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Induction of nitric oxide synthase by protein synthesis inhibition in aortic smooth muscle cells

Nándor Marczin, Carolyn Y. Go, Andreas Papapetropoulos & [1]John D. Catravas

Vascular Biology Center and Department of Pharmacology and Toxicology, Medical College of Georgia, Augusta, GA 30912-2500, U.S.A.

1 The role of *de novo* protein synthesis in inducible NO synthase (iNOS) activation was investigated *in vitro* by evaluating the effects of protein synthesis inhibitors cycloheximide (CH) and anisomycin (ANI) on iNOS activity, protein and mRNA levels in rat aortic smooth muscle cells (RASMC).

2 As determined by cyclic GMP accumulation, substrate (L-arginine)- and inhibitor (N^G-monomethyl-L-arginine, NMMA)-sensitive iNOS activity was significantly elevated in CH- or ANI-treated RASMC after 24 h.

3 Lipopolysaccharide (LPS) produced a time-dependent increase in cyclic GMP levels with maximal stimulation at 6 h and a decline to near baseline at 24 h. CH attenuated LPS-induced cyclic GMP accumulation at 3 and 6 h. However, cyclic GMP levels were superinduced at later times by CH. The concentration-dependence of cyclic GMP stimulation by cycloheximide was biphasic both in the absence and presence of LPS, with maximal stimulation at 10 μM and inhibition at higher concentrations.

4 Increased iNOS activity by CH was associated with elevated levels of immunoreactive iNOS protein as judged by Western blotting in LPS- and CH-treated cells.

5 CH-induced iNOS activity and superinduction of iNOS by CH in cells treated with LPS were both significantly inhibited by actinomycin D, a transcription inhibitor.

6 RT-PCR revealed elevated iNOS mRNA levels after 12 h of exposure to CH. The combination of LPS and CH caused a significant increase in iNOS gene expression relative to LPS- or CH stimulation alone.

7 These results show that partial protein synthesis inhibition by CH alone upregulates iNOS mRNA and superinduces iNOS mRNA in cytokine-treated RASMC, which is translated to the functional enzyme generating biologically active NO. Thus iNOS activation in these cells not only requires new protein synthesis but it also appears to be negatively regulated by newly synthesized proteins.

Keywords: Nitric oxide; cycloheximide; anisomycin; smooth muscle cells; cyclic GMP; repressor

Introduction

The role of vascular cell derived nitric oxide (NO) in normal and pathological cardiovascular regulation is now well established (Moncada & Higgs, 1991; Nathan, 1992). Under physiological conditions basal and stimulated release of endothelium-derived NO modulates vascular smooth muscle tone via a paracrine mechanism. Although NO release has been shown from human cultured vascular smooth muscle cells, most smooth muscle cells lack constitutive NO synthesis (Bernhardt *et al*., 1991; Fleming *et al*., 1991; Schmidt *et al*., 1993; Knowles & Moncada, 1992). However, smooth muscle cells have been shown to express an inducible NO synthase when challenged with inflammatory mediators such as bacterial lipopolysaccharide (LPS) and interleukin-1 (IL-1) (Fleming *et al*., 1991; Beasley *et al*., 1991). These agents inhibit contractile responses in endothelium-denuded vessels, activate soluble guanylate cyclase and produce prolonged generation of guanosine 3′:5′-cyclic monophosphate (cyclic GMP) via transcriptional activation of the NO synthase gene and persistent production of NO (Beasley *et al*., 1989; Beasley, 1990; Busse & Mulsch, 1990; Fleming *et al*., 1990; Rees *et al*., 1990). This mechanism is believed to be responsible for the systemic hypotension and cardiovascular collapse associated with septic shock and may contribute to vasodilatation in local infection and inflammation (Kilbourn *et al*., 1990a,b; Petros *et al*., 1991).

Protein synthesis inhibition by cycloheximide (CH) prevents reduced pressor responsiveness of cytokine exposed vessels and prevents the release of biologically active NO and cyclic GMP accumulation (Beasley *et al*., 1989; 1991; Busse & Mulsch, 1990; Rees *et al*., 1990), suggesting that cytokine-induced expression of NO synthase activity requires new protein synthesis. However, while investigating cytokine-induction of iNOS in rat aortic smooth muscle cells (RASMC), we accidentally observed elevated cyclic GMP levels after CH exposure (Go *et al*., 1994). We hypothesized that CH-induced cyclic GMP accumulation was due to iNOS activation. Here, we present pharmacological and biochemical evidence in support of this hypothesis.

Methods

Cell culture

Wistar rat aortic smooth muscle cells (RASM) were isolated by enzymatic dissociation with standard methods (Geisterfer *et al*., 1988). The cells were positively identified and maintained as previously described (Marczin *et al*., 1993). Smooth muscle cells derived from three different isolations were used in this study, 3–5 days after confluence and at passages lower than 10.

[1] Author for correspondence.

LPS and cytokine-induced activation of NO synthase in RASM cells

We and others have previously provided evidence that lipopolysaccharide (LPS) and interleukin-1 (IL-1) induces a prolonged activation of the inducible NO pathway with L-arginine-dependent activation of guanylate cyclase in cultured vascular smooth muscle cells (Geisterfer *et al*., 1988; Beasley *et al*., 1991; Schini *et al*., 1992). However, under normal conditions cyclic GMP steady state levels are only minimally increased in response to cytokines, due to rapid breakdown of cyclic GMP by phosphodiesterases (Beasley *et al*., 1991). This breakdown can be prevented by the presence of a phosphodiesterase inhibitor and, under these conditions, cyclic GMP accumulation better reflects the rate of cyclic GMP production and thus the extent of NO synthase activity and NO-mediated activation of guanylate cyclase. Thus, RASM cells were challenged with increasing concentrations of either LPS or IL-1 or tumour necrosis factor-α (TNF-α for 3–24 h before the determination of intracellular levels of cyclic GMP in the presence of a phosphodiesterase inhibitor.

Assay of cyclic GMP

After the various experimental protocols, culture medium was removed and the cells were incubated in Earle's balanced salt solution (ES) containing 1 mM 3-isobutyl-1-methylxanthine (IBMX), a cyclic nucleotide phosphodiesterase inhibitor, for 15 min, to prevent the breakdown of accumulated cyclic GMP. Unless otherwise specified, 1 mM L-arginine was also included to standardize substrate availability. After 15 min, the medium was rapidly aspirated, and 300 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 the acid-extracted cultures were solubilized by 1.0 N NaOH. The HCl extract was directly analysed for cyclic GMP by radioimmunoassay by use of the automated Gammaflow system and protein concentration of the NaOH-solubilized samples was determined by the Bradford method, as described previously (Marczin *et al*., 1992).

Cycloheximide exposure

RASM cells were incubated in complete medium supplemented with 10 μM CH for 24 h in the presence of serum. After 24 h the medium was removed, washed with 2 ml ES. In experiments addressing L-arginine-dependence of cyclic GMP accumulation the cells were further incubated for 30 min with an inhibitor of NO synthase, N^G-monomethyl-L-arginine (L-NMMA, 1 mM) alone or in combination with increasing concentrations of L-arginine (1–10 mM) before the addition of IBMX for 15 min. The concentration-dependence of CH-induced cyclic GMP accumulation was investigated by incubating the cells with increasing concentrations of CH (1–100 μM) for 24 h and measuring cyclic GMP accumulation in the presence of IBMX and L-arginine. In time course experiments, cells were exposed to 10 μM CH for increasing periods of time (3–24 h). To investigate the effects of CH on LPS-induced cyclic GMP accumulation, increasing concentrations of CH were added together with 100 ng ml^{-1} LPS for 3–24 h before the determination of cyclic GMP.

Bioassay for NO release from CH-treated RASM cells

To correlate intracellular cyclic GMP accumulation in CH-treated RASM cells with release of biologically active NO, bioassay experiments were performed where extracellular NO was detected by using control and untreated reporter RASM cells, as previously described (Marczin *et al*., 1992). For these experiments, cells grown on 12 mm diameter glass coverslips in 24 multiwell plates were treated as control or with 10 μM CH in the absence or presence of 100 ng ml^{-1} LPS for 24 h. These 'donor' cells were then washed with 2 ml of ES and incubated for 30 min in the presence of either 1 mM L-arginine or 1 mM N^G-nitro-L-arginine methyl ester (L-NAME). Control and untreated 'reporter' RASM cells were incubated with IBMX for 15 min. The coverslips containing CH and LPS-treated cells were then gently transferred into 'reporter' cell containing wells to share the same bathing media. After 15 min of coincubation of the coverslips with the 'donor' cells, the medium was removed from the wells and intracellular cyclic GMP was extracted separately from 'donor' and 'reporter' cells by HCl.

Analysis of total protein synthesis by cellular incorporation of [^{35}S]methionine

Confluent RASM cultures were incubated in methionine-free medium containing 10% dialysed foetal bovine serum supplemented with trans-S^{35}-label, which contained $\geqslant$70% L-[^{35}S]methionine and other ^{35}S-labelled compounds, for 24 h with 1–100 μM cycloheximide (CH) alone or combined with 100 ng ml^{-1} LPS. Thereafter, the culture was processed for cyclic GMP accumulation, as described above. HCl insoluble fractions were rinsed with ethanol and scraped with 1 N NaOH and an aliquot was taken for scintillation counting of total ^{35}S-labelled compounds.

Protein isolation and Western hybridization

After the experimental protocol, cells were rinsed twice with PBS and 250 μl lysis buffer was added (20 mM Tris HCl pH 7.4, 2.5 mM EDTA, 1% Triton, 10% glycerol, 1% deoxycholate, 0.1% SDS, 50 mM NaF, 10 mM $Na_4P_2O_7$ and 100 μM phenylmethylsulphonylfluoride). The total cell lysate was obtained after the dish had been left on ice for 10 min. Protein concentration was determined in a 25 μl aliquot of the lysate. A 25 μg protein lysate aliquot in 50 μl sample buffer was loaded onto a 7.5% polyacrylamide mini-gel (Biorad) and separated by electrophoresis for one hour with 150 V. The gel was transferred onto an immobilon PVDF transfer membrane (Millipore Corp., Bedford, MA) with a semi-dry system (Trans-Blot, Biorad) for 45 min with 100 V. The PVDF membrane was processed for immunoblotting by using anti-iNOS polyclonal antibody (1:4000; 1 h incubation) followed by incubation with horseradish peroxidase-conjugated with anti-rabbit IgG secondary antibodies (Bio-Rad, 1:2000 for one hour). Band detection was done with the ECL system (Amersham) before film exposure.

Isolation of total RNA

Total RNA was isolated from RASM by a commercially available kit (RNAzol or Ultraspec RNA, Biotecx Laboratories Inc, Houston, TX), according to the manufacturer's instructions including chloroform extraction and isopropanol precipitation. The pellet was washed with 75% ethanol and dried under vacuum for a final solubilization in a RNAse-free solution containing 0.1% sarkosyl and 0.1 M mercaptoethanol. The RNA content was quantified by absorbance at 260 nm (Shimadzu). The integrity of the RNA was checked by visualizing ribosomal RNA after electrophoresis on a 1.5%

agarose gel in Tris acetate EDTA buffer and 0.5 μg ml^{-1} ethidium bromide.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was resuspended in RNAse-free 10 mM Tris, 1 mM EDTA (TE), pH 8 solution. Three units of DNAse I (Gibco BRL) were added, followed by a phenol-chloroform extraction, ethanol wash and final resuspension in RNAse-free TE with 10 mM NaCl, pH 8. The amount of RNA in the solution was quantified by absorbance at 260 nm (Shimadzu Corp., Japan). In each reverse transcription reaction, 2 ng of the RNA sample and 1.25 pg of exogenous RNA standards (prepared according to methods described below) were reverse transcribed in a 20 μl-reaction mixture of commercially available reagents (GeneAmp RNA PCR kit, Perkin Elmer, NJ) containing 2.5 u M-MLV reverse transcriptase, 1 u RNase inhibitor, 2.5 μM random hexamers, 1 mM each dNTP, 5 mM $MgCl_2$ and 1 × PCR buffer II in 1 cycle of 18 min at 42°C, 5 min at 99°C and 5 min at 5°C in a Perkin-Elmer Cetus DNA thermal cycler. To check for the presence of DNA contamination, duplicate reactions without reverse transcriptase were also performed. As negative control, RT-PCR reaction was performed in the absence of known template. To each 20 μl-RT reaction, an 80 μl-PCR reaction solution containing a final concentration of 0.3 μM of each primer, 2.5 u Taq polymerase, 2 mM $MgCl_2$ and 1 × PCR buffer II was added. The primers used to target iNOS mRNA were 5′-CTGGCAGCAGCGGCTCCATG-3′ at base position 2987–3006 and 5′-GAAAAGACCGCACCGAAGAT-3′ at base position 3389–3409 of rat iNOS cDNA (Nunokawa *et al*., 1994). PCR amplification was achieved by 1 cycle of 94°C for 2 min, followed by 29 cycles of 94°C, 60°C, 72°C for 30 s, 30 s and 1 min, respectively; and a single extension step at 72°C for 4 min at 4°C for 3 min. For visualization and quantification by densitometry of each RT-PCR reactions, a 10 μl aliquot was electrophoresed in 1–1.5% agarose gel in 1 × TE containing 0.5 μg ml^{-1} ethidium bromide to allow u.v.-induced fluorescence (Transilluminator, Fisher Scientific). Densitometric analysis of the PCR products was performed with IS 1000 Digital Imaging System (Alpha Innotech Corp., San Leandro, CA). Preliminary experiments were performed to determine the range of amplification cycles and starting RNA substrate within the linear phase of the exponential increase of PCR products of each particular primer pair.

Preparation of exogenous RNA standards

Construction of RT-PCR standards was based on previously published methods with modifications (Borriello & Lederer, 1995). To differentiate between the RT-PCR products of RNA standards and iNOS mRNA, a plasmid containing a segment bigger than iNOS (743 bp) between T3 and T7 promoters (courtesy of Dr Gregory Liou, Medical College of Georgia) was used for PCR amplification with T3 and T7 primers linked to a 20-mer iNOS sequence at each 5′ end (underlined). The primer sequences were 5′-CTGGCAGCAGCGGCTCCATGAATTAAACCCTCACTAAAGG-3′ and 5′-GAAAAGACCGCACCGAAGATTTAATACGACTCACTATAGGG-3′. For the 100 μl-PCR reaction, 0.3 μM of each primer were added to a solution containing 2 mM $MgCl_2$, 1 × PCR buffer, 200 μM of each dNTP, 2.5 ng template (containing the desired 832 bp region) and 1 u of Taq polymerase. The reaction was performed at 94°C for 5 min, 55°C for 2 min followed by 29 cycles of 72°C for 2 min, 92°C for 1 min, 55°C for 2 min and a single extension step at 72°C for 10 min. Ten microlitre aliquots of the PCR reaction were electrophoresed in 1% agarose in 1 × TE to visualize the PCR product (830 bp). The PCR product was purified by gel electroelution, followed by cloning with the pGEM-T vector system II (Promega Corp., Madison, WI). For transformation of the ligation reaction, JM109 competent cells (Promega Corp., Madison, WI) were used. A 1:1 (vector:insert) ligation reaction provided the most positive colonies. Screening of the insert was performed by 'Easypreps' (Berghammer & Auer, 1993). Large scale cloning was performed in 250 ml of liquid broth (DIFCO laboratories, Detroit, MI) supplemented with 100 μg ml^{-1} ampicillin (Sigma), overnight at 37°C in a shaking incubator. Plasmid isolation was performed according to the protocol of Wizard maxipreps DNA purification system (Promega Corp., Madison, WI). For the *in vitro* transcription reaction, 1.5 μg Nco I-linearized plasmid in 20 μl TE, 25.5 μl of RNAse-free water, 20 μl of 5 × transcription buffer (at room temperature) containing 200 mM Tris HCl pH 7.9, 30 mM $MgCl_2$, 10 mM spermidine, 50 mM NaCl; 100 u RNAse inhibitor, 10 mM dithiothreitol (DTT), 25 μM NTP mix and 40 u Sp6 polymerase were combined in this sequence for a total of 100 μl, and subsequently incubated (dry incubator) at 37°C for 2 h. Thereafter, cRNA concentration was measured at A260 followed by purification by phenol/chloroform extraction and gel electroelution.

Materials

Carrier-free ^{125}I was from Du Pont NEN (Boston, MA); ^{32}P-d(NTP) was from Amersham Corp. (Arlington Heights, IL); trans ^{35}S-label was from ICN Biomedicals, Inc. (Irvine, CA). Ecoscint A scintillation solution was from National Diagnostics (Atlanta, GA). RNAzol was from Biotecx Laboratories Inc. (Houston, TX); GeneAmp RNA PCR kit was from Perkin Elmer (Norwalk, CT); ECL detection system was from Amersham International (Buckinghamshire, U.K.); PCR primers were synthesized by Genosys Biotechnologies, Inc., (The Woodlands, TX). Protein binding dye, mini-gels, PVDF membranes, dry milk, Tween 20 and other Western blotting reagents were purchased from Bio-Rad (Richmond, CA). Other chemicals including cycloheximide, LPS, actinomycin D, penicillin, streptomycin, succinyl tyrosine cyclic GMP methyl ester, isobutyl methylxanthine, bovine serum albumin, sodium nitroprusside (SNP), EDTA, were from Sigma Chemical Co. (St. Louis, MO). Recombinant human IL-1β and anisomycin were from Boehringer Mannheim (Indianapolis, IN). Recombinant human TNF-α was from R&D systems (Minneapolis MN). Monoclonal cyclic GMP antibody was a generous gift of Dr Ferid Murad (Lake Bluff, IL). Anti-iNOS polyclonal antibody was purchased from Transduction Laboratories (Lexington, KY).

Analysis of data

For all *in vitro* experiments, 4–6 replications of experiments were performed. Data were calculated and analysed with an IBM-computer by use of an appropriate spreadsheet programme (Lotus 123) and statistical software package (SYSTAT/SYSGRAPH). Comparisons within and between groups were performed with one-way analysis of variance (ANOVA) followed by Dunnett's or Neuwman-Keul's, or two-way ANOVA, when appropriate. Differences were considered significant when $P < 0.05$.

Results

Effects of cycloheximide on intracellular cyclic GMP levels in RASM cells

Baseline cyclic GMP levels were 13 ± 1 pmol mg^{-1} protein 15 min^{-1} in control RASM cells. Treatment with NMMA or L-arginine had no significant effects on these levels in control cells, suggesting the absence of basal iNOS activity in RASM. Exposure to CH for 24 h significantly increased cyclic GMP levels (265 ± 55 pmol mg^{-1} protein 15 min^{-1}). In the presence of NMMA, CH-stimulated cyclic GMP accumulation was significantly reduced by 70%. Addition of 1 or 10 mM L-arginine potentiated the CH-stimulated cyclic GMP accumulation and prevented the inhibition by NMMA (Figure 1).

Concentration- and time-dependent effects of CH and anisomycin on intracellular cyclic GMP levels and on total protein synthesis in RASM cells

To determine the concentration-dependent effects of CH on iNOS activity, confluent cells were exposed to increasing concentrations of CH (0–100 μM) for 24 h. CH alone significantly increased cyclic GMP levels at 1–50 μM, reaching maximum levels at 10 μM (Table 1). In contrast, cyclic GMP levels returned to baseline at 100 μM CH. To determine the concentration-dependent effects of CH on LPS-induced NOS (iNOS) activity, cells were exposed to increasing concentrations of CH in the presence of LPS (100 ng ml^{-1}) for 24 h. CH again produced a similar biphasic dose-response curve, but levels of cyclic GMP accumulation were 100 fold higher than levels attained by LPS stimulation alone (Table 1).

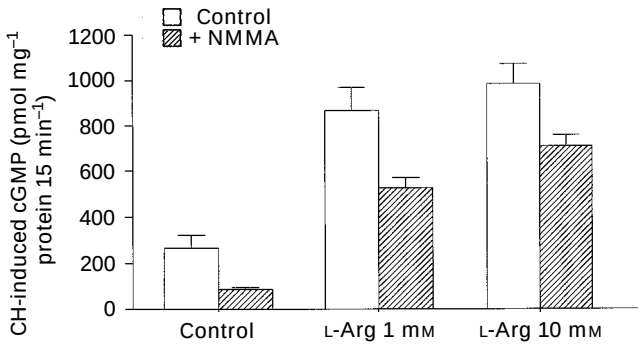

Figure 1 Modulatory effects of L-arginine (L-Arg) and its inhibitory analogue N-monomethyl-L-arginine (NMMA) on cycloheximide (CH)-stimulated cyclic GMP accumulation in RASM. Cells were stimulated with 10 μM CH for 24 h, followed by a 30 min incubation with or without (control) 1 mM NMMA in combination with increasing concentrations of L-Arg (0–10 mM). Means ± s.e.mean; $n=4$ wells. $^*P<0.05$ compared to cyclic GMP levels in appropriate control cultures. $\#P<0.05$ compared to cyclic GMP levels in the absence of NMMA.

To investigate the correlation between CH-stimulated cyclic GMP accumulation and total protein synthesis inhibition, protein synthesis was measured by [^{35}S]methionine incorporation in confluent RASM cells incubated for 24 h. Total [^{35}S]-methionine incorporation was 7,065,000 c.p.m. in control cells. In the presence of 10 μM CH, total [^{35}S]-methionine incorporation was significantly reduced to 13% (Table 1). Addition of LPS did not alter CH-dependent inhibition of protein synthesis.

To determine the time-dependent effects of CH on iNOS activity, confluent cells were exposed to 10 μM CH (maximum iNOS activity was achieved at this dose), for increasing lengths of time. Vehicle-treated groups (Figure 2a), did not produce significant cyclic GMP accumulation at any time throughout the entire experiment. However, in the presence of 10 μM CH (Figure 2a), cyclic GMP levels were significantly elevated from baseline values after 12 h, reaching peak levels after 24 h incubation with CH. Moreover, in response to LPS-stimulation alone, iNOS activity reached a peak after 6 h and steadily declined over the next 18 h (Figure 2b). When LPS was combined with CH the short-term ($\leqslant 6$ h) LPS-stimulated NOS activity was inhibited. However, after prolonged exposure to both CH and LPS, iNOS activity increased beyond the peak reached by LPS stimulation alone and remained elevated for 24 h (Figure 2b).

To determine whether other protein synthesis inhibitors can reproduce the effects of CH on iNOS activity, similar protocols were followed to determine the kinetics of anisomycin-induced cyclic GMP accumulation in RASM. Confluent cells were exposed to increasing concentrations (0–1 μg ml^{-1}) of anisomycin for 24 h. A biphasic pattern of cyclic GMP accumulation in response to increasing concentrations of anisomycin was observed, similar to that after CH stimulation (Figure 3a). A maximum 6 fold increase from baseline levels was observed with 250–750 ng ml^{-1} anisomycin. Higher concentrations did not significantly increase basal cyclic GMP levels but instead reduced cyclic GMP levels. Similar to CH, LPS-stimulated cyclic GMP levels were also markedly elevated (i.e. 6–7 fold) by 100–250 ng ml^{-1} anisomycin (Figure 3b).

To investigate the time-dependent effects of anisomycin, cells were exposed to 250 ng ml^{-1} anisomycin for 0–24 h. Cyclic GMP levels remained at basal levels at all times between vehicle-treated groups (Figure 4a). In contrast, cells exposed to anisomycin for a longer time ($\geqslant 18$ h) produced a significant increase in cyclic GMP levels (Figure 4a). iNOS activity after 6 h exposure to LPS and anisomycin was inhibited, whereas iNOS activity after 12 h exposure to LPS and anisomycin was markedly elevated compared to LPS-stimulation alone (Figure 4b).

Table 1 Concentration-dependent effects of cycloheximide (CH) on vehicle- or lipopolysaccharide (LPS)-induced cyclic GMP accumulation and trans^{35}S-label incorporation in RASM

	Vehicle		*LPS* (100 ng ml^{-1})	
	Radioactivity (c.p.m. × 10^3)	*Cyclic GMP* (pmol/well)	*Radioactivity* (c.p.m. × 10^3)	*Cyclic GMP* (pmol/well)
Control	7065 ± 242	1.6 ± 0.3	6405 ± 665	15 ± 3
CH 1 μM	4267 ± 194^a	16.3 ± 1.7^a	3882 ± 371^a	162 ± 17^a
CH 5 μM	1520 ± 33^a	21.4 ± 1.5^a	1340 ± 64^a	213 ± 14^a
CH 10 μM	951 ± 171^a	25.0 ± 1.0^a	708 ± 74^a	250 ± 9^a
CH 50 μM	360 ± 17^a	14.6 ± 0.9^a	380 ± 19^a	146 ± 8^a
CH 100 μM	523 ± 11^a	2.2 ± 0.2	279 ± 22^a	21 ± 2

Means ± s.e.mean; $n=4$ wells. $^aP<0.05$ compared to control values in the absence of CH.

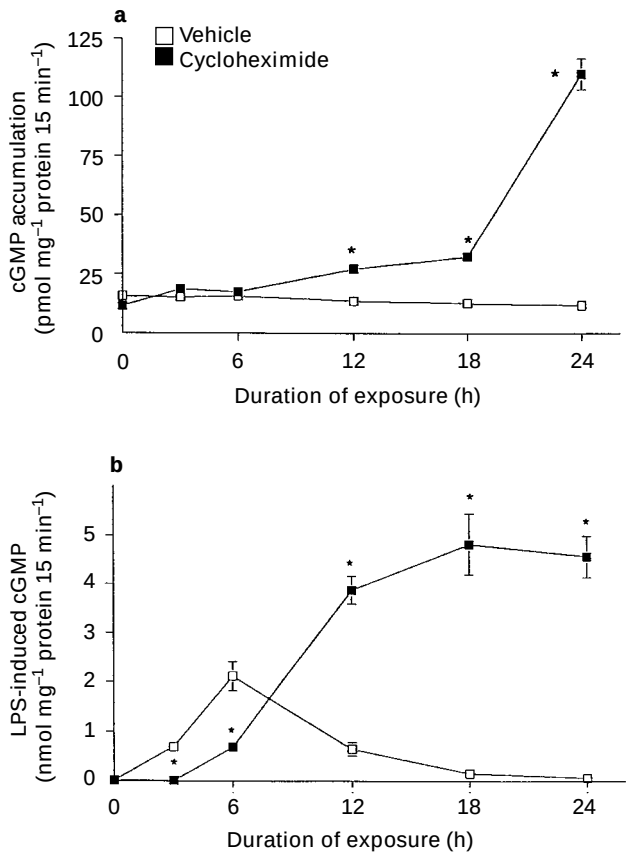

Figure 2 Time-dependent effects of cycloheximide (CH) on vehicle-stimulated (a) or lipopolysaccharide (LPS)-stimulated (b) cyclic GMP accumulation in RASM. Cells were incubated with either medium (a) or medium containing 100 ng ml^{-1} LPS (b) alone (vehicle) or in combination with 10 μM CH. Means with vertical lines showing s.e.mean; $n=4$ wells. *$P<0.05$ compared to cyclic GMP levels of the corresponding group stimulated by vehicle alone.

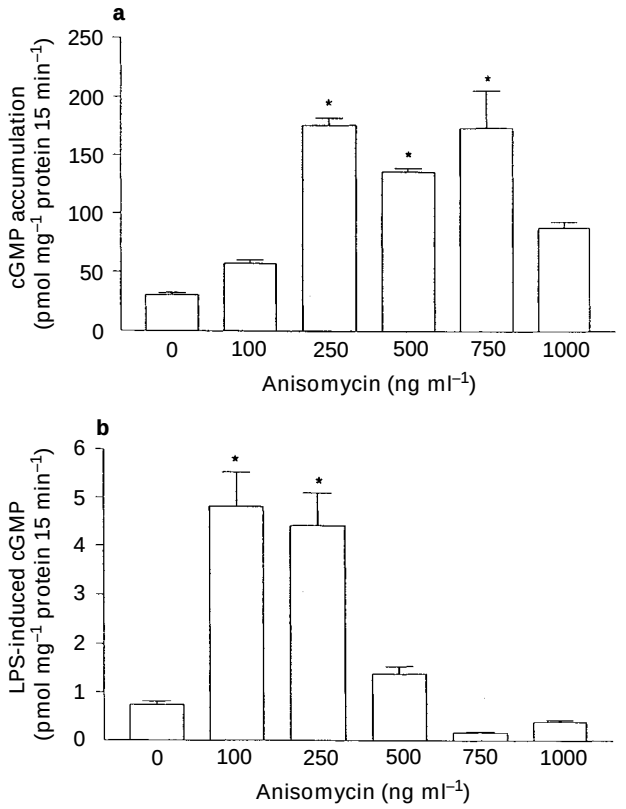

Figure 3 Concentration-dependent effects of anisomycin on vehicle-stimulated (a) or lipopolysaccharide (LPS)-stimulated (b) cyclic GMP accumulation in RASM. Cells were incubated for 24 h in medium containing 0.1 μg ml^{-1} anisomycin either in the absence (a) or presence of 100 ng ml^{-1} LPS (b). Means$\pm$s.e.mean; $n=4$ wells. *$P<0.05$ compared to cyclic GMP levels of the corresponding group without anisomycin.

Effects of CH on extracellular release of NO

To investigate the influence of CH on release of biologically active NO from RASM cells, bioassay experiments were performed as described under Methods. CH treatment (10 μM, 24 h) produced extracellular release of biologically active NO and superinduced NO release evoked by LPS, as judged from L-arginine-dependent and L-NAME-inhibitable cyclic GMP accumulation in reporter RASM cells under bioassay conditions (Figure 5).

Effects of CH on iNOS protein levels

Western blot analysis revealed the absence of iNOS protein in control cells. LPS-induced iNOS protein was detectable after 12 h. CH alone induced detectable iNOS protein at 24 h and appeared to superinduce LPS-induced iNOS induction at 18 and 24 h (Figure 6).

Effects of transcription inhibition on iNOS activity by CH, alone or in combination with LPS

To determine whether CH-stimulated iNOS activity and CH superinduction of LPS-induced NOS are dependent on continuous transcription, cells were incubated with 100 ng ml^{-1} LPS or vehicle alone for 18 h (when iNOS activity is on the decline), then stimulated with medium or 10 μM CH alone, or in combination with 5 μg ml^{-1} actinomycin-D, a transcription inhibitor, for another 12 h (Figure 7). Among the vehicle-treated groups, the group exposed to CH alone for a total of 12 h produced a significant 4 fold increase in cyclic GMP levels. Exposure to actinomycin D caused a significant but very small (2 fold) increase in basal cyclic GMP levels. The combination of actinomycin D and CH prevented the 4 fold increase in cyclic GMP levels observed with CH alone. LPS-treatment caused a 7 fold increase from baseline value, which was increased further by 6 fold (71 ± 6 vs 440 ± 43) when CH was added for the next 12 h. When actinomycin D was added instead of CH, there was no increase in cyclic GMP levels observed. Similarly, when actinomycin D and CH were added together, there was no significant increase in LPS-stimulated iNOS activity. These data suggest that CH-induced NOS and superinduction of LPS-induced NOS are both dependent on transcription.

NO synthase activity in CH-treated RASM cells after CH washout

CH can be readily washed out of cells, allowing translation of preformed mRNA that has been accumulated during protein synthesis arrest and allowing expression of induced enzyme activity (Ghersa *et al*., 1992). To explore the possibility that inhibition of protein synthesis by CH treatment could activate a signalling mechanism leading to iNOS mRNA transcription, we initially inhibited protein synthesis with high concentrations of CH before the restoration of translation for a short period of time by removal of CH. RASM cells were exposed to 500 μM CH for 12 h either under control conditions or in the

presence of either LPS (100 ng ml^{-1}) or cytokines IL-1 (10 u ml^{-1}) and TNFα (1000 u ml^{-1}). After 12 h, the cells were extensively washed with Earle's balanced salt solution to remove CH (3 times 2 ml) and new medium without CH and cytokines was added for 2 h before determination of IBMX-induced cyclic GMP accumulation. Cells in which the CH treatment was continued for 2 h to prevent protein synthesis served as negative controls, whereas cells cultured in the absence of CH were used as controls for superinduction studies. In the absence of CH, LPS and IL-1 produced 2- and 4 fold increases in cyclic GMP, respectively, whereas TNFα had no significant effect (Figure 8). These increases were abolished

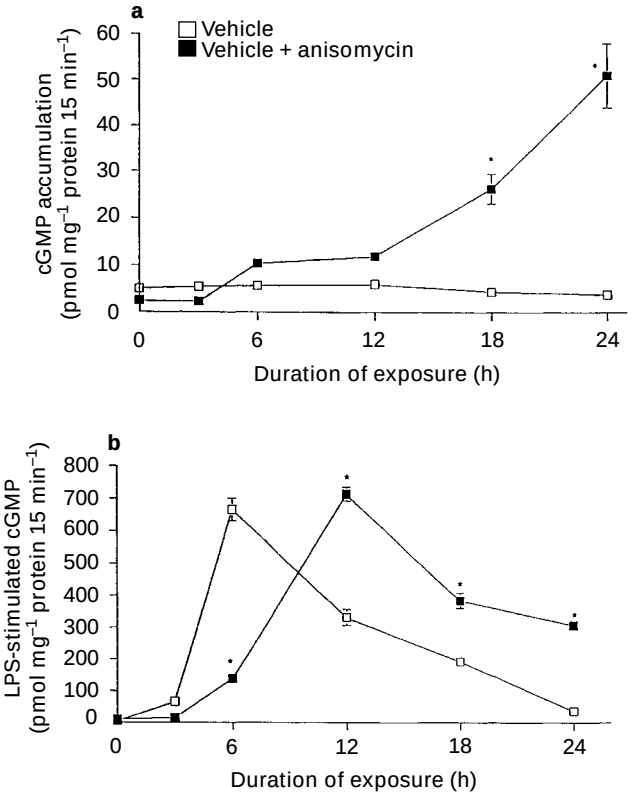

Figure 4 Time-dependent effects of anisomycin on vehicle-stimulated (a) or lipopolysaccharide (LPS)-stimulated (b) cyclic GMP accumulation in RASM. Cells were incubated in either medium alone (a) or medium containing 100 ng ml^{-1} LPS (b), alone or in combination with 250 ng ml^{-1} anisomycin. Means with s.e.mean shown by vertical lines; $n=4$ wells. *$P<0.05$ compared to cyclic GMP levels of the corresponding group stimulated by vehicle or LPS alone.

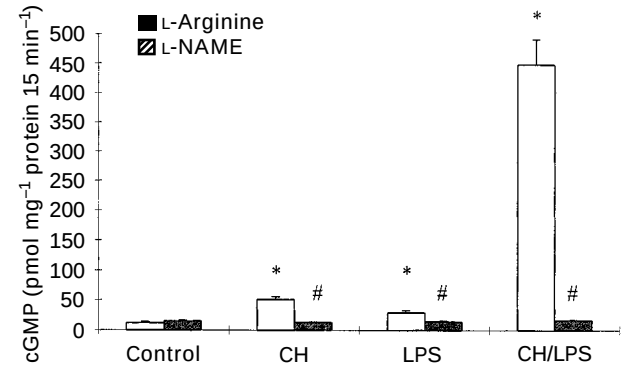

Figure 5 Bioassay determination of extracellular release of biologically active NO from cycloheximide (CH)- and lipopolysaccharide (LPS)-stimulated RASM. 'Donor' cells were incubated in either medium (control) or medium containing 100 ng ml^{-1} LPS alone or in combination with 10 μM CH before incubation with 1 mM L-arginine or 1 mM L-NAME for 30 min. Bioassay was subsequently performed as described under Methods. Data depict cyclic GMP accumulation in the 'reporter' RASM cells. Means±s.e.mean; $n=4$ wells. *$P<0.05$ compared to cyclic GMP levels in control cells; #*$P<0.05$ compared to cyclic GMP levels in appropriate L-arginine-treated cells.

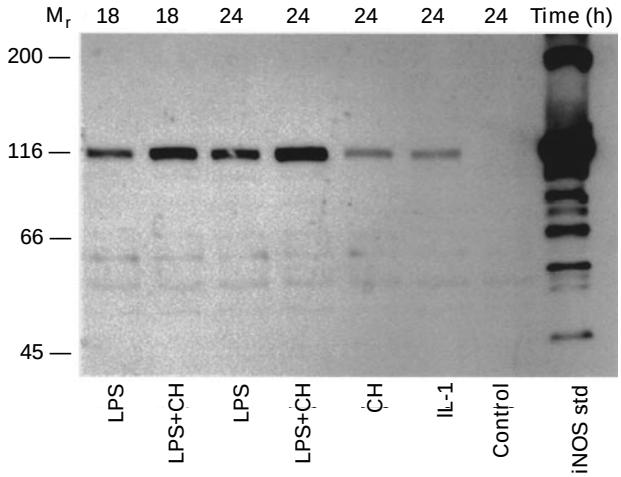

Figure 6 Effect of cycloheximide (CH) on iNOS protein levels in RASM. Confluent RASM were exposed to either medium (control), LPS (100 ng ml^{-1}) or CH (10 μM) alone or with LPS for 19 or 24 h. After each time indicated, total protein was isolated and analysed by Western blot hybridization, as discussed in Methods.

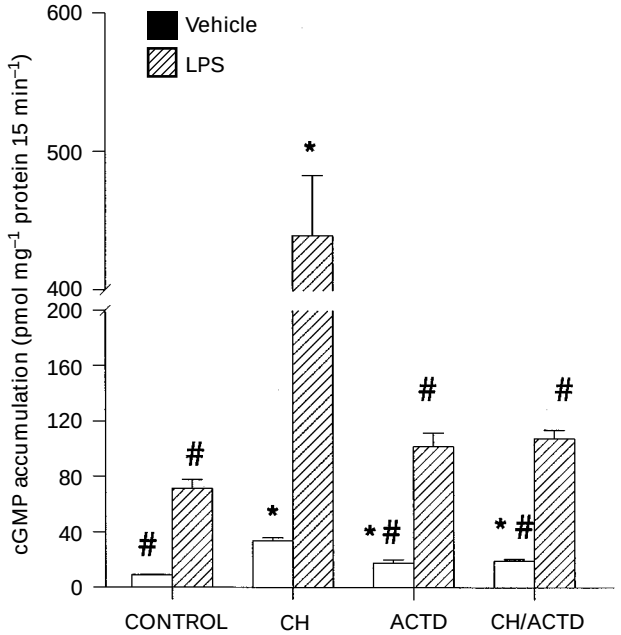

Figure 7 Transcriptional regulation of the superinduction by cycloheximide (CH) of lipopolysaccharide (LPS)-induced cyclic GMP accumulation in RASM. Cells were stimulated with vehicle or 100 ng ml^{-1} LPS for 18 h, followed by a 12 h incubation without (control) or with 10 μM CH (CH), with 5 μg ml^{-1} actinomycin-D (ACTD) alone or with cycloheximide (CH/ACTD). At the end of the 30 h treatment, cyclic GMP accumulation over a 15 min period was measured. *$P<0.05$ compared to cyclic GMP levels in appropriate control cultures. #$P<0.05$ compared to cyclic GMP levels in appropriate CH-treated cultures. Means±s.e.mean; $n=6$ wells.

in the continuous presence of CH, but sodium nitroprusside-induced cyclic GMP accumulation was maintained in these cells (data not shown). Treatment of RASM cells with CH in the absence of cytokines for 12 h followed by washout for 2 h resulted in a 5 fold increase in cyclic GMP (212 ± 27 vs 52 ± 3 pmol mg^{-1} protein). This treatment protocol with CH also superinduced cyclic GMP accumulation by LPS, (789 ± 30 vs 110 ± 7 pmol mg^{-1} protein), IL-1 and TNFα (Figure 8).

Effects of CH on iNOS gene expression

By utilizing RT-PCR analysis of gene expression in groups exposed to LPS and CH combined for 12 h, mRNA levels were significantly higher than mRNA levels from either CH- or LPS-stimulated cells (Figure 9). CH-treated groups showed a significant increase in mRNA levels, whereas LPS-stimulated cells showed the least amount of iNOS mRNA at this time point. There was no iNOS mRNA detected from groups exposed to medium alone.

Discussion

This study demonstrates that the protein synthesis inhibitors CH and anisomycin exhibit multiple effects on iNOS induction and associated cyclic GMP formation in cultured RASM cells. Similar to other studies, we observed that high concentrations of CH and anisomycin were capable of blocking LPS and cytokine-induced NO synthesis, suggesting that iNOS expression depends on new protein synthesis. However, the major novel finding of this study was that lower concentrations of CH alone induced iNOS mRNA and iNOS activity, and superinduced cytokine-evoked iNOS expression in a variety of conditions.

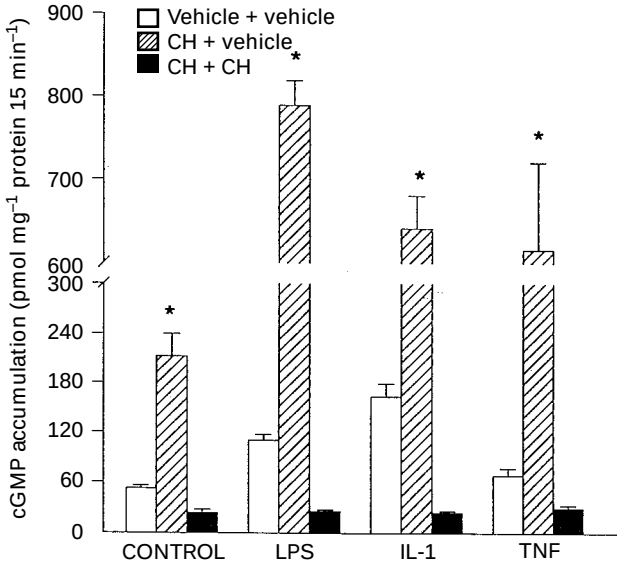

Figure 8 Effects of cycloheximide (CH) on cytokine-stimulated iNOS activity and reversibility of the CH effect. Cells were treated with medium alone (control) or supplemented each with LPS (100 ng ml^{-1}), IL-1β (10 u ml^{-1}) or TNFα (1000 u ml^{-1}) in the absence (vehicle) or presence of 500 μM CH for 12 h. After 12 h, each group was washed extensively with Earle's balanced salt solution and incubated for 2 h more in medium alone (vehicle) or medium containing 500 μM CH. This was followed by a 15 min period of cyclic GMP accumulation in the presence of IBMX and L-arginine (1 mM each). Means $\pm$ s.e.mean; $n=6$ wells. *$P<0.05$ compared to cyclic GMP values in appropriate vehicle 12 h + > vehicle 2 h and CH 12 h + > CH 2 h cultures.

The prolonged effect on intracellular cyclic GMP accumulation induced by CH reflects enhanced formation of NO because (i) it was reversed by L-NMMA and L-NAME, inhibitors of NO synthases; (ii) it was potentiated by L-arginine, the substrate of NO synthases, and (iii) L-arginine concentration-dependently reversed the inhibitory effects of NO synthase inhibitors. In addition, intracellular cyclic GMP accumulation in CH-treated cells was accompanied by extracellular release of biologically active NO capable of stimulating guanylate cyclase in reporter RASM cells.

The effects of CH appear to be due to iNOS gene expression since (i) resting RASM in culture lack constitutively active NOS (Kanno *et al*., 1993; Koide *et al*., 1993) and (ii) CH induced iNOS mRNA in RASM cells.

It is interesting to note that transcriptional activation of inducible genes by protein synthesis inhibition is not unprecedented. CH and other inhibitors of protein synthesis have been shown to activate transcription of immediate early response genes and expression of LPS and cytokine-inducible inflammatory genes such as E-selectin, cyclo-oxygenase and plasminogen activator inhibitor (Edwards & Mahadevan, 1992; Ghersa *et al*., 1992). Generally, it is assumed that these genes are normally kept inactive by the continuous synthesis of labile repressors, which rapidly disappear following inhibition of protein synthesis. We hypothesize that such repressor-regulated mechanism is responsible for the inactive state of the iNOS gene and lack of NO production in quiescent RASM cells.

The increased iNOS activity reflected as cyclic GMP accumulation after prolonged incubation with CH exhibited a characteristic biphasic concentration-dependence. Our interpretation of these data is that prolonged treatment with lower concentrations of CH induced iNOS mRNA and only partially blocked protein synthesis, which allowed cellular accumulation of relatively low amount of iNOS protein leading to production of low levels of NO causing guanylate cyclase activation and increase in cyclic GMP levels. This hypothesis is consistent with the observation that total protein synthesis was not completely eliminated, as judged by [^{35}S]-

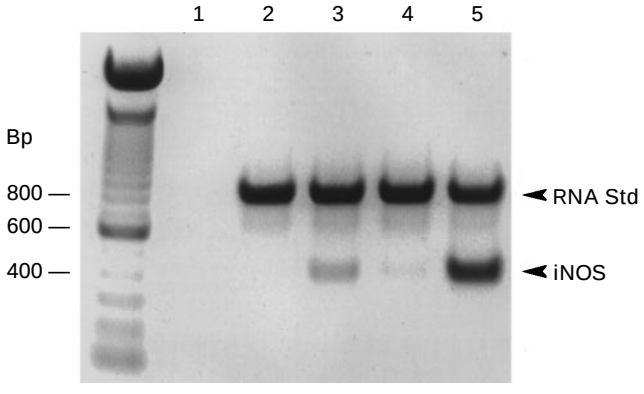

Figure 9 Superinduction of lipopolysaccharide (LPS)-induced NOS mRNA by cycloheximide (CH) in RASM. Total RNA were extracted from RASM exposed to medium (lane 2), 10 μM CH (lane 3), 100 ng ml^{-1} LPS (lane 4) or a combination of CH and LPS (lane 5) for 12 h. From each group, 2 ng RNA was obtained for semi-quantitative RT-PCR analysis in the presence of 1.25 pg exogenous RNA standard and an aliquot was electrophoresed in 1.5% agarose gel in 1 × TAE. Arrows point to 830 bp bands from RNA standards and 423 bp bands from iNOS mRNA. Negative controls include a reaction without RNA (lane 1) and identical reactions from each group with the exception of reverse transcriptase (data not shown). Data are representative of 2 RT-PCR reactions from 2 independent experiments.

methionine incorporation, and that iNOS mRNA and protein were detected in CH-treated cells. Although NO generating enzyme activity was not observed after continuous exposure to 500 μM CH, there was evidence for the accumulation of iNOS mRNA which could be translated upon restoration of protein synthesis by removal of CH. Taken together, these results suggest that CH exhibits dual actions on the NO pathway in cultured RASM: it acts as an inducer of the NO pathway but also restricts NO generation by inhibiting translation of the inducible NO synthase.

While our studies were in progress, Oguchi *et al.* (1994), showed that the iNOS promoter from the mouse macrophage transfected into a mouse macrophage cell line was fully functional in response to low concentrations of CH. Our results are in agreement with that study and may reflect some shared similarities in the regulation of the iNOS promoter between rat vascular smooth muscle cells and mouse macrophages.

CH also modulated LPS- and cytokine-induced NO generation in a time- and concentration-dependent manner. Continuous incubation of RASM cells with 500 μM CH completely blocked LPS- and cytokine-induced cyclic GMP accumulation at any time point tested, whereas the responsiveness of RASM cells to sodium nitroprusside was maintained. Similarly, even lower concentrations of CH appeared to block LPS-induced NO generation at relatively early time points, confirming earlier indications that protein synthesis is required for induction of NO synthesis. However, dramatic changes occurred after prolonged incubations in cytokine-stimulated RASM cells both in the absence and presence of CH. Cytokine induced iNOS mRNA and NO formation gradually declined after 12 h of stimulation followed by a return to near baseline levels by 24 h post induction, suggesting the existence of mechanisms to shut off NO synthase induction. However, in the presence of CH iNOS mRNA, protein and cyclic GMP accumulation reached higher levels and the decline in NO synthesis was prevented, a phenomenon which is more appropriately referred to as superinduction.

Protein synthesis inhibitors have been shown to accentuate and prolong the induction of a number of genes such as c-fos, c-myc, E-selectin, tissue factor, cyclo-oxygenase and TNFα in a variety of cells, including vascular endothelial and smooth muscle cells. With regard to iNOS induction, two other studies addressed modulation of iNOS expression by protein synthesis inhibition in vascular smooth muscle cells. Evans *et al.* (1994) demonstrated that CH upregulated iNOS mRNA by LPS and IFNγ, and similar data were obtained by Sirsjo *et al.* (1994). However, the latter group found opposite effects of CH *in vitro* and *in vivo*.

Superinduction caused by protein synthesis inhibitors has generally been attributed to messenger RNA stabilization through inhibition of short-lived nucleases and/or to an increase in the rate of transcription which may be due to depletion of labile repressor (McCarthy & Kollmus, 1995). Since CH increased NO formation alone and also in the presence of TNFα (a weak stimulus alone), stabilization of messenger RNA alone, although it may contribute to superinduction, cannot fully explain induction and superinduction of iNOS by CH. Thus, our hypothesis is that CH prolonged cytokine activation of iNOS by interfering with a negatively regulating repressor mechanism that is normally responsible for offsetting cytokine-induced NO synthesis in cultured RASM cells. Alternatively, protein synthesis inhibitors might directly upregulate the intracellular signal transduction pathway which leads to transcriptional activation of the iNOS gene in RASM cells. Such effects of anisomycin and cycloheximide have been described in activating p45 and p55 kinases, distal events in the activation of the mitogen-activated protein kinase cascade (Cano *et al.*, 1994; 1995; 1996).

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