Possible role of protein kinase C- ϵ isoenzyme in inhibition of interleukin 1 β induction of nitric oxide synthase in rat renal mesangial cells

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In cultured glomerular mesangial cells, interleukin 1 β (IL-1 β) has been shown to induce a dose- and time-dependent accumulation of nitrite, a stable metabolite of nitric oxide (NO). In parallel, increased levels of mRNA of an inducible macrophage-type of nitric oxide synthase (iNOS) were observed after incubating mesangial cells with IL-1 β . Here we report that addition of the biologically active phorbol esters, phorbol 12-myristate 13-acetate (PMA) and phorbol 12,13-dibutyrate (PDBu), dose-dependently inhibited the IL-1 β -stimulated increase in iNOS mRNA levels and nitrite production. In contrast, the biologically inactive phorbol ester 4 α -phorbol 12,13-didecanoate, had no effect on cytokine induction of iNOS and nitrite formation. Incubation of mesangial cells with PMA or PDBu alone, in the absence of IL-1 β , did not trigger any iNOS expression. Time-

course studies indicated that phorbol ester needs to be added for only 1 h in order to maximally inhibit cytokine-induced nitrite production. Down-regulation of protein kinase C (PKC)- α and - δ isoenzymes by 8 h PMA or PDBu treatment before stimulation with IL-1 β still resulted in full inhibition of iNOS induction. In contrast, a 24 h treatment of mesangial cells with PMA or PDBu, a regimen that also causes depletion of PKC- ϵ , abolished inhibition of IL-1 β -induced iNOS expression and nitrite production. In addition, the selective PKC inhibitor calphostin C potentiated IL-1 β induction of iNOS activity. In summary these data suggest that IL-1 β induction of iNOS expression is tonically suppressed by PKC and the ϵ -isoenzyme is the most likely candidate mediating this effect.

INTRODUCTION

Nitric oxide (NO) is well known as a mediator of blood vessel relaxation, but recent evidence indicates that NO has a much broader spectrum of functions and acts as an intercellular messenger in the central and peripheral nervous system and, additionally, NO is responsible for the non-specific cytotoxic activity of macrophages. Thus NO proves to be a highly versatile signalling molecule regulating a variety of diverse cellular functions (Moncada et al., 1991; Nathan, 1992).

Glomerular mesangial cells are a specialized type of vascular smooth muscle cells and take part in the regulation of the glomerular filtration rate (Pfeilschifter, 1989). These cells respond to endothelial-derived NO with increased levels of intracellular cyclic GMP. We and others have shown that cytokines, such as interleukin 1 (IL-1) or tumour necrosis factor α , induce a macrophage-type of NO synthase (NOS) in mesangial cells with subsequent elevation of cellular cyclic GMP concentrations (Pfeilschifter and Schwarzenbach, 1990; Marsden and Ballermann, 1990; Pfeilschifter et al., 1992). The excessive formation of NO and cyclic GMP in mesangial cells not only alters the contractile responses of the cells (Pfeilschifter et al., 1992), but may also cause tissue injury and thus contribute to the pathogenesis of certain forms of glomerulonephritis (Pfeilschifter et al., 1993; Cattell and Cook, 1993).

The careful control of this extremely reactive molecule is essential for the prevention of deleterious inflammatory reactions. Whereas the activity of the constitutive NOS is mainly regulated post-translationally by cytoplasmic Ca²⁺ levels or phosphorylation by a variety of protein kinases, inducible NOS (iNOS)

activity is regulated primarily at the transcriptional level (Nathan, 1992). To evaluate and establish new therapeutic concepts for the treatment of acute inflammatory reactions involving the production of NO it is essential to identify extracellular stimuli and intracellular signalling pathways that regulate iNOS expression. Recently, it has been reported that activation of protein kinase C (PKC; EC 2.7.1.37) by phorbol esters promotes the expression of iNOS in rat peritoneal macrophages (Hortelano et al., 1993) and rat hepatocytes (Hortelano et al., 1992). Unexpectedly, however, phorbol esters were also shown to inhibit lipopolysaccharide or interferon- γ induction of nitrite formation in macrophages and hepatocytes (Hortelano et al., 1992, 1993; Severn et al., 1992). As PKC exists as a family of at least 10 isoenzymes (Stabel and Parker, 1991; Nishizuka, 1992; Hug and Sarre, 1993), we felt that it is necessary to examine the role of PKC in the regulation of iNOS expression in mesangial cells, a cellular system where the expression and function of PKC isoenzymes has been well characterized (Huwiler et al., 1991a,b, 1992, 1993; Ochsner et al., 1993; Pfeilschifter and Huwiler, 1993).

MATERIALS AND METHODS

Cell culture

Rat glomerular mesangial cells were cultured as described previously (Pfeilschifter et al., 1992). In a second step, single cells were cloned by limited dilution using 96-microwell 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

Abbreviations used: IL-1, interleukin 1; NO, nitric oxide; iNOS, inducible nitric oxide synthase (EC 1.14.13.39); PDBu, phorbol 12,13-dibutyrate; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C (EC 2.7.1.37); DMEM, Dulbecco's modified Eagle's medium; SSC, 3 M NaCl/0.3 M sodium citrate; NF κ B, nuclear factor κ B.

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specific for myogenic cells, positive staining for Thy 1.1 antigen, negative staining for factor VIII-related antigen and cytokeratin excluded endothelial and epithelial contaminations respectively. The generation of inositol trisphosphate upon activation of the angiotensin II AT₁ receptor was used as a functional criterion for characterizing the cloned cell line (Pfeilschifter, 1990). The cells were grown in RPMI 1640 supplemented with 10% (v/v) fetal calf serum, penicillin (100 units/ml), streptomycin (100 µg/ml) and bovine insulin at 0.66 unit/ml (Sigma). For the experiments passages 9–16 were used. Confluent mesangial cells in 24-well plates were incubated with the indicated concentrations of compounds in Dulbecco's modified Eagle's medium (DMEM) without Phenol Red containing 0.1 mg/ml of BSA for the indicated time periods. Thereafter, the medium was removed for determination of nitrite concentration. Cell protein determination was performed according to the method of Lowry et al. (1951) with BSA (Sigma) as standard. For isolation of cellular RNA, cells were cultured in 150-mm diam. dishes.

Nitrite and nitrate analysis

Nitrite production by rat glomerular mesangial cells was measured as a read-out for NOS activity as described previously (Green et al., 1982). Confluent mesangial cells in 24-well plates were washed twice with PBS and incubated in DMEM without Phenol Red and supplemented with 0.1 mg/ml of fatty acid-free BSA, with or without agents, for the indicated time periods. Thereafter, the medium was withdrawn and centrifuged for 10 min at 9000 *g*-min in an Eppendorf laboratory centrifuge. Nitrite was measured by mixing 200 µl of the supernatant with 50 µl of Griess reagent. The absorbance at 550 nm was measured and the nitrite concentration was determined using a calibration curve with sodium nitrite standards. Nitrate was stoichiometrically reduced to nitrite by incubation of sample aliquots for 15 min at 37 °C, in the presence of 0.1 unit/ml nitrate reductase (EC 1.6.6.2; *Aspergillus* species; Boehringer–Mannheim), 50 µM NADPH and 5 µM FAD, in a final volume of 160 µl. When nitrate reduction was complete, the remaining NADPH (which would interfere with nitrite determination) was oxidized with 10 units/ml lactate dehydrogenase (Boehringer–Mannheim) and 10 mM sodium pyruvate, in a final volume of 170 µl for 5 min at 37 °C.

Northern-blot analysis

Confluent mesangial cells were cultured in 150-mm diam. culture dishes. For stimulation, cells were washed twice with PBS and incubated in DMEM without Phenol Red, supplemented with 0.1 mg/ml of fatty acid-free BSA, with or without agents, for the indicated time periods. Cells were washed twice with PBS and harvested using a rubber policeman. Total cellular RNA was extracted from the cell pellets using the guanidinium thiocyanate/caesium chloride method (Sambrook et al., 1989). Samples of 25 µg of RNA were separated on 0.8% agarose gels containing 0.66 M formaldehyde before transfer to gene screen membranes (New England Nuclear). After baking at 80 °C for 2 h and prehybridization for 4 h, the filters were hybridized for 16–18 h to a ³²P-labelled *Sma*I cDNA insert from pMac-NOS (Lyons et al., 1992). To correct for variations in RNA amount, the NOS probe was stripped with boiling 0.1 × NaCl/sodium citrate buffer (SSC, 3 M NaCl/0.3 M sodium citrate)/1% (w/v) SDS and the blots were rehybridized to the ³²P-labelled *Bam*HI/*Sal*I cDNA insert from clone pEX 6 coding for β-actin. DNA probes (approx.

2 × 10⁶ c.p.m./ml) were radioactively labelled with [α-³²P]dATP by random priming (Boehringer–Mannheim). Hybridization reactions were performed in 50% (v/v) formamide/5 × SSC/10 × Denhardt's solution/0.5% (w/v) SDS/250 µg/ml salmon sperm DNA. Filters were washed three times in 2 × SSC/0.1% SDS at room temperature for 30 min, and then in 2 × SSC/2% SDS at 65 °C for 30 min. Filters were exposed for 6–48 h to Kodak X-Omat XAR film using intensifying screens.

Chemicals

Recombinant human IL-1β and calphostin C were generously supplied by Dr. Klaus Vosbeck and Dr. Doriano Fabbro, Ciba-Geigy Ltd., Basel, Switzerland; recombinant human tumour necrosis factor α (TNFα) was from Boehringer, Mannheim, Germany; the different phorbol esters were purchased from Calbiochem, Lucerne, Switzerland. The cDNA clone pMac-Nos, coding for the inducible macrophage NOS was kindly provided by Dr. J. Cunningham, Boston, MA, U.S.A.; the cDNA clone pEX6, coding for human β-actin, was a gift from Dr. U. Aebi, Basel, Switzerland; nylon membranes (Gene Screen) were purchased from DuPont de Nemours International, Regensdorf, Switzerland; ³²P-dATP (specific radioactivity 3000 Ci/mmol) was from Amersham, Dübendorf, Switzerland; cell culture media and nutrients were from Gibco BRL, Basel, Switzerland and all other chemicals were either from Merck, Darmstadt, Germany, or Fluka, Buchs, Switzerland.

RESULTS

Incubation of mesangial cells for 24 h with IL-1β (2 nM) increased the content of nitrite in the culture supernatants (Table 1). Simultaneous incubation of IL-1β with the biologically active phorbol esters, PMA and PDBu, inhibited cytokine-stimulated nitrite generation, whereas the biologically inactive phorbol ester 4α-phorbol 12,13-didecanoate had no effect (Table 1). The amount of nitrate produced in IL-1β-stimulated cells was approx. 20–25% of that of nitrite and the relative amounts of nitrate and nitrite did not change upon phorbol ester treatment (results not shown). All phorbol esters, when given alone, did not stimulate nitrite production as shown in Table 1. The inhibitory action of PMA and PDBu was dose-dependent and half-maximal inhibition of IL-1β-stimulated nitrite formation was observed at 32 nM and 52 nM for PMA and PDBu respectively (Figure 1).

Table 1 Effects of different phorbol esters on IL-1β-stimulated nitrite production

Confluent mesangial cells were incubated with vehicle (Control), IL-1β (2 nM, IL-1), PMA (100 nM), PDBu (100 nM), 4α-phorbol 12,13-didecanoate (100 nM, PDD) or IL-1β (2 nM) plus the indicated phorbol esters (100 nM each) for 24 h. Thereafter, the medium was removed and used for nitrite determination. Results are means ± S.D. of four experiments. Significant differences from IL-1β stimulation in the absence of phorbol ester: ***P* < 0.01; n.s., not significant; ANOVA.

Addition	Nitrite (nmol/mg of protein)
Control	0 ± 0
IL-1	206 ± 43
PMA	0 ± 0
PDBu	0 ± 0
PDD	0 ± 0
IL-1 + PMA	67 ± 23**
IL-1 + PDBu	88 ± 20**
IL-1 + PDD	196 ± 21 ^{n.s.}

Figure 1 Inhibition of IL-1 β -induced nitrite production by different phorbol esters

Confluent mesangial cells were incubated with IL-1 β (2 nM) plus the indicated concentrations of PMA (■), PDBu (●) or 4 α -phorbol 12,13-didecanoate (PDD) (▲) for 24 h. Thereafter, the medium was removed and used for nitrite determination. Basal formation of nitrite in the absence of IL-1 β was 4 ± 2 nmol/mg of protein (means $\pm$ S.D., $n = 4$). Results are means of four experiments; S.D. ranged from 5 to 25%. Significant differences from IL-1 β stimulation in the absence of phorbol ester: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; n.s.; not significant; ANOVA.

Table 2 Time dependence of phorbol ester inhibition of IL-1 β -induced nitrite production

Confluent mesangial cells were incubated for 24 h with vehicle (Control), IL-1 β (2 nM, IL 1) or IL-1 β (2 nM) plus PDBu (500 nM). The indicated time points refer to the presence of PDBu in the stimulation medium (PDBu was added at time point 0). Cells were washed several times after the indicated time and incubation was continued in the presence of IL-1 β alone up to a total incubation time of 24 h. Thereafter, the medium was removed and used for nitrite determination. Results are the sum of nitrite produced over the 24 h stimulation period and are expressed as means $\pm$ S.D. of four experiments. Significant differences from IL-1 β stimulation in the absence of phorbol ester: *** $P < 0.001$; ANOVA.

Addition	Nitrite (nmol/mg of protein)
Control	$1 \pm 0^{***}$
IL-1	156 ± 30
IL-1 + PDBu (1 h)	$57 \pm 12^{***}$
IL-1 + PDBu (2 h)	$47 \pm 11^{***}$
IL-1 + PDBu (3 h)	$38 \pm 13^{***}$
IL-1 + PDBu (8 h)	$43 \pm 14^{***}$
IL-1 + PDBu (24 h)	$47 \pm 9^{***}$

These results suggest that PKC exerts an inhibitory action on cytokine-induced nitrite generation. In order to obtain information on the time course of inhibition, cells were incubated with IL-1 β for 24 h and PDBu (500 nM) was added for different periods during this stimulation. The data in Table 2 show that for maximal suppression of nitrite production PDBu need be present for only 1 h, suggesting that a short-term activation of PKC is sufficient to maximally block cytokine-induced iNOS activity. Furthermore, the data in Table 3 clearly demonstrate that activation of PKC just before the end of the stimulation period (16–20 h) is not sufficient to inhibit IL-1 β -stimulated nitrite synthesis. There is a marked synthesis of nitrite in mesangial cells in the time period between 16 h and 20 h after addition of IL-1 β (Table 3). Addition of PDBu (500 nM) in this very period (16–20 h) does not significantly attenuate cytokine-

Table 3 Critical time period for inhibition of IL-1 β -induced nitrite production by PDBu

Confluent mesangial cells were incubated with vehicle (Control) for 20 h, with IL-1 β (2 nM, IL-1) for 16 h or 20 h, as indicated, or with IL-1 β plus PDBu (500 nM) for 20 h. The indicated time periods refer to the presence of PDBu in the stimulation medium (IL-1 β was added at time point 0 and was present throughout the 20 h incubation period). PDBu was added either for the last 4 h of stimulation (4 h) or was present throughout the 20 h incubation period (20 h), as indicated. Thereafter, the medium was removed and used for nitrite determination. Results are the sum of nitrite produced over the 20 h stimulation period and are expressed as means $\pm$ S.D. of four experiments. Significant differences from IL-1 β stimulation (20 h) in the absence of phorbol ester: *** $P < 0.001$; n.s., not significant; ANOVA.

Addition	Nitrite (nmol/mg of protein)
Control	$4 \pm 0^{***}$
IL-1 (16 h)	$261.5 \pm 40.0^{***}$
IL-1 (20 h)	526.3 ± 31.8
IL-1 + PDBu (4 h)	$471.8 \pm 34.9^{n.s.}$
IL-1 + PDBu (20 h)	$89.2 \pm 13.9^{***}$

Figure 2 Time course of phorbol ester effects on IL-1 β -stimulated nitrite production

Confluent mesangial cells were pretreated for the indicated time periods with PMA (500 nM) (■), PDBu (500 nM) (●) or 4 α -phorbol 12,13-didecanoate (500 nM, PDD) (▲) and subsequently stimulated with IL-1 β (2 nM) for 24 h. Thereafter, the medium was removed and used for nitrite determination. Results are means of four experiments; S.D. ranged from 7 to 28%. Significant differences from IL-1 β stimulation in the absence of phorbol ester: *** $P < 0.001$; n.s., not significant; ANOVA.

stimulated nitrite production. In contrast, co-incubation of cells for 20 h with PDBu and IL-1 β resulted in a strong inhibition of IL-1 β -induced nitrite formation (Table 3). These results argue against a role for PKC-mediated covalent modifications of cytokine-induced NOS in the observed inhibition of nitrite production.

In an alternative approach, PKC down-regulation experiments were performed to obtain information on the PKC isoenzyme(s) mediating inhibition of cytokine-stimulated nitrite production. By immunoblot analysis we have shown previously that mesangial cells express four PKC isoforms, PKC- α , - δ , - ϵ and - ζ . No PKC- β 1, - β 2, - γ and - η isoforms were detected (Huwiler et al., 1991a, b, 1992, 1993). On exposure to PMA or PDBu these isoenzymes displayed distinctly different down-regulation kinetics. An 8 h treatment with phorbol ester was sufficient to completely deplete mesangial cells of PKC- α and - δ isotypes, and a 24 h incubation with phorbol esters was necessary to down-

Table 4 Effects of calphostin C on IL-1 β -stimulated nitrite synthesis in mesangial cells

Confluent mesangial cells were incubated for 24 h with vehicle (Control), IL-1 β (2 nM, IL 1) or IL-1 β (2 nM) plus the indicated concentrations of calphostin C. Thereafter, the medium was removed and used for nitrite determination. Results are means $\pm$ S.D. of four experiments. Significant differences from IL-1 β stimulation in the absence of inhibitor. * P < 0.05; ** P < 0.01, *** P < 0.001; by Student's *t*-test.

Addition	Nitrite (nmol/mg of protein)
Control	0 $\pm$ 0***
IL-1	118.6 $\pm$ 5.0
IL-1 + Calphostin C (0.1 μ M)	158.3 $\pm$ 27.3*
IL-1 + Calphostin C (0.3 μ M)	154.7 $\pm$ 15.5***
IL-1 + Calphostin C (1.0 μ M)	180.5 $\pm$ 30.2**

Figure 3 Time course of PDBu effects on IL-1 β -stimulated iNOS mRNA accumulation in mesangial cells

Confluent cells were incubated with vehicle (lane 1) or IL-1 β (2 nM, lanes 2–6) for 24 h. In some cases cells were pretreated with PDBu (500 nM) for 2 h (lane 3), 4 h (lane 4), 8 h (lane 5) or 24 h (lane 6) and subsequently stimulated with IL-1 β . Total cellular RNA (25 μ g) was hybridized successively to 32 P-labelled iNOS and β -actin cDNA probes as described in the Materials and methods section. For quantification, autoradiograms of the filters were scanned densitometrically. To correct for differences in loading, the signal density of each RNA sample hybridized to the iNOS probe was divided by that hybridized to the β -actin probe. The corrected density is expressed as the percentage of RNA found in IL-1 β -stimulated cells in the absence of phorbol ester. Lane 1, 0%; lane 2, 100%; lane 3, 1.2%; lane 4, 0.2%; lane 5, 7.9%; lane 6, 271%.

regulate PKC- ϵ . In contrast, phorbol ester treatment for 24 h did not induce any depletion of PKC- ζ . By comparing the down-regulation kinetics of the mesangial-cell PKC isoenzymes with the time-course of removal of the specific cellular functions, we have suggested that PKC- α may negatively regulate phospho-

Figure 4 Time course of PMA effects of IL-1 β -stimulated iNOS mRNA accumulation in mesangial cells

Confluent cells were incubated with vehicle (lane 1) or IL-1 β (2 nM, lanes 2–6) for 24 h. In some cases cells were pretreated with PMA (500 nM) for 2 h (lane 3), 4 h (lane 4), 8 h (lane 5) or 24 h (lane 6) and subsequently stimulated with IL-1 β . Total cellular RNA (25 μ g) was hybridized successively to 32 P-labelled iNOS and β -actin cDNA probes as described in the Materials and methods section. For quantification, autoradiograms of the filters were scanned densitometrically. To correct for differences in loading, the signal density of each RNA sample hybridized to the iNOS probe was divided by that hybridized to the β -actin probe. The corrected density is expressed as the percentage of RNA found in IL-1 β -stimulated cells in the absence of phorbol ester. Lane 1, 0%; lane 2, 100%; lane 3, 10%; lane 4, 11.7%; lane 5, 16.2%; lane 6, 181%.

inositide turnover in response to angiotensin II (Huwiler et al., 1991a; Ochsner et al., 1993), whereas PKC- ϵ is a candidate for regulation of phospholipases A $_2$ and D in mesangial cells (Huwiler et al., 1991a; Pfeilschifter and Huwiler, 1993). Using this type of down-regulation regimen to differentially deplete PKC isoenzymes from mesangial cells we wanted to address the question as to which of the four isotypes present in the cells mediates the inhibitory effect on cytokine-induced iNOS expression. A pretreatment of mesangial cells with PMA for up to 8 h, that causes depletion of α - and δ -isoenzymes of PKC in mesangial cells (Huwiler et al., 1991a, 1992), was not sufficient to eliminate the negative regulation of the subsequent IL-1 β -stimulated nitrite production (Figure 2). It was necessary to preincubate the cells for 24 h with PMA or PDBu, a regimen that also causes depletion of PKC- ϵ (Huwiler et al., 1991a), to prevent fully inhibition of IL-1 β -induced nitrite production (Figure 2). Actually, down-regulation of PKC- ϵ resulted in a potentiation of nitrite synthesis after exposure to IL-1 β , suggesting that PKC- ϵ exerts a tonic negative regulation on cytokine induction of iNOS. In line with this view, the selective PKC inhibitor, calphostin C (Kobayashi et al., 1989), potentiated IL-1 β -evoked nitrite generation (Table 4). In order to evaluate whether this inhibition of iNOS activity is due to regulation of iNOS gene expression, we performed Northern-blot analysis. The data in Figure 3 show that iNOS mRNA was present in IL-1 β -stimulated cells as a single band of approx. 4.5 kb when using a cDNA probe for the inducible

mouse macrophage NOS. In unstimulated cells there was no detectable iNOS mRNA. Preincubation of cells with PDBu for 2 h (Figure 3, lane 3), 4 h (lane 4) or 8 h (lane 5) completely abolished IL-1 β induction of iNOS mRNA. In contrast, preincubation of mesangial cells with PDBu for 24 h (lane 6), a regimen that causes down-regulation of PKC- ϵ , resulted in a superinduction of iNOS mRNA (Figure 3). Comparable data were obtained for mesangial cells treated with PMA (Figure 4).

DISCUSSION

We report here for the first time that phorbol ester treatment of mesangial cells inhibits IL-1 β induction of iNOS mRNA and subsequent nitrite production, thus suggesting that PKC may negatively regulate iNOS expression. Our results confirm previous observations in macrophages and hepatocytes showing that PKC activation antagonizes lipopolysaccharide-induced nitrite production (Hortelano et al., 1992, 1993). However, there is one inherent problem with experiments using long-term exposure of cells to phorbol esters in that such a treatment can activate PKC, but may also result in an opposite effect, i.e. the down-regulation of PKC (Stabel and Parker, 1991; Nishizuka, 1992; Hug and Sarre, 1993). Indeed, data in the literature comprise quite contradictory interpretations of data on a modulatory role of PKC in cytokine- or lipopolysaccharide induction of iNOS (Hortelano et al., 1992, 1993; Severn et al., 1992). The situation is even more complicated considering the fact that PKC exists as a family of at least 10 molecular species, all having closely related structures but differing in tissue and cellular distribution and in their sensitivity to phorbol ester-induced down-regulation (Stabel and Parker, 1991; Nishizuka, 1992; Hug and Sarre, 1993). By Western-blot analysis we have reported previously that mesangial cells express four PKC isoenzymes, PKC- α , - δ , - ϵ and ζ . No PKC- β_1 , - β_2 , - γ and - η isoforms were detected. These isoenzymes are differentially regulated by biologically active phorbol esters and display distinctly different depletion kinetics (Huwiler et al., 1991a, b, 1992, 1993). By comparing the depletion kinetics of the various PKC isoforms present in mesangial cells with the time-course of removal of specific cell responses, individual PKC isoenzymes can be appointed to specific cellular functions. Using this approach we have provided evidence that PKC α is a negative feedback regulator of hormone-triggered phosphoinositide turnover in mesangial cells (Huwiler et al., 1991a, b; Ochsner et al., 1993). Moreover, we were able to show that PKC- ϵ is responsible for the stimulation of phospholipase A $_2$ and phospholipase D observed after exposure of mesangial cells to angiotensin II or extracellular nucleotides (Pfeilschifter and Huwiler, 1993; Pfeilschifter and Merriweather, 1993). In our present study using this experimental approach, we provide now strong evidence that PKC- ϵ counteracts the IL-1 β -stimulated iNOS expression in mesangial cells. The results in Figures 2–4 show that an 8 h treatment with phorbol ester was not sufficient to reverse inhibition of IL-1 β -induced iNOS expression and nitrite synthesis, thus clearly ruling out a contribution of PKC- α and - δ isoenzymes to this cell response. In order to fully restore the responses to IL-1 β stimulation and to get even a potentiation of iNOS induction, a 24 h preincubation with PMA or PDBu was required, suggesting that PKC- ϵ triggers a signalling pathway that is able to antagonize IL-1 β action in mesangial cells. To the best of our knowledge this is the first report tracing the action of phorbol esters on iNOS expression back to the level of the individual PKC isoenzyme involved. At the present state we cannot exclude that PKC- ζ also contributes to the inhibition of iNOS induction. However, this possibility seems rather unlikely as PKC- ζ is not able to bind

phorbol esters such as PMA or PDBu and, therefore, does not respond to phorbol ester treatment (Huwiler et al., 1992; Ways et al., 1992; Liyanage et al., 1992). Bredt et al. (1992) reported that phosphorylation of constitutive brain NOS by PKC in intact cells leads to a dramatic inhibition of NOS catalytic activity. In contrast with this post-translational modification of the constitutive NOS activity by PKC, iNOS activity is regulated at a pretranslational level by PKC. This is clearly shown by the data in Table 3 because short-term activation of PKC, after iNOS has been induced by IL-1 β treatment, does not significantly affect iNOS activity. In contrast, the results in Figures 3 and 4 show that PKC activation before IL-1 β stimulation potentially decreased iNOS mRNA levels and subsequent nitrite synthesis (Figure 2).

The results presented in this paper do not address the molecular mechanism by which phorbol esters alter iNOS mRNA steady-state levels. Changes in the transcription rate of the iNOS gene or in the stability of the iNOS mRNA may contribute to the observed changes in iNOS mRNA. Preliminary data from our laboratory show that phorbol esters do not decrease the half-life of iNOS mRNA in mesangial cells stimulated with IL-1 β (H. Mühl and J. Pfeilschifter, unpublished work), suggesting that the decreased expression of iNOS observed after phorbol ester treatment is not attributed to decreased mRNA stability. Therefore, inhibition of the transcription rate of the iNOS genes seems to be the most likely mechanism involved. Possible targets for phorbol ester-stimulated PKC that eventually could result in inhibition of iNOS gene expression are (i) the IL-1 receptor; (ii) the signalling cascade triggering the activation of nuclear factor κ B (NF κ B) or other transcription factors involved in iNOS gene expression; (iii) induction of an inhibitory factor that suppresses iNOS gene transcription. There is indeed evidence in several cell systems that PKC down-regulates the receptors for IL-1 as well as for tumour necrosis factor α (Horuk and Gross, 1990; Hohmann et al., 1992) and thus negatively affects NF κ B activation. However, PKC has also been reported to increase IL-1 receptor mRNA in lung fibroblasts (Aksamit et al., 1993) and thus modulation of IL-1 receptors by PKC is cell-type specific and may depend on the set of PKC isoenzymes present in a certain cell line. The transcription factor NF κ B has been reported to mediate cytokine induction of iNOS in rat alveolar macrophages (Sherman et al., 1993) and vascular smooth muscle cells (Mülsch et al., 1993). Furthermore, Xie et al. (1993) reported on the presence of a potential NF κ B-binding site in the 5'-untranslated region of the iNOS gene. NF κ B is located in the cytoplasmic fraction of cells and is complexed with an inhibitor, I κ B (Grimm and Baeuerle, 1993). The mechanisms leading to NF κ B activation have not been fully defined, but phosphorylation of I κ B has been identified as one step in the activation process. It has been suggested that PKC may be responsible for the phosphorylation of I κ B and subsequent liberation of active NF κ B (Grimm and Baeuerle, 1993). Thus PKC may have a dual effect on cytokine-induced NF κ B activation, it may directly liberate NF κ B from its inhibitor, I κ B, and it may counteract the effects of IL-1 and TNF α by down-modulating the cytokine receptors at the cell surface. This scenario could explain why Hortelano et al. (1992, 1993) reported an induction of iNOS in hepatocytes and macrophages by PMA when given alone and simultaneously observed an inhibition of lipopolysaccharide-induced iNOS activity by PMA. In contrast, in mesangial cells there is no effect of phorbol esters on iNOS expression when given alone, but a strong inhibition of IL-1 β induction of iNOS by PMA and PDBu. Obviously it depends on the cell type which of the suggested actions of PKC dominates and possibly these two functions may be mediated by different PKC isoforms. Work is going on in our laboratory to evaluate this hypothesis.

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