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Induction of nitric oxide synthase in cultured vascular smooth muscle cells: the role of cyclic AMP

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1 Interleukin-1β (IL-1β) is a potent stimulant of inducible nitric oxide synthase (iNOS) mRNA and nitric oxide (NO) production in vascular smooth muscle (VSM) cells in culture. These studies investigate the role of adenosine 3':5'-cyclic monophosphate (cyclic AMP) in this process.

2 Dibutyryl cyclic AMP (db cyclic AMP, 0.1–1 mM), forskolin (1–10 μM) and the phosphodiesterase inhibitor, Ro 20-1724 (1–10 μM), all of which increase intracellular cyclic AMP, had no effect on NO production when added alone but markedly enhanced NO production by IL-1β-stimulated VSM cells in a dose-dependent manner. Consistent with a cyclic AMP-mediated action, isoprenaline (1–10 μM) increased NO production from IL-1β-stimulated cells. Dibutyryl cyclic GMP (db cyclic GMP) had no effect at concentrations up to 1 mM.

3 Pursuing these observations, iNOS protein levels were examined by Western blot analysis and iNOS mRNA levels were measured by reverse transcription and amplification of the resultant cDNA using the polymerase chain reaction. In addition to enhancing NO production, db cyclic AMP increased iNOS protein and mRNA above that produced by IL-1β alone.

4 These data demonstrate a major effect of cyclic AMP on cytokine-induced NOS activity in VSM cells, mediated at least in part by regulating synthesis of iNOS, and has implications for the pathogenesis and management of septic shock.

Keywords: Nitric oxide; nitric oxide synthase; cyclic AMP; mRNA; polymerase chain reaction; vascular smooth muscle

Introduction

Nitric oxide (NO) is a potent vasodilator produced locally in the vessel wall from L-arginine by the action of nitric oxide synthase (NOS) (Moncada *et al.*, 1991). Two major subtypes of NOS have been described. One subtype is calcium-dependent, expressed constitutively in endothelium and produces picomol amounts of NO. A second form of NOS is calcium-insensitive and can be induced in endothelium and vascular smooth muscle cells by endotoxin and some cytokines to generate nanomol quantities of NO.

It is now clear that induction of NOS synthesis and markedly increased NO production play a primary role in the pathogenesis of septic shock. This condition is characterized by pronounced hypotension which is resistant to treatment with vasoconstrictor agents. In contrast, inhibition of NO production with the L-arginine analogue, L-monomethyl-arginine, has been shown to increase blood pressure in this condition (Petros *et al.*, 1991) and has indicated a novel therapeutic strategy. Reservations have been expressed, however, about the value of global inhibition of NO synthesis (Nava *et al.*, 1991) as NO released from other cells, such as macrophages, may be important in combating infection. Specific inhibition of iNOS activity in vascular tissues requires a better understanding of the factors governing the expression and function of this enzyme. Some progress has been made in elucidating factors regulating iNOS activity in endothelial, macrophage and neuronal cell lines (Nakane *et al.*, 1991; Brune & Lapetina, 1991; Gaillard *et al.*, 1991; 1992; Bredt *et al.*, 1992; Severn *et al.*, 1992; Marotta *et al.*, 1992; Hortelano *et al.*, 1992; 1993; Feinstein *et al.*, 1993). One factor that appears to play a role in regulating NO production is adenosine 3':5'-cyclic monophosphate (cyclic AMP) but the effect of increasing cyclic AMP levels appears to differ in different tissues such that it is not possible to extrapolate from these studies to VSM cells. The purpose of this study was to examine the effect of increasing cyclic AMP on cytokine-stimulated iNOS activity in vascular smooth muscle (VSM) cells.

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Methods

Isolation and primary culture of rat aortic smooth muscle cells

VSM cells were harvested from enzymatically dissociated rat thoracic aorta according to the method of Beasley *et al.* (1991) with some minor modifications. The thoracic aorta was excised under sterile conditions from male Wistar rats (300–350 g) and placed in ice cold Dulbecco's Phosphate Buffered Saline (PBS, Ca^{2+} and Mg^{2+}-free) with penicillin-streptomycin 200 u ml^{-1} and amphotericin B 25 μg ml^{-1}. The aorta was washed and transferred into a petri dish containing Hanks Balanced Salt Solution (HBSS). Fat, connective tissue, and adventitia were removed by blunt dissection. The cleaned aortae were opened and the endothelium carefully scraped off. Fine pieces of aorta were transferred into a 15 ml plastic centrifuge tube and incubated for 90 min at 37°C in HBSS containing $CaCl_2$ (0.2 mM), HEPES (15 mM), collagenase (type II-S, 1 mg ml^{-1}), elastase (type III, 0.125 mg ml^{-1}), and bovine serum albumin (2 mg ml^{-1}). The digested tissue was triturated 10 times through an 18-gauge needle, sieved through nylon mesh, and the resulting cell suspension centrifuged at 200 *g* for 5 min. The cells were resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 20% foetal calf serum, L-glutamine (2 mM), penicillin-streptomycin (50 μl ml^{-1}) and amphotericin B (2.5 μg ml^{-1}) and seeded into 25 cm^2 flasks. After reaching confluence, cells were passaged by harvesting with trypsin-EDTA, seeded at a ratio of 1:5 into 96-well plates, and grown in DMEM supplemented with 10% foetal calf serum, L-glutamine (2 mM), penicillin-streptomycin (50 u ml^{-1}) and amphotericin B (2.5 μg ml^{-1}). The cells were characterized as smooth muscle cells by morphology and immunostaining with antibodies to smooth muscle α-actin.

Experimental protocol and measurement of NO production

All studies were carried out on VSM cells obtained between the 4th and 10th passage. Cells were stimulated by incuba-

tion with interleukin-1β (IL-1β) at different concentrations for varying periods of time up to 48 h. In preliminary experiments we observed that IL-1β-stimulated nitrite production was reduced in the presence of foetal calf serum (Figure 1a). Foetal calf serum also stimulates mitosis. In order to maximise the nitrite signal and to study quiescent cells, all subsequent experiments were conducted in the absence of foetal calf serum. Thus, 24 h prior to study, the medium was changed to DMEM without phenol red containing 0.1% bovine serum albumin (low endotoxin) in place of foetal calf serum; each well contained 100 μl of culture medium.

NOS activity was assessed by measurement of nitrite production (Griess reaction) over a timed period according to the method of Zembowicz & Vane (1992) with some modifications. Supernatants (100 μl) were mixed with 100 μl of Griess reagent (1% sulphanilamide in 5% H_3PO_4 and 0.1% naphthylethylenediamine dihydrochloride in a ratio of 1:1). Following a 10 min incubation at room temperature, the absorbance was read at 540 nm in a Titertek Multiscan Plus MKII 96 well plate spectrophotometer. Standard curves were determined by use of known concentrations of sodium nitrite. Nitrite levels reflect NOS activity in this system since no other potential source of this ion exists.

This assay was used to examine the effect of a variety of agents on NOS activity. The agents used were selected on the basis of their known actions on protein kinases. Unless indicated, all experiments were carried out using IL-1β 10 ng ml^{-1} and were repeated on at least 4 occasions. Appropriate controls were included in each 96-well plate to overcome variation produced by differences in cell density. All wells in each experiment were judged microscopically to be of similar confluence.

Western blotting

VSM cells were cultured in 75 cm^2 culture flasks with serum-free DMEM without phenol red containing antibiotics, L-glutamine (2 mM) and 0.1% bovine serum albumin (fatty acid-free and low endotoxin) for 24 h and then stimulated with IL-1β (10 ng ml^{-1}) in the presence or absence of db cyclic AMP for periods of time up to 48 h. Culture supernatant was then removed, divided into aliquots and stored for the measurement of nitrite. The cells were washed with PBS (5 ml) 3 times and released by treatment with trypsin-EDTA (2 ml). The cells were then pelleted by centrifugation (500 *g*, 5 min), resuspended in PBS and recentrifuged. The final cell pellet was resuspended in 300 μl lysis buffer (Tris HCl, 50 mM, pH 7.4) containing pepstatin A (5 μg ml^{-1}), chymostatin (1 μg ml^{-1}), aprotinin (5 μg ml^{-1}), leupeptin (1 μg ml^{-1}), dithiothreitol (1 mM) and phenylmethylsulphonyl fluoride (100 μM) and then lysed by quick freeze and thaw 3 times. The lysate was centrifuged at 16,000 *g* for 10 min and the resultant clear supernatant stored at −70°C until further analysis.

Aliquots of cells lysate were used for protein assay (BCA protein assay reagent, Pierce, Illinois, U.S.A.) and Western blot analysis. VSM cell lysate containing 25 to 400 μg of protein was reduced and separated on 7.5% SDS–PAGE using prestained molecular weight markers (Sigma). Proteins were electroblotted in 20% methanol, Tris (25 mM), glycine (192 mM) pH 8.3 on nitrocellulose membranes (ECL-Hyperbond, Amersham International plc, Amersham, UK). The membranes were blocked with 4% low fat milk in PBS for 2 h, washed 3 times in PBS containing 0.05% BSA, 0.05% Tween-20, then incubated with rabbit anti-rat iNOS antibody (diluted 1:5,000 in PBS, 0.1% BSA, Riveros-Moreno *et al.*, 1993) for 2 h, washed and finally incubated with a 1:20,000 dilution of goat anti-rabbit IgG conjugated to horseradish peroxidase (ICN Flow, Oxford, UK) for 2 h. After successive washes as before, the immunocomplexes were developed using an enhanced horseradish peroxidase/luminol chemiluminescence reaction (ECL Western blotting detection reagents, Amersham International plc, Amersham, UK) and detected with X-ray film.

Extraction of total RNA from VSM cells and analysis of iNOS mRNA by reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from VSM cells according to the method of Chomczynski & Sacchi (1987). VSM cells cultured in 96-well plates were homogenized in 1.5 ml Eppendorf tubes using 0.5 ml of a solution containing guanidium isothiocyanate (4 M), sodium citrate (25 mM, pH 7.0), 0.5% N-lauroyl-sarcosinate and 2-mercaptoethanol (0.1 M). In order, homogenates were mixed thoroughly with 50 μl sodium acetate (2 M, pH 4.0), 0.5 ml water-saturated phenol and finally 0.2 ml chloroform/isoamyl alcohol (49:1 v/v). Homogenates were left on ice for 15 min and then centrifuged at 16,000 *g* for 15 min at 4°C. The upper aqueous layer was transferred to a new tube and RNA was precipitated twice with isopropanol. The RNA pellet was finally washed with 70% ice-cold ethanol, dried, and dissolved in 20 μl of nuclease-free water.

Quantitative RT-PCR was performed using a method which has previously been extensively validated (Nunez *et al.*, 1992). Two μl aliquots of total RNA were mixed with 25 pmol of a 15 mer random sequence primer before incubation for 2 h at 37°C with 10 u Moloney murine leukaemia virus reverse transcriptase (Pharmacia). The cDNA samples were then diluted with nuclease-free water to a volume of 100 μl and stored at −20°C. Both iNOS and the 'housekeeper' gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were measured in each sample. The primers used were as follows: iNOS, sense 5'-CCTACCAAGGTGACCTGAAAG and antisense 5'-TAATGAATTCAATGGCTTGA (Nunokawa *et al.*, 1993), GAPDH sense 5'-GGTGTGAAACACGAGAAATATGAC and antisense 5'-TTGCAGGCTGGGTCCTCATTGTCA (Fort *et al.*, 1985). PCR was performed using 2.5 μl of the diluted cDNA sample in a total reaction volume of 25 μl with 1 μM iNOS or GAPDH primers, 1 unit of Taq DNA polymerase (Promega Corporation, Madison, U.S.A.) and 0.5 μCi of [α-^{32}P]-dCTP (> 5000 Ci $mmol^{-1}$; Amersham International plc) (Brown *et al.*, 1993). Preliminary experiments were performed to ensure that the PCR was terminated during the exponential phase of amplification (data not shown). The amplification conditions were: 26 cycles for GAPDH, 36 cycles for iNOS; each cycle comprised 93°C for 30 s, 60°C for 30 s, 73°C for 1 min. An aliquot (15 μl) of each PCR reaction was then separated by electrophoresis through a 6% polyacrylamide gel. The gels were exposed to Kodak-X-Omat XAR5 autoradiography film to locate the specific product bands (of the expected size) on the gel. These were excised and the amount of ^{32}P incorporated quantified by liquid scintillation counting. The specificity of the iNOS band was verified by DNA sequencing using the deoxy chain termination method (Sanger *et al.*, 1977). The relative amounts of template cDNA at the start of the PCR was assessed by measuring the quantity of PCR product during the exponential phase of amplification. For each cDNA sample, the counts (in c.p.m.) incorporated into iNOS DNA were divided by the amount in the GAPDH band to correct for variation in the extraction of RNA and the efficiency of reverse transcription.

Materials

Interleukin-1β (IL-1β) was obtained from British Biotechnology (Oxford, UK), L-NMMA (N^G-monomethyl-L-arginine) and RO-20-1724 ([4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone]) from Calbiochem (Nottingham, UK). Dibutyryl cyclic AMP (db cyclic AMP) was a kind gift from Dr Une, Daiichi Pharmaceutical Co Ltd (Tokyo, Japan). Anti-rat iNOS antiserum was provided by Drs Riveros-Moreno and Moncada, Wellcome Reserach Laboratories (Beckenham,

Kent). All culture media, antibiotics, antifungal agents, trypsin and EDTA were obtained from ICN Flow (Oxford, UK). Plastics for culture were purchased from Falcon (Oxford, UK). Fetal calf serum was from Imperial laboratories (West Portway, UK). Unless stated otherwise, all reagents for RT-PCR and all other reagents were obtained from Sigma Ltd (Poole, UK).

Statistics

Where appropriate, data were analysed by ANOVA (Complete Statistical System, StatSoft software) and statistical significance assessed by Student's unpaired 2-tailed t test. Differences were considered statistically significant when the P-value was less than 0.05.

Results

Production of nitrite by VSM cells stimulated with IL-1β

Mean nitrite concentration in media from unstimulated cells, cultured in 96 well plates for 24 h and used in the studies described here, was $2.9 \pm 0.22\,\mu M$ ($n = 28$ plates). Exposure of cultured VSM cells to IL-1β increased nitrite concentration in the supernatant in both a dose- and time-dependent manner (Figure 1a and b). Mean nitrite concentration in media from VSM cells cultured for 24 h with IL-1β (10 ng ml^{-1}) was $24.2 \pm 2.6\,\mu M$ ($n = 28$ plates). The variation between plates was largely due to differences in the density of plated cells; therefore for all experiments described, each 96 well plate contained controls appropriate to the treatment examined.

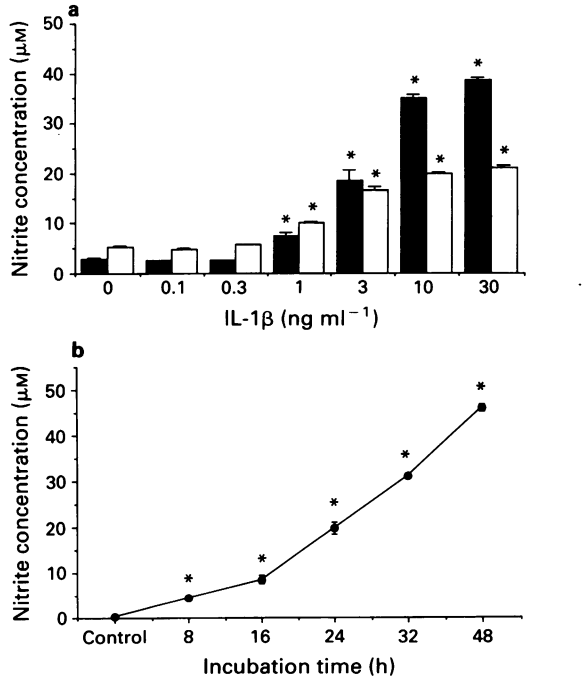

Figure 1 (a) Incubation of vascular smooth muscle (VSM) cells with interleukin-1β (IL-1β) for 24 h results in a dose-dependent increase in nitrite concentration in the culture medium. This response was greater in the presence of 0.1% BSA (solid columns) than with 10% foetal calf serum (open columns). Data are mean ± s.e.mean of 4 wells. *$P<0.01$, IL-1β-treated cells relative to cells not exposed to this cytokine. (b) Increase in nitrite concentration in culture medium with time during incubation of VSM cells with IL-1β (10 ng ml^{-1}). Control represents cells cultured for 24 h without IL-1β. Data are mean ± s.e.mean of 4 wells at each time point. *$P<0.01$, IL-1β-treated cells relative to control cells.

Accumulation of nitrite following incubation of cells with IL-1β (10 ng ml^{-1}) was inhibited by cycloheximide at all concentrations and by L-NMMA in a dose-dependent manner (Figure 2). Consistent with other reports (Szabo *et al.*, 1993), dexamethasone also had a significant inhibitory effect at 10 μM (results not shown).

Effect of agents that increase cyclic AMP levels

Once the cellular response to IL-1β was characterized, we studied the effect of a variety of agents that act to increase intracellular cyclic AMP levels. Specifically, we examined the effect of direct addition of cyclic AMP using the stable analogue, db cyclic AMP; stimulating adenylyl cyclase (e.g. with forskolin, isoprenaline); and inhibiting cyclic AMP phosphodiesterase (e.g. with Ro 20-1724). These observations were compared with the effect of increasing intracellular cyclic GMP levels by the addition of db cyclic GMP.

db cyclic AMP increased nitrite levels in a dose-dependent manner (0.01 – 1 mM) when co-incubated with IL-1β (10 ng ml^{-1}) for 24 h, in contrast to db cyclic GMP which had no effect (Figure 3). The mean increase in nitrite levels with

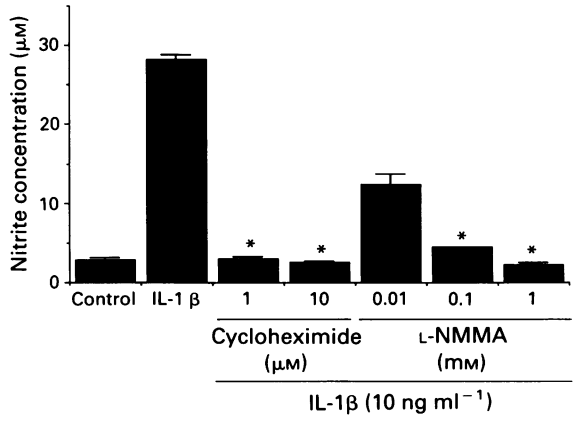

Figure 2 Effect of cycloheximide and the L-arginine analogue, N^G-monomethyl-L-arginine (L-NMMA), on nitrite concentration in culture medium during incubation of VSM cells with interleukin-1β (IL-1β, 10 ng ml^{-1}) for 24 h. Data are mean ± s.e.mean of 4 wells. *$P<0.01$ compared to IL-1β alone.

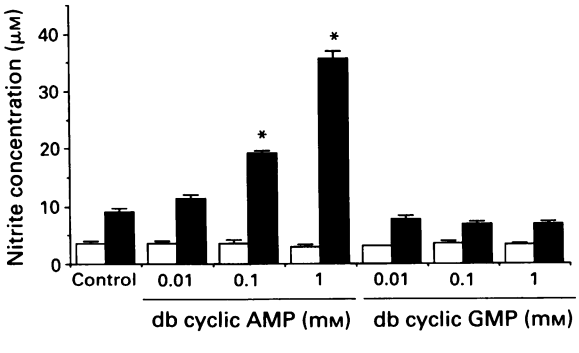

Figure 3 Effects of db cyclic AMP (0.01 – 1 mM) and db cyclic GMP (0.01 – 1 mM) on nitrite concentration in culture medium from VSM cells incubated in the presence (solid columns) and absence (open columns) of interleukin-1β (IL-1β, 10 ng ml^{-1}) for 24 h. The cyclic nucleotides were present throughout the 24 h incubation period. Increasing cyclic AMP levels with db cyclic AMP increased nitrite concentration in the presence of IL-1β above that produced by the cytokine alone (control, solid). In contrast, db cyclic GMP did not affect the response to IL-1β. Neither db cyclic AMP nor db cyclic GMP had any measurable effect on nitrite production in the absence of IL-1β. Data are mean ± s.e.mean of 4 wells. *$P<0.01$ compared to control cells incubated with IL-1β alone.

IL-1β (10 ng ml^{-1}) plus db cyclic AMP (1 mM) over that produced by IL-1β (10 ng ml^{-1}) alone was 393 ± 71% ($n = 8$ plates). Further experiments showed that potentiation of IL-1β induced elevation of nitrite levels could be produced by short, early exposure of the cells to db cyclic AMP i.e. for the first 4 h of a 28 h incubation with IL-1β (Figure 4a). To examine the effects of cyclic AMP on the activity of the induced enzyme, VSM cells were incubated with IL-1β for 20 h, then washed and incubated with media containing db cyclic AMP (1 mM) for 8 h; the final nitrite concentration in the media was not significantly different from that in media from cells similarly treated but incubated in the absence of db cyclic AMP (Figure 4b). These data support a role for cyclic AMP in the regulation of iNOS induction rather than the activity of the enzyme itself.

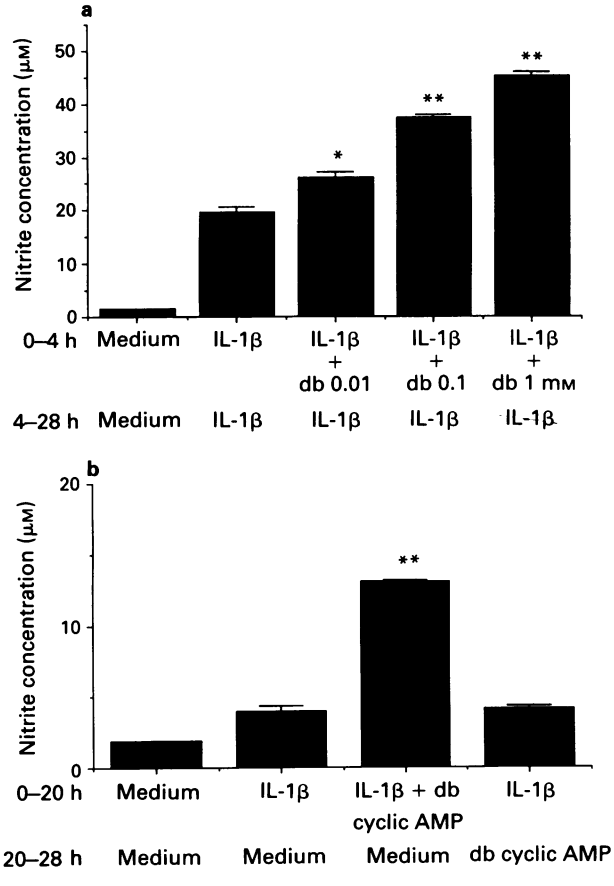

Figure 4 (a) VSM cells were incubated with interleukin-1β (IL-1β, 10 ng ml^{-1}) in the presence and absence of db cyclic AMP (0.01–1 mM) for 4 h; cells were then washed and incubated with IL-1β (10 ng ml^{-1}) alone for a further 24 h. Control cells were incubated without cytokine or cyclic nucleotide for 4 + 24 h. Nitrite concentration therefore represents nitrite accumulation in culture media during the last 24 h incubation period. Co-incubation with db cyclic AMP resulted in a dose-dependent increase in nitrite concentration in the medium above that produced by the cytokine alone. Data are mean ± s.e.mean of 4 wells and are representative of 4 experiments. *$P < 0.01$, **$P < 0.001$ compared to control cells incubated with IL-1β alone. (b) VSM cells were incubated with IL-1β (10 ng ml^{-1}) in the presence or absence of AMP (1 mM) for 20 h; cells were then washed and cultured for a further 8 h without IL-1β. One group of wells previously exposed to IL-1β only for 0–20 h received db cyclic AMP (1 mM) during last 8 h incubation (20–28 h). Control cells were incubated without cytokine or cyclic nucleotide for 20 + 8 h. Nitrite concentration reflects nitrite accumulation in the media during the last 8 h incubation period. The presence of db cyclic AMP during the first 20 h incubation period but not the subsequent 8 h period enhanced nitrite levels above that produced by the effect of the cytokine alone. Data are mean ± s.e.mean of 4 wells and are representative of 4 experiments. *$P < 0.01$, **$P < 0.001$ compared to control cells incubated with IL-1β alone.

Forskolin (0.1–10 μM) and Ro 20-1724 (1–10 μM) had no detectable effect on nitrite levels in the absence of IL-1β but enhanced the production of nitrite in response to IL-1β in a dose-dependent manner (Figure 5a). The mean increase in nitrite concentration with IL-1β (10 ng ml^{-1}) and forskolin (10 μM) above that produced by the cytokine alone was 259 ± 48% ($n = 5$ plates). An inactive derivative of forskolin, 1,9-dideoxyforskolin (0.1–10 μM), had no effect on IL-1β-stimulated nitrite production. The Ro 20-1724 potentiated the effect of forskolin on IL-1β stimulated nitrite production (Figure 5a), in keeping with cyclic AMP as the common mediator of this effect.

To pursue these observations further, the effect of receptor-mediated changes in intracellular cyclic nucleotide levels on nitrite levels was examined. The β-adrenoceptor agonist, isoprenaline which elevates cyclic AMP, enhanced nitrite production by IL-1β-stimulated VSM cells dose-dependently (Figure 5b) but had no detectable effect in the absence of the cytokine. The mean increase in nitrite levels with IL-1β (10 ng ml^{-1}) and isoprenaline (10 μM) above that produced by the cytokine alone was 142 ± 15% ($n = 4$ plates). Ro 20-1724 increased further the effect of isoprenaline on IL-1β-stimulated nitrite production (Figure 5b). The α-adrenoceptor agonist, phenylephrine, had no effect on nitrite levels with or without IL-1β (data not shown).

Measurement of iNOS protein levels by Western blotting

Incubation of VSM cells with IL-1β (10 ng ml^{-1}) produced a single band (~130 kDa) on immunoblotting with anti-iNOS antibody, consistent with iNOS protein. This band was not detected in control cells (cultured in the absence of cytokine) and increased in a time-dependent manner during culture with IL-1β from 0–48 h (Figure 6a). Co-incubation of IL-1β (10 ng ml^{-1}) and db cyclic AMP (1 mM) increased the density of staining with anti-iNOS antibody at any given protein concentration compared to IL-1β alone, while db cyclic AMP alone produced no detectable band (Figure 6b). These data

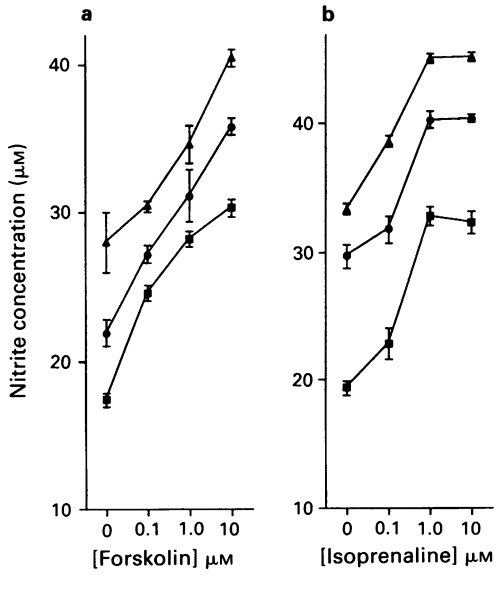

Figure 5 Effect of 2 doses of Ro 20-1724 (●, 1 μM and ▼, 10 μM), a cyclic AMP phosphodiesterase inhibitor, on nitrite levels in media from VSM cells incubated with interleukin-1β (IL-1β, 10 ng ml^{-1}) for 24 h together with increasing doses of (a) forskolin (0.1–10 μM) or (b) isoprenaline (0.1–10 μM): (■) represents cells incubated without Ro 20-1724. Forskolin, isoprenaline and Ro 20-1724 alone caused dose-dependent enhancement of IL-1β-induced nitrite production. Both doses of Ro 20-1724 increased significantly the effect of forskolin or isoprenaline on IL-1β-induced nitrite production ($P < 0.05$, ANOVA). Data are mean ± s.e.mean of 4 wells and are representative of 4 experiments.

are consistent with cyclic AMP enhancing IL-1β-induced iNOS protein synthesis.

Measurement of iNOS mRNA in VSM cells

In unstimulated VSM cells, iNOS mRNA was not detectable after 36 cycles of PCR. However, after incubation with IL-1β (10 ng ml^{-1}), iNOS mRNA levels were readily detectable at 3 h and continued to increase up to 48 h (data not shown). Consistent with the effect of cyclic AMP on IL-1β-stimulated nitrite production, incubation of VSM cells for 4 h with IL-1β and db cyclic AMP (1 mM) markedly enhanced mRNA levels above that produced by IL-1β alone (Figure 7). IL-1β did not affect the prevalence of GADPH mRNA.

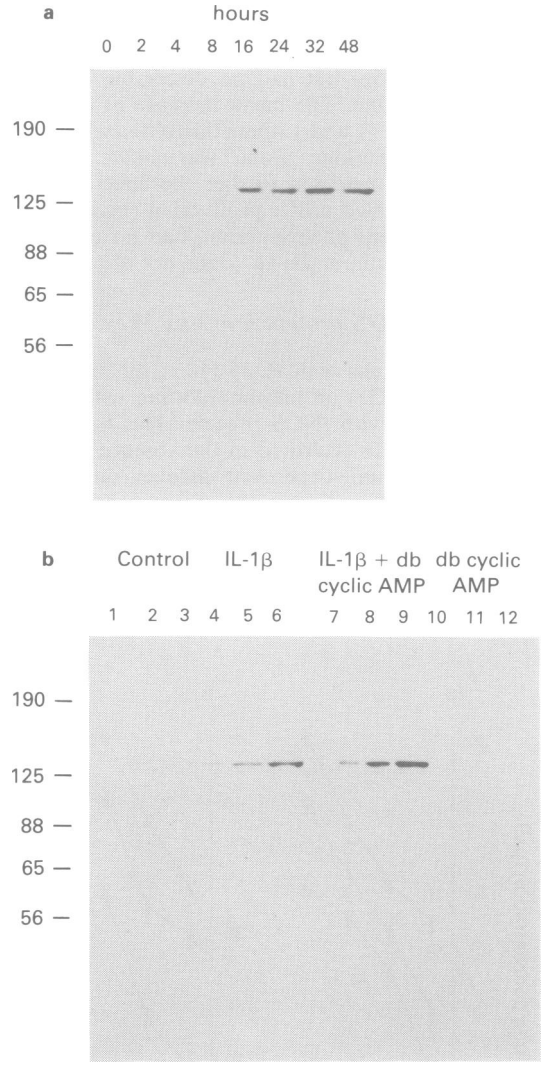

Figure 6 Western blot analysis of iNOS in VSM cells. Supernatant fraction of cell lysate was separated by SDS–PAGE, transferred to nitrocellulose and blotted with anti-iNOS antibody. A single band was seen at ~130 kDa, of appropriate molecular weight for iNOS protein. (a) Time-dependent increase in iNOS protein in VSM cells incubated with interleukin-1β (IL-1β, 10 ng ml^{-1}) for 0, 2, 4, 8, 16, 24, 32 and 48 h (lanes 1–8 respectively, 100 μg protein per lane). (b) Enhancement of IL-1β-induced increase in iNOS protein levels by db cyclic AMP. VSM cells were incubated with media alone (lane 1, 2), IL-1β (10 ng ml^{-1}, lanes 3–6), IL-1β plus db cyclic AMP (1 mM, lanes 7–9), or db cyclic AMP alone (1 mM, lanes 10–12) for 24 h. Different amounts of protein were subjected to SDS–PAGE (25 μg, lanes 3, 7; 50 μg, lanes 4, 8; 100 μg, lane 1, 5, 9, 10; 200 μg, lanes 2, 6, 11; 400 μg, lane 12) to enable semi-quantitation of iNOS protein. iNOS was readily detected in the 25 μg of protein of cells incubated with IL-1β plus db cyclic AMP (lane 7) but required 100 μg of protein from cell incubated with IL-1β alone (lane 5), consistent with db cyclic AMP enhancing iNOS protein synthesis. Data are representative of 3 experiments.

Discussion

Several groups have reported that IL-1β is a powerful stimulant of NO production in VSM cells in culture (Busse & Mulsch, 1990; Beasley *et al.*, 1991; Nakayama *et al.*, 1992; Scott-Burden *et al.*, 1992). This effect is thought to be mediated largely by increased synthesis of NOS. In support of this we have shown that exposure to IL-1β is accompanied by increased iNOS protein and mRNA levels and that the effect of this cytokine on NO production is inhibited by cycloheximide.

More importantly, however, these studies demonstrate a clear effect of cyclic AMP in IL-1β-stimulated NO production in VSM cells. Increasing intracellular cyclic AMP concentration directly by the addition of db cyclic AMP produced a marked shift to the left of the dose-response curve for IL-1β. Similarly, indirect elevation of cyclic AMP levels with forskolin, a phosphodiesterase inhibitor and isoprenaline augmented significantly the production of NO at a given concentration of IL-1β. Insight into some of the mechanisms underlying this effect is provided by the measurements of iNOS protein and mRNA levels. The effects of db cyclic AMP on IL-1β-stimulated NO production were accompanied by a further increase in iNOS protein levels and

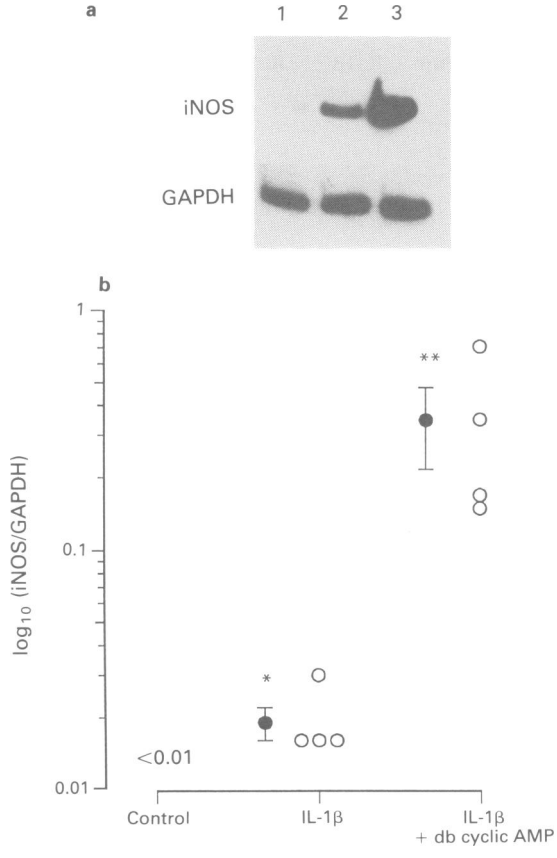

Figure 7 Measurement of mRNA levels for iNOS and GAPDH by RT-PCR in VSM cells cultured for 4 h without interleukin-1β (IL-1β) (1) control, with IL-1β 10 ng ml^{-1} (2) and with IL-1β 10 ng ml^{-1} plus db cyclic AMP 1 mM (3). (a) Inducible NOS mRNA was not detected at 36 cycles of PCR in control cells. IL-1β increased iNOS mRNA levels and this was increased further in the presence of db cyclic AMP. GAPDH measured at 26 cycles did not increase with IL-1β with or without db cyclic AMP. (b) $\log_{10}$ ratio of iNOS to GAPDH mRNA levels. Data (●) are mean ± s.e.mean for 4 experiments. *$P<0.01$ compared to control; **$P<$ compared to IL-1β.

mRNA transcripts for iNOS, above that produced by IL-1β alone. This would suggest that cyclic AMP acts at least in part by influencing iNOS synthesis by altering the steady-state level of iNOS mRNA (but whether this is by increasing transcription or reducing degradation rate is unknown). Since submission of this paper Koide *et al.* (1993) have shown that cyclic AMP-elevating agents such as forskolin and prostaglandin E_1 enhance NO production by interferon-γ, IL-1β and tumour necrosis factor in VSM cells at the level of iNOS mRNA expression, findings in agreement with our own results.

Cyclic AMP can regulate the expression of a number of genes through a conserved cyclic AMP-response element, CRE (Yamamoto *et al.*, 1988). The CRE is a palindromic octanucleotide (TGACGTCA) which binds a number of structurally related proteins constituting the ATF/CREB family. These proteins usually bind as homodimers and can exert both positive and negative effects on transcription (Yamamoto *et al.*, 1988; Karpinski *et al.*, 1992). Evidence for the existence of CRE domains in association with the iNOS gene is currently lacking (there is no CRE sequence present in the 5'-flanking region of mouse iNOS, Xie *et al.*, 1993). The absence of an effect of db cyclic AMP alone on nitrite production suggests, however, that if CRE-dependent transcription is operating in our system then this must be conditional on the activation of the numerous cytokine-response elements which have been identified in the iNOS promoter region (Xie *et al.*, 1993). The interactions are complex and appear to vary between different cell types. Thus, isoprenaline, 8-bromo cyclic AMP, db cyclic AMP and PGE_2 have been reported to potentiate cytokine-stimulated NO production in endothelial cells and Kupffer cells (Gaillard *et al.*, 1992; Durieu-Trautmann *et al.*, 1993), whereas db cyclic AMP inhibits this effect in macrophage and astroglial cells (Marotta *et al.*, 1992; Feinstein *et al.*, 1993). Although species differences may contribute to these apparent discrepancies, it is also possible that there are differences between tissues in the regulation of inducible NOS synthase activity which it may be possible to exploit therapeutically.

Our data show that cyclic AMP enhances IL-1β-stimulated elevation of iNOS protein and mRNA levels but this does not exclude the possibility that cyclic AMP-mediated phosphorylation of iNOS protein itself may also be involved in regulating its activity. Both the constitutive and inducible forms of NOS exhibit sites for cyclic AMP-dependent phosphorylation (Dinermann *et al.*, 1993). Against this, addition of db cyclic AMP to VSM cells in which synthesis of iNOS had already been induced (by incubation with IL-1β for 20 h) did not increase NO levels above that produced by IL-1β alone, suggesting little effect of cyclic AMP on the activity of the enzyme. It is noteworthy that phosphorylation of purified constitutive NOS from brain or the recombinant enzyme in transfected cells by protein kinase A does not alter its activity (Brune & Lapetina, 1991; Bredt *et al.*, 1992). Phosphorylation by other protein kinases may regulate iNOS activity although the effect of phosphorylation by protein kinase C remains unclear; one study has reported activation of protein kinase C decreases (Bredt *et al.*, 1992) and another that it increases (Nakane *et al.*, 1991) iNOS activity. Recent studies have shown that phosphorylation of constitutive NOS affects the site of action of this enzyme, as phosphorylation influences translocation of the enzyme from membrane to cytosol (Michel *et al.*, 1993).

In summary, induction of NOS activity in VSM cells by IL-1β is enhanced by cyclic AMP. Moreover, the effect of cyclic AMP is mediated, at least in part, by an increase in iNOS protein levels and mRNA prevalence. These data have implications for the pathogenesis and treatment of septic shock. Specifically, endogenous factors such as adrenaline that elevate cyclic AMP and are increased in septic shock may facilitate the production of NO and hypotension. Similarly, therapeutic agents, for example dobutamine, that act by increasing cyclic AMP may be best avoided in the management of septic shock.

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