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Induction by staurosporine of nitric oxide synthase expression in vascular smooth muscle cells: role of NF-κB, CREB and C/EBPβ

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1 The effect of different protein kinase inhibitors on the expression of the inducible isoform of nitric oxide (NO) synthase (iNOS) was investigated in cultured vascular smooth muscle cells (VSMC) isolated from the rat aorta.

2 The non-selective protein kinase C (PKC) inhibitor, staurosporine, but not the more selective PKC inhibitors, calphostin C and Ro 31-8220, or the tyrosine kinase inhibitors, genistein and erbstatin analogue (erbstatin A), elicited a distinct (up to six fold) up-regulation of iNOS gene expression in these cells, as demonstrated by a parallel increase in iNOS mRNA and protein abundance as well as an accumulation of nitrite (NO_2^-) in the conditioned medium. Actinomycin D and cycloheximide inhibited the effect of staurosporine, suggesting an involvement of both DNA transcription and *de novo* protein synthesis.

3 Staurosporine also synergistically potentiated the stimulating effect of interleukin-1β (IL-1β), but not that of the adenylyl cyclase activator, forskolin, on NO_2^- production and iNOS protein abundance. Staurosporine, on the other hand, had no effect on the IL-1β-mediated increase in iNOS mRNA abundance. The effect of staurosporine on both basal and IL-1β-stimulated NO_2^- production was concentration-dependent with an apparent maximum at 3 nM. Among the other protein kinase inhibitors tested, only calphostin C also enhanced the stimulant effect of IL-1β approximately two fold, while genistein, erbstatin A and Ro 31-8220 inhibited rather than potentiated it.

4 Staurosporine did not influence basal activity of the transcription factors CREB and nuclear factor κB (NF-κB), but increased that of C/EBP. Moreover, staurosporine significantly augmented the activation of C/EBP by IL-1β and forskolin.

5 These findings suggest that in cultured VSMC a staurosporine-sensitive protein kinase exists, which is unlikely to be related to PKC, that prevents iNOS gene expression presumably by suppressing basal C/EBP activity. They also indicate that NF-κB and a member of the C/EBP family of transcription factors, presumably C/EBPβ, act synergistically under basal conditions and possibly also following exposure to IL-1β in the up-regulation of iNOS gene expression in these cells. Targeting of the activation of C/EBPβ may thus represent an interesting approach to interfere selectively with the cytokine-induced over-production of NO in acute and chronic inflammatory conditions.

Keywords: Staurosporine; protein kinase C; tyrosine kinase(s); inducible NO synthase; gene expression; interleukin-1β; cyclic AMP; NF-κB; C/EBP; CREB; vascular smooth muscle cells

Introduction

Nitric oxide (NO) is a highly effective autacoid with a multitude of biological effects ranging from vasodilatation to cytotoxicity. Expression of the inducible NO synthase (iNOS) in vascular smooth muscle cells (VSMC), endothelial cells and monocyte/macrophages following exposure to cytokines, microbes or microbial products is frequently associated with a generalized or localized inflammatory response resulting from infection or tissue injury. Cells expressing this enzyme release high amounts of NO for several days with potentially harmful consequences, e.g. in the pathogenesis of arthritis, colitis or septic shock.

Since iNOS activity does not seem to be controlled once the enzyme is expressed, regulation of NO release from iNOS-expressing cells must occur at the level of DNA transcription and/or protein stability (Morris & Billiar, 1994). The promoter region of the iNOS gene contains several binding sites for nuclear factor κB (NF-κB) and AP-1 (Jun/Fos) as well as for various members of the C/EBP, ATF/CREB and STAT family of transcription factors (Lowenstein *et al*., 1993; Xie *et al*., 1993; Chartrain *et al*., 1994).

Thus far, only activation of NF-κB has been shown to mediate the enhanced expression of the iNOS gene in macrophages exposed to bacterial lipopolysaccharides (LPS) (Xie *et al*., 1994) as well as in interleukin-1β (IL-1β)-stimulated VSMC (Schini-Kerth *et al*., 1995; Hecker *et al*., 1996b) and mesangial cells (Eberhardt *et al*., 1994). Moreover, the synergistic effect of interferon-γ (IFNγ) on iNOS expression in LPS-stimulated macrophages is mediated by the activation of interferon regulatory factor-1 (Kamijo *et al*., 1994; Martin *et al*., 1994; Hecker *et al*., 1996a).

Nuclear cytokine signalling proceeds via a cascade of phosphorylation (and dephosphorylation) reactions involving various serine/threonine and tyrosine kinases, some of which have not yet been identified. Ultimately, one or several latent transcription factors become activated either by direct phosphorylation (e.g., C/EBPβ, Jun, STAT-1) or that of associated regulatory proteins (e.g., NF-κB, CREB, STAT-2). It does not come as a surprise, therefore, that various protein kinase inhibitors are capable of attenuating the cytokine-induced expression of the iNOS gene. However, their inhibitory action varies markedly between different types of cells. In VSMC, for example, tyrosine kinase inhibition has consistently been shown to inhibit the IL-1β-mediated increase in NO production (Marczin *et al*., 1993; Moritoki *et al*., 1995). However, it remains to be clarified whether tyrosine kinase inhibitors affect iNOS gene expression upstream or downstream from activation of the transcription factors (Kwon *et al*., 1995; Tetsuka *et al*., 1996). Activation of protein kinase C (PKC), on the other hand, has been shown to be either of no

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consequence (Kanno *et al*., 1993), to up-regulate (Scott-Burden *et al*., 1994) or to down-regulate (Geng *et al*., 1994; Nakayama *et al*., 1994) the cytokine-induced increase in iNOS gene expression in VSMC.

Our laboratory has a long-standing interest in the regulation of iNOS gene expression in VSMC, and we have recently demonstrated that activation of NF-κB is essential for the up-regulation of iNOS gene expression both in native and cultured aortic SMC of the rat exposed to IL-1β (Schini-Kerth *et al*., 1995; 1996; Hecker *et al*., 1996b). In the course of these studies we noticed that the non-selective PKC inhibitor, staurosporine, paradoxically potentiated rather than inhibited both the basal and IL-1β-induced NO_2^- formation in these cells. Here we have attempted to characterize the effect of staurosporine on iNOS gene expression in VSMC in more detail and to elucidate the nuclear signalling mechanism involved therein.

Methods

Cell culture

VSMC were isolated from the thoracic aorta of male Wistar-Kyoto rats (300–350 g body weight) by elastase/collagenase digestion and characterized by positive immunostaining with monoclonal antibodies raised against smooth muscle α-actin (Gordon *et al*., 1986). They were serially cultured in Waymouth medium (PAN Systems, Aidenbach, Germany) containing non-essential amino acids (Biochrom, Berlin, Germany), 100 units ml^{-1} of both penicillin and streptomycin, and 7.5% (v/v) foetal calf serum (PAN Systems), and passaged by using trypsin-EDTA (0.05/0.02%, w/v). Experiments were performed with cells from passages 8–16 seeded into 24-well multiwell plates (4×10^5 cells/well) or 60 mm i.d. Petri dishes (5×10^6 cells/dish).

Experimental protocol

Confluent VSMC were incubated in 0.3 ml (multiwell plates) or 3 ml (Petri dishes) of serum-free Waymouth medium containing 0.1% (w/v) BSA in the absence (control cells) or presence of 60 units ml^{-1} IL-1β or 10 μM forskolin. When incubations were performed in the presence of the protein kinase inhibitors, these were added together with IL-1β or forskolin at time zero. After 20 h in the incubator, the cells were harvested and the concentration of NO_2^- in the conditioned medium (200 μl plus 80 μl Griess reagent) was determined photometrically at 570 nm as described by Saville (1958). In some experiments, incubations were terminated after 30 min (NF-κB) or 2 h (C/EBPβ and CREB) to prepare nuclear extracts for electrophoretic mobility shift analyses (Schreiber *et al*., 1989). Cell viability was assessed microscopically and by trypan blue exclusion.

Western blot analysis

Immunoblot analysis of iNOS protein was performed essentially as previously described (Hecker *et al*., 1996a). Protein extracts (10,000 *g* supernatant) were separated by electrophoresis (10–50 μg protein per lane) on 8% polyacrylamide gels in the presence of sodium dodecylsulphate and then transferred onto nitrocellulose membranes. The immobilized iNOS protein was visualized by subsequent incubation with a polyclonal anti-iNOS antibody (kindly provided by Dr M. Marletta, University of Michingan, Ann Arbor, MI, U.S.A.) and a secondary polyclonal peroxidase-conjugated anti-rabbit antibody (Amersham), followed by staining with the enhanced chemiluminescence (ECL) technique developed by Amersham. Densitometry (Pharmacia densitometer equipped with a Kappa CCD video camera and the ImageMaster software) was employed to quantify the intensity of the iNOS-specific bands.

RT-PCR analysis

Total RNA was isolated as described by Chomczynski and Sacchi (1987). Reverse transcription (RT) with 2 μg of total RNA per sample was performed in a total volume of 20 μl as previously described (Hecker *et al*., 1996a). By use of the polymerase chain reaction (PCR), the sequence of the two iNOS-specific primers (Biometra, Göttingen, Germany) was 5′-CCC GGC AGA CTG GAT TTG-3′ (sense) and 5′-GAG GGT ACA TGC TGG AGC C-3′ (antisense), and the predominant cDNA amplification product was predicted to be 527 bp in length. The sequence of the elongation factor 2 (EF-2)-specific primers (Biometra) was 5′-GAC ATC ACC AAG GGT GTG CAG-3′ (sense) and 5′-GCG GTC AGC ACA CTG GCA TA-3′ (antisense) and the predominant cDNA amplification product was predicted to be 209 bp in length. RT and PCR of EF-2 served as a positive control. In a separate series of experiments, the optimum number of cycles for amplification of the iNOS- and EF-2-specific cDNA transcripts was determined to be 25 and 19 cycles, respectively (not shown). The PCR reaction was started with 6 μl of the RT reaction mixture, 5 μl 10 $\times$ reaction buffer (Pharmacia, Freiburg, Germany), 1 μl deoxynucleotide mixture (dATP, dCTP, dGTP, dTTP; 2.5 mM), 20 pmol each of the two iNOS-specific primers and 0.8 units Taq DNA polymerase (Pharmacia) in a total volume of 50 μl. The samples were placed in an Omn-E thermal cycler equipped with a heated (100°C) lid (Hybaid, Teddington, U.K.) which was programmed as follows: 5 min at 95°C (initial melt) followed by 6 cycles of 30 s at 90°C (denaturation), 20 s at 52°C (annealing) and 60 s at 72°C (extension). Thereafter, the two EF-2-specific primers (20 pmol) were added to the samples and the PCR reaction continued for an additional 19 cycles as before, followed by 5 min at 72°C (final extension). The PCR products (20 μl) were size-fractionated by agarose (1.5%) gel electrophoresis, stained with ethidium bromide and visualized by using an ultraviolet transilluminator (Bachofer, Reutlingen, Germany). The intensity of the iNOS- and EF-2-specific bands was quantified by densitometry.

Electrophoretic mobility shift analysis (EMSA)

Aliquots of nuclear protein (3 μg) were incubated with the C/EBP- (5′-TGC AGA TTG CGC AAT CTG CA-3′; Santa Cruz Biotechnology, Heidelberg, Germany), CREB- (5′-AGA GAT TGC CTG ACG TCA GAG AGC TAG-3′; Santa Cruz Biotechnology) or NF-κB-specific (5′-AGT TGA GGG GAC TTT CCC AGG C-3′; Promega from Serva, Heidelberg, Germany) double-stranded oligonucleotides which had been end-labelled with ^{32}P. Binding experiments were performed with 1–4 μl nuclear extract, 3 μl 5 $\times$ binding buffer (50 mM HEPES, pH 7.5, 500 mM NaCl, 25% glycerol (w/v), 5 mM EDTA), 1 μg poly dI-dC (Pharmacia) as non-specific competitor DNA, and the oligonucleotide (5000 c.p.m.) in a total volume of 15 μl for 30 min at ambient temperature. Non-denaturing polyacrylamide gel (4%) electrophoresis was performed with 1 $\times$ TBE buffer, pH 8.0 followed by autoradiography. To monitor the specificity of the binding reaciton, the assay was performed in parallel with the same samples in the presence of a 100 to 1,000 fold excess of the non-labelled oligonucleotide. Densitometry was used to quantify the intensity of the labelled DNA-protein complexes.

Materials

Calphostin C, erbstatin analogue (erbstatin A), genistein and (3-[1-[3-(amidinothio)propyl-1H-indolyl-3-yl]-3-(1-methyl-1H-indolyl-3-yl) maleimide methane sulfonate (Ro 31-8220) were obtained form Calbiochem-Novabiochem (Bad Soden, Germany), forskolin from Sigma (Deisenhofen, Germany), IL-1β from Collaborative Research (Bedford, MA, U.S.A.), and the isotopes from Amersham (Braunschweig, Germany). Staurosporine was obtained from both Calbiochem-Novabiochem and Sigma to account for contaminants in the bath potentially

being responsible for the observed biological effect. However, no differences between the two batches of staurosporine were observed throughout the study (not shown).

Statistical analysis

Unless indicated otherwise, all data in the figures and text are expressed as means ± s.e.mean of n observations. Statistical evaluation was performed by one-way analysis of variance followed by a Bonferroni t test for multiple comparisons with a P value <0.05 considered statistically significant.

Results

Effects of staurosporine on iNOS expression

NO_2^- production Incubation of the cultured VSMC with IL-1β or forskolin produced a marked and comparable (7.2 and 7.3 fold, respectively) increase in the concentration of NO_2^- in the conditioned medium, indicative of an up-regulation of iNOS gene expression in these cells (Figure 1a and b). Exposure of the cells to staurosporine (10 nM) for the same period of time also caused a significant, approximately three fold increase in NO_2^- production. This effect of staurosporine was significantly and to a similar extent (72% and 65% inhibition) attenuated by actinomycin D (20 nM) and cycloheximide (10 μM) (Figure 1c). In comparison, actinomycin D and cycloheximide inhibited the IL-1β-induced increase in NO_2^- formation by 97% and 100%, respectively.

Combined treatment of the cells with staurosporine and IL-1β resulted in a prominent supra-additive increase in NO_2^- formation (Figure 1a) which in some experiments corresponded to a more than six fold potentiation of the IL-1β effect (cf. Figure 2). In contrast, the combination of staurosporine with forskolin led to a less than additive effect on NO_2^- production which was not significantly different from the effect of forskolin alone (Figure 1b).

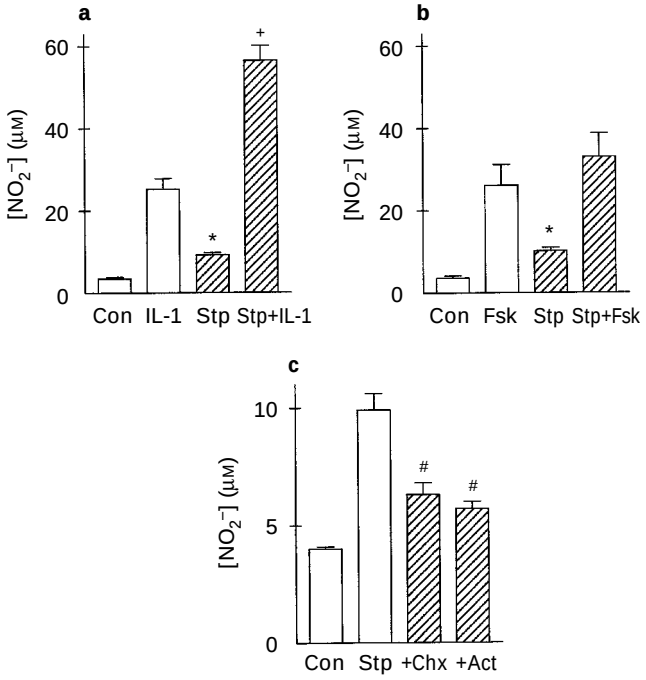

Figure 1 Effects of staurosporine (10 nM), in the absence and presence of (a) IL-1β (IL-1; 60 units ml^{-1}; $n=12$) or (b) forskolin (Fsk; 10 μM; $n=7$), on the accumulation of nitrite (NO_2^-) over 20 h in the conditioned medium of rat aortic VSMC cultured in 24-well multiwell plates. (c) Inhibition by actinomycin D (Act; 20 nM) and cycloheximide (Chx; 10 μM) of the stimulating effect of staurosporine on NO_2^-formation ($n=3$; $^*P<0.05$ vs. con, $^+P<0.05$ vs. IL-1, $^\#P<0.05$ vs Stp).

iNOS protein abundance Similar findings were obtained when iNOS protein expression was examined (Figure 2). Both IL-1β and forskolin caused a marked increase in iNOS protein abundance, whereas in control cells no iNOS-specific immunoreactivity was detectable. Staurosporine alone also caused a distinct increase in iNOS protein expression which was strongly inhibited in the presence of cycloheximide and, albeit less effectively, also by actinomycin D (Figure 2). Moreover, staurosporine markedly enhanced the IL-1β-mediated increase in iNOS protein abundance (414 ± 59% of IL-1β, $n=3$, $P<0.05$) in a supra-additive manner, but did not significantly affect iNOS protein expression induced by forskolin (128 ± 28% of forskolin, $n=3$). Although the effect of forskolin on iNOS protein expression appeared to be more pronounced than that of IL-1β in the experiment shown in Figure 2, this was not the case in the other experiments where its effect was comparable to that of IL-1β (not shown).

iNOS mRNA level According to RT-PCR analysis, both IL-1β and forskolin caused a marked increase in iNOS mRNA abundance (Figure 3), although the effect of IL-1β was consistently more pronounced (approximately 40% greater). In contrast to the iNOS protein level, there was a weak but distinct iNOS mRNA signal detectable in unstimulated control cells (Figure 3a). Staurosporine alone elicited a marked increase in iNOS mRNA abundance, the magnitude of which was similar to the effect caused by IL-1β. However, the protein kinase inhibitor failed to augment the increase in iNOS gene expression induced by IL-1β or forskolin (Figure 3b).

Effects of other protein kinase inhibitors

The induction of iNOS gene expression by staurosporine and its potentiating effect on the IL-1β-mediated increase in NO_2^- production and iNOS protein abundance was concentration-dependent with an apparent maximum at 3 nM (Figure 4a). The reason for the bell-shaped curve is that in most experiments, cell viability was already decreased by 10–20% in the presence of 10 nM staurosporine, most likely due to an induction of apoptosis by the protein kinase inhibitor as indicated both by DNA laddering and expression of p53 (not shown). Concentrations of staurosporine exceeding 30 nM were clearly cytotoxic for the SMC and therefore could not be tested.

The more selective PKC inhibitors, Ro 31-8220 and calphostin C, differed in their effects. Ro 31-8220 did not affect basal or IL-1β-stimulated NO_2^- production at 0.01–0.1 μM (Figure 4b), but inhibited the IL-1β-dependent accumulation of NO_2^- in the conditioned medium at concentrations greater

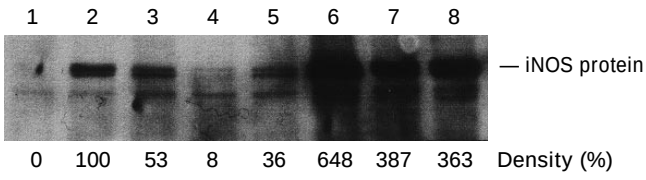

Figure 2 Western blot analysis of the effects of IL-1β (60 units ml^{-1}; 2), staurosporine (10 nM; 3), staurosporine plus cycloheximide (10 μM; 4), staurosporine plus actinomycin D (20 nM; 5), staurosporine plus IL-1β (6), forskolin (10 μM; 7), and staurosporine plus forskolin (8) on iNOS protein abundance (1; non-stimulated control cells). Typical Western blot, qualitatively similar results were obtained in two further experiments with different batches of VSMC. Changes in iNOS protein abundance according to densitometry are expressed as percentage of the level of the IL-1β-stimulated cells.

than 0.3 μM (e.g., 89% inhibition at 1 μM). Calphostin C (10 nM), on the other hand, had no effect on basal NO_2^- production, but enhanced the stimulant effect of IL-1β two fold (Figure 4c).

The PKC activator, phorbol 12-myristate 13-acetate (PMA, 0.1 μM), had no effect on basal NO_2^- formation, but weakly attenuated the stimulant effect of IL-1β (Figure 4d). The tyrosine kinase inhibitors, genistein and erbstatin A, showed only a moderate inhibitory effect on the IL-1β-mediated increase in NO_2^- formation at concentrations of 100 μM and 30 μM, respectively (Figure 4e).

Effects of staurosporine on transcription factor activation

In the cultured VSMC, NF-κB activation by IL-1β was near-maximal after 15–30 min (cf. Schini-Kerth *et al*., 1996). Two NF-κB-specific DNA-protein complexes were detected in nuclear extracts of these cells which, as previously shown (Hecker *et al*., 1996b), represent the p50/p50 homodimer and p65/p50 heterodimer, respectively. Staurosporine had no effect on basal NF-κB activity (122$\pm$23% of the intensity of the NF-κB-specific complexes in nuclear extracts from control cells, $n=5$) and did not affect the IL-1β-stimulated increase in NF-κB activity (279$\pm$89% of control, $n=5$ in the presence of IL-1β and 269$\pm$107% of control, $n=5$ in the presence of both IL-1β and staurosporine). Forskolin had no appreciable effect on NF-κB activity (Figure 5).

A study of the time course of both C/EBP and CREB activation by forskolin in the cultured VSMC revealed a near-maximum effect after 2 h (not shown). Only one C/EBP-specific DNA-protein complex was detected in non-stimulated cells, the intensity of which varied depending on the batch of VSMC used (Figure 6). Staurosporine, IL-1β and forskolin all significantly increased C/EBP activity with forskolin being approximately twice as effective as staurosporine and four times as effective as IL-1β. In four of five experiments, the protein kinase inhibitor clearly augmented the stimulant effect of IL-1β and forskolin on C/EBP activity in a supra-additive manner, albeit to a varying degree (Figure 6).

Three CREB-specific DNA-protein complexes were detected in nuclear extracts of forskolin-stimulated VSMC, one of which was induced in the presence of the adenylyl cyclase

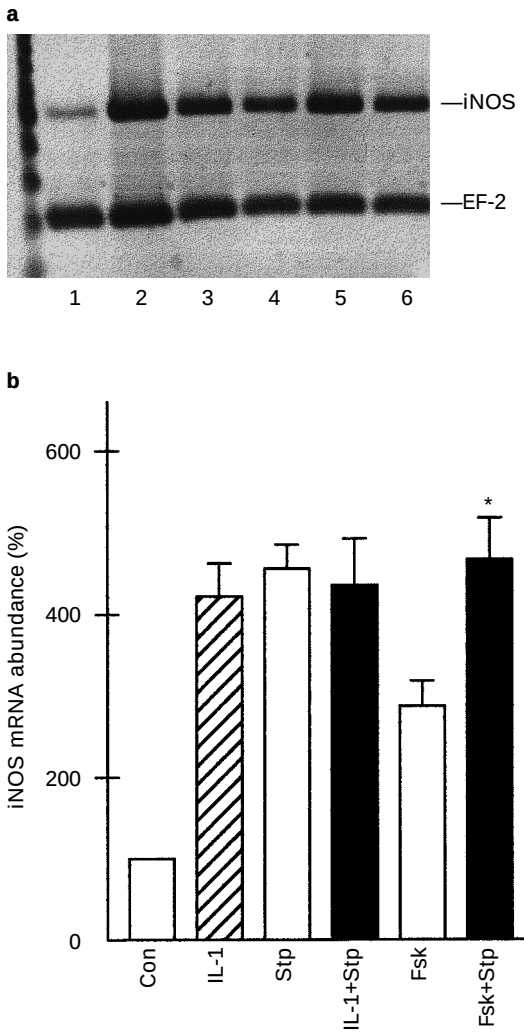

Figure 3 RT-PCR analysis of iNOS mRNA abundance in VSMC treated with IL-1β (IL-1; 60 units ml^{-1}; 2), staurosporine (Stp; 10 nM; 3), forskolin (Fsk; 10 μM; 4), staurosporine plus IL-1β (5), and staurosporine plus forskolin (6). (a) Typical RT-PCR analysis. (b) Statistical summary of three individual experiments with different batches of VSMC (*$P<0.05$ vs forskolin).

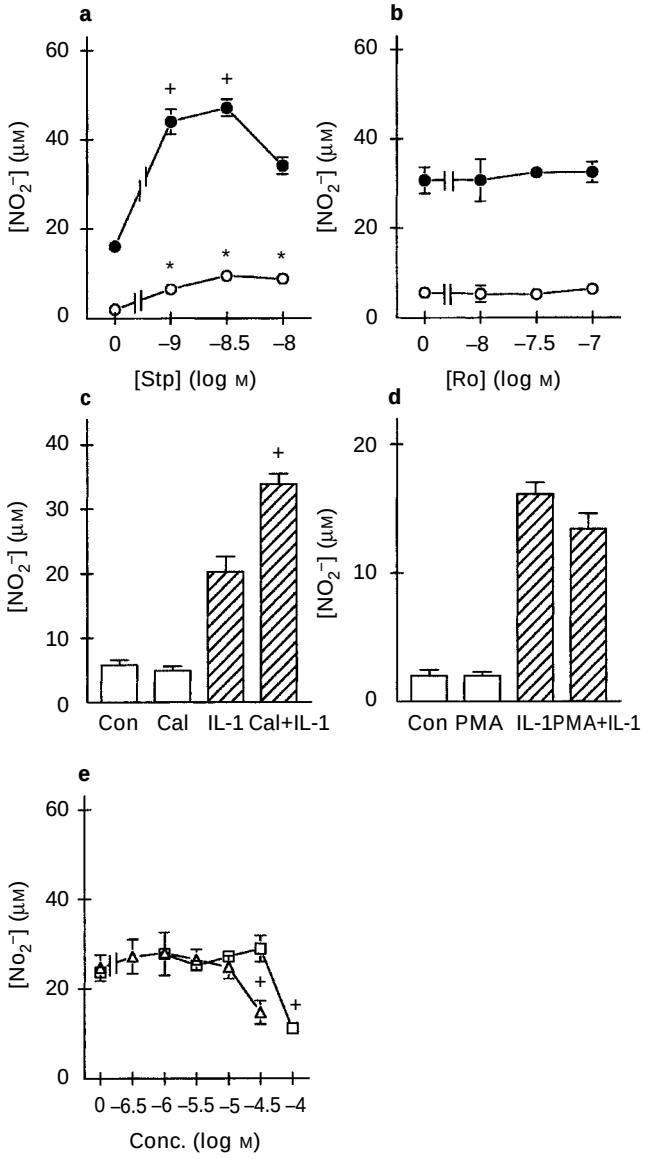

Figure 4 (a,b) Concentration-dependent effects of (a) staurosporine and (b) Ro 31-8220 on the accumulation of NO_2^- in the conditioned medium of rat aortic VSMC incubated for 20 h in the absence (○) or presence (●) of IL-1β (60 units ml^{-1}). (c–e) Effects of (c) calphostin C (Cal; 10 nM), (d) phorbol 12-myristate 13-acetate (PMA; 0.1 μM), and (e) genistein (□) or erbstatin A (△) on basal and/or IL-1β-stimulated NO_2^- formation. For reasons of clarity, the lack of effect of the two tyrosine kinase inhibitors on basal NO_2^- production is not shown (means$\pm$s.d. from experiments with a single batch of VSMC performed in triplicate; *$P<0.05$ vs control, $^+P<0.05$ vs IL-1).

activator (Figure 7). Staurosporine treatment did not affect the intensity of the two constitutive complexes, caused no induction of the forskolin-specific complex, and failed to enhance the intensity of this DNA-protein complex in the presence of forskolin (Figure 7). IL-1β also did not induce the formation of this DNA-protein complex (not shown).

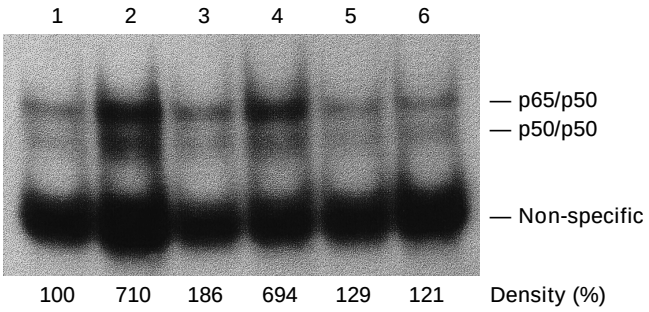

Figure 5 Effects of IL-1β (60 units ml^{-1}; 2), staurosporine (10 nM; 3), staurosporine plus IL-1β (4), forskolin (10 μM; 5), and staurosporine plus forskolin (6) on the NF-κB-specific DNA-protein complex formation in nuclear extracts of VSMC cultured in 60 mm Petri dishes and incubated for 30 min. Typical EMSA, qualitatively similar results were obtained in four additional experiments with different batches of VSMC. Changes in the intensity of the two NF-κB-specific complexes (p65/p50 heterodimer, p50/p50 homodimer) according to densitometry were normalized on the basis of the intensity of the non-specific complex and expressed as percentage of the level of non-stimulated control cells (1).

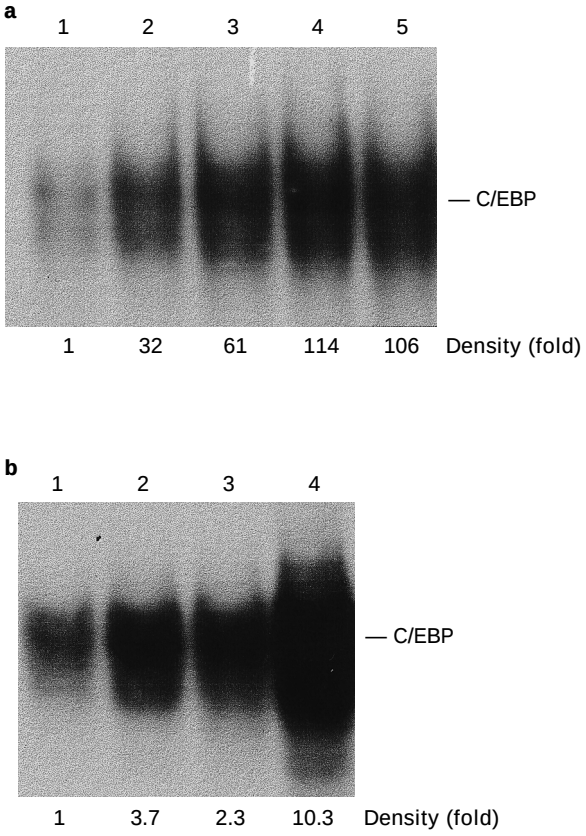

Figure 6 (a) Effects of IL-1β (60 units ml^{-1}; 2), staurosporine (10 nM; 3), staurosporine plus IL-1β (4), and forskolin (10 μM; 5) on the C/EBP-specific DNA-protein complex formation in nuclear extracts of VSMC cultured in 60 mm Petri dishes and incubated for 2 h. (b) Effects of forskolin (2), staurosporine (3), and forskolin plus staurosporine (4) on C/EBP activity. Typical EMSA, qualitatively similar results were obtained in four additional experiments with different batches of VSMC. Changes in the intensity of the C/EBP-specific complex according to densitometry were expressed as percentage of the level of non-stimulated control cells (1).

Discussion

The present findings demonstrate that in rat cultured aortic VSMC the non-selective PKC inhibitor staurosporine is capable of inducing iNOS gene expression in the absence of any other stimulus. When combined with IL-1β, staurosporine caused a supra-additive increase in NO_2^- production and iNOS protein abundance, indicative of a synergistic mechanism of action. While the latter effect was mimicked to some extent by the more selective PKC inhibitor calphostin C, Ro 31-8220, another selective PKC inhibitor, had no effect on basal NO_2^- production and inhibited rather than augmented the stimulating effect of IL-1β at higher concentrations. Long-term exposure (20 h) of the SMC to the PKC activator, PMA, did not affect basal NO_2^- formation and only marginally attenuated the stimulant effect of IL-1β.

PKC activation by PMA or a membrane-permeable diacylglycerol analogue has previously been shown to attenuate the increase in iNOS gene expression in VSMC in response to IL-1β (Nakayama *et al*., 1994) or interferon γ (IFNγ) plus tumour necrosis factor α (TNFα; Geng *et al*., 1994), while basal iNOS gene expression remained unaffected. Together with the amplification by calphostin C and staurosporine of the IL-1β-mediated increase in NO_2^- formation and iNOS protein abundance, it would be reasonable to conclude, as previously suggested for iNOS gene expression in rat renal mesangial cells (Mühl & Pfeilschifter, 1994), that the cytokine-induced expression of the enzyme in VSMC is tonically suppressed by a constitutively active isoform of PKC, e.g. PKCε, which is resistant to depletion by phorbol esters.

However, the findings by Scott-Burden *et al*. (1994) of an inhibition of the IL-1β-mediated increase in iNOS gene ex-

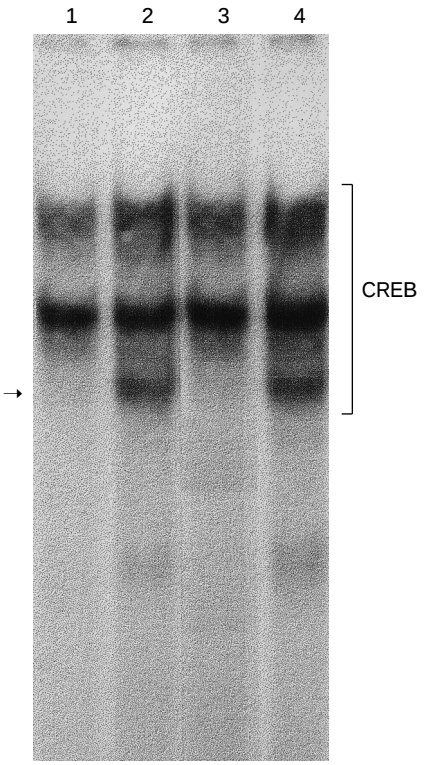

Figure 7 Effects of forskolin (10 μM; 2), staurosporine (10 nM; 3), and forskolin plus staurosporine (4) on CREB-specific DNA-protein complex formation in nuclear extracts of VSMC cultured in 60 mm Petri dishes and incubated for 2 h (1; non-stimulated control cells). Typical EMSA, qualitatively similar results were obtained in two additional experiments with different batches of VSMC. The arrow indicates the position of the forskolin-induced DNA-protein complex. Due to the clear lack of effect of staurosporine, densitometry was not performed.

pression in VSMC by the specific PKC inhibitor, bisindolylmaleimide, and our own findings that effective PKC blockade by $\geqslant 0.3$ μM Ro 31-8220 caused a down-regulation of the IL-1β-stimulated NO_2^- production, while a presumably ineffective PKC blockade by 10 nM calphostin C caused the opposite effect, question this hypothesis. Moreover, results obtained with PKC activators or inhibitors in one cell type cannot necessarily be compared with observations made in a different type of cell. Thus, in the murine macrophage cell line RAW 264.7, the same batch of staurosporine which was employed in the present study attenuated the LPS/IFNγ-induced increase in iNOS gene expression by 53% at a concentration of 10 nM (Hecker *et al*., unpublished observation). Taken together, the available data therefore suggest that PKC inhibition *per se* does not play a role in the staurosporine-induced increase in iNOS gene expression in VSMC or in the potentiation by staurosporine of the IL-1β-mediated increase in NO_2^- production and iNOS protein abundance. In fact, it seems more likely that staurosporine targets another, as yet unidentified protein kinase which is involved in the negative control of iNOS gene expression in these cells.

This notion is also supported by the finding that two different tyrosine kinase inhibitors, genistein and erbstatin A, failed to mimic the effects of staurosporine on iNOS gene expression. Their inhibitory profile rather resembled that of Ro 31-8220. Moreover, recent work from this laboratory shows that the selective protein kinase A (PKA) inhibitor, Rp-8-CPT-cyclic AMPS, does not induce an expression of the enzyme in VSMC itself, but strongly attenuates the increase in iNOS gene expression in response to IL-1β (Boese *et al*., 1996).

The paradoxical effect of staurosporine on iNOS gene expression, on the other hand, is not unprecedented. While this study was under way, staurosporine was shown to cause an increase in both basal and dibutyryl-cyclic AMP and/or TNFα-stimulated NO_2^- production in rat peritoneal macrophages. Moreover, as shown in the present study, the apparent induction of iNOS gene expression by staurosporine in these cells was sensitive to treatment with cycloheximide (Sowa & Przewlocki, 1995). Staurosporine, in contrast to many other known protein kinase inhibitors, has also been shown to upregulate expression of the 5-hydroxytryptamine (5-HT) transporter in human choricarcinoma cells, an effect which did not involve either an increase in intracellular cyclic AMP or cyclic GMP. Staurosporine, similar to its lack of effect on the forskolin-dependent increase in iNOS gene expression in the present study, also failed to potentiate the cyclic AMP-mediated effect of cholera toxin on the expression of the 5-HT transporter in these cells (Ramamoorthy *et al*., 1995). Finally, calphostin C has been shown to augment the IL-1β-stimulated increase in NO_2^- formation in rat renal mesangial cells (Mühl & Pfeilschifter, 1994).

What is the mechanism underlying the stimulant effect of staurosporine on iNOS gene expression? In the presence of staurosporine alone, iNOS mRNA levels were markedly increased, indicative of an effect of the protein kinase inhibitor at the level of DNA transcription. This notion is supported by the finding that actinomycin D significantly attenuated the staurosporine-induced increase in NO_2^- formation. Moreover, the finding that cycloheximide had a similar inhibitory effect could be interpreted as indicating that *de novo* protein synthesis, e.g. the expression of an inducible transcription factor, plays a role in the stimulant effect of staurosporine on iNOS gene expression.

Combined treatment of the VSMC with staurosporine and IL-1β, on the other hand, did not result in a further increase in iNOS mRNA abundance as compared to cells which had been exposed to staurosporine alone. One explanation for this finding is that the protein kinase inhibitor itself already caused a maximum increase in transcription of the iNOS gene or that after 20 h of stimulation iNOS mRNA levels were already approaching the steady-state. The fact that the protein kinase inhibitor significantly enhanced the forskolin-induced rise in iNOS mRNA abundance, but only up to the level attained with staurosporine and/or IL-1β, supports both possibilities. Another explanation for the lack of synergism between staurosporine and IL-1β is that the signal transduction pathways responsible for their effects on iNOS gene expression converge proximal to or at the level of DNA transcription. However, this interpretation of the RT-PCR data does not explain why staurosporine had such a pronounced effect on the IL-1β-mediated increase in NO_2^- formation and iNOS protein abundance. We would therefore favour the hypothesis that in addition to its effect at the level of transcription, staurosporine affects iNOS protein stability. This interesting possibility warrants further investigation.

To address the question as to how staurosporine up-regulates iNOS gene expresion, we have investigated its effects on the activity of three major transcription factors, NF-κB, CREB and C/EBP, which are likely to be involved in the increase in iNOS gene expression in VSMC in response to IL-1β and/or agents, such as forskolin, which cause an increase in the intracellular concentration of cyclic AMP. Indeed, activation of NF-κB appears to be essential for the cytokine-mediated induction of iNOS gene expression in VSMC (Schini-Kerth *et al*., 1995; 1996; Hecker *et al*., 1996b) as well as in other iNOS-expressing cells, such as macrophages and mesangial cells (Xie *et al*., 1994; Eberhardt *et al*., 1994). In the VSMC employed for this study, a distinct basal activity of NF-κB was detectable, as characterized by retardation of the p65/p50 heterodimer in the electrophoretic mobility shift analysis. The intensity of this NF-κB-specific DNA-protein complex was significantly enhanced in the presence of IL-1β, indicative of an activation of the transcription factor by the cytokine. However, staurosporine had no effect either on basal or IL-1β-stimulated NF-κB activity, hence ruling out an activation of this transcription factor as the mechanism responsible for the staurosporine-induced increase in iNOS gene expression.

Cyclic AMP regulation of iNOS gene expression has not been shown for human or mouse iNOS, and appears to be unique for the rat iNOS promoter which has recently been cloned and sequenced (Eberhardt *et al*., 1996). There are two candidate CREB binding sites in the promoter, of which the one closest to the transcription start site and adjacent to one of the two NF-κB binding sites could be shared by members of the C/EBP family of transcription factors. It may be this cyclic AMP response element which confers cyclic AMP inducibility of iNOS gene expression in VSMC (Hecker *et al*., 1996b), macrophages (Sowa & Przewlocki, 1995) and renal mesangial cells (Eberhardt *et al*., 1994) of rat origin. In the present study, only forskolin, but not staurosporine or IL-1β, caused an activation of CREB, as demonstrated by the appearance of an inducible CREB-specific DNA-protein complex in nuclear extracts of forskolin-treated VSMC, presumably because staurosporine and IL-1β elicited either no or only a modest increase in intracellular cyclic AMP in these cells (Boese *et al*., 1996). It is thus unlikely that staurosporine exerts its effect on iNOS gene expression via activation of CREB.

The C/EBP proteins form a family of transcription factors with at least seven members of which C/EBPβ in the rat appears to be the only C/EBP protein which can be induced, e.g. by cytokines and microbial products (Wedel & Ziegler-Heitbrock, 1995). Moreover, the activity of C/EBPβ can be enhanced by phosphorylation (Wegner *et al*., 1992), and a synergism between C/EBPβ and NF-κB, e.g. in the activation of IL-8 gene expression has been shown (Stein & Baldwin, 1993). The rat iNOS promotor contains four binding sites for members of the C/EBP family of transcription factors, two of which are located within less than 200 bp upstream from the transcription start site and close to one of the two NF-κB binding sites (Eberhardt *et al*., 1996). Although the functional importance of these C/EBP binding sites for IL-1β regulation of iNOS gene expression in VSMC remains to be demonstrated, the present findings suggest that a C/EBP protein, presumably C/EBPβ, is involved therein.

Thus, staurosporine itself caused a distinct increase in basal C/EBPβ activity and, in a supra-additive manner, enhanced the activation of C/EBPβ both by IL-1β and forskolin. If one

considers the low level activation of NF-κB already in non-stimulated SMC, it is conceivable that an up-regulation of the activity of C/EBPβ by staurosporine may lead to a synergistic induction of iNOS gene expression under basal conditions. The more pronounced activation of C/EBPβ in the presence of both IL-1β and staurosporine together with that of NF-κB may also promote a supra-additive induction of iNOS gene expression. However, as discussed above, this potential mechanism was not revealed by a corresponding increase in the level of iNOS mRNA in the present study. C/EBPβ and CREB, on the other hand, may target the same binding site on the iNOS promter conferring cyclic AMP inducibility either individually or via dimerization (Wedel & Ziegler-Heitbrock, 1995). The increased activity of C/EBPβ in the presence of both staurosporine and forskolin may thus result in a similar increase in iNOS gene expression as compared to stimulation with forskolin alone.

It remains to be determined how staurosporine enhances the DNA-binding activity of C/EBPβ. Two mechanisms could be involved: (a) an indirect effect of staurosporine on the phosphorylation, hence activation of C/EBPβ (Wegner *et al*., 1992), e.g. by altering the activity of the corresponding protein kinase (or phosphatase); or (b) an increased expression of C/EBPβ following exposure of the VSMC to staurosporine. The latter hypothesis seems to be supported by the findings that activation of C/EBPβ by IL-1β or forskolin required a significantly longer period of time (2 h) than that of NF-κB (30 min), and that the induction of iNOS gene expression by staurosporine may depend on *de novo* protein synthesis.

In summary, the aforementioned data demonstrate that the non-selective PKC inhibitor, staurosporine, unlike various other protein kinase inhibitors, causes an induction of iNOS gene transcription in VSMC which appears to be mediated by an increase in the basal activity of CEBPβ. It is conceivable, therefore, that a staurosporine-sensitive, as yet unidentified protein kinase exists in cultured VSMC which prevents iNOS gene expression by tonically suppressing basal C/EBPβ activity. Although this remains to be demonstrated by reporter gene analyses, we postulate that under basal conditions and possibly also following exposure to IL-1β, NF-κB and C/EBPβ co-operate in the up-regulation of iNOS gene expression in VSMC. Targeting of C/EBPβ (or NF-IL-6) activation may thus represent an interesting approach for interfering selectively with the cytokine-induced over-production of NO in acute and chronic inflammatory conditions.

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