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# Effect of cyclic GMP-dependent vasodilators on the expression of inducible nitric oxide synthase in vascular smooth muscle cells: role of cyclic AMP

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**1** In the present study we examined whether interleukin-1$\beta$ (IL-1$\beta$) increases the activity of adenylyl cyclase in vascular smooth muscle cells and determined its role in the cytokine-induced expression of the inducible nitric oxide synthase (iNOS) and activation of nuclear transcription factor-$\kappa$B (NF-$\kappa$B). In addition the interaction between cyclic AMP- and cyclic GMP-elevating agonists on the IL-1$\beta$-stimulated expression of iNOS was examined.

**2** Exposure of vascular smooth muscle cells to IL-1$\beta$ stimulated the formation of cyclic AMP but not of cyclic GMP. The intracellular level of cyclic AMP reached a maximum within 1 h and then gradually declined over the next 5 h. This IL-1$\beta$ (60 u ml$^{-1}$)-stimulated formation of cyclic AMP was modest (about 3 fold at 60 u ml$^{-1}$ for 1 h) compared to that evoked by isoprenaline (about 9 fold at $3 \times 10^{-6}$ M for 2 min).

**3** The IL-1$\beta$ (60 u ml$^{-1}$ for 24 h)-stimulated accumulation of nitrite, which was taken as an index of NO production, was concentration-dependently increased by preferential inhibitors of cyclic AMP-dependent phosphodiesterases (rolipram and trequinsin). This effect was reproduced by a specific activator of the cyclic AMP-dependent protein kinase(s) A, Sp-8-CPT-cAMPS ($10^{-4}$ M) but was prevented by a specific inhibitor of cyclic AMP-dependent protein kinase(s) A, Rp-8-CPT-cAMPS ($10^{-4}$ M). These compounds alone [rolipram ($10^{-6}$ M), trequinsin ($3 \times 10^{-6}$ M) and Sp-8-CPT-cAMPS ($10^{-4}$ M)] slightly but significantly increased the release of nitric oxide while Rp-8-CPT-cAMPS elicited no such effect.

**4** Inducible NOS protein was expressed in IL-1$\beta$ (30 u ml$^{-1}$, 24 h)-stimulated smooth muscle cells as assessed by Western blot analysis. The level of iNOS protein was markedly increased in smooth muscle cells which had been exposed to IL-1$\beta$ in combination with either rolipram ($3 \times 10^{-6}$ M) or Sp-8-CPT-cAMPS ($10^{-4}$ M) but was reduced in those exposed to IL-1$\beta$ and Rp-8-CPT-cAMPS ($10^{-4}$ M). A weak expression of iNOS protein was found in smooth muscle cells which had been exposed to either Sp-8-CPT-cAMPS or rolipram alone for 24 h while Rp-8-CPT-cAMPS elicited no such effect.

**5** Exposure of smooth muscle cells to IL-1$\beta$ (30 u ml$^{-1}$) for 30 min increased the level of NF-$\kappa$B-DNA complexes in nuclear extracts as detected by electrophoretic mobility shift assay. Similar levels of NF-$\kappa$B-DNA complexes were found in cells which had been exposed to IL-1$\beta$ in combination with either Sp-8-CPT-cAMPS ($10^{-4}$ M), trequinsin ($10^{-5}$ M) or rolipram ($10^{-5}$ M). None of the modulators alone affected the basal level of NF-$\kappa$B binding activity.

**6** NO-donors [sodium nitroprusside (SNP) $10^{-4}$ M; dinitrosyl-iron-di-L-cysteine-complex (DNIC), $10^{-4}$ M; 3-morpholino-sydnonimine (SIN-1), $10^{-4}$ M] and atrial natriuretic factor ($10^{-6}$ M) significantly increased the IL-1$\beta$ (30 or 60 u ml$^{-1}$, 24 h)-stimulated expression of iNOS protein and activity as assessed indirectly by the conversion of oxyhaemoglobin to methaemoglobin. In the absence of IL-1$\beta$, SNP ($10^{-4}$ M, 24 h) but not the other cyclic GMP-dependent vasodilators caused a modest expression of iNOS protein. No such effect was found in smooth muscle cells exposed to SNP in combination with Rp-8-CPT-cAMPS ($10^{-4}$ M) while an increased level of iNOS protein was found in those exposed to SNP in combination with either Sp-8-CPT-cAMPS ($10^{-4}$ M) or rolipram ($3 \times 10^{-6}$ M).

**7** Exposure of vascular smooth muscle cells to either S-nitroso-L-cysteine (Cys-SNO, $10^{-4}$ M), SNP ($10^{-4}$ M) or SIN-1 ($10^{-4}$ M) for 35 min affected minimally the basal activation of NF-$\kappa$B but abolished that evoked by IL-1$\beta$ (30 u ml$^{-1}$ added during the last 30 min). However, addition of Cys-SNO following the stimulation with IL-1$\beta$ (during the last 5 min of the 30 min exposure period) reduced the level of NF-$\kappa$B-DNA complexes only slightly.

**8** These data indicate that the cyclic AMP-dependent pathway plays a decisive role in the signal transduction cascade initiated by the activation of the IL-1$\beta$-receptor and leading to iNOS expression in vascular smooth muscle cells. The stimulatory effect of the cyclic AMP pathway on iNOS expression appears not to be related to the activation of NF-$\kappa$B. In addition, cyclic GMP-dependent vasodilators potentiate the cytokine-stimulated expression of iNOS probably by interaction with the cyclic AMP effector pathway.

**Keywords:** Inducible nitric oxide synthase; cyclic AMP; nitric oxide donor; interleukin-1 $\beta$; phosphodiesterase inhibitor; protein kinase(s) A; atrial natriuretic factor; nuclear factor-$\kappa$B

## Introduction

Blood vessel injury resulting in dysfunction or disruption of the endothelial layer such as occurs after endotoxaemia or balloon catheterization, is associated with the generation of nitric oxide (NO) by the injured vascular smooth muscle (Julou-Schaeffer *et al.*, 1990; Joly *et al.*, 1992). This response to injury reflects the expression of an inducible type of nitric oxide synthase (iNOS) in smooth muscle cells and possibly also

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in other types of resident cells following the exposure to inflammatory mediators such as interleukin-1$\beta$ (IL-1$\beta$), tumour necrosis factor and lipopolysaccharide (Busse & Mülsch, 1990; Schini *et al.*, 1991; Beasley *et al.*, 1991; Fleming *et al.*, 1991). Unlike the endothelial nitric oxide synthase, the activity of the inducible enzyme is $Ca^{2+}$-independent and is regulated at the transcriptional level by several nuclear transcription factors including nuclear factor-$\kappa$B (NF-$\kappa$B; Busse & Mülsch, 1990; Xie *et al.*, 1993; 1994). Therefore once the enzyme is expressed, it generates large amounts of NO over a prolonged period. Vascular smooth muscle-derived NO appears to play a dual role in the vascular response to injury. Initially, the continuous release of discrete amounts of NO may be protective as it inhibits vasospasm, maintains vascular smooth muscle cells in a quiescent state, and prevents platelet activation at sites of injury (Durante *et al.*, 1991; Joly *et al.*, 1992; Scott-Burden *et al.*, 1992). However, when the expression of iNOS becomes excessive, the release of large amounts of NO may become deleterious causing loss of vascular tone and cytotoxic responses (Hibbs *et al.*, 1988; Julou-Schaeffer *et al.*, 1990). Thus, elucidation of the intracellular mechanisms regulating the expression of the iNOS by cytokines and lipopolysaccharide is of major importance.

Since the cytokine-stimulated formation of NO in vascular smooth muscle cells is sensitive to inhibition of either protein kinase C or protein tyrosine kinases (Marczin *et al.*, 1993; Scott-Burden *et al.*, 1994), the activation of both protein kinase C and protein tyrosine kinases seem to be events regulating the expression of iNOS. In addition, the cytokine-stimulated expression of iNOS mRNA and synthesis of NO are potentiated by cyclic AMP-elevating vasodilators in vascular smooth muscle cells (Koide *et al.*, 1993; Hirokawa *et al.*, 1994; Schini-Kerth *et al.*, 1994a; Imai *et al.*, 1994). Moreover, in the absence of a cytokine, high concentrations of the cyclic AMP elevating agents are able to induce the expression of iNOS in some (Koide *et al.*, 1993; Imai *et al.*, 1994) but not all cultures of vascular smooth muscle cells (Hirokawa *et al.*, 1994; Scott-Burden *et al.*, 1994; Schini-Kerth *et al.*, 1994a). Thus, besides protein kinase C and protein tyrosine kinases adenosine 3', 5'-cyclic monophosphate (cyclic AMP) is another important regulator of iNOS gene expression. Previous investigations have shown that the IL-1 receptor is linked to a pertussis toxin-sensitive G protein that stimulates the activity of adenylyl cyclase in human and murine cell lines (Shirakawa *et al.*, 1988; Chedid *et al.*, 1989), but not in mesangial cells (Kunz *et al.*, 1994), indicating that the IL-1$\beta$-elicited signal transduction via cyclic AMP may be cell type specific. Therefore, the purpose of the present study was to clarify the role of cyclic AMP in the IL-1$\beta$-stimulated expression of iNOS in vascular smooth muscle cells, and to determine whether the stimulatory effect of cyclic AMP on the expression of iNOS occurs at the level of NF-$\kappa$B. In addition, since cyclic GMP-dependent vasodilators mimic the actions of cyclic AMP-dependent vasodilators and both cyclic GMP- and cyclic AMP-dependent vasodilators synergistically affect vascular functions such as inhibition of vascular tone and growth, and platelet activation (Levin *et al.*, 1982; Maurice *et al.*, 1991; Assender *et al.*, 1992), the influence of cyclic GMP-dependent vasodilators on the IL-1$\beta$-stimulated expression of iNOS in vascular smooth muscle cells was examined.

## Methods

### *Cell culture*

Smooth muscle cells were isolated by elastase and collagenase digestion of thoracic aortae from male Wistar-Kyoto rats and characterized by immunocytochemical techniques by use of a monoclonal antibody against smooth muscle $\alpha$-actin (Gordon *et al.*, 1986). Cells were cultured in Waymouth medium containing non-essential amino acids, penicillin 50 u $ml^{-1}$, streptomycin (50 $\mu$g $ml^{-1}$) and 7.5% (v/v) foetal bovine serum. Confluent cultures of smooth muscle cells were serially passaged with 0.05% trypsin-0.02% EDTA. All experiments were performed with confluent cultures of cells (passage 4 and higher). The cells were seeded into either 24-well multiwell plates for the measurement of cyclic AMP content and NO production (nitrite and methaemoglobin formation), or into petri dishes (60 mm diameter) for the detection of iNOS protein by Western blot analysis and NF-$\kappa$B binding activity. When cells reached confluence the culture medium was replaced with serum-poor Waymouth medium containing 0.1% foetal calf serum. After 6 to 24 h, the incubation medium was replaced just before use.

### *Measurement of cyclic nucleotides*

Smooth muscle cells were incubated in serum-poor Waymouth medium in the presence of trequinsin ($10^{-5}$ M) to prevent the degradation of cyclic nucleotides by phosphodiesterases for at least 30 min before the addition of IL-1$\beta$ or solvent. The incubation medium was removed and the cells were harvested and denatured by scraping in 0.6 ml of ice-cold 6% trichloroacetic acid. The cell lysates were centrifuged at 10,000 *g* for 15 min and the supernatant was then extracted 4 times with 5 volumes of water-saturated ethylether. The cyclic AMP and cyclic GMP contents of the aqeuous phase were determined by use of commercial radioimmunoassays (DuPont, Bad Homburg, Germany).

### *Release of nitrite*

Nitrite was determined by diazotation with Griess reagent and absorbance reading at 570 nm in a Dynatek MR 600 microplate reader (Dynatech, Alexandria, Virginia, U.S.A.). Aliquots (150 $\mu$l) of conditioned medium were collected and mixed with an equal volume of Griess reagent (1% sulphanilamine and 0.1% N-(1-naphtyl)ethylenediamine di-hydrochloride in 2% phosphoric acid). The mixture was incubated at 20°C for 10 min. Concentrations were determined relative to a standard curve by use of an aqueous solution of sodium nitrite.

### *Measurement of iNOS activity*

NO production by smooth muscle cells was assessed by measuring the NO-induced conversion of oxyhaemoglobin ($HbO_2$) to methaemoglobin (MetHb; Wolf & Werringloer, 1987). The spectral change associated with this conversion at 402 nm and 420 nm was taken for calculation, with an extinction coefficient of 120 mM $cm^{-1}$ per haem for the differences of absorbances at both wavelengths (Wolf & Werringloer, 1987). The conditioned medium from confluent cells was replaced with 0.8 ml of HEPES-Tyrode solution (composition in mM: $CaCl_2$ 1.8, KCl 2.7, $MgCl_2$ 0.23, NaCl 137, $NaH_2PO_4$ 3.6, glucose 5.0, HEPES 10, pH 7.4) containing 2 $\mu$M $HbO_2$ and 1 mM L-arginine or 1 mM $N^G$-nitro-L-arginine. After one hour the extinction at 402 nm and 420 nm was measured in a UV/VIS spectrophotometer (Uvikon 941+, Kontron, München, Germany). In order to account for auto-oxidation of $HbO_2$ and non-NO-mediated oxidation, the absorbance readings obtained with L-arginine-supplemented cell supernatants were corrected by subtraction of readings obtained with $N^G$-nitro-L-arginine containing supernatants.

### *Expression of iNOS protein*

Cells were washed twice with cold HEPES-Tyrode solution, harvested by scraping, and collected by microcentrifugation at 3,000 *g* for 3 min at 4°C. The cell pellets were resuspended in bidistilled water and lysed by 5 cycles of freeze-thaw. An equal volume of homogenization buffer (Tris 100 mM (pH 7.4); KCl 2.3% (w/v), ethylene diaminetetraacetic acid (EDTA) 2 mM; (±)-dithiothreitol (DTT) 0.2 mM; phenylmethylsulphonyl fluoride 8.8 $\mu$g $ml^{-1}$ and 2 $\mu$g $ml^{-1}$ each of leupeptin, pepstatin A, trypsin inhibitor, antipain, chymostatin, and aproti-

nin) was added to cell homogenates, and cell cytosols were clarified by centrifugation at 10,000 *g* for 10 min at 4°C. The resulting supernatants contained 1 to 5 mg $ml^{-1}$ protein, as assessed by the Bradford assay using bovine serum albumin as a standard. The cytosolic fractions were subjected to SDS-polyacrylamide gel electrophoresis [8% (w/v) gradient gel] and then electrophoretically transferred to nitrocellulose membranes (Bio-Rad laboratories GmbH, München, Germany). Nitrocellulose blots were incubated overnight first at 4°C with a 1:2000 diluted polyclonal rabbit antibody directed against murine iNOS (kindly provided by Dr J. Pfeilschifter, JWG-University Clinic, Frankfurt, Germany). After 5 successive washes (5 min) with Tris (50 mM, pH 7.5) containing 20 mM NaCl and 0.3% (w/v) Tween 20, followed by one wash without Tween, a secondary polyclonal donkey anti-rabbit Ig antibody conjugated to horseradish peroxidase (Amersham International, Braunschweig, Germany) was added for 1 h at 20°C. Nitric oxide synthase immunoreactivity was visualized by exposing an X-ray film to blots incubated with the ECL reagent (Amersham). Prestained molecular mass markers (Bio-Rad) were used as standards for SDS-PAGE immunoblot analysis. The autoradiographs were analyzed by scanning densitometry (Image Master, Pharmacia, Freiburg, Germany).

### *Measurement of NF-κB-DNA binding activity*

Cells were washed twice with cold HEPES-Tyrode solution, harvested by scraping, and incubated in 200 μl of buffer A (HEPES 10 mM, pH 7.9, KCl 10 mM, EDTA 0.1 mM, ethylene glycol-bis (β-aminoethylether) N,N,N'N',-tretraacetic acid (EGTA) 0.1 mM DTT, 1 mM, leupeptin 2 μg $ml^{-1}$, pepstatin A 2 μg $ml^{-1}$, trypsin inhibitor 10 μg $ml^{-1}$, phenylmethylsulphonyl fluoride 88 μg $ml^{-1}$) for 15 min at 4°C. After addition of 0.6% Nonidet P-40, the nuclei were collected by centrifugation at 15,000 *g* for 30 s. The nuclear pellets were resuspended in 50 μl of buffer B (HEPES 20 mM, pH 7.9, KCl 400 mM, EDTA 1 mM, EGTA 1 mM, glycerol 10% (w/v), DTT 1 mM, phenylmethylsulphonyl fluoride 88 μg $ml^{-1}$, and 20 μg $ml^{-1}$ each of leupeptin, pepstatin A, trypsin inhibitor, antipain, chymostatin, and aprotinin). The nuclear suspension was shaken for 15 min at 4°C and clarified by centrifugation at 15,000 *g* for 5 min. The resulting supernatant contained 1–5 mg $ml^{-1}$ protein. Nuclear protein extracts were frozen in liquid nitrogen and stored at −70°C. A double stranded 22-mer oligonucleotide containing the most common NF-κB consensus sequence (underlined 5' AGTTGAGGGGACTTTCCCAGGC 3'; Santa Cruz Biotechnology, Inc., Santa Cruz, California, U.S.A.) was end-labelled with γ-[$^{32}$P]-adenosine 5'-triphosphate (3,000 Ci $mmol^{-1}$; Amersham-Buchler, Braunschweig, Germany), with T4 polynucleotide kinase. Binding reactions were set up with 10,000 cpm $^{32}$P-labelled DNA, HEPES 5 mM (pH 7.5), NaCl 100 mM, DTT 1 mM, 5% glycerol, EDTA 1 mM, 1 μg poly dI-dC (Pharmacia), and 10–15 μg of nuclear protein extract. The binding reactions proceeded at 20°C for 30 min. The mixture was then electrophoresed for 2 h at 135 V on a 6% non-denaturing polyacrylamide gel (Tris-borate 89 mM and EDTA 1 mM). Subsequently the gel was dried and DNA-protein complexes were localized by autoradiography. The relative amounts of NF-κB-DNA complexes were determined by scanning densitometry.

### *Materials and drugs*

Interleukin-1β was obtained from Collaborative Research Inc., Bedford, Massachusetts, U.S.A.; isoprenaline, atrial natriuretic factor (ANF), sodium nitroprusside (SNP), potassium hexacyano-ferrat, sulphanilamide, N-(1-naphthyl)ethylenediamine dihydrochloride from Sigma Aldrich Chemie GmbH, Deisenhofen, Germany; 3-morpholino sydnonimine (SIN-1) from Cassella, Frankfurt, Germany; rolipram and trequinsin from Biomol Feinchenmikalien GmbH, Hamburg, Germany, Rp-8-(4-chlorophenylthio)adenosine 3',5' cyclic monophosphorothioate Rp-8-CPT-cAMPS and Sp-8-(4-chlorophenylthio)adenosine 3',5' cyclic monophosphorothioate Sp-8-CPT-cAMPS from Biolog Life Science Institute (Bremen, Germany). Dinitrosyl-iron-(±)-L-cystein-complex (DNIC) was prepared as described previously (Boese *et al.*, 1995). The Waymouth medium was purchased from PAN Systems, Chemische Produkte GmbH, Aidenbach, Germany, and foetal bovine seum and antibiotics were obtained from Boehringer Mannheim (Mannheim, Germany).

### *Statistical analysis*

The results are expressed as means ± s.e.mean. Statistical analysis was performed by Student's paired *t* test (two-tailed) or analysis of variance followed by Fisher's protected least significant difference test where appropriate. A value of *P* less than 0.05 was considered to be statistically significant; *n* represents the number of different experiments.

## Results

### *Role of the cyclic AMP pathway in IL-1β-stimulated iNOS expression*

*Production of cyclic AMP* Exposure of rat aortic smooth muscle cells to IL-1β (60 μ $ml^{-1}$) in the presence of a high concentration of trequinsin in order to block the degradation of both cyclic AMP and cyclic GMP by phosphodiesterases, caused a time-dependent increase in the production of cyclic AMP (Figure 1) but not in cyclic GMP (data not shown). The production of cyclic AMP reached a maximum within 1 h and then declined gradually over the next 5 h. However, the IL-1 β-stimulated production of cyclic AMP was modest (about 3 fold at 60 u $ml^{-1}$ for 1 h) compared to that evoked by isoprenaline (about 9 fold at $3 \times 10^{-6}$ M for 2 min).

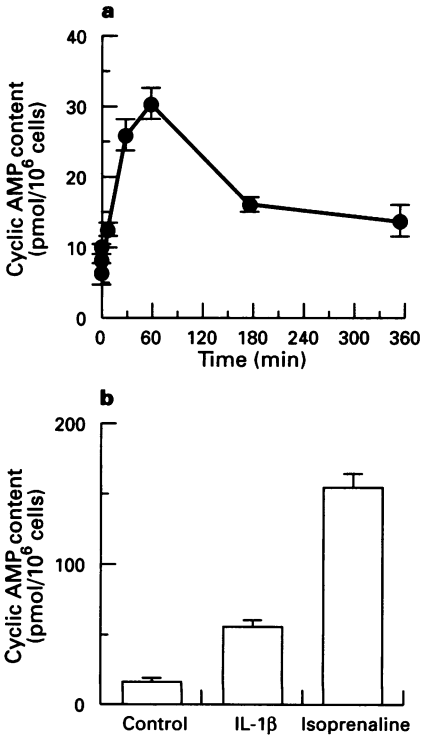


**Figure 1** (a) Interleukin-1β (IL-1β; 60 u $ml^{-1}$; ●) caused a time-dependent formation of cyclic AMP in rat aortic smooth muscle cells. (b) Comparison of the formation of cyclic AMP evoked by IL-1β (60 u $ml^{-1}$ for 60 min) and isoprenaline ($3 \times 10^{-6}$ M for 2 min) in rat aortic smooth muscle cells. All experiments were performed in the presence of trequinsin ($10^{-5}$ M). Results are shown as mean ± s.e.mean (vertical lines) of a representative experiment performed in quadruplicate.

*Release of nitrite*

The IL-1$\beta$ (60 u ml$^{-1}$ for 24 h)-stimulated release of nitrite by vascular smooth muscle cells was increased in a concentration-dependent manner by rolipram a specific inhibitor of phosphodiesterase IV, and also by trequinsin at concentrations which preferentially inhibit phosphodiesterases III and IV (Figure 2). The stimulating effect of rolipram was significant at concentrations greater than $10^{-7}$ M and reached a maximum at $10^{-6}$ M (about 2.2 fold) while an effect of trequinsin was only apparent at concentrations greater than $10^{-6}$ M and amounted to a 4.5 fold increase in nitrite release at $10^{-5}$ M. The limited solubility of trequinsin precluded the completion of a concentration-effect curve. In the absence of IL-1$\beta$ both phosphodiesterase inhibitors elicited a small increase in nitrite release, which became significant at $10^{-6}$ M with rolipram, and at $3 \times 10^{-6}$ M with trequinsin (Figure 2a and b). The IL-1$\beta$ (60 u ml$^{-1}$ for 24 h)-stimulated nitrite release was enhanced by the selective activator, Sp-8-CPT-cAMPS ($10^{-4}$ M) and diminished by the selective inhibitor of protein kinase(s) A, Rp-8-CPT-cAMPS ($10^{-4}$ M; Figure 3a and b). Sp-8-CPT-cAMPS ($10^{-4}$ M for 24 h) in the absence of IL-1$\beta$ increased the release of nitrite by vascular smooth muscle cells by 3 fold (Figure 3a), while Rp-8-CPT-cAMPS had no effect (Figure 3b).

*Expression of iNOS protein* Western blot analysis with a polyclonal iNOS antibody directed against murine iNOS revealed a protein band at about 130 kDa in soluble extracts from IL-1$\beta$ (60 u ml$^{-1}$)-stimulated but not from unstimulated smooth muscle cells after 24 h incubation (Figure 4). The level of iNOS protein was markedly increased in cells which had been exposed to IL-1$\beta$ (60 u ml$^{-1}$) in combination with either rolipram ($3 \times 10^{-6}$ M, by $1.8 \pm 0.4$ fold, $n=3$) or Sp-8-CPT-cAMPS ($10^{-4}$ M, by $2.5 \pm 0.2$ fold, $n=3$), but was abolished in cells which had been exposed to the cytokine in combination with Rp-8-CPT-cAMPS ($10^{-4}$ M) for 24 h (Figure 4). Although not clearly visible in the blot shown, weak iNOS-positive bands were detected in cells which had been exposed to either rolipram ($3 \times 10^{-6}$ M) or Sp-8-CPT-cAMPS ($10^{-4}$ M) alone for 24 h, as recognized by longer exposure to the ECL reagent (data not shown), while Rp-8-CPT-cAMPS had no such effects.

*Nuclear factor-κB activation* Nuclear protein extracts prepared from rat aortic smooth muscle cells exhibited basal NF-κB-binding activity, as assessed by electrophoretic mobility shift assay (Figure 5). Preliminary experiments have indicated that IL-1$\beta$ increased in a time-dependent manner the level of NF-κB reaching a peak level of $2.1 \pm 0.3$ fold ($n=3$) after a 30 min exposure of the cells to IL-1$\beta$ (Figure 5 and data not shown). Exposure of smooth muscle cells to either Sp-8-CPT-cAMPS ($10^{-4}$ M), trequinsin ($10^{-5}$ M) or rolipram ($10^{-5}$ M) did not significantly affect NF-κB binding activity in control and IL-1$\beta$-stimulated cells ($n=5$, Figure 5).

*Effect of cyclic GMP-dependent vasodilators on IL-1β-stimulated expression of iNOS*

*Expression of iNOS protein* The IL-1$\beta$ (30 u ml$^{-1}$ for 24 h)-stimulated expression of iNOS protein in vascular smooth muscle cells was markedly increased by activators of soluble guanylyl cyclase such as SNP ($10^{-4}$ M), SIN-1 ($10^{-4}$ M) and DNIC ($10^{-4}$ M) (Figure 6a), and also by an activator of particulate guanylyl cyclase atrial natriuretic factor (ANF,

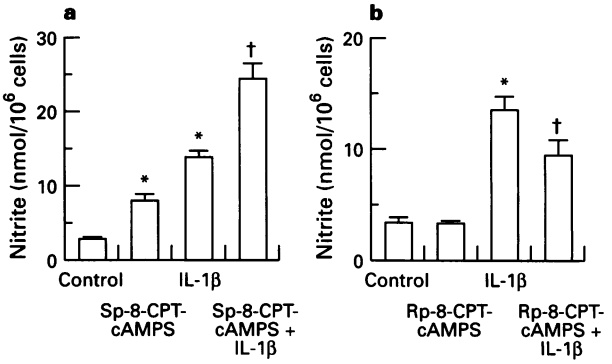


**Figure 3** Effect of (a) Sp-8-CPT-cAMPS ($10^{-4}$M), a selective activator of protein kinase(s) A and (b) Rp-8-CPT-cAMPS ($10^{-4}$M), a selective inhibitor of protein kinase(s) A on both basal and interleukin-1$\beta$ (IL-1$\beta$, 60 u ml$^{-1}$)-stimulated release of nitrite from rat aortic smooth muscle cells. The level of nitrite in the incubation medium was determined after a 24 h incubation period of the cells at 37°C. Results are expressed as mean ± s.e.mean of (a) three and (b) two experiments performed in triplicate. (*$P<0.05$ and †$P<0.05$; ANOVA).

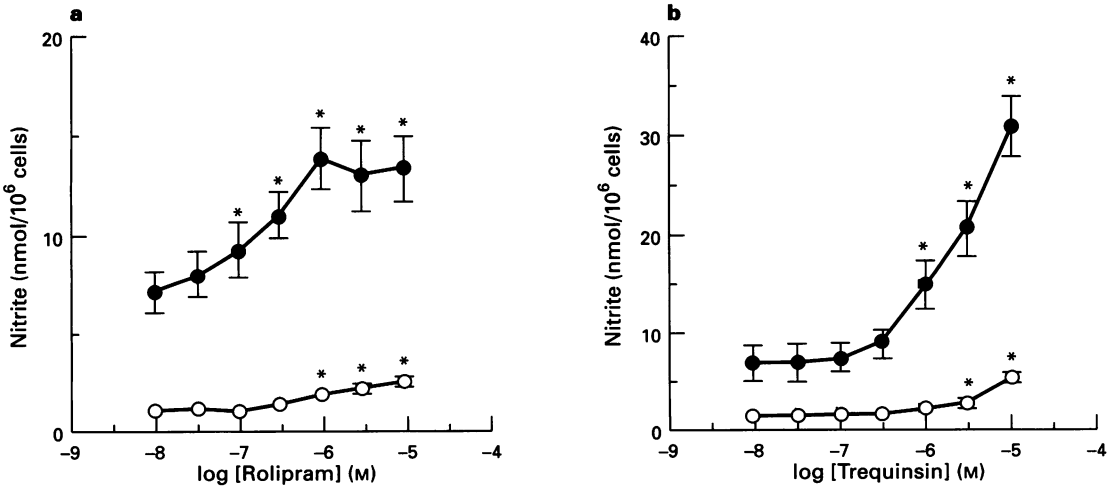


**Figure 2** Effects of (a) rolipram, a specific inhibitor of phosphodiesterase IV and (b) trequinsin, a preferential inhibitor of phosphodiesterase III and IV, on both basal (○) and interleukin-1$\beta$ (IL-1$\beta$) (60 u ml$^{-1}$; ●)-stimulated release of nitrite from rat aortic smooth muscle cells. The level of nitrite in the incubation medium was determined after a 24 h incubation period at 37°C. Release of nitrite from unstimulated cells and from cells exposed to IL-1$\beta$ alone were in (a) $1.35 \pm 0.16$ nmol/million cells and $6.21 \pm 0.69$ nmol/million cells, and in (b) $1.99 \pm 0.47$ nmol/million cells and $6.71 \pm 1.36$ nmol/million cells, respectively. Results are expressed as mean ± s.e.mean of (a) five and (b) three experiments performed in triplicate. (*$P<0.05$; ANOVA).

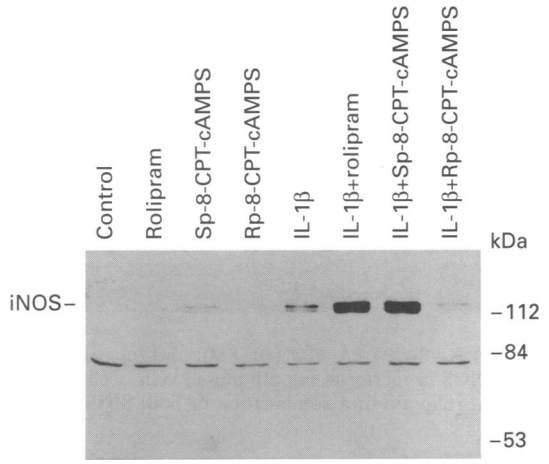


**Figure 4** Western blot showing the effects of interfering with the cyclic AMP-effector pathway on the interleukin-1$\beta$ (IL-1$\beta$, 30 u ml$^{-1}$)-induced expression of iNOS protein in rat aortic smooth muscle cells. Cells were incubated in the absence and presence of either IL-1$\beta$, rolipram ($3 \times 10^{-6}$ M), Sp-8-CPT-cAMPS ($10^{-4}$ M), Rp-8-CPT-cAMPS ($10^{-4}$ M) or a combination of both IL-1$\beta$ and a modulator of the cyclic AMP pathway for 24 h at 37°C.

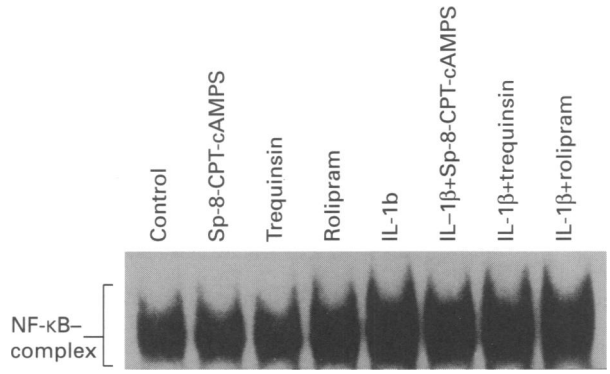


**Figure 5** Electrophoretic mobility shift assay showing the effects of interfering with the cyclic AMP-effector pathway on the activation of nuclear transcription factor-$\kappa$B (NF-$\kappa$B) elicited by interleukin-1$\beta$ (IL-1$\beta$, 30 u ml$^{-1}$) in rat aortic smooth muscle cells. Cells were incubated in the absence and presence of IL-1$\beta$, Sp-8-CPT-cAMPS ($10^{-4}$ M), trequinsin ($10^{-5}$ M), rolipram ($10^{-5}$ M) or a combination of both IL-1$\beta$ and a modulator of the cyclic AMP pathway for 30 min at 37°C.

$10^{-6}$ M, Figure 6c). Densitometric analysis revealed an average increase in the level of iNOS protein by $1.98 \pm 0.39$ fold, $1.68 \pm 0.08$ fold, $2.08 \pm 0.26$ fold, and $1.37 \pm 0.06$ fold, respectively ($n = 3$). The stimulating effect of SNP was concentration-dependent and was unrelated to its iron-cyanide complex structure as indicated by the fact that potassium hexacyanoferrat ($K_3[Fe(CN)_6]$, $10^{-4}$ M) minimally affected the response to IL-1$\beta$ (Figure 6b). A high concentration of SNP ($10^{-4}$ M) elicited a low but detectable level of iNOS protein when added to vascular smooth muscle cells in the absence of IL-1$\beta$ for 24 h while the other cyclic GMP elevating agents had no such effect (Figure 6a and b). The modest expression of iNOS protein evoked by SNP was greatly reduced by the Rp-8-CPT-cAMPS ($10^{-4}$ M) but was synergistically increased by either rolipram ($3 \times 10^{-6}$ M) or Sp-8-CPT-cAMPS ($10^{-4}$ M; Figure 7).

*Expression of iNOS activity* IL-1$\beta$ (30 u ml$^{-1}$ for 24 h)-stimulated vascular smooth muscle cells released significant amounts of NO as assessed by the $N^G$-nitro-L-arginine-sensitive oxidation of haemoglobin to methaemoglobin (Figure 8). This response was significantly increased from smooth muscle

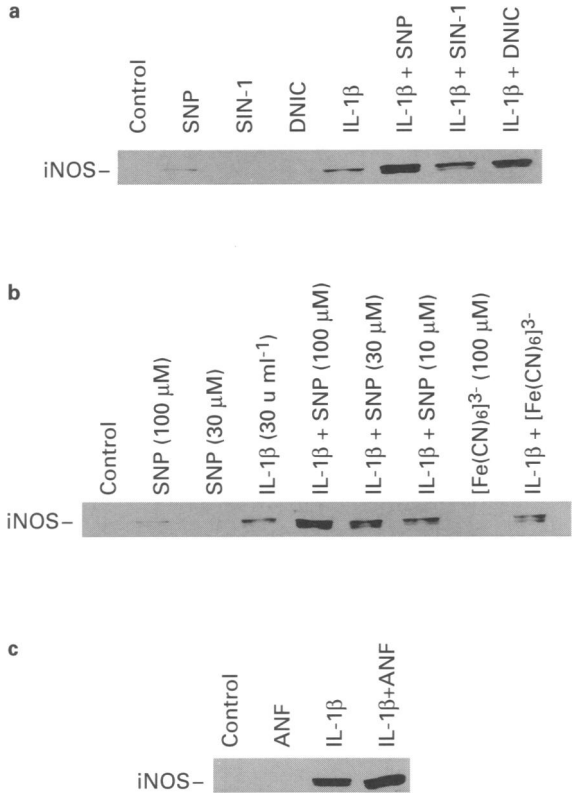


**Figure 6** Western blot showing that both NO-donors (a and b) and atrial natriuretic factor (ANF; c) increase the expression of iNOS protein elicited by interleukin-1$\beta$ (IL-1$\beta$, 30 u ml$^{-1}$) in rat aortic smooth muscle cells. Cells were incubated in the absence and presence of IL-1$\beta$, sodium nitroprusside (SNP, $10^{-4}$ M or specified), SIN-1 ($10^{-4}$ M), dinitrosyl-iron-di-L-cysteine (DNIC, $10^{-4}$ M), ANF ($10^{-6}$ M) or a combination of both IL-1$\beta$ and a cyclic GMP-dependent vasodilator for 24 h at 37°C. In addition, the effect of a structurally related FeCN-containing compound is shown in (b).

cells which had been exposed to the cytokine in the presence of either SNP ($10^{-4}$ M; by $53.6 \pm 18.6\%$, $n = 8$) or ANF ($10^{-6}$ M, by $86.6 \pm 20.6\%$, $n = 8$; Figure 8). Neither SNP nor ANF alone stimulated the expression of iNOS activity in the absence of IL-1$\beta$ (Figure 8).

*Nuclear factor-$\kappa$B activation* Exposure of vascular smooth muscle cells to either S-nitroso-L-cysteine (Cys-SNO, $10^{-4}$ M), SNP ($10^{-4}$ M) or SIN-1 ($10^{-4}$ M) for 35 min minimally affected the basal activation of NF-$\kappa$B but attenuated that evoked by IL-1$\beta$ (30 u ml$^{-1}$ added during the last 30 min) by $37 \pm 7\%$ ($n = 5$), $45 \pm 15\%$ ($n = 4$), and $46 \pm 26\%$ ($n = 3$), respectively (Figure 9 and data not shown). The inhibitory effect of Cys-SNO on NF-$\kappa$B activation was greatly reduced by the subsequent addition of the NO-donor to IL-1$\beta$ treated cells (during the last 5 min of the 30 min incubation period; Figure 9).

## Discussion

Cytokines and lipopolysaccharide can activate vascular smooth muscle cells to release copious amounts of NO that have been implicated in the severe loss of vascular tone in septic shock (Busse & Mülsch, 1990; Julou-Schaeffer *et al.*, 1990). This response to the inflammatory mediators is a slowly developing process that reflects the transcriptional expression of the iNOS gene as indicated by the time-dependent appearance of iNOS mRNA and protein (Kanno *et al.*, 1993; Hirokawa *et al.*, 1994; Schini-Kerth *et al.*, 1994a). IL-1$\beta$, a 17.5 kDa cytokine produced by macrophages as well as by

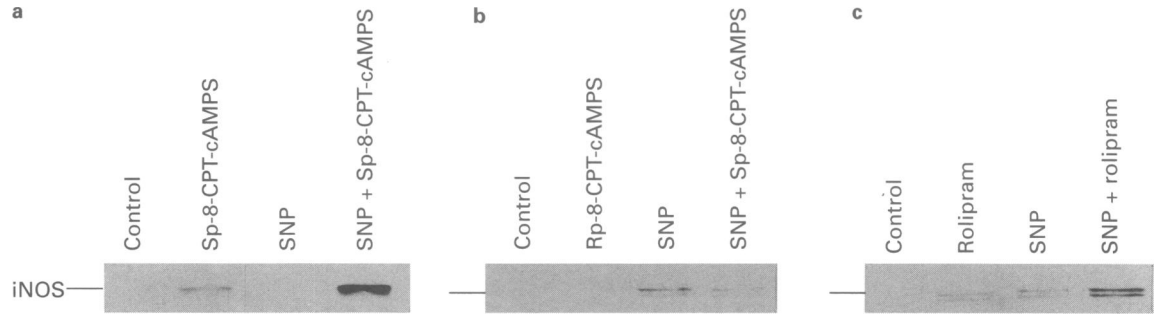


**Figure 7** Western blot showing the effect of (a) Sp-8-CPT-cAMPS ($10^{-4}$ M), (b) Rp-8-CPT-cAMPS ($10^{-4}$ M), and (c) rolipram ($3 \times 10^{-6}$ M) on the sodium nitroprusside (SNP, $10^{-4}$ M)-stimulated expression of iNOS in rat aortic smooth muscle cells. Cells were incubated in the absence and presence of SNP, a modulator of protein kinase(s) A, rolipram or a combination of both SNP and a modulator of the cyclic AMP pathway for 24 h at 37°C.

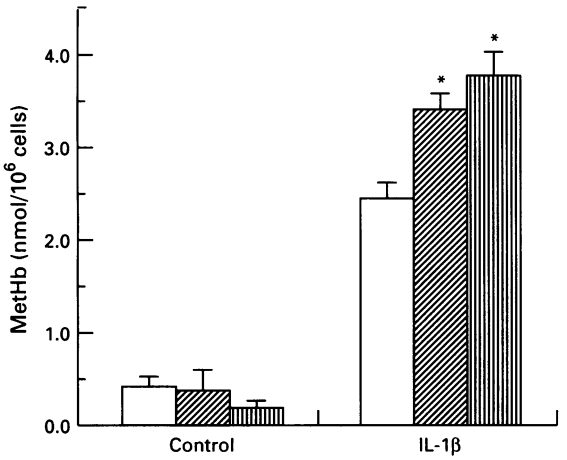


**Figure 8** Effect of sodium nitroprusside (SNP, $10^{-4}$ M; hatched columns) and atrial natriuretic factor (ANF; $10^{-6}$ M; vertically-hatched columns) on both basal (control) and interleukin-1$\beta$ (IL-1$\beta$)-stimulated release of nitric oxide from rat aortic smooth muscle cells as assessed by the NO mediated conversion of oxyhaemoglobin to methaemoglobin. Cells were incubated in the absence and presence of IL-1$\beta$ (30 u ml$^{-1}$), a cyclic GMP-dependent vasodilator or a combination of both IL-1$\beta$ and a cyclic GMP-dependent vasodilator for 24 h at 37°C. Results are shown as mean ± s.e.mean of eight experiments. (*$P < 0.05$; ANOVA).

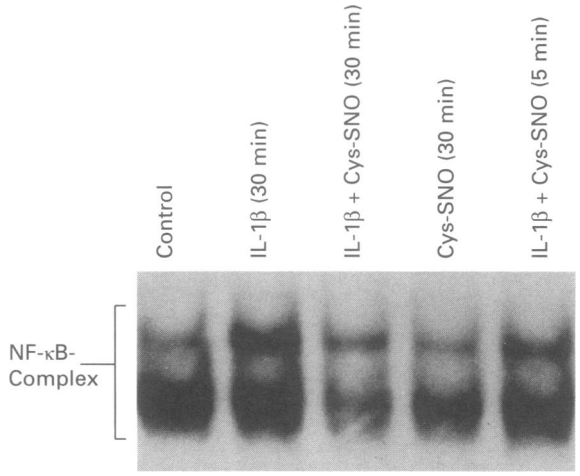


**Figure 9** Electrophoretic mobility shift assay showing that the interleukin-1$\beta$ (IL-1$\beta$, 30 u ml$^{-1}$ for 30 min)-induced nuclear transcription factor (NF)-$\kappa$B-DNA binding activity in rat aortic smooth muscle cells was abolished by pretreatment of the cells with S-nitroso-L-cysteine (Cys-SNO; $10^{-4}$ M) for 5 min while the addition of Cys-SNO to the cytokine-stimulated cells during the last 5 min of the 30 min incubation period had only minimal effects.

cells from the vascular wall in response to lipopolysaccharide, is one of the most potent inducers of iNOS expression in the vascular smooth muscle (Schini-Kerth & Vanhoutte, 1995), and is thought to be a major mediator of the severe hypotension associated with endotoxaemia.

The molecular mechanisms mediating the expression of the iNOS gene in response to IL-1$\beta$ remain largely unknown. However, this action of IL-1$\beta$ is presumably initiated by the binding of the cytokine to the 80 kDa type I IL-1-receptor which has been identified on a variety of cells including vascular cells and T-cells while the 68 kDa type II receptor is found predominantly on B-cells (see review by Dinarello, 1991). Activation of the type I IL-1 receptor leads to increases in the intracellular level of cyclic AMP via a G-protein coupled activation of adenylyl cyclase and activation of protein kinase(s) A in murine fibroblasts and thymocytes (Zhang *et al.*, 1988; Shirakawa *et al.*, 1988; Chedid *et al.*, 1989). A potentiation of the activity of adenylyl cyclase by IL-1$\beta$ was also found in vascular smooth muscle cells as indicated by the time-dependent accumulation of cyclic AMP (Scott-Burden *et al.*, 1994; this study). The generation of cyclic AMP occurred after a delay of 10 min, reached a peak level within one hour and thereafter declined progressively during the next couple of hours. In contrast the formation of cyclic AMP elicited by $\beta$-adrenoceptor agonists in vascular smooth muscle cells reached a maximum within one min and declined within 10 min (Schoeffter & Stoclet, 1982). Furthermore, IL-1$\beta$ was only a poor activator of adenylyl cyclase when compared to $\beta$-adrenoceptor agonists. The long-lasting formation of cyclic AMP by IL-1$\beta$ was not affected by indomethacin in cultural synovial cells and lymphocytes and hence cannot be explained by IL-1$\beta$-induced production of prostaglandins with subsequent activation of adenylyl cyclase by prostaglandins $E_2$ and $I_2$ (Shirakawa *et al.*, 1988; Chedid *et al.*, 1989). The present findings showing that a selective inhibitor of protein kinase(s) A, Rp-8-CPT-cAMPS, significantly inhibited the IL-1$\beta$-stimulated expression of iNOS protein and synthesis of NO indicate that the formation of cyclic AMP via activation of protein kinase(s) A plays a decisive role in the expression of iNOS in response to IL-1$\beta$. Furthermore, the positive regulatory role of the cyclic AMP pathway on iNOS expression was also evidenced by the observation that an increase in cyclic AMP level either by cyclic AMP-elevating agents or by preventing the degradation of cyclic AMP by selective inhibitors of phosphodiesterase III and IV potentiated the action of the cytokine (Koide *et al.*, 1993; Hirokawa *et al.*, 1994; Scott-Burden *et al.*, 1994; Schini-Kerth *et al.*, 1994a; present study). A similar amplifying effect was also found with liposoluble analogues of cyclic AMP and with direct activators of protein kinase(s) A (Koide *et al.*, 1993; Durante *et al.*, 1994; Schini-Kerth *et al.*, 1994a; present study).

An increased transcription of the iNOS gene rather than a decreased destabilization of the iNOS mRNA seems to account for the stimulating effect of the cyclic AMP pathway on iNOS expression (Imai *et al.*, 1994; Schini-Kerth *et al.*, 1994a). However a certain amount of conflicting evidence on the role of cyclic AMP on iNOS expression exists (Koide *et al.*, 1993; Kunz *et al.*, 1994; Hirokawa *et al.*, 1994; Durante *et al.*, 1994; Schini-Kerth *et al.*, 1994a; Imai *et al.*, 1994; present study). One possible explanation is that in different cell batches there is a differential activation of complimentary signalling pathways, which are necessary to elicit maximal gene expression. Such a cross-talk could involve protein kinase C and protein tyrosine kinase pathways both of which have been implicated in the regulation of IL-1$\beta$-induced iNOS expression in cultured vascular smooth muscle cells (Marczin *et al.*, 1993; Scott-Burden *et al.*, 1994). Consistent with such an idea are the findings that forskolin in combination with phorbol esters led, in the absence of a cytokine, to the release of significant amounts of nitric oxide from cultured vascular smooth muscle cells while each agent alone had only minimal effects (Scott-Burden *et al.*, 1994). Little is known about the mechanisms by which the IL-1$\beta$ activator signal is transmitted from the cytosol to the nucleus but this pathway probably involves the activation of transcription factors that bind to their specific enhancer elements in the promoter region flanking the iNOS gene.

Amongst the 24 consensus sequences that have been identified in the promoter region of the murine iNOS gene (Xie & Nathan, 1994) are putative binding sites for transcription factors including NF-$\kappa$B and NFIL-6 whose activity can be stimulated by protein kinase(s) A, protein kinase C and protein tyrosine kinase (Shirakawa *et al.*, 1989; Ghosh & Baltimore, 1990; Metz & Ziff, 1991; Link *et al.*, 1992). Although the role of NFIL-6 still remains to be clarified, activation of NF-$\kappa$B seems to be a necessary event for the expression of iNOS in lipopolysaccharide-stimulated macrophages and in IL-1$\beta$-activated vascular smooth muscle cells (Xie *et al.*, 1993; 1994; Sherman *et al.*, 1993; Mülsch *et al.*, 1993; Schini-Kerth *et al.*, 1994b). Therefore, the stimulating effect of cyclic AMP on iNOS expression in vascular smooth muscle cells could occur at the level of NF-$\kappa$B activation. However, such a possibility is not supported by the present findings since the IL-1$\beta$-induced NF-$\kappa$B-DNA binding activity was potentiated neither by an activator of protein kinase(s) A nor by selective inhibitors of cyclic AMP-dependent phosphodiesterases.

Besides cyclic AMP elevating agents, NO-donors which stimulate the formation of cyclic GMP by activating soluble guanylyl cyclase, also potentiated the IL-1$\beta$-stimulated expression of iNOS protein and NO synthesis in vascular smooth muscle cells. A similar amplifying effect was observed with a membrane-permeable analogue of cyclic GMP, 8-bromo cyclic GMP, and with atrial, brain and C-type natriuretic peptides which stimulate the formation of cyclic GMP by activating the particulate guanylyl cyclase (Inoue *et al.*, 1995; Marumo *et al.*, 1995; present study). In addition, in the absence of IL-1$\beta$ high concentrations of SNP and 8-bromo cyclic GMP were able to induce the synthesis of NO in vascular smooth muscle cells (Inoue *et al.*, 1995; present findings). These findings suggest that intracellular levels of cyclic GMP also modulate the expression of iNOS gene in vascular smooth muscle cells. Most actions of cyclic GMP on vascular cells are due to the activation of its major intracellular target cyclic GMP-dependent protein kinase (Lincoln & Cornwell, 1993). Cyclic GMP may also affect vascular functions via interaction with the cyclic AMP signalling pathway, due to its ability to activate protein kinase(s) A at high concentrations which is closely related in structure and function to the cyclic GMP-dependent protein kinase (Forte *et al.*, 1992; Cornwell *et al.*, 1994a). Moreover cyclic GMP inhibits the activity of the vascular smooth muscle phosphodiesterase III which preferentially hydrolyzes cyclic AMP (Beavo & Reifsnyder, 1990). The expression of the cyclic GMP-dependent protein kinase decreases with increasing passage of rat aortic smooth muscle cells (Cornwell *et al.*, 1994b) whereas the activity of protein kinase(s) A is maintained in cultured vascular smooth muscle cells (Lincoln & Cornwell, 1993); hence the stimulating effect of cyclic GMP on iNOS expression (as observed here) could be due to an interaction with the cyclic AMP signalling pathway. This conclusion is supported by the findings that the expression of iNOS by SNP was abolished by a selective inhibitor of protein kinase(s) A and that the NO-donor and a selective activator of protein kinase(s) A or a specific inhibitor of phosphodiesterase IV synergistically increased the expression of iNOS. Such an assumption is also supported by the finding that NO-donors inhibit rather than potentiate the IL-1$\beta$-stimulated activation of NF-$\kappa$B in vascular smooth muscle cells. An inhibitory effect of NO on the redox-dependent NF-$\kappa$B activation has already been demonstrated in endothelial cells and may be attributed to the scavenging of $O_2^-$ by NO (Zeiher *et al.*, 1995). In addition, NO has also been shown to induce the expression and prolong the half-life of I$\kappa$B, the inhibitory subunit of NF-$\kappa$B (Peng *et al.*, 1995).

In conclusion the IL-1$\beta$-stimulated formation of cyclic AMP in vascular smooth muscle cells represents one step in the pathway linking cytokine receptor occupancy to the enhanced expression of the iNOS gene. The role of cyclic AMP as an intracellular transducing system is dependent on the activation of protein kinase(s) A and to the subsequent activation, in coordination with other signalling pathways, of transcription factors regulating the iNOS gene. Furthermore, the expression of iNOS by cytokines is amplified by cyclic GMP elevating agents, an effect which may involve the ability of cyclic GMP to interact with the cyclic AMP effector pathway. Such a positive regulatory effect of cyclic GMP would suggest that both endothelium-derived NO and atrial natriuretic peptides, the circulating levels of which are elevated in endotoxaemia (Schuller *et al.*, 1992), may contribute to excessive formation of NO by blood vessels in septic shock.

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