, group II PLA_2 protein was immunoprecipitated and samples were analysed as described in the Methods section. Similar results were obtained in three independent experiments.

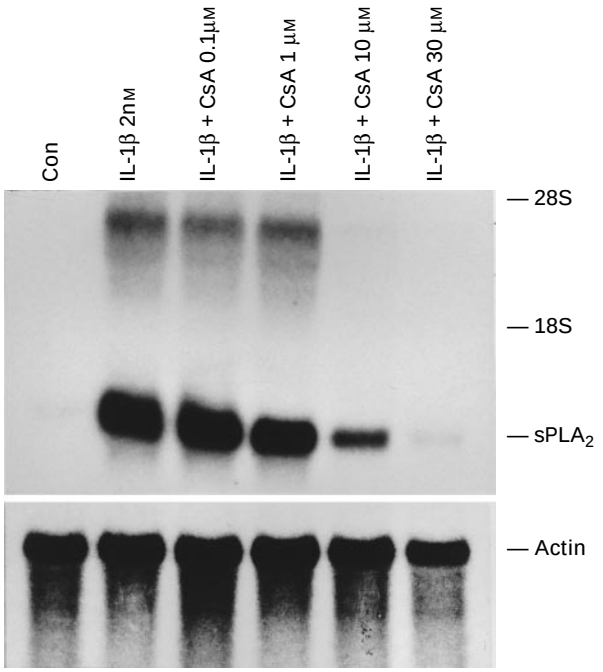

Figure 2 Inhibition of IL-1β-stimulated induction of group II PLA_2 mRNA in mesangial cells by cyclosporin A. Mesangial cells were incubated for 24 h with vehicle (Con), IL-1β (2 nM) or IL-1β (2 nM) plus the indicated concentrations of cyclosporin A (CsA). Total cellular RNA (20 μg) was successively hybridized to ^{32}P-labelled group II PLA_2 and β-actin cDNA probes as described in the Methods section. Similar results were obtained in four independent experiments.

Figure 3, IL-1β markedly increased the transcription rate of the PLA_2 gene to about 6.4 fold compared to unstimulated cells. However, upon coincubation of mesangial cells with IL-1β (2 nM) and cyclosporin A (10 μM) the transcription rate of the PLA_2 gene was increased only to about 3 fold compared to control cells. Thus, cyclosporin A reduced the PLA_2 gene

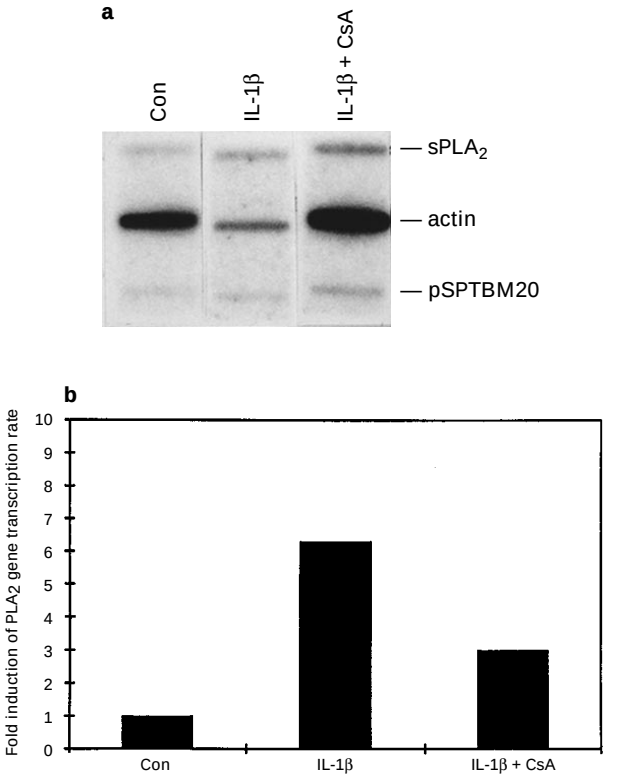

Figure 3 (a) PLA_2 gene transcription in mesangial cells. Mesangial cells were stimulated with vehicle (Con), IL-1β (2 nM), or IL-1β (2 nM) plus cyclosporin A (CsA, 10 μM) for 6 h. The rate of transcription of the genes of group II PLA_2 or β-actin by isolated nuclei was determined by hybridizing the elongated, labelled RNA transcripts to group II PLA_2-, β-actin cDNA probes or pSPTBM20 immobilized onto nitrocellulose membranes. The densitometrical evaluation of the run-on assay is shown in (b). Similar data were obtained in three independent experiments.

5′ - **GGGRNNPPCC** - 3′
- 194 GTATGA **GGGCTTTTCC** GCCCT - 174

Figure 4 NFκB DNA-binding element derived from the group II PLA_2 promoter sequence. R: purine, P: Pyrimidine, N: any nucleotide.

transcription by about 53% when compared to cells stimulated with IL-1β alone. In order to determine whether cyclosporin A affects the DNA binding activity of nuclear factor NFκB, we performed electrophoretic mobility shift analyses (EMSA). For these experiments a radioactively labelled oligonucleotide containing a putative NFκB binding site in the PLA_2 promoter from -188 to -179 was used (Figure 4). In control mesangial cells no DNA binding activity was determined (Figure 5a). Stimulation of mesangial cells with IL-1β (2 nM) induced the formation of NFκB complexes (Figure 5a). These were composed mainly of p50-p50 homodimers, as revealed by the fact that specific antisera against p50, but not against p65 and c-Rel significantly reduced the formation of DNA binding complexes in electrophoretic mobility shift analyses (Figure 5a). Moreover, stimulation of mesangial cells with IL-1β (2 nM) strongly increased the formation of NFκB-DNA binding complexes in a time-dependent fashion (Figure 5b). Complex-formation was inhibited by a specific oligonucleotide containing the NFκB-binding site from the promoter of the rat inducible nitric oxide synthase gene (Figure 5b). Under the action of cyclosporin A the binding of nuclear factor NFκB to its *cis*-regulatory element was diminished as shown in Figure 6. These data strongly suggest that nuclear factor NFκB is a main target site of action for cyclosporin A.

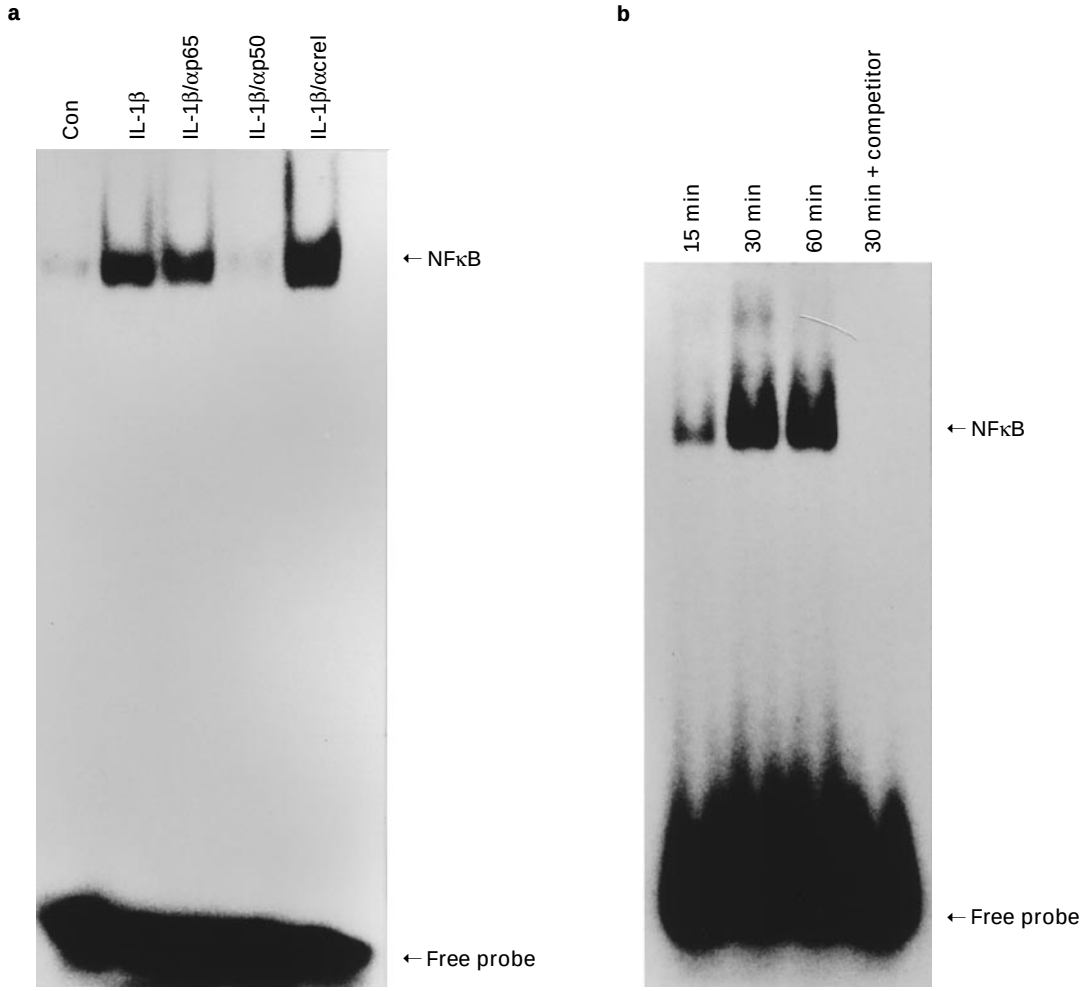

Figure 5 (a) Supershift analysis of NFκB binding complexes. Mesangial cells were stimulated with vehicle (Con) or IL-1β (2 nM) for 30 min. Radiolabelled oligonucleotides were incubated with nuclear extracts prepared from stimulated mesangial cells in the presence of the antibodies as indicated. DNA-protein complexes were analysed by use of low ionic strength 4% acrylamide gel. (b) Time-course of induction of NFκB binding in IL-1β-stimulated mesangial cells. Mesangial cells were stimulated with IL-1β (2 nM) for 15 min, 30 min and 1 h. Radiolabelled oligonucleotides were incubated with nuclear extracts prepared from stimulated mesangial cells. For competion, a 10 fold excess of nonlabelled oligonucleotide containing the NFκB binding site derived from the rat iNOS promoter was used in the assay. Similar results were obtained in three independent experiments.

Discussion

Cyclosporin A is a widely clinically used immunosuppressive drug for the prevention of graft rejection following organ transplantation. However, the therapeutic application of the drug is limited by considerable side-effects, notably nephrotoxicity and neurotoxicity (Mason, 1989). Numerous studies on nephrotoxic activities of cyclosporin A suggest that these effects are likely to be due to the action of the drug on renal cells, thus interfering with important regulatory mechanisms in the kidney (Pfeilschifter, 1988).

Among the cell types present in the glomerulus, mesangial cells are a major determinant in the regulation of the glomerular filtration rate and blood flow. The cells are an abundant source of vasoactive substances, such as prostaglandins and platelet-activating factor. These may act as intracellular or intercellular messengers, playing an important role in the cross-communication between glomerular cells (for review see Pfeilschifter, 1994; 1995). Thus mesangial cells help to preserve structure and function of the glomerulus. Under pathological conditions mesangial cells may be activated by factors released from immune cells, such as macrophages and neutrophils invading the glomerulus, or by serum factors. Such activated mesangial cells have the potential to produce a myriad of inflammatory mediators including eicosanoids, nitric oxide and other reactive oxygen species, as well as growth factors. The aberrant production of such mediators may sustain connective tissue accumulation, resulting in irreversible alteration of glomerular structure and function and finally ending up in glomerulosclerosis. Previously we observed that cyclosporin A reduces PLA_2 secretion from cytokine-stimulated mesangial cells (Pfeilschifter *et al*., 1989c).

The aim of this study was to evaluate which step involved in the cytokine-dependent expression of group II PLA_2 in mesangial cells might be a target for the action of cyclosporin A. We confirm here that cyclosporin A suppresses the induction of IL-1β-induced group II PLA_2 activity in mesangial cell culture supernatants with an IC_{50} of 4.3 μM. This IC_{50} value is much higher than that obtained for inhibition of calcineurin phosphatase activity, thus suggesting that additional intracellular signalling pathways are affected at higher drug concentrations. The reduction of secreted PLA_2 activity is the consequence of reduced PLA_2 protein levels as assessed by immunoprecipitation of radiolabelled enzyme. Northern blot analysis suggests that the observed reduction of PLA_2 protein levels is due to decreased steady state levels of PLA_2 mRNA. Moreover, we demonstrate that this phenomenon is caused by a reduction of the transcriptional activity of the PLA_2 gene, as demonstrated by nuclear run on experiments.

Recently, we have shown that the nuclear transcription factor NFκB is an absolute requirement for the IL-1β-dependent group II PLA_2 gene transcription in mesangial cells. By

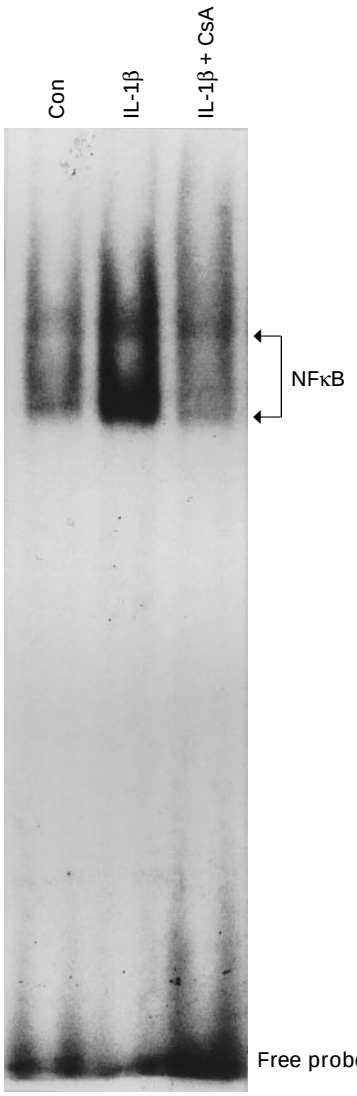

Figure 6 Cyclosporin A (CsA) inhibited the Il-1β-induced activation of NFκB in mesangial cells. Mesangial cells were stimulated with vehicle (Con), IL-1β (2 nM) or IL-1β (2 nM) plus cyclosporin A (10 μM) for 6 h. Radiolabelled oligonucleotides were incubated with nuclear extracts prepared from stimulated mesangial cells. DNA-protein complexes were analysed by use of low ionic strength 4% acrylamide gel. Similar results were obtained in three independent experiments.

using pyrrolidine dithiocarbamate, which is known to suppress the activation of NFκB in stimulated cells, we showed that this compound efficiently blocked IL-1β induced PLA_2 expression (Walker *et al*., 1995). Furthermore, sequence analysis of the 5′-flanking region of the rat group II PLA_2 gene revealed the existence of a potential NFκB-binding site within the proximal region of the putative promoter, at positions -188 to -179 (Walker *et al*., unpublished observations).

NFκB is a multisubunit transcription factor that is activated in response to IL-1β, TNFα, bacterial endotoxin and various other stimuli and plays a pivotal role in the development of cellular immune and inflammatory responses (Baeuerle & Henkel, 1994). NFκB is constitutively present in cells as a heterodimer, consisting of p50 DNA-binding subunit and a Rel A (p65) transactivation subunit. This NFκB complex is maintained in a latent cytoplasmatic form by the physical association with its inhibitory subunit IκB (Baeuerle & Henkel, 1994). The mechanism leading to NFκB activation is not fully defined, but phosphorylation and degradation of IκB seem to be important steps in the activation process which causes dissociation of IκB from NFκB and subsequent nuclear translocation of NFκB and specific gene transcription (Baeuerle & Henkel, 1994).

The data presented here provide strong evidence that cyclosporin A suppresses IL-1β-induced group II PLA_2 expression in renal mesangial cells by inhibition of the transcriptional machinery involved in the upregulation of the PLA_2 gene. The results obtained with electrophoretic mobility shift analysis show that the DNA-binding activity of NFκB, the main trigger involved in the transcriptional regulation of inflammatory mediators, like the inducible nitric oxide synthase in mesangial cells exposed to IL-1β, is drastically reduced under the action of cyclosporin A. Whether the dissociation of NFκB from its inhibitor IκB or the transloaction of the p50- and p65- subunits into the nucleus is the cause for reduced DNA-binding activity remains to be demonstrated. Nevertheless, the inhibition of DNA-binding activity of NFκB may be the key-step in the suppression of cytokine-induced group II PLA_2 expression by cyclosporin A in mesangial cells and may contribute to the effectiveness of cyclosporin A in glomerular diseases associated with nephrotic syndrome, such as immunoglobulin A nephropathy, systemic lupus erythematosus and membranoproliferative glomerulonephritis (Niaudet & Habib, 1994).

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