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Inhibition of NFκB-mediated pro-inflammatory gene expression in rat mesangial cells by the enolized 1,3-dioxane-4,6-dione-5-carboxamide, CGP-43182

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1 CGP-43182 has been described as a potent inhibitor of group IIA secreted phospholipase A_2 (group IIA $sPLA_2$) activity *in vitro*. In rat mesangial cells, inhibition of group IIA $sPLA_2$ activity by CGP-43182 results in a 70% reduction of cytokine-stimulated prostaglandin E_2 biosynthesis, suggesting that group IIA $sPLA_2$ participates in arachidonic acid release and eicosanoid formation. Under these conditions the cytosolic phospholipase A_2 is not affected.

2 In mesangial cells, in addition to inhibition of catalytic activity, the membrane-permeant CGP-43182 completely blocked interleukin 1β (IL1β)-stimulated group IIA $sPLA_2$ gene expression.

3 A further action of CGP-43182 was a complete inhibition of cyclo-oxygenase-2 gene expression, resulting in a drastic reduction of prostaglandin formation in mesangial cells.

4 Moreover, CGP-43182 completely blocked IL1β-induced gene expression of the inducible nitric oxide synthase, leading to an inhibition of cytokine-stimulated nitric oxide formation.

5 In contrast, the stimulatory effect of the cell-permeant cyclic AMP-analogue, dibutyryl-cAMP, on the induction of these enzymes was not inhibited by CGP-43182. These data indicate that CGP-43182 interferes with IL1β- but not cyclic AMP-activated transcriptional regulation.

6 By studying components of the upstream transcription machinery, we observed an inhibition of NFκB activation by CGP-43182 in IL1β-treated cells. Moreover, we observed that CGP-43182 prevented the phosphorylation and proteolytic degradation of the endogenous NFκB inhibitor, IκB, a process necessary for NFκB activation.

7 From our data, we propose that CGP-43182 is a potent anti-inflammatory drug useful for preventing the consequences of a concerted action of cytokine-stimulated pro-inflammatory genes mediated by NFκB.

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Keywords: Cyclic AMP; cyclo-oxygenase; IκB; inducible nitric oxide synthase; interleukin 1β; mesangial cells; NFκB; phospholipase A_2; secretory; transcription factors

Abbreviations: BSA, bovine serum albumin; cDNA, complementary DNA; Cox, cyclo-oxygenase; db-cAMP–N^6,2′-O-dibutyryl cyclic AMP; DMEM, Dulbecco's modified Eagle medium; DTT, dithiothreitol; ECL, enhanced chemiluminescence; EMSA, electrophoretic mobility shift analysis; IκB, inhibitor of NFκB; IKK, IκB kinase; iNOS, inducible NO synthase; NFκB, nuclear factor κB; PBS, phosphate-buffered saline; PDTC, pyrrolidine dithiocarbamate; PMSF, phenylmethylsulphonyl fluoride; PVDF, polyvinylidene difluoride; SDS–PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; $sPLA_2$, secreted phospholipase A_2

Introduction

The development of inflammatory processes involves a concerted action of several mediators, such as histamine, 5-HT, prostaglandins, cytokines and nitric oxide (NO). In the renal glomerulus, these mediators trigger the progression of glomerulosclerosis and irreversible loss of renal function. In particular, mesangial cells play an important role in the inflammatory response to glomerular injury (Pfeilschifter 1994, 1995). Once stimulated by cytokines or other exogenous pro-inflammatory signals these cells produce a broad spectrum of mediators. The production of these molecules occurs after activation of a special group of enzymes, which are not detectable in resting, unstimulated cells, but which are induced at the transcriptional level after stimulation by several pro-inflammatory agonists.

To this group of enzymes belongs the group IIA secreted phospholipase A_2 (group IIA $sPLA_2$). This enzyme is present in high amounts in synovial fluid from patients with rheumatoid arthritis (Jamal *et al*., 1998), in the blood of patients with acute pancreatitis and septic shock (Green *et al*., 1991; Gronroos & Nevalainen 1992), in Crohn's disease (Van Dullemen *et al*., 1998), in psoriatic skin (Forster *et al*., 1983) and in colorectal adenomas from familial adenomatous polyposis patients, where it is thought to exert pro-inflammatory actions (Kennedy *et al*., 1998). Intense studies are necessary for understanding the function of group IIA $sPLA_2$ in these diseases.

Also in glomerular mesangial cells, used as a model system to study mechanisms involved in glomerulonephritis, several investigations concerning stimulation of group IIA $sPLA_2$ have been performed (Pfeilschifter 1994; 1995;

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Pfeilschifter *et al*., 1997; Wada *et al*., 1997; Pruzanski *et al*., 1998; Walker *et al*., 1998). This $sPLA_2$ subtype is induced at the transcriptional level and is secreted from the cells after stimulation with pro-inflammatory cytokines such as interleukin 1β (IL1β) as well as cyclic AMP-elevating agents such as forskolin (Pfeilschifter *et al*., 1997). We have described previously that exogenous group IIA $sPLA_2$ has the potential to activate several pro-inflammatory signalling mechanisms in mesangial cells, including protein kinase C and the mitogen-activated protein kinase cascade, which result in the stimulation of cytosolic phospholipase A_2, release of arachidonic acid and subsequent synthesis of prostaglandin E_2 (PGE_2) (Pfeilschifter *et al*., 1993; Huwiler *et al*., 1997).

Recently, we and others have shown that the gene expression of group IIA $sPLA_2$ and cyclo-oxygenase 2 (Cox-2) in mesangial cells is strongly dependent on endogenous NO formed by the action of the inducible nitric oxide synthase (iNOS) (Rupprecht *et al*., 1999; Salvemini, 1997). This enzyme, in turn, is regulated at the transcriptional level by cytokines (Eberhardt *et al*., 1998). An inhibition of IL1β-stimulated iNOS activity and NO formation results in a nearly complete inhibition of group IIA $sPLA_2$ mRNA expression (Rupprecht *et al*., 1999).

The intention of this study was to elucidate further the potential cross-talk between group IIA $sPLA_2$ and the other pro-inflammatory enzymes, Cox-2 and iNOS, in ligand-stimulated signal transduction in mesangial cells. For this purpose we used the enolized 1,3-dioxane-4,6-dione-5-carboxamide, CGP-43182, which has been described as a potent inhibitor of secreted PLA_2 activity from synovial fluid – now called group IIA $sPLA_2$. CGP-43182 was originally termed 'compound 1a' in the publication of Breitenstein *et al*. (1994). It exerts a clear preference for group IIA $sPLA_2$ *in vitro* ($IC_{50} = 2.8\ \mu M$), whereas the pancreatic group IB $sPLA_2$ was inhibited with an IC_{50} of 27 μM and the cytosolic PLA_2 with an IC_{50} over 30 μM. In crystallization studies it was shown that the enolized β-dicarbonyl moiety of CGP-43182 acts as a scavenger for the calcium ion located at the catalytic site of the $sPLA_2$, thereby forming a stable calcium complex. Inhibition of group IIA $sPLA_2$ *in vitro* is calcium-dependent with an IC_{50} of about 1 mM calcium. In *in vivo* models, such as the pertussis pleurisy model, repeated oral or intraperitoneal administration of CGP-43182 inhibits exudate volume, cell infiltration and eicosanoid production (PGE_2, LTC_4). In experimental models of adjuvant arthritis and carrageenan-induced oedema formation CGP-43182 was shown to have anti-inflammatory activity, suggesting that group IIA $sPLA_2$ is involved in the pathogenesis of these diseases. Earlier studies in rat mesangial cell cultures have shown that CGP-43182 inhibits PGE_2 formation at concentrations where the cytosolic PLA_2 is not affected, suggesting that group IIA $sPLA_2$ is responsible for this effect (Pfeilschifter *et al*., 1993). Use of CGP-43182 as a therapeutic agent requires an exact knowledge of the mode of action of this substance on pro-inflammatory signalling mechanisms, which have been investigated in this study in more detail.

The data presented here indicate that CGP-43182 is not only a specific inhibitor of group IIA $sPLA_2$ activity *in vitro* but that, in addition, it prevents the activation of the transcription factor NFκB, which regulates the IL1β-induced transcription of group IIA $sPLA_2$, iNOS and Cox-2 genes in rat mesangial cells, thereby inhibiting the synthesis of NO and prostaglandins as important mediators of inflammation.

Methods

Cell culture

Rat renal mesangial cells were cultivated as described previously (Pfeilschifter *et al*., 1984). The cells were grown in RPMI 1640 supplemented with 10% foetal calf serum, penicillin (100 units ml^{-1}), streptomycin (100 $\mu g\ ml^{-1}$) and bovine insulin (0.66 units ml^{-1}). For experiments, cells were cultured in 3.5 or 10-cm plastic Petri dishes (Greiner Labortechnik, Frickenhausen, Germany) to near confluence. Cells were then incubated for 24 h in serum-free Dulbecco's modified Eagle medium (DMEM) containing 0.1 mg ml^{-1} fatty acid-free bovine serum albumin (BSA). After this period cells were treated for the indicated time points with the compounds to be tested.

Phospholipase A_2 assay

Phospholipase A_2 activity in the supernatant of mesangial cell cultures was determined with 1-^{14}C-oleate-labelled *Escherichia coli* as substrate, as described previously (Märki & Franson, 1986). Briefly, assay mixtures (1 ml) contained 100 mM Tris/HCl (pH 7.0), 1 mM $CaCl_2$, 1-^{14}C-oleate-labelled *E. coli* ($\approx$5000 c.p.m.) and 5 μl of the enzyme-containing supernatants of the cell cultures, which is sufficient to produce less than 5% substrate hydrolysis to be within the linear range. Reaction mixtures were incubated for 1 h at 37°C in a thermomixer. Extraction of lipids was performed by the Dole method, exactly as described (Pfeilschifter *et al*., 1993). Liberated [1-^{14}C]-oleate was measured in a β-scintillation counter.

Western blot analysis

Group IIA $sPLA_2$ protein secretion by cells was assayed by precipitating 1 ml of culture supernatant with 400 μl of 20% trichloroacetic acid. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS – PAGE) using a 15% polyacrylamide gel was performed under non-reducing conditions according to Laemmli (1970). The proteins were transferred to polyvinylidine difluoride (PVDF) membranes for 2 h at 2 mA cm^{-2}. Non-specific binding was blocked with 0.1% (w v^{-1}) milk powder in phosphate-buffered saline (PBS) for 1 h at room temperature followed by incubation with primary antibody at a 1 : 100 dilution. The monoclonal antibodies against rat group IIA $sPLA_2$ (hybridoma supernatants 2E7 and 2B9 dissolved in 0.01% milk powder) were a generous gift of Professor Henk van den Bosch, University of Utrecht, Netherlands. The blot was incubated with horseradish peroxidase-conjugated goat anti-mouse IgG. After washing, peroxidase activity was detected by developing the blots by the enhanced chemiluminescence method (ECL; Amersham-Pharmacia Biotech, Freiburg, Germany).

Detection of IκBα protein was performed in total cell lysates, which were obtained by homogenizing cells in a buffer consisting of (mM) Tris/HCl 20, pH 7.5, EDTA 1, EGTA 1, dithiothreitol (DTT) 2, phenylmethylsulphonyl fluoride (PMSF) 1 and 25 $\mu g\ ml^{-1}$ leupeptin. Separation of total cellular protein was performed by 12% SDS – PAGE under reducing conditions. After transfer of proteins to the PVDF membranes, blocking was performed with 3% milk powder in PBS for 1 h at room temperature followed by incubation with a specific antibody against human IκBα (Santa Cruz, U.S.A.) at a 1 : 1000 dilution. The blot was incubated with horseradish peroxidase-conjugated goat anti-mouse IgG. After washing,

peroxidase activity was detected by developing the blots by the ECL method.

Northern blot analysis

Confluent mesangial cells were cultured in 10-cm diameter culture dishes. After stimulation, cells were washed twice with PBS and harvested using a rubber policeman. Total cellular RNA was extracted from the cell pellets using the guanidinium isothiocyanate/phenol/chloroform method (Sambrook *et al*., 1989). Samples of 10 μg of RNA were separated on 1.4% agarose/formaldehyde gels and transferred to a gene screen membrane. After u.v. cross-linking and pre-hybridization for 4 h the filters were hybridized for 16 h at 42°C to ^{32}P-labelled complementary DNA (cDNA) inserts from group IIA $sPLA_2$, iNOS, Cox-2 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). DNA probes were radioactively labelled with α-^{32}P-dCTP by random priming. Finally, the filters were washed twice with $2\times$SSC/0.1% SDS for 2×20 min and several times at 65°C with $0.2\times$SSC/1% SDS. The signal was detected and quantified with a phosphorimager BAS 1500 (Fuji, Straubenhardt, Germany). To correct for variations in RNA loading the respective cDNA probes were stripped and the blots were rehybridized to the α-^{32}P-dCTP-labelled cDNA insert for GAPDH.

Determination of 6-keto-prostaglandin $F_{1\alpha}$

6-Keto-prostaglandin $F_{1\alpha}$ was measured in aliquots of the cell culture supernatants by enzyme immunoassay (EIA) according to the manufacturer's instructions (Biotrend, Cologne, Germany).

Nitrite analysis

Nitrite concentration was determined by the Griess reaction (Green *et al*., 1982). Cell culture supernatants were collected and 200 μl was added to a 96-well plate, mixed with 20 μl sulphanilamide (dissolved in 1.2 M HCl) and 20 μl N-naphthylethylenediamine dihydrochloride. After 5 min at room temperature the absorbance was measured at 540 nm with a microplate-reader (Biorad, Munich, Germany). Nitrite concentrations were calculated using sodium nitrite as standard.

Electrophoretic mobility shift analysis (EMSA)

Nuclear extracts from cultured mesangial cells were prepared as described (Schreiber *et al*., 1989). The consensus sequence of the double-stranded oligonucleotide for NFκB used for EMSA (Stratagene, Heidelberg, Germany) was as follows (coding strand): 5′-GATCGAGGGGACTTTCCCTAGC-3′. The complementary DNA strands were endlabelled by T4 polynucleotide kinase using [γ-^{32}P]-ATP (3000 Ci mmol^{-1}). Binding reactions were performed for 30 min on ice with 5–10 μg of protein in 20 μl binding buffer containing (mM) HEPES 20 (pH 7.9), KCl 50, EDTA 1, DTT 1, PMSF 1, 4% Ficoll, 0.25 mg ml^{-1} BSA, 2 μg poly(dI-dC) and 20,000 to 25,000 d.p.m. ^{32}P-labelled oligonucleotide. DNA-protein complexes were separated from unbound oligonucleotide by electrophoresis through native 4% polyacrylamide gels using $0.5\times$Tris-borate-EDTA. Gels were fixed in 10% acetic acid/10% isopropanol for 20 min and were vacuum dried afterwards. Radioactive signals were detected by a phosphorimager BAS 1500.

Data analysis and statistics

$sPLA_2$ activity is shown as c.p.m. released [^{14}C]-oleic acid measured in 5 μl of cell culture supernatants. 6-keto-$PGF_{1\alpha}$ is shown as pg ml^{-1}. NO formation is shown as μM nitrite measured in the cell culture supernatants.

Unless otherwise indicated, the data represent the means of four independent determinations $\pm$ s.e.mean. Data shown in the figures are representative of at least three individual experiments with similar results. Statistical analysis was performed by repeated-measures ANOVA followed by Dunnett's test, comparing all CGP-43182 concentrations with control. A probability <0.05 was defined as significant.

Materials

Recombinant human IL1β was obtained from Cell Concepts (Umkirch, Germany). CGP-43182 (2-Hydroxy-4-oxo-1,5-dioxyspiro[5,5] undec-2-ene-3-N-(2,4-dichlorophenyl)-carboxamide) was kindly provided by Dr W. Breitenstein (Novartis, Basel, Switzerland). [1-^{14}C]-oleic acid and [^{32}P]-deoxynucleotides were from Amersham-Buchler, Frankfurt, Germany. Immobilone PVDF membranes were from Millipore (Eschborn, Germany) and Nylon membranes (Gene Screen) were purchased from NEN Life Science (Cologne, Germany). All cell culture media and nutrients were from Gibco BRL (Eggenstein, Germany) and all other chemicals used were from Merck (Darmstadt, Germany), Sigma or Fluka (Deisenhofen, Germany).

Results

Effect of CGP-43182 on group IIA $sPLA_2$ induction, secretion and activity

Mesangial cells were treated for 24 h with IL1β in the presence of different concentrations of CGP-43182 ranging from 1 to 10 μM. Within this concentration range CGP-43182 inhibited group IIA $sPLA_2$ activity without affecting the cytosolic phospholipase A_2 activity (Pfeilschifter *et al*., 1993; Breitenstein *et al*., 1994). Subsequently, cell culture supernatants were used for precipitation of group IIA $sPLA_2$ protein. Western blot analysis was performed with a specific antibody against rat group IIA $sPLA_2$ to show whether the inhibition of this enzyme in the cell culture supernatant is due to changes in group IIA $sPLA_2$ protein levels.

After treatment of mesangial cells with different concentrations of CGP-43182 the secreted group IIA $sPLA_2$ protein completely disappeared from the supernatant of IL1β-stimulated cells at 5 μM (Figure 1A). At this concentration of CGP-43182 the $sPLA_2$ activity measured in the cell culture supernatant was also completely abolished (Figure 2). At 3 μM the amount of group IIA $sPLA_2$ protein was clearly diminished by about 70–80%, as evaluated by densitometric analysis of the spots corresponding to group IIA $sPLA_2$ protein. At this concentration an 80% reduction of $sPLA_2$ activity was also observed. Interestingly, at 1 μM, CGP-43182 induces an increase in levels of the protein secreted into the cell culture supernatant by about 20–50% in three independent experiments. The reason for this biphasic effect of CGP-43182 on group IIA $sPLA_2$ protein levels is unknown. Surprisingly, this increase in group IIA $sPLA_2$ protein was not associated with an increase in $sPLA_2$ activity. To exclude the possibility that this effect is simply due to an *in vitro* inhibitory effect of CGP-43182 on $sPLA_2$ catalytic activity, we performed a $sPLA_2$

activity assay with supernatants from cell cultures treated for 24 h with CGP-43182 and purified rat group IIA $sPLA_2$. We did not observe any inhibitory effect on enzyme activity suggesting that after a 24 h incubation there is not enough CGP-43182 left in the supernatants to reduce $sPLA_2$ activity (data not shown).

In the absence of cytokine stimulation CGP-43182 alone had no effect on group IIA $sPLA_2$ expression and was also not cytotoxic as measured by LDH release (data not shown). From these data we conclude that the observed decrease in $sPLA_2$ activity after 24 h incubation is mainly due to the reduction in group IIA $sPLA_2$ protein and not to an inhibition of $sPLA_2$ activity in the cell culture supernatant.

An alternative pathway to trigger group IIA $sPLA_2$ expression is mediated by cyclic AMP. Cyclic AMP-elevating agents such as forskolin, which use signalling pathways and regulatory mechanisms different from IL1β, potently induce group IIA $sPLA_2$ expression and secretion in mesangial cells (Pfeilschifter *et al*., 1991; Mühl *et al*., 1992; Walker *et al*., 1995). To study the effect of CGP-43182 on the cyclic AMP-mediated group IIA $sPLA_2$ induction we used the cell-permeant cyclic AMP-analogue N^6,2′-O-dibutyryl-cAMP (db-cAMP) for stimulation.

Concentrations of CGP-43182 up to 10 μM had no significant inhibitory effect on group IIA $sPLA_2$ protein secretion (Figure 1B) or $sPLA_2$ activity (Figure 2) stimulated by 0.5 mM db-cAMP. Again, at 1 μM but also at 10 μM we observed an increase in group IIA $sPLA_2$ protein. The reason for this biphasic effect is unknown but may point to additional modes of action of CGP-43182.

Since group IIA $sPLA_2$ is induced at the transcriptional level by IL1β, we next evaluated whether the decrease of group IIA $sPLA_2$ protein seen with CGP-43182 might result from an inhibitory effect of IL1β-stimulated group IIA $sPLA_2$ mRNA induction. Northern blot analysis was performed from mesangial cells that were incubated either with IL1β or db-cAMP plus CGP-43182 for 24 h.

As shown in Figure 3, IL1β-stimulated group IIA $sPLA_2$ mRNA expression was concentration-dependently reduced by CGP-43182 and completely inhibited by 5 μM, whereas the db-cAMP-stimulated induction was not affected at all. These data correspond to the concentration-response relationship obtained for the effects of CGP-43182 on group IIA $sPLA_2$ protein secretion and activity.

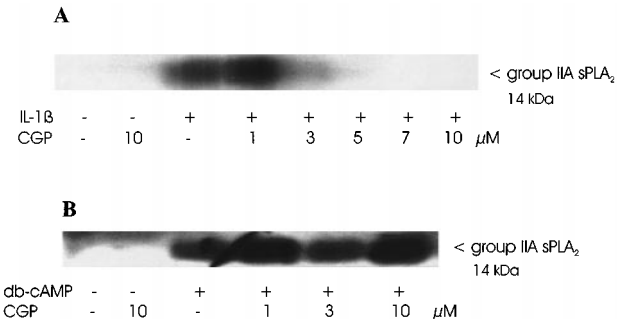

Figure 1 Effect of CGP-43182 on IL-1β and db-cAMP-stimulated group IIA $sPLA_2$ secretion. Cells were pre-incubated for 30 min with the indicated concentrations of CGP-43182 and then treated for 24 h with 1 nM IL-1β (A) or 0.5 mM db-cAMP (B) and cell culture supernatants were collected for Western blot analysis with a specific antibody against group IIA $sPLA_2$, as described in Methods. Data are representative for three independent experiments.

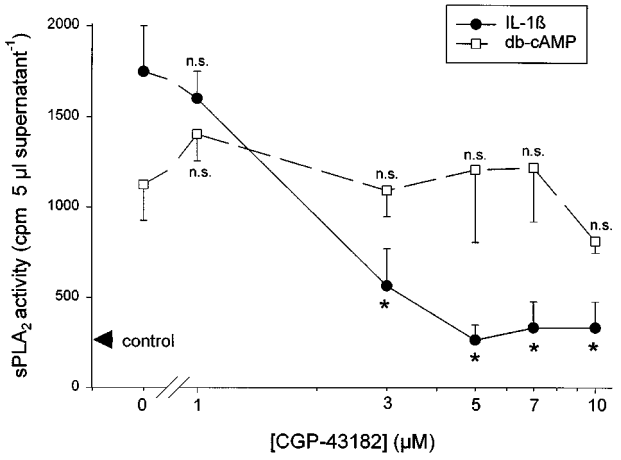

Figure 2 Effect of CGP-43182 on IL-1β- and db-cAMP-stimulated group IIA $sPLA_2$ activity. Mesangial cells were pre-incubated for 30 min with the indicated concentrations of CGP-43182 and then treated for 24 h with IL-1β (1 nM) or db-cAMP (0.5 mM). $sPLA_2$ activity was determined as described in Methods. Enzyme activity in control cells incubated in the absence of stimuli is indicated by an arrow. Data are mean$\pm$s.e.mean from four experiments. $^*P<0.05$ compared to control cells; n.s. not significant.

Effect of CGP-43182 on Cox-2 induction and prostacyclin formation

Pfeilschifter *et al*. (1993) described an inhibitory effect of CGP-43182 on cytokine-induced PGE_2 formation and interpreted the data as a result of inhibition of group IIA $sPLA_2$ enzyme activity. In order to examine whether CGP-43182 also affects the IL1β-stimulated synthesis of other prostanoids in mesangial cells, we measured the formation of 6-keto-$PGF_{1\alpha}$, the stable degradation product of prostacyclin, another major eicosanoid produced by mesangial cells (Klein *et al*., 1994).

First it was observed that 6-keto-$PGF_{1\alpha}$ formation measured as pg ml^{-1} reached much lower levels after stimulation with db-cAMP than after IL1β stimulation (Figure 4). A similar difference between IL1β and db-cAMP was also found for the levels of PGE_2 (data not shown). CGP-43182 concentration-dependently inhibited IL1β-stimulated formation of 6-keto-$PGF_{1\alpha}$, whereas the db-cAMP-stimulated formation of this prostaglandin was not significantly influenced (Figure 4).

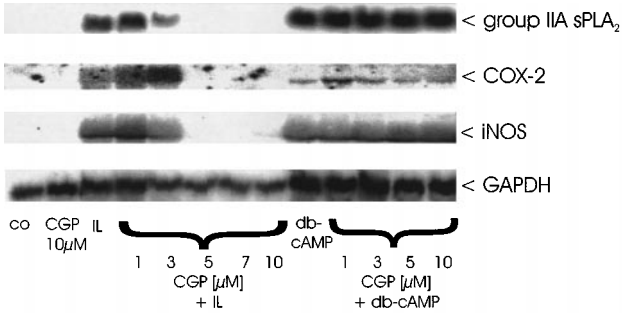

Figure 3 Effect of CGP-43182 on IL-1β- and db-cAMP-stimulated mRNA induction of $sPLA_2$-IIA, Cox-2 and iNOS. Cells were incubated for 24 h with IL-1β (1 nM) or db-cAMP (0.5 mM) in the absence or presence of the indicated concentrations of CGP-43182. Northern blots were performed from the RNA extracts of the cells as described in Methods and were sequentially hybridized with the cDNA probes for group IIA $sPLA_2$, Cox-2, iNOS and GAPDH. Data are representative for three independent experiments giving comparable results.

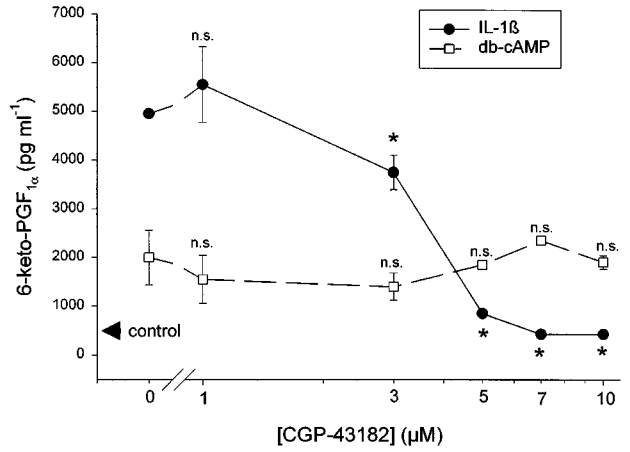

Figure 4 Effect of CGP-43182 on IL-1β- and db-cAMP-stimulated prostacyclin formation in mesangial cells. Cells were incubated for 24 h with IL-1β (1 nM) or db-cAMP (0.5 mM) in the presence of the indicated concentrations of CGP-43182. Cell culture supernatants were collected for measurement of the formation of 6-keto-$PGF_{1\alpha}$, the stable end product of prostacyclin, as described in Methods. The level of 6-keto-$PGF_{1\alpha}$ found in vehicle-treated cells (control) is indicated by an arrow. Statistical analysis was performed as described in legend of Figure 2. *$P<0.05$; n.s. not significant.

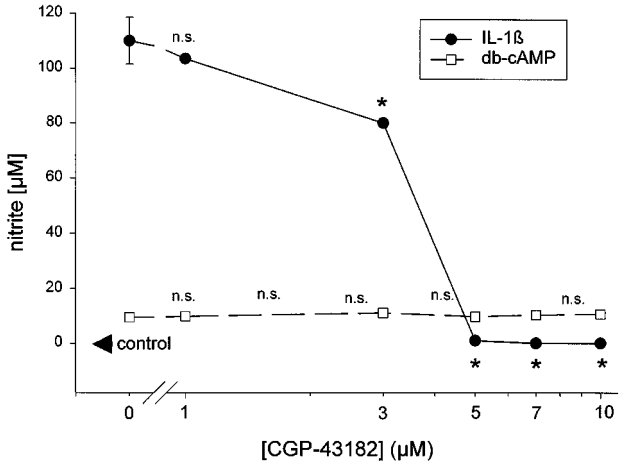

Figure 5 Effect of CGP-43182 on IL-1β- and db-cAMP-stimulated nitrite formation. Cells were incubated for 24 h with IL-1β (1 nM) or db-cAMP (0.5 mM) in the presence of the indicated concentrations of CGP-43182. Cell culture supernatants were collected and used for measurement of the formation of nitrite as described in Methods. The level of nitrite found in vehicle-treated cells (control) is indicated by an arrow. Statistical analysis was performed as described in legend of Figure 2. *$P<0.05$; n.s. not significant.

Cytokines (Salvemini, 1997), as well as cyclic AMP-elevating agents (Nüsing *et al*., 1996), are known to induce gene expression of Cox-2 in rat mesangial cells. In order to study effects of CGP-43182 on mRNA induction and product formation of Cox-2 we rehybridized the Northern blots used for detection of group IIA $sPLA_2$ mRNA with the cDNA probe for Cox-2. A representative blot is shown in Figure 3.

Induction of Cox-2 mRNA was markedly increased after a 24 h treatment with IL1β. Moreover, the Cox-2 mRNA levels were found to be much lower after db-cAMP than after IL1β stimulation, thus corresponding to data obtained for 6-keto-$PGF_{1\alpha}$. IL1β-induced Cox-2 mRNA expression was completely inhibited in the presence of 5 μM CGP-43182. Interestingly, at 1 μM of CGP-43182 a moderate increase in Cox-2 mRNA was observed, which indicates a biphasic effect of CGP-43182 on mechanisms involved in the transcription of Cox-2. In contrast to IL1β, the db-cAMP-stimulated Cox-2-mRNA expression was not reduced by CGP-43182.

These data indicate that CGP-43182 potently inhibited prostaglandin formation by simultaneously preventing IL1β-induced expression of Cox-2 and group IIA $sPLA_2$.

Effect of CGP-43182 on iNOS induction and nitrite formation

Recently we described that the IL1β-stimulated gene expression of group IIA $sPLA_2$ is strongly dependent on NO produced by iNOS in mesangial cells (Rupprecht *et al*., 1999). Since group IIA $sPLA_2$ and Cox-2 were inhibited at the transcriptional level by CGP-43182, we hypothesized that the induction of iNOS in mesangial cells might also be affected by this compound. As in the case of group IIA $sPLA_2$ and Cox-2, we compared the effects of CGP-43182 on IL1β- as well as db-cAMP-stimulated iNOS mRNA induction by rehybridization of the Northern blots with a cDNA probe for iNOS.

CGP-43182 concentration-dependently reduced IL1β-stimulated iNOS mRNA expression (Figure 3). Nitrite formation determined in the supernatants of IL1β-stimulated cell cultures was completely inhibited by 5 μM CGP-43182. A 30% reduction was obtained with 3 μM (Figure 5). In contrast, induction of iNOS mRNA by db-cAMP treatment of mesangial cells was not affected by concentrations up to 10 μM CGP-43182 (Figure 3).

Db-cAMP-stimulated nitrite formation, which is approximately 10 times lower than IL1β-stimulated nitrite production, was not inhibited by CGP-43182 (Figure 5). These data indicate that CGP-43182 is a potent inhibitor of the IL1β- but not cyclic AMP-mediated iNOS induction.

Effect of CGP-43182 on IL1β-stimulated NFκB binding

Nuclear factor κB (NFκB) is known to be involved in IL1β-induced group IIA $sPLA_2$, Cox-2 and iNOS gene expression in mesangial cells (Walker *et al*., 1995; 1997; Martin *et al*., 1994; Beck & Sterzel 1996; Eberhardt *et al*., 1994; 1998) and thus seems to be a common denominator of cytokine-induced production of inflammatory mediators (Barnes & Karin 1997; Mercurio & Manning 1999; Pfeilschifter & Mühl 1999).

In order to determine whether CGP-43182 affects NFκB activation, we performed electrophoretic mobility shift analysis (EMSA). To this end a radioactively labelled oligonucleotide containing a consensus NFκB binding site was used. A 30-min treatment of mesangial cells with IL1β strongly induced the formation of NFκB-containing complexes, whereas in control mesangial cells only a weak DNA binding activity was observed (Figure 6). In the presence of 10 μM CGP-43182 the cytokine-stimulated binding of NFκB to its regulatory element was completely blocked. CGP-43182 alone had no effect (data not shown). These data strongly suggest that IL1β-stimulated NFκB activation is suppressed by CGP-43182.

Effect of CGP-43182 on IκBα-degradation

Activation of NFκB occurs by degradation of the endogenous inhibitor IκBα *via* ubiquitination and degradation by the 26S proteasome (Baeuerle, 1998; Mercurio & Manning, 1999). An important step in the initiation of proteolytic degradation of IκBα is its stimulus-dependent phosphorylation by an IκB-kinase (IKK) (Brown *et al*., 1995).

We examined whether CGP-43182 prevents the activation of NFκB by interfering with the phosphorylation and proteolytic degradation of IκBα. With protein lysates from cells treated for 15 min with IL1β in the presence of different concentrations of CGP-43182, Western blot analysis was performed using a specific antibody against IκBα. This antibody recognizes both the unphosphorylated and the phosphorylated forms of IκBα, which are discernible by different migration behaviour during PAGE, resulting in a band shift (Mercurio & Manning, 1999).

After IL1β treatment of mesangial cells the level of unphosphorylated IκBα protein (lower band) decreased compared to the control, whereas the upper band, which reflects phosphorylated IκBα, was clearly enhanced (Figure 7). Treatment of cells with CGP-43182 resulted in a concentration-dependent increase in the unphosphorylated form of IκBα protein and a corresponding decrease in the phosphorylated form. However, at higher concentrations of CGP-43182 (5 and 10 μM) there was a pronounced increase in both the phosphorylated and the unphosphorylated bands, thus suggesting different modes of action of CGP-43182.

We concluded that CGP-43182 interferes with the degradation process of IκBα by inhibiting its phosphorylation by an IKK as well as by possibly interfering with the ubiquitine-proteasome complex. This prevents NFκB activation and subsequent gene expression of pro-inflammatory enzymes.

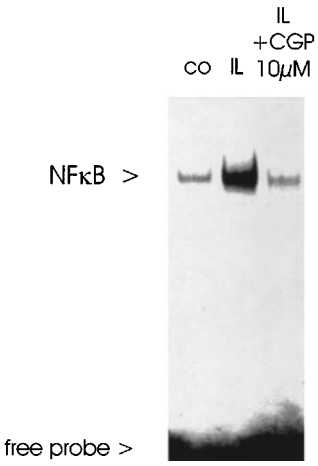

Figure 6 Electrophoretic mobility shift assay demonstrating the effect of CGP-43182 on the activation of NFκB by IL-1β. Cells were treated for 30 min with IL-1β (1 nM) or vehicle either in the absence or presence of 10 μM CGP-43182 as indicated. Radiolabelled oligonucleotides were incubated with nuclear extracts prepared from stimulated mesangial cells as described in Methods. This experiment was performed twice with comparable results.

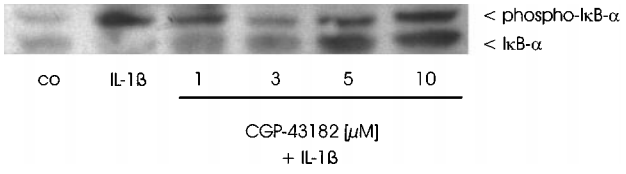

Figure 7 Effect of CGP-43182 on the degradation of IκBα-protein. Cells were treated for 15 min with IL-1β (1 nM) or vehicle in absence or presence of the indicated concentrations of CGP-43182. Cells were homogenized and proteins were separated by 12% SDS–PAGE. Western blot analysis was performed with a specific antibody against human IκBα as described in Methods. This experiment was performed three times with comparable results.

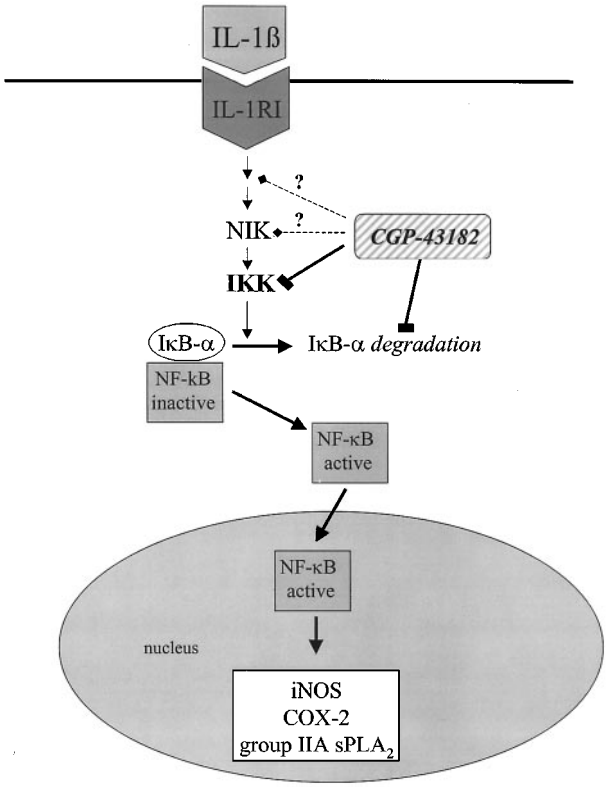

Figure 8 Schematic representation of IL-1β-stimulated signal transduction and inflammatory gene expression and possible interaction points of CGP-43182. Abbreviations: IL-1RI, interleukin 1-receptor type I; IKK, IκB-kinase; NIK, NFκB-inducing kinase.

Figure 8 summarizes the proposed modulation of the IL1β signalling cascade by CGP-43182 resulting in a decreased expression of pro-inflammatory mediators such as group IIA $sPLA_2$, Cox-2 and iNOS.

Discussion

The aim of this study was to investigate the mechanisms of the anti-inflammatory activity of CGP-43182 in mesangial cells, which was originally described to be a selective inhibitor of group IIA $sPLA_2$ activity *in vitro* (Breitenstein *et al*., 1994). In animal models of inflammation (carrageenan-induced oedema, adjuvant arthritis) CGP-43182 showed potent anti-inflammatory activity. To further elucidate the molecular mechanisms of CGP-43182 action we investigated the effect of this compound on gene expression of different enzymes thought to be critical for inflammatory processes in the kidney.

We report here that CGP-43182 is a potent inhibitor of IL1β-induced group IIA $sPLA_2$ gene expression in mesangial cells. As a consequence, the protein levels of group IIA $sPLA_2$ and the enzyme activity in the cell culture supernatant were completely abolished at 10 μM of the compound. From these data we conclude that the inhibitory effect of CGP-43182 is not due to the *in vitro* inhibition of activity of secreted group IIA $sPLA_2$, but that CGP-43182 interferes with the preceding expression of the enzyme. In mesangial cells the group IIA $sPLA_2$ protein secretion starts about 8–12 h after cytokine treatment (Pfeilschifter *et al*., 1997). After this incubation period probably most of the cell-permeant CGP-43182 is taken up by the cells or is metabolized, since supernatants taken from cell cultures at this time point did not any longer inhibit rat

group IIA $sPLA_2$ activity *in vitro*. Furthermore, the db-cAMP-induced $sPLA_2$ activity, which reached levels similar to that observed after IL1β, was not affected by CGP-43182, supporting the loss of enzyme inhibiton after long-term incubations with the compound.

We can exclude that the $sPLA_2$ activity measured in rat mesangial cell cultures is derived from other $sPLA_2$ subtypes such as the group V $sPLA_2$. Using an antibody raised against a peptide sequence specific for the rat group V $sPLA_2$ and recognizing recombinant rat group V $sPLA_2$ protein, we could not detect any signal by Western blot analysis. From this experiment and from earlier sequencing studies performed by Schalkwjik *et al*. (1992) we conclude that, in rat mesangial cells, the group IIA $sPLA_2$ represents the major $sPLA_2$ subtype secreted upon cytokine stimulation.

Recently, we have obtained evidence that group IIA $sPLA_2$ gene expression in mesangial cells is strongly dependent on NO formation by iNOS (Rupprecht *et al*., 1999). Similarly, Cox-2 mRNA expression and enzyme activity are stimulated by NO in mesangial cells (Salvemini, 1997). It is becoming clearer that many inflammatory processes are associated with co-induction of active iNOS and Cox-2 (Salvemini, 1997). In addition to the inhibition of group IIA $sPLA_2$ expression we found a complete suppression of iNOS and Cox-2 mRNA induction by CGP-43182, strongly indicating that CGP-43182 modulates a common regulatory event in the transcription of these inducible enzymes.

Earlier studies have shown that cyclic AMP-elevating agents are able to mimic cytokine-induced group IIA $sPLA_2$ expression and that they synergically interacted with IL1β or tumour necrosis factor α to promote group IIA $sPLA_2$ and iNOS induction in mesangial cells by using different signalling pathways (Mühl *et al*., 1992; Pfeilschifter *et al*., 1991; Kunz *et al*., 1994; Eberhardt *et al*., 1998). Investigating the effects of CGP-43182 on db-cAMP-mediated gene expression and activity of group IIA $sPLA_2$, Cox-2 and iNOS, we did not find any reduction in the expression levels of these enzymes. By contrast, CGP-43182 even slightly increased the mRNA expression of Cox-2 and iNOS by a mechanism which is currently not understood.

Other compounds, such as pyrrolidine dithiocarbamate (PDTC), cyclosporin A and glucocorticoids have already been described to differentially affect cytokine- and cyclic AMP-induced expression of group IIA $sPLA_2$ and iNOS in rat mesangial cells, suggesting that IL1β and cyclic AMP trigger separate signalling cascades (Walker *et al*., 1995; 1997; Vervoordeldonk *et al*., 1996). Similar observations were made for the induction of iNOS by cytokines and cyclic AMP (Pfeilschifter & Schwarzenbach 1990; Kunz *et al*., 1994; Eberhardt *et al*., 1998). This separate regulation of iNOS and group IIA $sPLA_2$ expression by different stimuli seems to be due to the involvement of separate sets of transcription factors, which control the induction of these enzymes by cyclic AMP and IL1β (Eberhardt *et al*., 1998). IL1β is known to stimulate the activation of NFκB-mediated gene transcription (Baeuerle & Henkel, 1994), whereas cyclic AMP acts *via* cyclic AMP-responsive element-binding (CREB) protein and CCAAT/enhancer-binding protein (C/EBP) transcription factors (Gonzalez & Montminy, 1989; Ray & Ray, 1994). In rat mesangial cells NFκB is a crucial transcription factor for IL1β-mediated iNOS gene induction but is not involved in the cyclic AMP-dependent pathway of iNOS transcription (Eberhardt *et al*., 1998). Inhibition of NFκB activation by PDTC was shown to affect IL1β- but not cyclic AMP-stimulated group IIA $sPLA_2$ gene expression (Walker *et al*., 1995).

Since CGP-43182 only inhibited the IL1β- but not the cyclic AMP-induced gene expression, we speculated whether CGP-43182 interferes with the activation of NFκB. NFκB is constitutively present in cells as a heterodimer consisting of a p50 DNA-binding subunit and a Rel A (p65) transactivation subunit. In the cytoplasm this NFκB complex is kept inactive by association with its inhibitory subunit IκBα (Baeuerle, 1998). Phosphorylation and degradation of IκBα seem to be crucial steps in the activation process leading to dissociation of IκBα from NFκB and subsequent translocation of NFκB to the nucleus. The results obtained with EMSA show that CGP-43182 drastically reduced the DNA binding activity of NFκB. Analysis of the IκBα protein 15 min after IL1β treatment shows that CGP-43182 prevents its phosphorylation and subsequent degradation. In addition to the inhibition of the IKK complex by CGP-43182, the compound also seems to have an inhibitory effect on the proteasome-mediated degradation of phospho-IκBα.

Whether CGP-43182 interferes directly with the proteolytic degradation of IκBα, the ubiquitination of the responsible ubiquitin-proteasome complex or the kinases upstream of IKK (see Figure 8), is under current investigation.

In summary, this study shows that CGP-43182 is a potent inhibitor of the activation of NFκB-mediated gene transcription, which seems to be a key step in expression of group IIA $sPLA_2$, Cox-2 and iNOS. Moreover, there is no doubt that CGP-43182 has marked *in vivo* activities, as shown in experimental models of adjuvant arthritis and carrageenan-induced oedema formation. Whether the capacity of CGP-43182 to inhibit cytokine-stimulated induction of potent pro-inflammatory enzymes actually explains its potent anti-inflammatory activity *in vivo* remains to be proven. In any case CGP-43182 is a useful tool to study NFκB-mediated signalling cascades.

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