British Journal of Pharmacology (1996) 118, 1359-1366 

# Downregulation of nitrovasodilator-induced cyclic GMP accumulation in cells exposed to endotoxin or interleukin-1β

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1 Induction of nitric oxide synthase (iNOS) results in overproduction of nitric oxide (NO), which may be a principal cause of the massive vasodilatation and hypotension observed in septic shock. Since NO-induced vasorelaxation is mediated via the soluble isoform of guanylate cyclase (sGC), the regulation of sGC activity during shock is of obvious importance, but yet poorly understood. The aim of the present study was to investigate the activation of sGC by sodium nitroprusside (SNP) before and after exposure of rat aortic smooth muscle cells to endotoxin (LPS) or interleukin-1β (IL-1β).

2 Exposure of rat aortic smooth muscle cells to SNP (10 μM) elicited up to 200 fold increases in cyclic GMP. This effect was attenuated by 30–70% in IL-1β- or LPS-pretreated cells, in a pretreatment time- and IL-1β- or LPS-concentration-dependent manner. When, however, cells were exposed to IL-1β or LPS and then stimulated with the particulate guanylate cyclase activator, atriopeptin II, no reduction in cyclic GMP accumulation was observed.

3 Pretreatment of rats with LPS (5 mg $kg^{-1}$, i.v.) for 6 h led to a decrease in aortic ring SNP-induced cyclic GMP accumulation.

4 The IL-1β-induced reduction in SNP-stimulated cyclic GMP accumulation in cultured cells was dependent on NO production, as arginine depletion abolished the downregulation of cyclic GMP accumulation in response to SNP.

5 Reverse-transcriptase-polymerase chain reaction analysis revealed that the ratio of steady state mRNA for the $\alpha_1$ subunit of sGC to glyceraldehyde phosphate dehydrogenase was decreased in LPS- or IL-1β-treated cells, as compared to vehicle-treated cells.

6 Protein levels of the $\alpha_1$ sGC subunit remained unaltered upon exposure to LPS or IL-1β, suggesting that the early decreased cyclic GMP accumulation in IL-1β- or LPS-pretreated cells was probably due to reduced sGC activation. Thus, the observed decreased responsiveness of sGC to NO stimulation following cytokine or LPS challenge may represent an important homeostatic mechanism to offset the extensive vasodilatation seen in sepsis.

**Keywords:** Endotoxin; interleukin-1; guanylate cyclase; cyclic GMP; smooth muscle; polymerase chain reaction; sodium nitroprusside

## Introduction

Furchgott & Zawadski (1980) first described the obligatory role of endothelium in eliciting vascular relaxation in response to acetylcholine (ACh) and proposed the release of a humoral substance termed endothelium-derived relaxing factor. This was later shown to share a common mechanism of action with nitric oxide (NO) and organic nitrates, i.e., by activating soluble guanylate cyclase (sGC) and elevating guanosine 3':5'-cyclic monophosphate (cyclic GMP) levels in the vascular smooth muscle (Katsuki *et al.*, 1977; Holzmann, 1982). The source of endothelial NO generation was found to be one of the two chemically equivalent terminal guanidino nitrogen atoms of L-arginine (Palmer *et al.*, 1988). Today three isoforms of the NO synthase (NOS) have been identified in a variety of tissues. Type I NOS is soluble, constitutively expressed in neurones and has a molecular weight of 155 kDa (Bredt *et al.*, 1991). Endothelial cells constitutively express a particulate (type III) isoform which is shorter (135 kDa) than the one expressed by neuronal cells (Janssens *et al.*, 1992). Finally, macrophages, as well as other cell types including smooth muscle cells, express an inducible isoform, (type II NOS or iNOS) upon stimulation with cytokines and bacterial lipopolysaccharide (LPS; Xie *et al.*, 1992).

In the normal vascular wall, NOS activity is localized in the endothelium, with NO being released both under basal conditions and following increases in intracellular calcium brought about by stimulation with calcium ionophores or agonists, such as ACh, bradykinin or substance P (Moncada *et al.*, 1991). NO has been shown to play a key role in vascular homeostasis by contributing to the regulation of vascular tone, the inhibition of smooth muscle proliferation and the antithrombogenicity of the vascular wall (Moncada *et al.*, 1991). Inhibition of basally released NO in animals leads to elevation in blood pressure and increased adherence of blood elements to the vascular wall (Aisaka *et al.*, 1989; Kubes *et al.*, 1991). While underproduction of NO has been associated with a number of pathological conditions, including hypertension (Lüscher *et al.*, 1991) and atherosclerosis (Harrison *et al.*, 1991), overproduction can also be detrimental. In vascular smooth muscle cells, inflammatory mediators, such as interleukin-1, tumour necrosis factor-α and LPS, activate a pathway that leads to transcription of a gene coding for the iNOS through *de novo* protein synthesis (Busse & Mulsch, 1991). Since calmodulin is tightly bound to the iNOS, this enzyme is maximally active at all times producing large amounts of NO, as opposed to the relatively low quantities released by the cNOS (Nathan, 1992). Appearance of the iNOS is accompanied by impairment in contractility, activation of sGC and cyclic GMP accumulation and elevation of nitrites and nitrates in the serum of animal models of septic shock, as well as in patients with sepsis (Kilbourne *et al.*, 1990; Moncada *et al.*, 1991; Nava *et al.*, 1991; Nathan, 1992; Marczin *et al.*, 1993b). Although inhibitors of NO synthesis have been shown to in-

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hibit the development of hypotension in animals and man, it is still unclear whether complete inhibition of NO synthesis will be of therapeutic benefit (Kilbourne *et al.*, 1990; Nava *et al.*, 1991).

In spite of the abundance of information on the action of cytokines and LPS on iNOS activity and NO production, relatively little is known about the effects of these agents on sGC. Since sGC mediates the vasodilator action of NO in vascular smooth muscle, the severity of NO-induced hypotension during septic shock strongly depends on both the amount of NO produced and on sGC activity. In the present study, the effects of exposure of cultured cells or conscious animals to LPS or IL-1β on sGC were investigated.

## Methods

### *Isolation and culture of smooth muscle cells*

Rat aortic smooth muscle cells were isolated from 325–350 g male Wistar rats by previously published procedures (Geisterfer *et al.*, 1988) and 4–5 rats per isolation. Cells were positively identified as smooth muscle cells by indirect immunofluorescent staining for α-actin, using mouse anti-α-actin antibody and anti-mouse IgG FITC conjugate. Smooth muscle cells were grown in T-75 tissue culture flasks in 50% F12 and 50% Dulbecco's Modified Eagle Medium supplemented with 10% foetal bovine serum, 0.2 g $l^{-1}$ L-glutamine, penicillin (100 u $ml^{-1}$) and streptomycin (0.1 mg $ml^{-1}$). For the present experiments, cells between passages 1–5 were used.

### *Radioimmunoassay for cyclic GMP*

The radioligand ([$^{125}$I]-succinyl cyclic GMP-tyrosine methyl ester) was prepared in our laboratory and, using a monoclonal antibody for cyclic GMP, radioimmunoassay was performed in the Gammaflo automated RIA system, as previously described (Papapetropoulos *et al.*, 1995).

### *Determination of intracellular cyclic GMP in cultured cells*

The experimental protocol consisted of a pre- and a post-treatment period. The pretreatment served to expose the cells to LPS or IL-1β and was followed by a 15 min post-treatment period to assess either NO production or guanylate cyclase activity, using cyclic GMP accumulation as an index. Measurement of intracellular cyclic GMP in the presence of IBMX, while not synonymous with sGC activity, is considered to be an accurate index of sGC activity since, in intact cells, substrate and cofactor availability are not limiting factors. To determine cyclic GMP accumulation as an index of guanylate cyclase activity, cells were washed at the end of the pretreatment period with Earle's balanced salt solution (ES) and incubated for 30 min with 100 μM NG-nitro-L-arginine methylester (L-NAME), to inhibit cyclic GMP accumulation due to induction of NOS; cells were then stimulated with ES containing SNP (0.01–10 μM) or ANF (1–1000 nM) for 15 min in the presence of IBMX (0.3 mM) to prevent cyclic GMP breakdown unless otherwise noted. To determine cyclic GMP accumulation, as an index of NO production from cytokine- or LPS-induced NOS, no L-NAME was used; post-treatment in this case consisted of a 15 min exposure to IBMX + arginine (1 mM). For either case, after the 15 min post-treatment period, medium was rapidly aspirated, and 500 μl of 0.1 N HCl was added to each well to stop enzymatic reactions and to extract cyclic GMP. Thirty minutes later, the HCl extract was collected and cell remnants removed from the wells with 0.5 ml hot 1.0 N NaOH and scraping the well with a rubber policeman. The HCl extract was analysed for cyclic GMP by radioimmunoassay and NaOH-solubilized samples were used for protein determination.

### *Cyclic GMP determination in aortic rings*

Under anaesthesia (55 mg $kg^{-1}$ pentobarbitone sodium, i.p.) rats were equipped with indwelling venous catheters in the femoral vein for LPS administration. One to two days later, conscious rats were used. The control group consisted of untreated, non-operated animals, since initial experiments showed that the surgical procedure and anaesthetics used did not affect cyclic GMP accumulation in response to SNP. LPS was administered at a dose of 5 mg $kg^{-1}$, i.v. and after 6 h, animals were killed and thoracic aortae harvested. The endothelium was removed by inserting a glass rod through the lumen of the aorta; aortae were then flushed to remove blood, cleaned of periadventitial fat and cut into rings (3–4 mm long). Rings were blotted to remove excess fluid, weighed and placed in ES in the presence or absence of 50 μM L-NAME. After the 30 min period, rings were stimulated with 10 μM SNP in the presence of IBMX for 15 min and were then placed in 500 μl 0.1 N HCl to extract the cyclic GMP.

### *Protein determination*

Protein content of the supernatant of the centrifuged (2,000 r.p.m. for 5 min at room temperature) NaOH solubilized samples was measured by the Bradford (1976) method.

### *Determination of protein bound and non-protein bound sulphydryls*

Sulphydryl concentration in trypsinized VSMC was determined by the Ellmann method (Sedlak & Lindsay, 1968). Reduced glutathione, in TCA or SDS-EDTA, was used to construct standard curves for non-protein and protein thiols, respectively.

### *Reverse transcriptase-polymerase chain reaction (RT-PCR)*

Using published sequences, primers for the sGC $\alpha_1$ (forward, base position 1071 5'GAAATCTTCAAGGGTTATG3' and reverse, base position 1896 5'CACAAAGCCAGGACAGTC3'), and glyceraldehyde phosphate dehydrogenase (GAPDH; forward, base position 35 5'TGAAGGTCGGTGTCAACGGATTTGGC3' and reverse, base position 1017 5'CATGTAGGCCATGAGGTCCACCAC3') were synthesized on an automated DNA synthesizer using phosphoramidite chemistry. RNA was reverse transcribed and amplified using a commercially available kit (GeneAmp RNA PCR kit) in a DNA Thermal Cycler 480 (Perkin Elmer). RNA samples were precipitated and resuspended in 10 mM Tris, 10 mM NaCl, 10 mM EDTA, pH 8.0, and incubated with 3 u RNAse-free DNAse per 35 μg total RNA, for 30 min at 37°C to digest traces of genomic DNA. GAPDH (50 ng) or 200 ng ($\alpha_1$) total RNA were combined with 50 u Moloney murine leukaemia virus reverse transcriptase (RT) in 5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, 1 mM dNTPs, 10 u RNAse inhibitor and 2.5 μM random hexamers to prime the cDNA formation, in a reaction volume of 20 μl, for 15 min at 42°C. Samples were then heated for 5 min at 99°C to destroy RT activity before the PCR reaction. cDNA was amplified for 28 cycles 92°C for 1 min, 58°C for 1.5 min and 72°C for 3 min (melting, annealing and extension temperatures, respectively), as previously determined (Papapetropoulos *et al.*, 1995). For the PCR reaction, primers were used at 1 μM for the $\alpha_1$ subunit and 0.2 μM for GAPDH. $MgCl_2$ concentration was 2 mM and 2.5 u of polymerase was used per reaction. After the amplification, 10 μl of the PCR reaction mixture was electrophoresed on 0.9% agarose gels stained with ethidium bromide, visualized on a u.v. transilluminator and photographed. A molecular weight standard consisting of 100 base pair increments between 100–2600 base pairs (Pharmacia, LKB Biotechnology) was used to confirm the predicted PCR product size.

*H.p.l.c. quantification of RT-PCR products*

PCR reaction mixture (5.25 μl) was used for h.p.l.c. analysis (Wei *et al.*, 1995) in a Bio-Rad model 1350 HPLC system with an on line 1706 u.v.-visible photometer, driven by a Bio-Rad Series 800, HRLC software package to control flow rate, gradient production and calculate the peak area based on the u.v. absorbance (260 nm). The mobile phase consisted of two buffers: A (1 M NaCl in 25 mM Tris-HCl, pH 9.0) and B (25 mM Tris-HCl, pH 9.0). Flow through the Perkin Elmer TSK DEAE-NPR column was set at 1 ml $min^{-1}$. The gradient employed was 35% A in B for 2 min, 35–54% A for 0.1 min, 54–60% A for 2.9 min, 60–75% A for 1 min, 75–100% A for 2 min and 100–35% A for 0.1 min. The relative amounts of RT–PCR products were calculated as the area under the curve of the absorbance at 260 nm in arbitrary units and are presented as the ratio $\alpha_1$/GAPDH.

*Immunoblotting*

Rat aortic smooth muscle cells were cultured in 60 mm dishes, pretreated and lysed in lysis buffer (1%NP40, 150 mM NaCl, 20 mM HEPES, 1 mM EDTA, 1% aprotinin and 1 mM PMSF, pH 7.0). Cell lysates were centrifuged at 20,000 r.p.m., the supernatant fraction was collected and protein concentration measured by the Bradford method: 35 μg per lane were electrophoresed in SDS–7.5% polyacrylamide gels and transferred to a PVDF membrane at 60 V for 1.5 h at 4°C in a buffer containing 25 mM Tris and 700 mM glycine. Membranes were incubated overnight at 4°C with 5% dried milk in buffer containing 0.1% (v/v) Tween 20 in TBS (TTBS) to block nonspecific binding. The following day, membranes were incubated with 1:750 of a monoclonal antibody ($H_6$) against the $\alpha_1$ subunit of sGC (Lewicki *et al.*, 1983) in 5% milk in TTBS with 1 M glucose and 10% glycerol, for 1 h at room temperature, washed three times with TTBS for 20 min each time, blocked for an additional hour with 5% milk in TTBS and finally incubated for 1 h with a horseradish peroxidase conjugated anti-mouse IgG (1:10,000 dilution, Bio-Rad). Immunoreactive protein bands were visualized by the ECL system after 15 min exposure to X-ray film. To check for equality in loading and transfer, membranes were subsequently incubated with a monoclonal antibody against tubulin and immunoreactive bands visualized after exposure to X-ray film for 30 s.

*Materials*

Rats were purchased from Harlan, Sprague-Dawley, Inc. Indianapolis, IN, U.S.A. Tissue culture plasticware was from Corning Glass Inc., Corning, N.Y., U.S.A., growth medium was from GIBCO Laboratories, Grand Island, NY, U.S.A., and foetal calf serum from Hyclone Laboratories Inc., Logan, Utah, U.S.A. $^{125}I$ was from Du Pont, NEN, Boston, MA, U.S.A. RNAzol from Biotecx Laboratories Inc, Houston, TX, U.S.A., the GeneAmp RNA PCR kit from Perkin Elmer, Norwalk, CT, U.S.A., and the ECL detection system from Amersham International, Buckinghamshire, England. IL-1β was purchased from Boehringer Mannheim. Protein binding dye, PVDF membranes, dry milk, Tween 20 and the other immunoblotting reagents were from Bio-Rad, Richmond, CA, U.S.A. RNAse-free DNAse was from GIBCO. X-ray film was from Kodak and all other chemicals, including LPS, penicillin, streptomycin, succinyl tyrosine cyclic GMP methyl ester, IBMX, SNP, bovine serum albumin, NP40, PMSF, aprotinin, tiron and EDTA were from Sigma Chemical Co, St. Louis, MO, U.S.A.

*Data analysis and statistics*

Data are means±s.e.mean of the indicated number of individual observations. Statistical comparisons between groups were performed using the one way ANOVA followed by the Dunnett's or Newman-Keuls *post hoc* test or the Student's *t* test, as appropriate. Differences among means were considered significant when $P<0.05$.

## Results

*Effects of exposure to LPS or IL-1β on SNP- or ANF-induced cyclic GMP accumulation*

To investigate whether cyclic GMP accumulation in response to SNP is altered, cultured rat aortic smooth muscle cells were pretreated with LPS (1 μg $ml^{-1}$) or IL-1β (10 u $ml^{-1}$) for 24 h, exposed to L-NAME for 30 min, and were then stimulated with 10 μM SNP for an additional 15 min in the presence or absence of IBMX. SNP-induced cyclic GMP accumulation was reduced to a similar degree both in the absence and presence of the phosphodiesterase (PDE) inhibitor, IBMX (Figure 1) as it decreased to 43±10% (no IBMX; $n=4$) and 66±8% (0.3 mM IBMX; $n=4$) of vehicle for LPS pretreated cells. Similar results were obtained with 1 mM IBMX. In all subsequent experiments 0.3 mM IBMX was used to inhibit the PDE, augment the cyclic GMP accumulation and increase the sensitivity of the method. Smooth muscle cells exposed to LPS or IL-1β showed a 30–70%, time-dependent decrease in SNP-induced cyclic GMP accumulation (Figure 2b) that lagged 2 h behind the appearance of iNOS (reflected in cyclic GMP accumulation in the absence of L-NAME; Figure 2a) The downregulation of the SNP response was also LPS (0.1–1000 ng $ml^{-1}$) and IL-1β (0.01–100 u $ml^{-1}$) concentration-dependent, reaching a plateau at 10 ng $ml^{-1}$ LPS (66.0±2.2% reduction; $n=4$) and 10 u $ml^{-1}$ IL-1β (52.8±3.6% reduction; $n=4$) after 12 h of incubation with LPS or IL-1β. Exposure of cells to IL-1β or LPS (Figure 3a and b, left panels) caused a decrease in cyclic GMP accumulation only when high SNP concentrations (1 and 10 μM) were used. On the other hand, cyclic GMP accumulation in response to the particulate GC activator atriopeptin II (ANF) was spared by LPS or IL-1β and, if anything, potentiated throughout the ANF concentration-range tested (Figure 3a and b, right panels). In these experiments, the 30 min exposure to L-NAME completely inhibited the LPS-induced cyclic GMP accumulation at 24 h and eliminated 90% of the cyclic GMP formed in the IL-1β-treated cells. In these experiments only, cyclic GMP values after L-NAME treatment of vehicle, IL-1β- or LPS-pretreated cells, were subtracted from the cyclic GMP that accumulated in response to SNP or ANF. This was done to ensure that

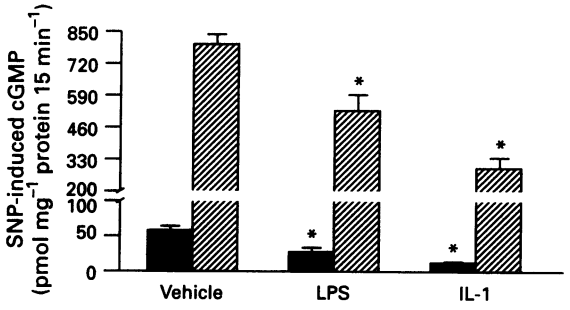


**Figure 1** Downregulation of cyclic GMP (cGMP) accumulation in response to sodium nitroprusside (SNP) stimulation in vehicle, lipopolysaccharide (LPS) or interleukin-1β (IL-1β)-pretreated cells. Smooth muscle cells were pretreated with vehicle (water), 1 μg $ml^{-1}$ LPS or 10 u $ml^{-1}$ IL-1β for 24 h. At the end of the pretreatment, and following 30 min L-NAME (100 μM) incubation, cyclic GMP accumulation was determined during a 15 min incubation with 10 μM SNP in the absence (solid columns) or presence (hatched columns) of phosphodiesterase inhibition with IBMX (0.3 mM). Means±s.e.mean; $n=4$ wells. *$P<0.05$ compared with respective vehicle.

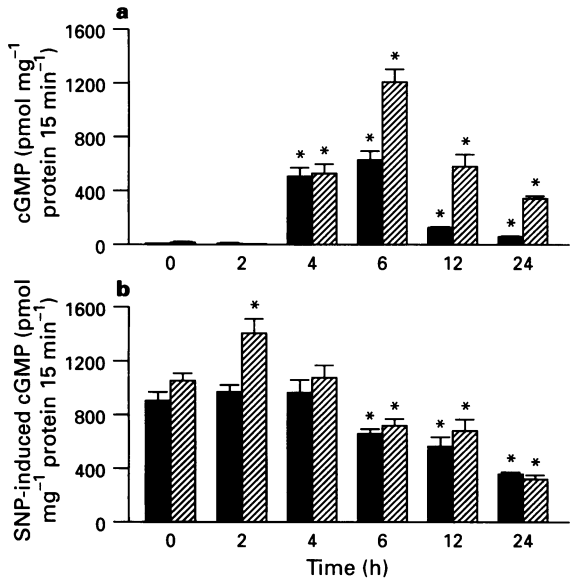


**Figure 2** (a) Time course of lipopolysaccharide (LPS)- and interleukin-1β (IL-1β)-induced NO production in rat aortic smooth muscle cells, as estimated by cyclic GMP (cGMP) accumulation. Cells were exposed to 1 μg ml$^{-1}$ LPS or 10 u ml$^{-1}$ IL-1β for the indicated time. After the end of the pretreatment cells were incubated with 1 mM arginine in the presence of IBMX. (b) Time course of LPS- and IL-1β-induced downregulation of cyclic GMP accumulation. Cells were exposed to 1 μg ml$^{-1}$ LPS or 10 u ml$^{-1}$ IL-1β for the indicated time. Cells were also treated with 100 μM L-NAME for the last 30 min to inhibit iNOS. At the end of this period, cyclic GMP accumulation was determined during stimulation of the sGC with 10 μM sodium nitroprusside (SNP). Solid columns: LPS, hatched columns: interleukin-1β. Means ± s.e.mean, $n=4$ wells *$P<0.05$ compared with 0 h.

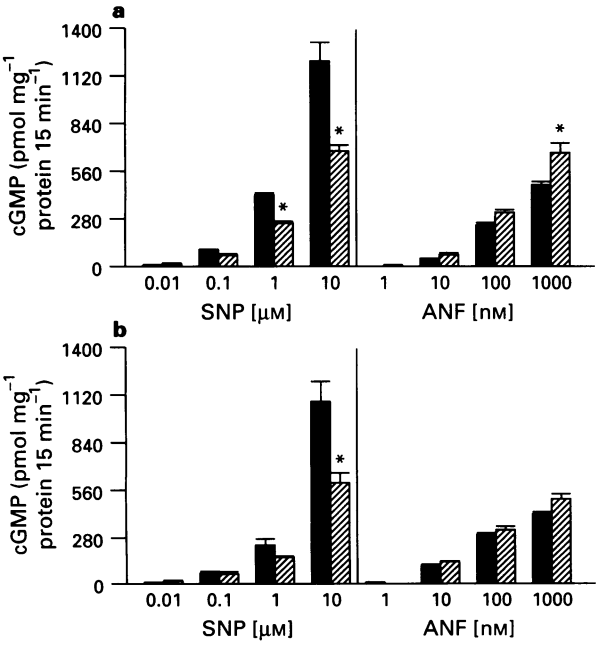


**Figure 3** Stimulation of NO production by interleukin-1β (IL-1β; a) or endotoxin (LPS; b) in smooth muscle cells results in downregulation of cyclic GMP (cGMP) accumulation upon subsequent exposure to sodium nitroprusside (SNP), but not atriopeptin (ANF). Smooth muscle cells were incubated with vehicle, 10 u ml$^{-1}$ IL-1β or 1 μg ml$^{-1}$ LPS for 24 h. Following that, cells were washed and incubated with 100 μM L-NAME for 30 min to prevent NO formation. Cyclic GMP content was then determined during a 15 min stimulation with SNP or ANF. Solid column: vehicle, hatched columns: interleukin-1β (a) or LPS (b). Means ± s.e.mean; $n=4$ wells. *$P<0.05$ compared with vehicle.

there is no masking of the downregulation in cyclic GMP accumulation at low SNP or ANF concentrations. In all other experiments, no such subtractions were done since the high concentration of SNP used (10 μM) led to large increases in cyclic GMP accumulation and failure to eliminate endogenously produced NO would only minimally underestimate the downregulation in cyclic GMP accumulation.

Conscious rats treated with 5 mg kg$^{-1}$ LPS i.v. for 6 h showed evidence of iNOS, as indicated by the increased cyclic GMP accumulation in aortic rings from these animals (Figure 4). When rings from the LPS-treated animals were incubated with L-NAME for 30 min and then stimulated with 10 μM SNP, cyclic GMP accumulation was reduced as compared to control, confirming the present *in vitro* observations of reduced sGC responsiveness to SNP in LPS- or cytokine-treated cells.

### *Involvement of free radicals on the LPS- and IL-1β-induced downregulation of SNP-induced cyclic GMP accumulation*

To investigate the role of oxygen radicals in the downregulation of SNP-induced cyclic GMP accumulation, free radical scavengers were tested for their ability to prevent the reduction in cyclic GMP accumulation in cells exposed to IL-1β. Scavengers for intracellular and extracellular superoxide (10 mM tiron and 300 u ml$^{-1}$ Cu/Zn superoxide dismutase; SOD, respectively) and extracellular hydrogen peroxide (2,000 u ml$^{-1}$ catalase; CAT) did not have protective effects on the IL-1β-induced downregulation in cyclic GMP accumulation (vehicle 54 ± 2%; tiron 50 ± 4%; SOD 70 ± 4% and CAT 47 ± 8% of control; $n=4$ each) when added for 30 min before determination of cyclic GMP accumulation. Similar results were obtained when scavengers were present throughout the 24 h IL-1β exposure. In separate experiments, induction of NOS activity was found not to be affected by the presence of SOD or CAT (117.5 ± 5.0% and 94.9 ± 9.7% of control; $n=4$, respectively). In addition, no change in sulphydryl group concentration was noted after treatment with either 1 μg ml$^{-1}$ LPS or 10u ml$^{-1}$ IL-1β for 24 h, as soluble thiol levels were 18 ± 4, 18 ± 3 and 16 ± 3 nmol mg$^{-1}$ protein for vehicle, IL-1β or LPS pretreated cells, respectively ($n=4$), and protein bound thiol levels were 48 ± 12, 62 ± 30 and 40 ± 6 nmol mg$^{-1}$ protein for vehicle, IL-1β and LPS pretreated cells, respectively ($n=4$). Parallel processed cultures pretreated with IL-1β or LPS showed a 40% and 32% reduction in SNP-induced cyclic GMP accumulation, respectively. Since IL-1β has been reported to increase intracellular cyclic AMP, we determined cyclic AMP levels in control and IL-1β-treated cells in the presence of IBMX. Incubation for 1–20 min with up to 100 u ml$^{-1}$ IL-1β did not alter cyclic AMP levels (96.1 ± 6.1

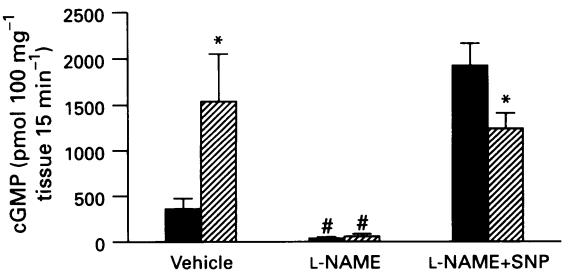


**Figure 4** Cyclic GMP levels in aortic rings from rats treated with 5 mg kg$^{-1}$ lipopolysaccharide (LPS) i.v. After 6 h, they were killed and thoracic aortae were removed. After removal of the endothelium, aortic rings were incubated for 30 min with vehicle or 50 μM L-NAME followed by a 15 min incubation with vehicle or 10 μM sodium nitroprusside (SNP) in the presence of IBMX. Solid columns: control, hatched columns: LPS. Means ± s.e.mean; $n=7-14$ animals. *$P<0.05$ compared with control; #$P<0.05$ compared with vehicle.

and 96.3±4.4 pmol $mg^{-1}$ protein 20 $min^{-1}$ for control and 100 u $ml^{-1}$ IL-1β treated cells; $n=4$).

To determine if the effects of IL-1β on SNP-induced cyclic GMP accumulation were due to the increased production of NO, cells were incubated with arginine-depleted medium for 24 h (medium was depleted of arginine by incubation with 5u $ml^{-1}$ arginase for 8 h at room temperature) and then challenged with IL-1β for another 24 h in the presence of 100 μM L-NAME to inhibit NO production completely (Figure 5). Culture of the cells in arginine-depleted medium prevented the NO production in cells treated with IL-1β. The presence of iNOS under these conditions was confirmed by replenishing the arginine in the medium bathing the cells for the last 15 min; this led to a substantial increase in cyclic GMP accumulation. SNP-induced cyclic GMP accumulation in vehicle-treated cells was lower when cells were arginine-depleted than when they were cultured in normal medium (984±58 vs 779±42 pmol $mg^{-1}$ protein 15 $min^{-1}$; $n=4$). Arginine depletion and elimination of NO production protected against the IL-1β-induced reduction in cyclic GMP accumulation (86±6 vs 56±1% of vehicle for arginine-depleted and non-depleted cells, respectively; $n=4$). When SNP was added as a source of NO to arginine-depleted cells (in the absence of IL-1β stimulation), cyclic GMP accumulation decreased as expected (37±18 and 39±6% of vehicle for arginine-depleted and non-depleted cells, respectively; $n=4$), indicating that SNP-induced cyclic GMP accumulation is downregulated in smooth muscle cells under arginine depletion. In separate experiments, addition of 10 mM L-NAME in normal medium during the exposure of cells to IL-1β had similar protective effects: it restored the SNP-induced cyclic GMP accumulation of IL-1β treated cells from 71.0±3.7% of control to 93.1±3.0% of control ($n=4$).

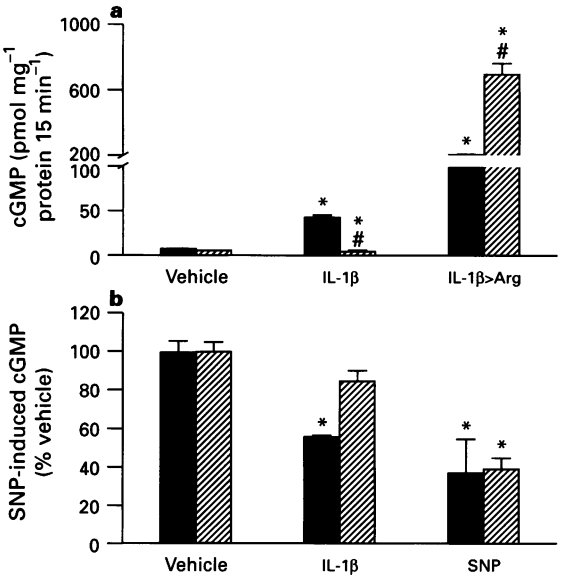


**Figure 5** Elimination of NO production protects against the interleukin-1β (IL-1β)-induced downregulation of cyclic GMP accumulation. (a) Smooth muscle cells were depleted of arginine (L-Arg) for 24 h and were then incubated with vehicle or 10 u $ml^{-1}$ IL-1β for 24 h. The ability of the cells to synthesize the inducible isoform of nitric oxide synthase was confirmed by adding 1 mM L-Arg during the subsequent 15 min IBMX period (IL-1β>Arg; L-Arg was added after the 24 h exposure to IL-1β). Solid columns: normal medium, hatched columns: L-Arg-depleted medium. Means±s.e.mean, $n=4$ wells. *$P<0.05$ compared with respective vehicle, #$P<0.05$ compared with normal medium. (b) Cells were depleted of L-Arg and incubated with IL-1β (10 u $ml^{-1}$, 24 h) or sodium nitroprusside (SNP; 10 μM, 24 h). sGC responsiveness to SNP was determined as previously described, following a 30 min L-NAME (100 μM) exposure. Solid columns: normal medium, hatched columns: arginine-depleted medium. Means±s.e.mean, $n=4$ wells. *$P<0.05$ compared with respective vehicle.

### *Effects of LPS and IL-1β on $\alpha_1$ sGC steady state mRNA and protein levels*

H.p.l.c. quantification of the RT-PCR products showed that the ratio $\alpha_1$/GAPDH was reduced in arginine-depleted cells, LPS- or IL-1β-pretreated cells as well as in arginine-depleted cells exposed to IL-1β (Figure 6). On the other hand, $\alpha_1$ sGC protein levels did not change in cells pretreated with LPS (1 μg $ml^{-1}$) or IL-1β (10 u $ml^{-1}$) for 24 h (Figure 7), but were decreased in arginine-depleted cells. Parallel processed cultures pretreated with IL-1β or LPS showed a 30% and 57% reduction in SNP-induced cyclic GMP accumulation, respectively.

## Discussion

Exposure of rat cultured aortic smooth muscle cells to LPS or IL-1β led to the induction of NOS activity, as indicated by the increase in cyclic GMP accumulation that was first evident at 4 h. We have previously reported that this increase in cyclic GMP can be inhibited by a variety of agents, such as the transcription inhibitor, actinomycin D (5 μg $ml^{-1}$), the protein synthesis inhibitor, cycloheximide (20 μM), dexamethasone (10 μM), NOS inhibitors and methylene blue (Marczin *et al.*, 1993b). Cells exposed to LPS or IL-1β displayed a concentration- and time-dependent reduction in SNP-stimulated

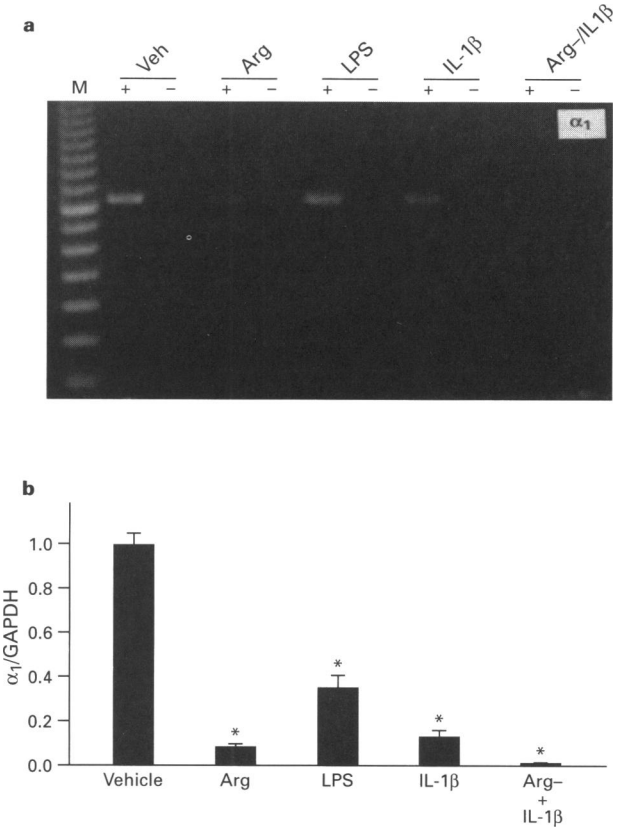


**Figure 6** (a) Analysis of RT-PCR products for the $\alpha_1$ subunit of soluble guanylate cyclase by agarose gel electrophoresis stained with ethidium bromide. Cells were pretreated with either vehicle (Veh), arginine-depleted medium for 24 h in the presence of 100 μM L-NAME (Arg), 1 μg $ml^{-1}$ lipopolysaccharide for 24 h (LPS), or 10 u $ml^{-1}$ interleukin-1β for 24 h (IL-1β) with (IL-1β/Arg−) or without (IL-1β) arginine depletion. Total RNA (200 ng) was reverse transcribed and amplified for 28 cycles. A marker (M) consisting of DNA fragments in increments of 100 bp (lower band 100 bp) was used to estimate the size of the RT-PCR products. +,−: with, without reverse transcriptase. (b) Quantification of the $\alpha_1$/GAPDH ratio by h.p.l.c. after RT-PCR. Means±s.e.mean, $n=3$. *$P<0.05$ compared with vehicle.

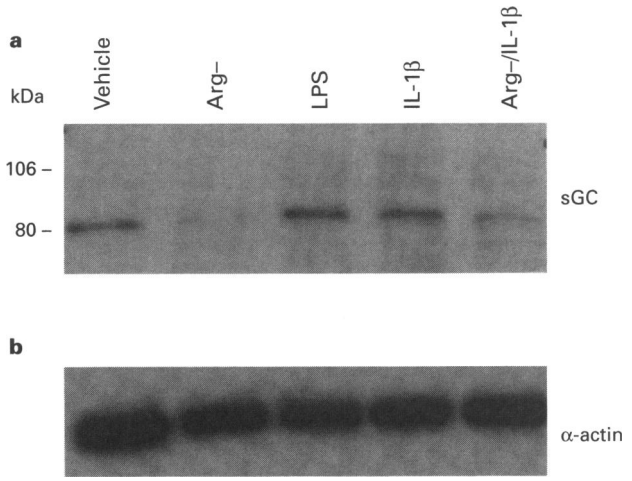


**Figure 7** (a) Levels of $\alpha_1$ sGC protein are not altered after incubation with interleukin-1$\beta$ (IL-1$\beta$) or lipopolysaccharide (LPS). Control of arginine-depleted (Arg−) smooth muscle cells were incubated with or without $10\,u\,ml^{-1}$ IL-1$\beta$ or $1\,\mu g\,ml^{-1}$ LPS for 24 h; 35 $\mu$g protein from each group was loaded and subjected to SDS-PAGE and transferred to a nylon membrane that was subsequently incubated with a 1:750 dilution of a monoclonal antibody for the $\alpha_1$ subunit. (b) Membranes were incubated with a monoclonal antibody against smooth muscle α-actin to check for consistency in loading and transfer. Autoradiographic signals were analysed by computer assisted densitometry. Relative optical density values (average of two separate experiments) yielded $\alpha_1$ sGC/α-actin ratios of 1, 0.3, 1.5, 1.2, 0.3 for vehicle, Arg−, LPS, IL-1$\beta$ and Arg−/IL-1$\beta$, respectively.

cyclic GMP levels, that reached a maximum at 24 h. Reduction in SNP-induced cyclic GMP accumulation did not begin until after 6 h exposure to LPS or IL-1$\beta$. This correlates well with the time necessary for NOS induction (3–4 h) and production of excessive amounts of NO. Persistence in the reduction of SNP-induced cyclic GMP accumulation beyond 6 h, at a time that iNOS activity seems to decline, may be explained by the following: (i) although iNOS is only transiently expressed (Evans *et al.*, 1994), iNOS activity in the present experiments is inferred from cyclic GMP accumulation and is thus underestimated due to desensitization of sGC and (ii) even though smaller amounts of NO may be released from the iNOS, they may still be enough to produce an effect on sGC activity. Similarly to cultured cells, SNP-induced cyclic GMP accumulation was decreased in aortic rings of animals treated with LPS for 6 h.

The LPS- or IL-1$\beta$-induced attenuation in cyclic GMP levels in response to SNP was due to decreased cyclic GMP production, rather than increased degradation, since cyclic GMP levels were reduced both in the presence and absence of PDE inhibition. Our findings are in agreement with recently published results that exposure of bovine isolated mesenteric arterial rings to interferon-γ (DeKimpe *et al.*, 1994) or rat aortic rings to endotoxin (Tsuchida *et al.*, 1994) inhibits SNP-induced vasodilatation and cyclic GMP accumulation.

LPS and IL-1$\beta$ are known prooxidant agents (Schreck *et al.*, 1991). Since oxidant stress reduces NO responses (Marczin *et al.*, 1993a), we determined the effects of free radical scavengers on the IL-1$\beta$-induced reduction in sGC activity. Cu/Zn superoxide dismutase, tiron and catalase, all failed to protect against the LPS- and IL-1$\beta$-induced downregulation of cyclic GMP accumulation. Similar free radical scavenger treatments partially or completely restored the endothelium-derived NO-mediated cyclic GMP accumulation by activated neutrophils (Marczin *et al.*, 1993a). It is well established that sGC activity is redox regulated with thiols being involved in the expression of enzymatic activity (Waldman & Murad, 1987). Mixed disulphide formation has been previously reported to inactivate the enzyme reversibly (Brandwein *et al.*, 1981) and massive oxidation has been shown to lead to a loss of enzymatic activity (Waldman & Murad, 1987). In addition, reducing agents, such as N-acetylcysteine and dithiothreitol, potentiate sGC activity presumably by preserving the haem iron at a reduced state, by facilitating the formation of NO-haem, or by preserving the sulphydryl groups on sGC in a reduced state (Waldman & Murad, 1987). The present observation that no reduction in soluble or protein-bound thiols was detected in LPS and IL-1$\beta$ pretreated cells, along with the finding that free radical scavengers failed to protect the reduction in cyclic GMP accumulation, suggest that free radicals are not involved in the downregulation of SNP-induced cyclic GMP accumulation under these conditions.

We and others have previously reported that exposure of cultured cells or vessels to non-nitrate nitrovasodilators, such as SNP, leads to the development of tolerance (Henry *et al.*, 1989; Kojda *et al.*, 1994; Papapetropoulos *et al.*, 1996). In rat cultured aortic smooth muscle cells, tolerance is evident as early as 2 h following exposure to SNP without affecting responses of the pGC (Papapetropoulos *et al.*, 1996). The time frame for the present LPS- and IL-1$\beta$-induced downregulation of SNP-induced cyclic GMP accumulation is consistent with the time course observed in the development of tolerance to exogenous nitrovasodilators. To investigate whether the effects of IL-1$\beta$ are mediated by the increased production of NO from the iNOS, cells were depleted of arginine and were then stimulated with IL-1$\beta$. Arginine-depleted cells exposed to IL-1$\beta$ showed no evidence of NO production, but were nevertheless able to synthesize iNOS, since replenishing L-arginine in the culture medium at the end of the experiment, led to an increase in cyclic GMP accumulation. The greater cyclic GMP accumulation observed in the arginine-depleted group (vs normal medium) treated with IL-1$\beta$ once arginine was replenished, can be explained by the negative feedback regulation exerted by NO on the activity of iNOS, as has been described in rat alveolar macrophages (Griscavage *et al.*, 1993). When arginine-depleted cells were challenged with IL-1$\beta$, no downregulation of cyclic GMP accumulation was evident. In addition, when cells cultured in normal medium were incubated with IL-1$\beta$ in the presence of L-NAME throughout the 24 h cytokine treatment, no impairment of the SNP-induced cyclic GMP accumulation was observed.

Rat smooth muscle cells have been reported to express the $\alpha_1$, $\alpha_2$ and $\beta_1$ subunits of sGC (Ujiie *et al.*, 1993). Recent cloning and expression experiments have revealed that although the $\alpha$ and $\beta$ subunits each appears to possess a catalytic domain, expression of enzymatic activity requires the presence of both subunits (Buechler *et al.*, 1991). On the other hand, NO and IL-1$\beta$ have been shown to alter protein levels of several enzymes and proteins by modulating gene expression in vascular and non-vascular cells (Magrinat *et al.*, 1992; Kouremembanas *et al.*, 1993). The ratio of steady state mRNA levels for $\alpha_1$ /GAPDH after 28 cycles of amplification was decreased in arginine-depleted cells, cells pretreated with LPS or IL-1$\beta$, as well as in arginine-depleted cells treated with IL-1$\beta$. This is in line with previously reported observations (Filippov & Bloch, 1993) that steady state mRNA levels for the $\beta_1$ sGC subunit decrease in cells exposed to cytokines or LPS. However, Western blot analysis revealed that $\alpha_1$ protein levels were decreased only in arginine-depleted cells irrespective of whether they were exposed to IL-1$\beta$ or not. The reduction in mRNA, but not protein, levels of the $\alpha_1$ subunit may be explained by a relatively long half life of the sGC (Shimouchi *et al.*, 1993; unpublished data). As no evidence was found for reduced $\alpha_1$ sGC protein levels in cells pretreated with LPS or IL-1$\beta$ for 24 h, and provided that protein levels for the $\beta_1$ subunit also remain unaffected, the LPS-and IL-1$\beta$-induced downregulation of SNP-induced cyclic GMP accumulation at 24 h appears to result from decreased responsiveness of sGC to NO. This is in line with the observation that cyclic GMP accumulation is reduced only when LPS or IL-1$\beta$ pretreated cells are exposed to high concentrations of SNP. The exact mechanism by which the reduction in sGC mRNA levels occurs is

still unclear. It is unlikely that this reduction is due to the increased NO production, since when SNP or S-nitroso-N-acetylpenicilamine were added exogenously both failed to reduce $\beta_1$ sGC mRNA. Some of the actions of IL-1$\beta$ have been proposed to be mediated through increases in cyclic AMP (Dinarello, 1991). In the present study, acute incubation (up to 20 min) of smooth muscle cells with IL-1$\beta$ did not result in increased cyclic AMP levels. Prolonged incubation with IL-1$\beta$ may lead to increases in cyclic AMP levels through the induction of cyclo-oxygenase-2 (Gierse *et al.*, 1995), which could then contribute to the reduction in sGC mRNA levels through increases in cyclic AMP (Shimouchi *et al.*, 1993; Papapetropoulos *et al.*, 1995). Should cyclic AMP cause the reduction in the sGC mRNA, the $\alpha_1$ protein levels should also be decreased after the 24 h incubation (Papapetropoulos *et al.*, 1995).

It should be noted that arginine-depleted cells showed decreased $\alpha_1$ sGC mRNA and protein levels, confirming our finding that arginine depletion leads to reduced SNP-induced cyclic GMP accumulation in vehicle treated-arginine depleted cells. The importance of this finding, as well as the mechanism by which it occurs, remain unclear, but seem to be unrelated to the arginine content of the $\alpha_1$ subunit, as arginine-depleted cells could express another protein (iNOS) with a greater number of arginine molecules (Xie *et al.*, 1992). It is possible that the $\alpha_1$ subunit is one of the proteins cells eliminate under arginine deficiency by stopping its mRNA and protein synthesis and/or increasing protein degradation, using it as a source of arginine.

Endotoxin activates protein kinase C in rat macrophages (Geisel *et al.*, 1991). Moreover, activation of PKC leads to desensitization of the pGC (GC-A receptor) responses (Potter & Garbers, 1994). On the other hand, Kishimoto *et al.* (1993) reported that increased levels of cyclic GMP reduce the steady state mRNA expression of the clearance, but not the GC-A or GC-B, receptor for the natriuretic peptides in rat cultured aortic smooth muscle cells. It is not known if cytokines, NO or the concomitant rise in cyclic GMP levels may lead to alterations in the pGC response to natriuretic peptides in vascular smooth muscle cells. Since downregulation of cyclic GMP accumulation following exposure to cytokines or LPS could be a part of a homeostatic mechanism to counteract the massive hypotension seen in shock, it was important to establish if both cyclic GMP generating pathways in the vasculature, sGC and pGC, are affected following treatment with LPS or IL-1$\beta$. Under the present experimental conditions, exposure of cells to LPS or IL-1$\beta$ failed to reduce ANF-induced cyclic GMP accumulation.

In conclusion, exposure of smooth muscle cells to either LPS or IL-1$\beta$ affects sGC at two levels: that of mRNA and that of activity, possibly by two distinct mechanisms. The downregulation of SNP-induced cyclic GMP accumulation, following exposure to LPS or IL-1$\beta$, at least initially, results from decreased responsiveness of the enzyme to NO rather than decreased amounts of sGC protein. Such a reduction in sGC activity would limit the biological effect of NO produced by the iNOS and would be beneficial in offsetting the extensive vasodilatation seen in septic shock.

We are pleased to acknowledge the expert technical assistance of Ms Connie Snead, Ms Ilona Dienes-Kadi and Mr Jim Parkerson. We thank Ms Annie Cruz and Ms Aretha Bogan for the preparation of the manuscript. This study was supported by HL52958.

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*(Received October 23, 1995*
*Revised March 18, 1996*
*Accepted March 26, 1996)*