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Cytoskeleton-dependent activation of the inducible nitric oxide synthase in cultured aortic smooth muscle cells

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1 Vascular endothelial and smooth muscle cells generate nitric oxide (NO) via different nitric oxide synthase (NOS) isozymes. Activation of the endothelial constitutive NOS (ecNOS) contributes to the maintenance of cardiovascular homeostasis, whereas expression of the endotoxin- and cytokine-inducible pathway (iNOS) within the vascular smooth muscle is thought to be responsible for the cardiovascular collapse which occurs during septic shock and antitumour therapy with cytokines. Since the cytoskeleton is involved in the activation of certain genes and in some effects of endotoxin in macrophages, we investigated the role of microtubules and microfilaments in the activation of the NO pathway in cultured vascular cells.

2 Depolymerization of microtubules by either nocodazole or colchicine prevented lipopolysaccharide (LPS)- and interleukin-1β-induction of NO-dependent cyclic GMP accumulation. Steady state levels of iNOS mRNA, assessed by Northern blot and RT-PCR, and iNOS protein, assessed by Western blotting, were also decreased by either colchicine or nocodazole treatment.

3 Taxol enhanced microtubule polymerization alone, and prevented microtubule depolymerization elicited by nocodazole and colchicine. Associated with its effect on microtubule assembly, taxol prevented the inhibitory effects of nocodazole and colchicine on cyclic GMP accumulation and iNOS mRNA levels.

4 Disruption of microfilaments by cytochalasins had no inhibitory effect on the activation of the inducible NO pathway.

5 In contrast to cytokine-stimulated smooth muscle cells, modulation of either microtubule or microfilament assembly did not affect the constitutive NO pathway in endothelial cells, as endothelial cell- and NO-dependent cyclic GMP accumulation in endothelial-smooth muscle co-cultures remained unchanged.

6 Our findings demonstrate that microtubules play a prominent role in the activation of the inducible NO pathway in response to inflammatory mediators in smooth muscle cells but not of the constitutive synthesis of NO in endothelial cells.

Keywords: Inducible NOS; endothelial NOS; microtubules; microfilaments; colchicine; nocodazole; taxol; smooth muscle cells; nitric oxide; cytochalasins

Introduction

Basal and stimulated release of nitric oxide (NO) and associated activation of guanylate cyclase with increased tissue content of guanosine 3':5'-cyclic monophosphate (cyclic GMP) are now an established endogenous regulatory mechanism of cardiovascular homeostasis (Moncada *et al.*, 1991). Under physiological conditions, the endothelial lining of vessels contributes to the regulation of vascular tone via NO production by the calcium/calmodulin-dependent constitutive NO synthase (ecNOS) (Furchgott & Zawadzki, 1980; Ignarro *et al.*, 1987; Palmer *et al.*, 1988; Vallance *et al.*, 1989; Rees *et al.*, 1989; Bredt *et al.*, 1991). There is increasing evidence, however, for a different mechanism of NO formation via the inducible and calcium-independent NO synthase (iNOS) in a variety of cells, including macrophages (Stuehr *et al.*, 1991; Xie *et al.*, 1992)) and vascular smooth muscle cells (Fleming *et al.*, 1990; Beasley, 1990; Beasley *et al.*, 1989; Busse & Mulsch, 1990). Such an endotoxin- and cytokine- inducible pathway synthesizing large amounts of NO and increasing cyclic GMP in the vasculature appears to be responsible for the hypotension, reduced responsiveness to vasoconstrictors and cardiovascular collapse associated with septic shock (Kilbourne & Belloni 1990; Kilbourne *et al.*, 1991), as well as for the side effects of anti-tumour therapy with cytokines (Moritz *et al.*, 1989; Hibbs *et al.*, 1992). Although endothelial cells respond to cytokines by increasing the expression of the iNOS (Kilbourne & Belloni, 1990; Radomski *et al.*, 1990), the diminished responsiveness to vasoconstrictors of endotoxin- and cytokine-exposed vascular smooth muscle is mainly endothelium- independent. The majority of the iNOS is found in the smooth muscle layer of the aorta from endotoxin-treated rats, and this enzyme can also be induced by cytokines in cultured vascular smooth muscle cells (Rees *et al.*, 1990; Knowles *et al.*, 1990; Szabó *et al.*, 1993; Schini *et al.*, 1992), suggesting that smooth muscle cells are both the source and target of NO in pathological conditions.

Less is known, however, about cellular constituents and molecular signalling mechanisms that regulate the activation of the NO pathway in smooth muscle cells. The cytoskeletal system plays a pivotal role in the regulation of cell shape, intracellular transport, secretion and signal transduction (Penman *et al.*, 1983; Birchmeier, 1984), Furthermore, there is evidence for the influence of the cytoskeletal architecture on gene activation. Alterations in the microfilament structure stimulate the expression of certain proteolytic enzymes in fibroblasts and LLC-PK1 cells (Unemori & Werb, 1986; Botteri *et al.*, 1990). Disassembly of microtubules enhances the mitogenic activity of growth factors (Otto *et al.*, 1979) and prevents gene expression of positive acute phase proteins in hepatocytes (Carter *et al.*, 1989). Relevant to the present studies, microtubules are involved in certain actions of endotoxin in macrophages, such as in the release of TNF α and in the inhibition of TNF-α binding (Ding *et al.*, 1990; 1992).

In light of this relationship between the organization of the

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filamentous apparatus of the cytoplasm and activation of certain genes, we have investigated the role of microfilaments and microtubules, two major components of the cytoskeleton, in the activation of the NO pathways in vascular cells. In a previous report (Marczin *et al.*, 1993), we showed that the microtubule depolymerizing agents nocodazole and colchicine dose-dependently reduce the interleukin-1β (IL-1)- and lipopolysaccharide (LPS)-induced cyclic GMP accumulation in rat aortic smooth muscle cells. Here we expand on this observation and show evidence that microtubules play an essential role in mediating endotoxin- and interleukin- inducible NO-responses in smooth muscle cells but not constitutive release of NO in endothelial cells.

Methods

Cell culture

Aortic smooth muscle cells (RASM) from Wistar rats (Harlan) were isolated by enzymatic dissociation using standard methods (Geinsterfer *et al.*, 1988). The cells were positively identified as smooth muscle by indirect immunofluorescent staining for α-actin, using mouse anti-α-actin antibody and anti-mouse IgG FITC conjugate. RASM cells were grown in T-75 tissue culture flasks (Corning, Corning, NY, U.S.A.) in 50% F12 nutrient medium and 50% Dulbecco's Modified Eagle Medium (GIBCO Laboratories, Grand Island, NY, U.S.A.) supplemented with 10% foetal bovine serum, glutamine, penicillin (Sigma, St. Louis, MO, U.S.A.; 10,000 ul^{-1}) and streptomycin (Sigma, St. Louis, MO, U.S.A.; 10,000 ul^{-1}). They were subcultured into 24-well tissue culture plates (Costar, Cambridge, MA, U.S.A.) at 5,000 cm^{-2} and reached confluence after 5–7 days. Bovine aortic endothelial cells (BAE) were harvested from fresh vessels obtained at the local abattoir by previously published methods (Ryan, 1984). They were grown as monolayers in T-75 tissue culture flasks (Corning, Corning, NY, U.S.A.) in Medium 199 (Mediatech, Washington, D.C., U.S.A.), supplemented with 10% foetal bovine serum (Hyclone Laboratories, Logan, UT, U.S.A.), penicillin and streptomycin. Long-term co-cultures of smooth muscle cells with endothelial cells were established by seeding endothelial cells (50,000/well) on top of a subconfluent smooth muscle cell layer, as described by Marczin *et al.* (1992; 1993).

Modulation of microtubule and microfilament assembly

Microtubule polymerization was modulated by either a depolymerization protocol using nocodazole or colchicine (Sigma Chemical Co.), or by promoting microtubule assembly with taxol, a plant-derived drug that has been reported to enhance microtubule assembly and stabilize microtubules against depolymerizing conditions (Garland, 1978; Schiff *et al.*, 1979; Lee *et al.*, 1980). To depolymerize microtubules, RASM cells or RASM-BAE co-cultures were chilled to 4°C and incubated with dimethyl sulphoxide (DMSO) as vehicle, or exposed to various concentrations of nocodazole or colchicine for 90 min at 4°C (Breitfeld *et al.*, 1990). Cells were then re-warmed at 37°C in the continued presence or absence of the agents for 30 min. The experiment of interest was then performed in the continued presence or absence of nocodazole or colchicine. The effects of taxol on microtubule polymerization were investigated by exposing the cells to taxol alone or in combination with nocodazole or colchicine. To depolymerize microfilaments, RASM cells, or RASM-BAE co-cultures were exposed to cytochalasin D or to cytochalasin B for 60 min at 37°C prior to stimulation of the NO responses.

Tubulin Western blotting

To quantify cytoskeletal tubulin content, cells were exposed to microtubule agents and, after the appropriate incubation, native cells were permeabilized and extracted with extraction buffer (PHEM, 60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM $MgCl_2$, pH 6.9) containing 0.5% Triton X-100 and protease inhibitors. The resulting supernatant containing unassembled monomeric tubulin was combined with an equal volume of 2 × lysis buffer (50 mM Tris, pH 7.4, 0.4 M NaCl, 1% SDS supplemented with protease inhibitors). Detergent resistant material was solubilized in 2 × lysis buffer prior to the addition of an equal volume of extraction buffer. All samples were made up in 0.1% β-mercaptoethanol and boiled for 5 min prior to centrifugation at maximum speed in a microfuge for 5 min. Aliquots of 25 μl were then electrophoresed in a 4–20% gradient SDS-polyacrylamide gel and transferred to a PVDF membrane at 60V for 3 h at 4°C in a buffer containing 25 mM Tris-HCl and 700 mM glycine. After blocking, the membranes were incubated with a monoclonal antibody against tubulin with immunoreactive protein bands visualized using the ECL detection system (Amersham Corp., Arlington Heights, IL) according to manufacturer's instructions with 30 s exposure to X-ray film.

Immunofluorescence studies

To visualize microtubules and microfilaments, cells grown and treated on glass coverslips were fixed with 4% formaldehyde, permeabilized with 0.2% Triton X-100 and processed for anti-tubulin indirect immunofluorescence using a mouse monoclonal antibody (TU-27) to β-tubulin and rhodamine-labelled polyclonal anti-mouse IgG (DAKO Corporation). Actin filaments were stained by treating the coverslips with fluorescein-labelled phalloidin (1 $\mu g\ ml^{-1}$, Sigma). Images were taken with a NU-200 series cooled CCD camera and relayed to a Macintosh computer. Digital enhancement and gray-scale normalization of 16 bit images were performed identically for each sample.

Determination of NO synthesis

NO synthesis was monitored by measuring L-arginine sensitive cyclic GMP accumulation, as one of the most sensitive and specific indices of NO action. We and others have already demonstrated that cytokines and endotoxin lipopolysaccharide (LPS) induce a prolonged activation of the inducible NO pathway in cultured vascular smooth muscle cells, which is reflected by increased cyclic GMP levels and which is sensitive to inhibitors of mRNA transcription, translation and NO synthesis inhibitors (Loeb *et al.*, 1985; Pollock *et al.*, 1991; Ishii *et al.*, 1991; Nunokawa *et al.*, 1993). To monitor activation of the inducible pathway, RASM were treated in the absence or presence of LPS or IL-1. After different experimental protocols, culture medium was removed and the cells were incubated in Earle's balanced salt solution 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 250 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 in 1.0 N NaOH. The HCl extract was directly analysed for cyclic GMP by radioimmunoassay using the automated Gammaflow system and the protein concentration of the NaOH-solubilized samples was determined, as described by Marczin *et al.* (1992, 1993).

Activation of the constitutive NO pathway in endothelial cells was investigated in long-term co-cultures of endothelial and smooth muscle cells, as described by Marczin *et al.* (1992; 1993). Briefly, after various pretreatment protocols, co-culture cyclic GMP accumulation was determined by a 15 min incubation in the presence of IBMX and L-arginine. To study the calcium-dependence of this pathway, the cultures were stimulated with an endothelium-dependent vasodilator (bradykinin, 10 μM) or calcium ionophore (A23187, 10 μM) for 10 min. To

determine the NO contribution to cyclic GMP accumulation, in each experiment the effect of N^G-nitro-L-arginine (L-NOARG), a potent inhibitor of endothelial NO synthase was determined. L-NOARG was present for 30 min prior to, and during the IBMX incubations.

iNOS mRNA studies

Isolation of total RNA Total RNA was isolated from RASM cells by using a commercially available kit (RNAzol, Biotecx Laboratories Inc, Houston, TX, U.S.A.), quantified by absorbance at 260 nm (Shimadzu Spectrophotometer) and stored at −70°C in a 0.1% mercaptoethanol, 0.1% sarcosyl, 70% ethanol and 2 M ammonium acetate solution.

Northern Blot analysis Aliquots (10 μg) of total RNA were glyoxylated for 1 h, electrophoresed in 1% agarose gel in 10 mM Na_2HPO_4, pH 6.5 and transferred to nylon membrane in 20 × SSC solution. A 645 bp iNOS cDNA from mouse macrophage iNOS (generously provided by Dr Carl Nathan from Cornell University Medical College, NY, U.S.A.) was labelled with [α-^{32}P]dCTP (NEN Research Products, Boston, MA, U.S.A.) by random primer labelling (oligolabelling kit from Pharmacia LKB Biotechnology, Piscataway, NJ, U.S.A.) to a specific activity of 10^6 c.p.m. μg^{-1}, as probe. Membranes were hybridized overnight in a 42°C water bath with 50% formamide, 5 × SSC, 0.2% SDS, 0.05 M $NaHPO_4$, 10% dextran sulphate, 50 μg poly-A RNA, 50 μg denatured salmon sperm DNA and the labelled probe (100,000 c.p.m. cm^{-2} of the membrane). Hybridized membranes were washed twice at room temperature with 2 × SSC-0.1% SDS for 10 min and once with 0.1 × SSC-0.1% SDS for 5 min prior to exposure to XOMAT film. To determine uniformity of sample loading and transfer, blots were stripped and rehybridized with a 1 Kb glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe.

Reverse transcription polymerase chain reaction After ethanol precipitation of RNA, the pellet was resuspended in 10 mM Tris, 1 mM EDTA, pH 8 solution and 3 u of DNase (Gibco BRL) was added for a further 30 min incubation at 37°C. The DNase-treated RNA was extracted by phenol-chloroform, ethanol washed and resuspended in RNase-free 1 mM EDTA, 10 mM NaCl, 10 mM Tris HCl pH 8.0 solution. The amount of RNA in solution was quantified by absorbance at 260 nm after which 400 ng were reverse transcribed in a 20 μl reaction mixture of commercially available reagents (GeneAmp RNA PCR kit) containing 2.5 u M-MLV reverse transcriptase, 1 u RNase inhibitor, 2.5 μM random hexamers, 1 mM dNTP, 5 mM $MgCl_2$ and 1 × PCR buffer II in 1 cycle of 15 min at 42°C, 5 min at 99°C and 5 min at 5°C (Perkin-Elmer Cetus DNA Thermal Cycle). For the PCR, each 20 μl reaction was supplemented with (final concentration) 0.2 μM of each primer, 2.5 u Taq polymerase, 2 mM $MgCl_2$, and 1 × PCR buffer II in a final total volume of 100 μl. The upstream and downstream primers for iNOS used were 5′-CCCTTCCGAAGTTTCTGGCAGCAG-3′ at base position 2952 and 5′-GGGCTCCTCCAAGGTGTTGCCC-3′ at base position 3426 (Ransom Hill Bioscience, Inc., Ramona CA, U.S.A.) to rat iNOS cDNA (43,44). Denaturation, annealing and elongation were done at 94, 55 and 72°C for 1, 1 and 1.5 min, respectively for 29 cycles. The number of cycles and amount of starting material was determined in preliminary experiments to fall within the linear phase of exponential increase in PCR end products. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was used as control. Primers for GAPDH are 5′-TGAAGGTCGGTGTCAACG-GATTTGGC-3′ at base position 35 and 5′-CATGTAGGC-CATGAGGTCCACCAC-3′ at base position 1017, synthesized on an automated DNA synthesizer using phosphoramidite chemistry. To determine the presence of genomic DNA, duplicates of each reverse transcription reaction mixture were done in the absence of reverse transcriptase. To visualize the PCR products, 10 μl from each reaction was electrophoresed in 1.5% agarose gel in 0.5 × TAE containing ethidium bromide to allow u.v.-induced fluorescence of DNA.

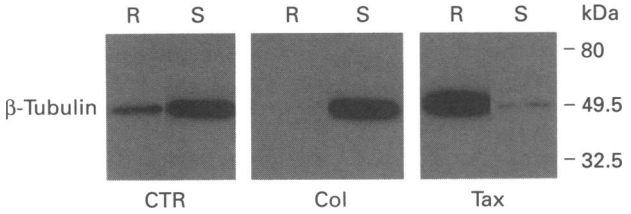

Figure 1 Western blot showing the effects of colchicine (Col, 5 μM) and taxol (Tax, 10 μM) on the distribution of tubulin between detergent resistant (R) and detergent soluble (S) compartments in rat aortic smooth muscle cells. Cells were treated with the microtubule agents prior to extraction of soluble materials with 0.5% Triton X-100 and solubilization of resistant structures with lysis buffer, as described in methods.

iNOS Western blotting

iNOS protein levels in RASM were estimated by Western blotting as described by Hirokawa *et al.* (1994). Three different cell treatments were employed: (a) control, (b) cytokine-exposed and (c) colchicine- and cytokine-exposed RASM, as discussed above. For reference, actin protein levels were also estimated in the same preparations.

Results

Cytoskeletal changes induced by nocodazole, colchicine and taxol in RASM cells

In control cells, a significant amount of tubulin was recovered from the detergent resistant fraction (Figure 1, CTR, R), whereas the majority of tubulin was present in the soluble fraction (CTR, S). Colchicine treatment resulted in an almost complete loss of tubulin from the detergent resistant fraction (Figure 1, Col, R). Taxol promoted microtubule polymerization, as judged by translocation of tubulin from the soluble to the detergent-resistant fraction (Figure 1, Tax, R and S). Immunofluorescent studies revealed that in vehicle-treated cells, microtubules were abundant and frequently formed bundles (Figure 2a). Cells treated with either nocodazole or colchicine, however, were largely depleted from microtubules; only a few colchicine-resistant microtubules were seen (Figure 2c), consistent with published reports (Dustin, 1984). Taxol alone significantly promoted microtubule assembly in RASM cells, as characteristic dense bundles of microtubules were seen following taxol treatment (Figure 2e). Taxol not only promoted microtubule polymerization but also prevented microtubule depolymerization induced either by nocodazole or colchicine. In the presence of both taxol and nocodazole or taxol and colchicine (Figure 2g) the microtubule staining was similar to that in control cells in the absence of nocodazole or colchicine. Staining of actin filaments with fluoresceinated phalloidin revealed that microfilament structures remained unaltered after treatment with nocodazole (not shown), colchicine (Figure 2d), taxol (Figure 2f), or with combination of taxol and colchicine (Figure 2h), when compared to untreated control (Figure 2b).

Effects of microtubule disassembly on the induction of NO synthesis in RASM cells

Nocodazole treatment, although having no effect on basal cyclic GMP levels, completely prevented LPS-induced cyclic GMP accumulation (Figure 3a). Similar treatment with nocodazole, on the other hand, had virtually no effect on the

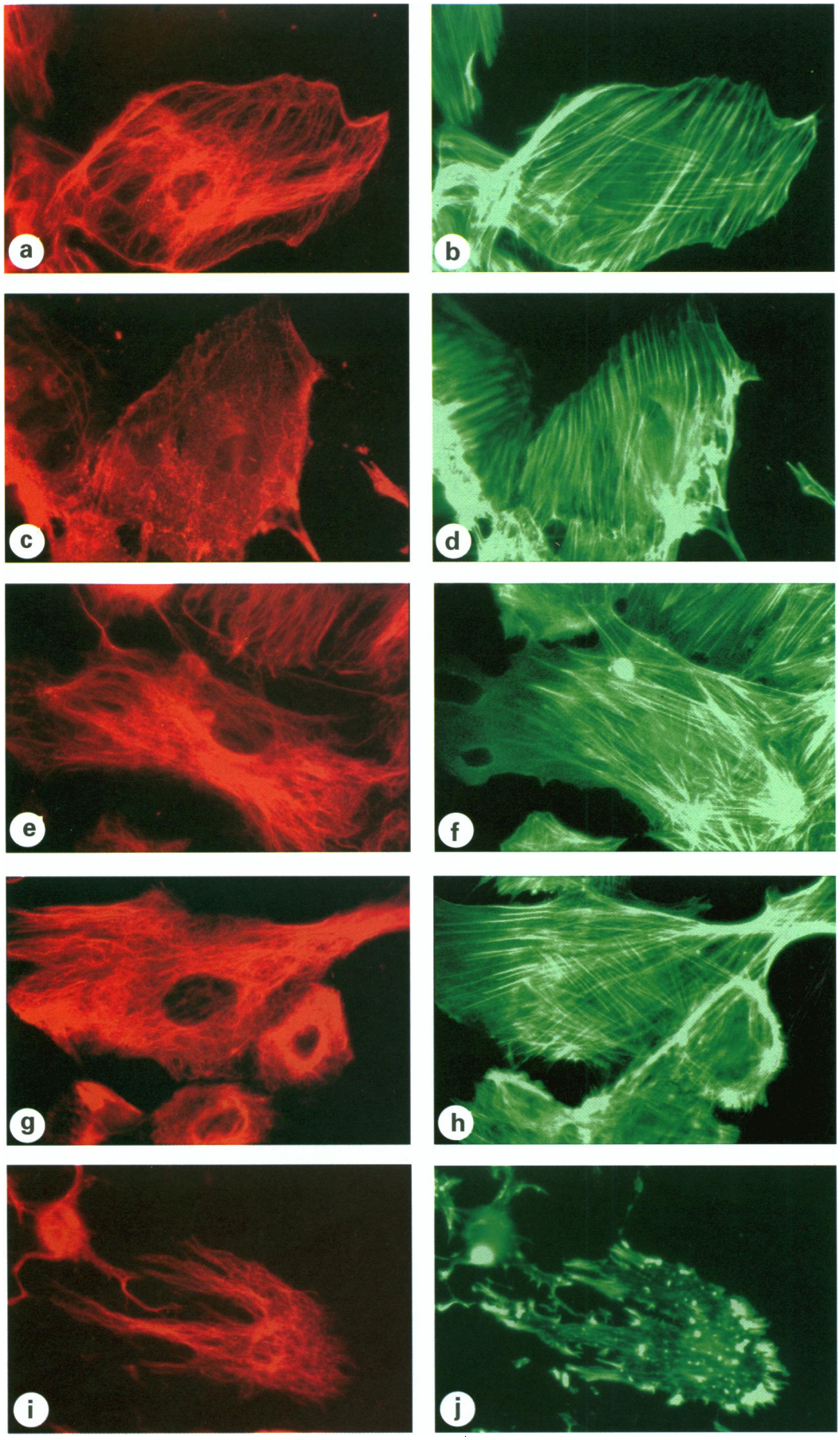

Figure 2 Modulation of microtubule and microfilament assembly in rat aortic smooth muscle cells. Shown are indirect anti-tubulin immunofluorescence images (a,c,e,g,i) and fluorescein phalloidin fluorescence images (b,d,f,h,j) of control RASM cells (a,b), or cells treated with colchicine (5 μM, c,d), taxol (10 μM, e,f), taxol + colchicine (g,h) and cytochalasin D (i,j). Treatments and staining were performed as described in methods.

cyclic GMP increase elicited by the NO-releasing sodium nitroprusside. This suggests that the effect of nocodazole was not related to a direct action on guanylate cyclase activity. Similarly to nocodazole, exposure of RASM cells to colchicine also diminished LPS-induced cyclic GMP accumulation, with no effect on that elicited by sodium nitroprusside (Figure 3b).

We also investigated the role of microtubules in the activation of the NO-pathway in response to interleukin-1 β (IL-1), a cytokine known to induce NO generation both in isolated vessels and in cultured RASM (Bredt *et al.*, 1991). The IL-1 induction of cyclic GMP accumulation (31 ± 3 fold increase) was also inhibited by both nocodazole and colchicine (Figure 3c).

Effects of microtubule stabilization by taxol on the induction of NO pathway in RASM cells

Although taxol alone had no effect on basal cyclic GMP levels and on those elicited by maximally effective concentrations of either LPS (1 μg ml^{-1}) or IL-1 (10 u ml^{-1}) (Figure 4b and c first set of columns), it significantly stimulated cyclic GMP accumulation induced by lower concentrations of LPS ($\leqslant 1$ ng ml^{-1}) in the presence of serum (Figure 4a). In addition, taxol also potentiated the relatively low cyclic GMP formation elicited by 10 μM LPS under serum-free conditions (206 ± 23 vs. 137 ± 7 pmol mg^{-1} protein 15 min^{-1}). These results suggest that promoting microtubule polymerization does not modulate cyclic GMP formation alone, but it might increase the sensitivity of RASM to LPS.

Taxol (10 μM) fully protected LPS-induced cyclic GMP

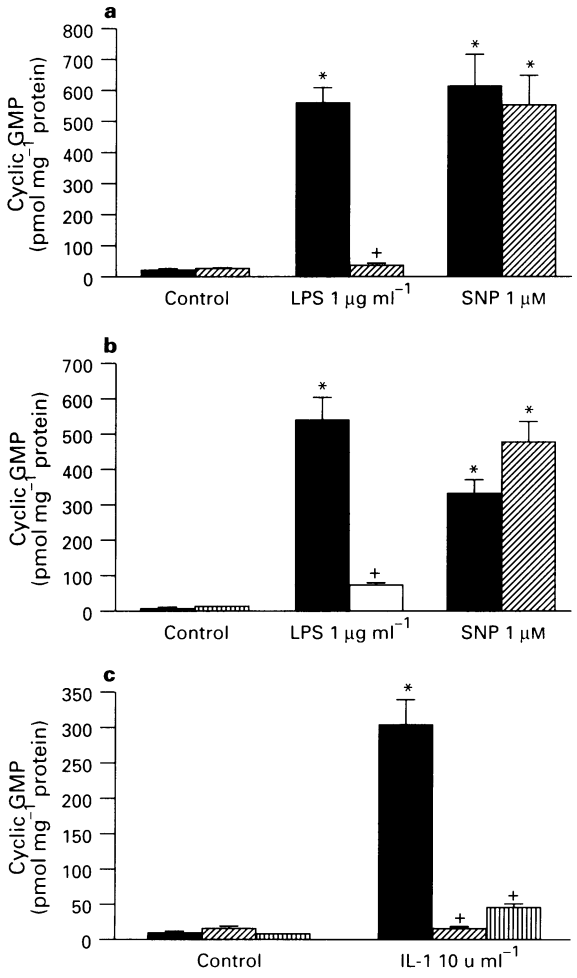

Figure 3 Effects of microtubule disassembly on the induction of cyclic GMP accumulation in rat cultured aortic smooth muscle cells. Effects of nocodazole (33 μM, a, hatched columns) or colchicine (5 μM, b, hatched columns) on LPS (1 μg ml^{-1}) and SNP (1 μM)-induced cyclic GMP levels. (c) Effects of nocodazole (diagonally hatched columns) or colchicine (vertically hatched columns) on interleukin-1β- (IL-1, 10 u ml^{-1})-induced cyclic GMP accumulation. All cells were chilled to 4°C and incubated in the absence (Vehicle, 0.2% DMSO, solid columns) or in the presence of nocodazole or colchicine for 90 min. Cells were then warmed to 37°C in the continued absence or presence of nocodazole or colchicine for 30 min, prior to stimulation with or without LPS or IL-1 for 3 h. $^{*}P<0.05$ from cyclic GMP levels in appropriate Control cultures. $^{+}P<0.05$ from cyclic GMP levels in appropriate Vehicle cultures. Data shown are means $\pm$ s.e. of 4–6 cultures.

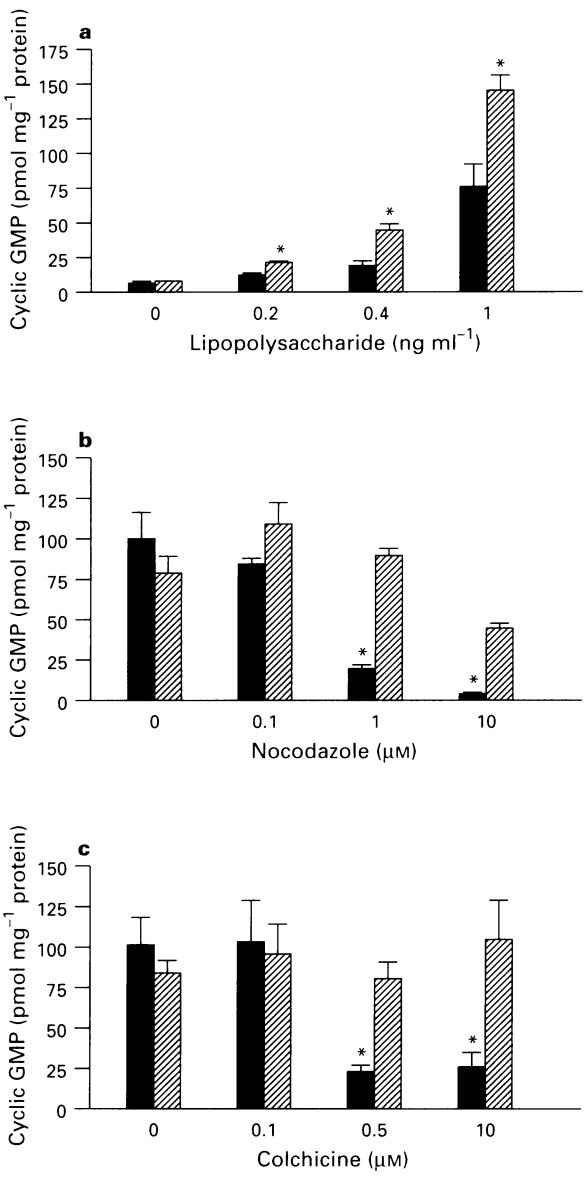

Figure 4 Effects of microtubule stabilization by taxol on the induction of cyclic GMP accumulation in rat cultured aortic smooth muscle cells. (a) Effects of taxol (10 μM) on LPS-induced cyclic GMP accumulation. $^{*}P<0.05$ from cyclic GMP levels in appropriate Vehicle cultures. (b) Effects of taxol on nocodazole-mediated inhibition of LPS (1 μg ml^{-1})-induced cyclic GMP accumulation. $^{*}P<0.05$ from cyclic GMP levels in Vehicle cultures in the absence of nocodazole. (c) Effects of taxol on colchicine-mediated inhibition of IL-1-(10 u ml^{-1}) induced cyclic GMP accumulation. $^{*}P<0.05$ from cyclic GMP levels in Vehicle cultures in the absence of colchicine. Cells were pretreated with DMSO as vehicle (solid columns) or with taxol (hatched columns) for 30 min prior to the microtubule disassembly protocol using either colchicine or nocodazole prior to LPS challenge for 3 h. Data shown are means $\pm$ s.e. of 4 individual cultures. (Data with nocodazole alone and colchicine alone were previously given in Marczin *et al.*, 1993).

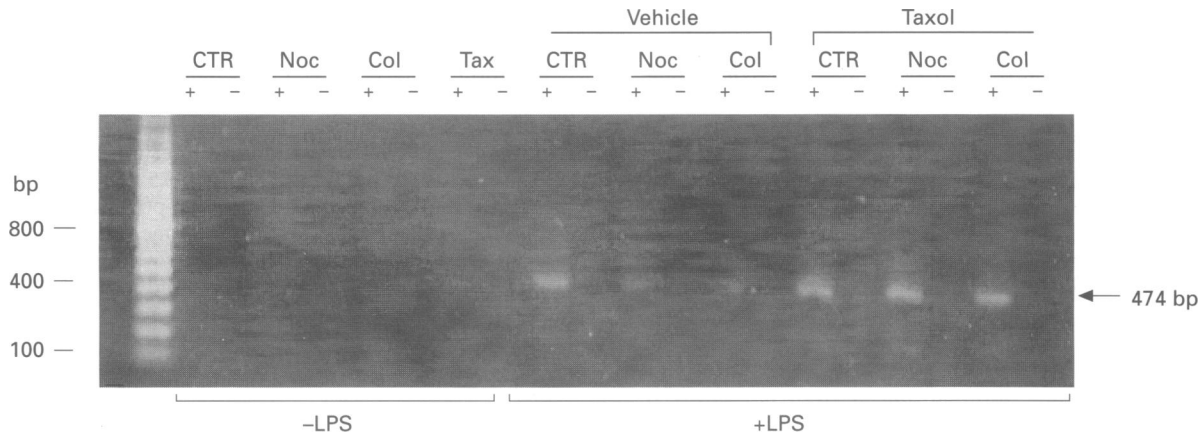

Figure 5 RT-PCR analysis of the effects of microtubule modulating agents on iNOS expression. Cells were pretreated with DMSO as vehicle or with taxol (Tax) for 30 min prior to the microtubule disassembly protocol using either colchicine (Col) or nocodazole (Noc) prior to LPS challenge for 3 h. After isolation of total RNA, 400 ng was amplified using iNOS primers for 29 cycles, as described in methods. Plus and minus signs represent presence (+) or absence (–) of reverse transcriptase.

Table 1 Effect of microtubule destabilizing agents on the induction phase of LPS-stimulated cyclic GMP accumulation in RASM cells

	Cyclic GMP (pmol mg^{-1} protein 15 min^{-1})			
RASM treatment	*Vehicle*		*LPS*	
A. Control	13.5±1.9	(3)	49.3±6.8	(6)*
Nocodazole	13.5±0.9	(3)	16.6±2.9	(6)†
B. Control	6.3±0.5	(6)	40.0±6.9	(6)*
Colchicine	5.9±0.6	(6)	9.9±0.9	(6)†
C. Control	19.9±5.5	(4)	400±78	(6)*
Nocodazole	19.2±2.1	(4)	298±49	(6)*
Colchicine	20.8±5.7	(4)	267±42	(6)*

RASM cells were first exposed to LPS (1 $\mu g\ ml^{-1}$) or medium alone for 15 min at 37°C and, after removal of LPS, the cells were treated with nocodazole (33 μM, **A**) or colchicine (5 μM, **B**) for 90 min at 4°C. Accumulation of cyclic GMP was determined after a subsequent 3 h incubation at 37°C in the continuous presence or absence of these agents. **C** Microtubular disassembly by cold and nocodazole or colchicine was performed after 4 h of LPS exposure. *$P < 0.005$ from corresponding vehicle value. †$P < 0.05$ from corresponding control value.

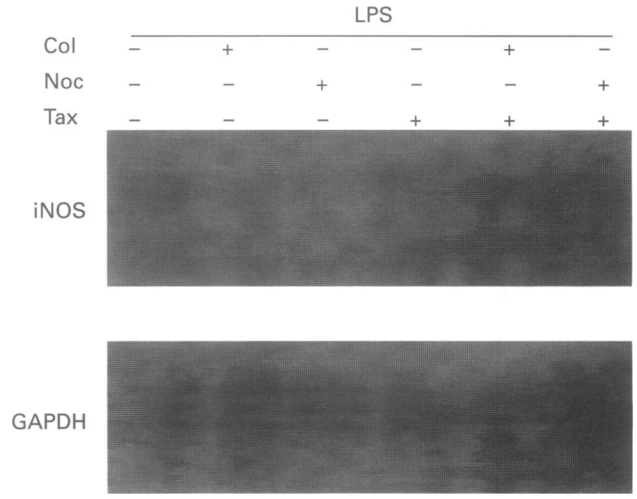

Figure 6 Northern blot analysis of the effects of microtubule modulating agents on iNOS and GAPDH expression. Cells were pretreated with DMSO as vehicle or with taxol (Tax) for 30 min prior to microtubule disassembly protocol using either colchicine (Col) or nocodazole (Noc) prior to LPS challenge for 3 h. After isolation of total RNA, 10 μg was loaded, electrophoresed, transferred to nylon membrane and hybridized with the 645 bp iNOS cDNA probe. Thereafter, the membrane was stripped and re-hybridized with a GAPDH cDNA probe.

production against the action of 1 μM nocodazole and partially protected against 10 μM nocodazole (Figure 4b). Moreover, taxol provided full protection against colchicine inhibition of IL-1-induced cyclic GMP formation in RASM cells (Figure 4c).

Delayed effects of microtubule depolymerization on the induction of NO pathway in RASM cells

To characterize further the role of microtubules in the transduction of immunomodulatory signals, we identified two phases of LPS-induced cyclic GMP accumulation. RASM cells were allowed to interact with LPS for 15 min only, followed by an additional 3 h incubation in LPS-free medium. Even under these conditions, LPS produced a 5–10 fold increase in cyclic GMP levels, suggesting that the continuous presence of LPS in the medium is not required to stimulate cyclic GMP generation. Thus, two phases of LPS-action can be identified, a short 'priming' phase in the presence of LPS and a subsequent 'induction' period in the absence of LPS. It is possible, of course, that even after rinsing, there is LPS remaining bound to the smooth muscle cell surface. To investigate the requirement for microtubules during the 'induction' phase of LPS action, we depolymerized microtubules at different time points after LPS exposure. As shown in Table 1 (A and B), exposure of RASM to either nocodazole or colchicine immediately after the 15 min LPS-treatment, significantly inhibited cyclic GMP accumulation. However, when the microtubule disassembly protocol was initiated 4 h after LPS exposure, LPS-induced cyclic GMP levels were not affected by nocodazole or colchicine (Table 1C).

Effects of microtubule modulating agents on the induction of iNOS mRNA in RASM cells

In the absence of inducing agents, there were no iNOS mRNA signals detected (Figure 5 lanes 1–8). Exposure to LPS along for 3 h induced the transcription of the iNOS gene (lane 9). In the presence of either nocodazole (10 μM) or colchicine (10 μM) the iNOS mRNA signals were reduced (lanes 11 and

13). Although taxol had only a marginal effect on LPS-induced iNOS mRNA levels, it prevented the reduction of the iNOS mRNA caused by both colchicine and nocodazole (lanes 17 and 19). There were no significant changes in GAPDH mRNA levels under these conditions. These results were further confirmed by Northern blot analysis (Figure 6).

Effects of colchicine on the induction of iNOS protein in RASM

In the absence of inducing agents, there was no iNOS protein detectable in RASM (Figure 7, lane 1). Following a 24 h exposure of RASM to a combination of IL-1 (135 u ml^{-1}) and TNFα (150 u ml^{-1}) (CMIX), a strong iNOS band was observed (Figure 7, lane 2). Similar stimulation with CMIX, but after a 30 min exposure to colchicine (10 μM), significantly reduced the intensity of the iNOS band (Figure 7, lane 3). There were no differences among the three treatment groups in the amounts of actin detected.

Effects of microtubule depolymerization on the activation of the constitutive NO pathway in BAE-RASM cultures

To detect basal or stimulated release of NO from BAE cells, BAE-RASM co-cultures were exposed to vehicle or to the endothelium-dependent vasodilator bradykinin or calcium ionophore, or to the potent inhibitor of the constitutive NO synthase, N^G-nitro-L-arginine (L-NOARG). None of these agents affected cyclic GMP accumulation in single cultures of either RASM or BAE. In co-culture, however, the high basal cyclic GMP levels of 747±68 pmol ng^{-1} protein were almost completely inhibited by L-NOARG, suggesting basal release of NO from BAE (Figure 8). Inclusion of bradykinin or A23187 (both at 10 μM) produced a 2 and 3 fold increase in cyclic GMP from basal levels, suggesting calcium-dependence of these responses. Nocodazole or taxol had no effect on either basal or stimulated NO release in these co-cultures.

Effects of cytochalasins on the cytoskeleton and on the induction of NO pathway in RASM cells

Cytochalasin D produced dramatic alterations of actin staining, by completely eliminating stress-fibre like structures (Figure 2j). The small amounts of remaining polymer actin (i.e. actin that is capable of phalloidin binding) were seen as small patches of fluorescence without any clear orientation (Figure 2j). Cell shape was dramatically altered by cytochalasin treatment. Even though mild effects on microtubule morphology were also present, still a large number of bundled microtubules were seen (Figure 2i). Cytochalasin B produced similar morphological changes, although to a lesser extent. Exposure of RASM cells to either cytochalasin B or cytochalasin D (both at 10 μM) had no effect on basal cyclic GMP levels (Table 2). Similarly, neither compound modulated cyclic GMP accumulation in response to 1 μM sodium nitroprusside (51.2±4.3, 56.0±5.2 and 57.1±2.7 fold increase for vehicle, cytochalasin B and cytochalasin D, respectively), or to LPS (Table 2).

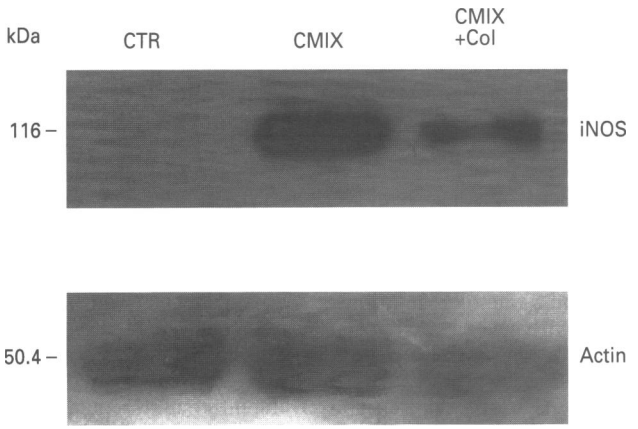

Figure 7 Western blot analysis of the effects of the microtubule modulating agent colchicine on iNOS and actin protein levels. Cells were exposed to a microtubule disassembly protocol using colchicine (Col, 10 μM) prior to a combined IL-1 (135 u ml^{-1}) and TNF (150 u ml^{-1}) (CMIX) challenge for 24 h.

Effects of cytochalasins on the activation of the constitutive NO pathway in BAE-RASM cultures

The effects of cytochalasin preincubation on basal and calcium ionophore-induced release of NO from endothelial cells was investigated in BAE-RASM co-cultures. Despite pronounced morphological changes, cytochalasin D or cytochalasin B had no significant effects on cyclic GMP accumulation in these co-cultures (Table 3).

Discussion

The major novel finding is that the cytoskeleton selectively modulates the activation of NO pathways in cultured vascular cells. This regulation appears to be selective in two ways: (1) the cytoskeleton plays an essential role in bringing about the

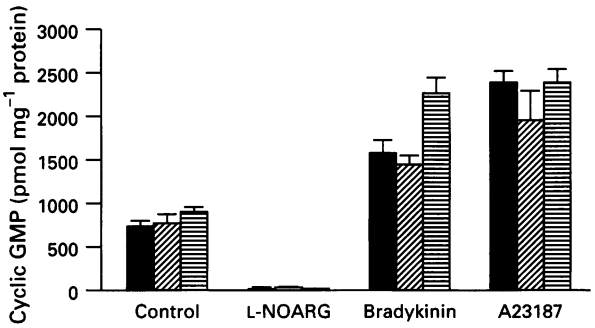

Figure 8 Effects of microtubule modulating agents on basal and stimulated release of NO from endothelial cells. BAE-RASM co-cultures were chilled to 4°C and incubated in the absence (Vehicle, 0.2% DMSO, solid columns) or in the presence of nocodazole (diagonally hatched columns) or taxol (horizontally hatched columns) for 90 min. Cells were then warmed to 37°C in the continued absence or presence of these agents for 30 min, prior to determination of cyclic GMP accumulation in the presence of IBMX and L-arginine (1 mM for 15 min). Accumulation of cyclic GMP was determined either under basal conditions (Vehicle) or in the presence bradykinin (10 μM, 10 min) or calcium ionophore (A23187, 10 μM, 10 min). L-NOARG was included 30 min prior to and during IBMX incubations.

Table 2 Effects of cytochalasins on LPS-induced cyclic GMP accumulation in RASM cells

	Cyclic GMP (pmol mg^{-1} protein 15 min^{-1})			
RASM treatment	*Vehicle*		*LPS*	
A. Control	17.4±3.6	(6)	1312±129	(6)*
Cytochalasin B	17.8±2.6	(6)	2246±400	(6)*
B. Control	11.7±1.8	(6)	1716±226	(6)*
Cytochalasin D	16.6±1.9	(6)	2603±615	(6)*

RASM cells were treated with 0.1% DMSO as vehicle or were exposed to cytochalasin B (10 μM, **A**) or cytochalasin D (10 μM, **B**) for 60 min prior to incubation with LPS (1 μg ml^{-1}) for 3 h. *$P<0.05$ from corresponding vehicle value.

Table 3 Effects of cytochalasins on basal and stimulated release of NO from endothelial cells

RASM treatment	*Cyclic GMP* (pmol mg^{-1} protein 15 min^{-1}) *L-NOARG*	*Vehicle*	*A23187*
Control	13.3±0.9 (4)*	118±7 (4)	586±33 (4)*
Cytochalasin D	18.1±0.7 (4)*	133±10 (4)	618±31 (4)*
Cytochalasin B	13.0±0.5 (4)*	134±2 (4)	753±59 (4)*

BAE-RASM co-cultures were treated with 0.1% DMSO as control, or were exposed to cytochalasin D or cytochalasin B (both at 10 μM) for 60 min prior to the determination of cyclic GMP accumulation in the presence of IBMX and L-arginine (1 mM for 15 min). Accumulation of cyclic GMP was determined under basal conditions (Vehicle) or in the presence of calcium inophore (A23187, 10 μM, 10 min). L-NOARG was included 30 min prior to and during IBMX incubations. *$P<0.05$ from corresponding vehicle value.

activation of the inducible NO pathway in smooth muscle cells, but not the activation of the constitutive NO pathway in endothelial cells, and (2) there is an absolute requirement for microtubules but not for microfilaments during induction of NO in smooth muscle cells.

There is increasing evidence that under different conditions, vascular cells generate NO either basally or in response to a variety of stimuli, via distinct mechanisms and isozymes. Under physiological conditions, a ecNOS operates within the endothelial cells and continuously utilizes L-arginine to synthesize and tonically release NO, which helps maintain the fluidity of blood and regulate vascular tone. This ecNOS can be further triggered by agonists that increase intracellular Ca^{2+} levels and activate calmodulin. In response to immunological stimuli, such as cytokines and endotoxin, a different pathway can be induced in both vascular smooth muscle cells and endothelial cells. This iNOS also uses L-arginine as substrate and is sensitive to inhibitory L-arginine analogues; furthermore, it does not appear to differ significantly from ecNOS on the basis of cofactor requirements. It can be distinguished, however, by the major characteristics of its activation. In contrast to the endothelial, rapidly responsive constitutive pathway, the activation of the inducible pathway is Ca^{2+} and calmodulin-independent, slow in onset, occurs after a delay of several hours, is transcriptionally regulated and sensitive to dexamethasone (Xie *et al.*, 1992).

Our results provide evidence for a further difference between the inducible vs. the constitutive NO pathway, on the basis of selective cytoskeletal requirements for their activation. In smooth muscle cells, the time course of endotoxin- and interleukin-stimulated responses and the sensitivity of the cyclic GMP responses to actinomycin D and cycloheximide is consistent with the role of transcription and *de novo* synthesis of an iNOS as an underlying mechanism for NO synthesis (Marczin *et al.*, 1993). This is further proved by the demonstration of iNOS mRNA following LPS treatment in these cells. In the present study, two chemically distinct agents, nocodazole and colchicine, disrupted microtubule assembly and prevented endotoxin-elicited and NO-mediated cyclic GMP accumulation and reduced iNOS mRNA and protein levels in vascular smooth muscle cells. These effects appear to correlate well with the polymerization state of microtubules, because the inhibitory actions of nocodazole and colchicine were absent, when microtubule depolymerization was prevented by taxol. Since nocodazole and colchicine did not affect microfilament assembly, and disruption of microfilaments had no inhibitory effects on LPS responses, we suggest that the activation of the inducible NO pathway is regulated by a microtubule-dependent system in vascular smooth muscle cells. This regulatory mechanism is not restricted to endotoxin alone, but functions during interleukin responses, as well, suggesting a potentially central role in the induction process in these cells.

There are several potential means by which cytoplasmic microtubules might influence the activation of the inducible NO pathway by immunomodulators. Microtubules may play a role in the interaction between the activators and the cell surface, in the internalization process, signal transduction leading to activation of the iNOS gene, or transcription of mRNA. Furthermore, microtubules might affect stability and sorting of mRNA and protein synthesis (Walker & Whitfield, 1985; Sundell & Singer, 1991). This regulatory mechanism is a unique phenomenon, since disruption of this component of the cytoskeleton reduces the expression of only certain genes such as positive acute phase proteins in hepatocytes and tissue plasminogen activator in endothelial cells, whereas it does not affect the expression of other genes such as negative acute phase reactants in hepatocytes or actually promotes protein expression such as urokinase in LLC-PK1 cells or plasminogen activator inhibitor in endothelial cells (Carter *et al.*, 1989; Botteri *et al.*, 1990; Santell *et al.*, 1992). Our initial characterization suggests that signalling mechanism(s) beyond the interaction of LPS with the RASM cell surface, but not the activity of the iNOS once it is induced, or the guanylate cyclase activated process, depend(s) upon microtubules. This conclusion is substantiated by experiments suggesting that either the signal transduction process or events related to enzyme formation might require intact microtubules in vascular smooth muscle cells activated by inflammatory mediators.

The increase in cyclic GMP levels in co-cultures of endothelial and smooth muscle cells in response to endothelium-dependent vasodilators and calcium ionophore is consistent with the activity of the constitutive NO synthase in the endothelial cells. In light of the shared sensitivity to Ca^{2+}-calmodulin of both the constitutive NO pathway and cytoskeleton assembly, we considered the possibility that alterations in cytoskeletal structures might influence NO generation and release from endothelial cells (Ignarro, 1990). It is remarkable that none of the cytoskeletal destabilizing agents had detectable effects on basal or agonist-induced release of NO in the present model, suggesting that in contrast to the activation of the inducible NO pathway in smooth muscle cells, the constitutive NO pathway is not under cytoskeletal control.

Clinical findings of increased serum levels of nitrites/nitrates, the stable degradation products of NO, in patients receiving interleukin therapy or suffering from sepsis, coupled with the ability of inhibitors of NO synthesis to inhibit hypotension in animal models of sepsis, suggest that these inhibitors may constitute an important class of drugs for the treatment of endotoxin and cytokine-mediated septic shock (Nava *et al.*, 1991; Petros *et al.*, 1991; Geroulanos *et al.*, 1992). Given the multiplicity of isozymes of NO synthase and the central role of the constitutive NO synthase in the regulation of cardiovascular homeostasis and in neuronal function, it may be presumed, that a therapy targeting the substrate and the relatively common cofactor regulation of these enzymes would be associated with severe side effects. In this regard, a selective mode of activation of the different isozymes may produce a more rational basis and more cautious approach to pharmacological therapy. Our observations concerning differential regulation of the constitutive and inducible pathways of NO synthesis via the dynamic assembly of the microtubule system might represent a basis for novel therapeutic potential in septic shock.

Agents capable of modulating microtubule assembly are already used in clinical practice as anti-inflammatory and antineoplastic drugs. For instance, colchicine is a well-toler-

ated drug used for the chronic treatment of gout and familial mediterranean fever (Malkinson, 1982; Dinarello *et al.*, 1974). Although the mechanisms of its anti-inflammatory action are not fully understood, it appears to inhibit leukocyte migration and degranulation (Malawista, 1968; Zurier *et al.*, 1974). Given the role of NO in mediating cytokine actions and immunecomplex-induced vascular injury (Mulligan *et al.*, 1991), inhibition of the inducible NO pathway may be an additional mechanism underlying the anti-inflammatory effects of colchicine. Since colchicine also inhibits TNF binding on macrophages and endothelial cells, it may target multiple events involved in cytokine-mediated septic shock (Ding *et al.*, 1990). It is noteworthy, that taxol, a plant-derived new antitumour drug, prevented the effects of colchicine in the present study and facilitated endotoxin-induced activation of the NO pathway in smooth muscle cells. Since taxol mimics endotoxin-elicited release of TNF from macrophages (Ding *et al.*, 1992), taxol might exhibit proinflammatory properties *in vivo*, as well.

In conclusion, the present study demonstrates that microtubules mediate the activation of an LPS- and interleukin-1β-inducible pathway to synthesize L-arginine-derived NO and increase cyclic GMP in vascular smooth muscle cells, a proposed pathophysiological mechanism underlying septic shock and side-effects of antitumour therapy with cytokines. This offers an experimental framework for further investigating the participation of the structural organization of cytoskeleton in vascular responses during immunological challenges.

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References

BEASLEY, D. (1990). Interleukin 1 and endotoxin activate soluble guanylate cyclase in vascular smooth muscle. *Am. J. Physiol.*, **259,** R38–R44.

BEASLEY, D., COHEN, R.A. & LEVINSKY, N.G. (1989). Interleukin-1 inhibits contraction of vascular smooth muscle. *J. Clin. Invest.*, **83,** 331–335.

BEASLEY, D., SCHWARTZ, J.H. & BRENNER, B.M. (1991). Interleukin 1 induces prolonged L-arginine-dependent cyclic guanosine monophosphate and nitrite production in rat vascular smooth muscle cells. *J. Clin. Invest.*, **87,** 602–608.

BIRCHMEIER, W. (1984). Cytoskeleton structure and function. *Trends Biochem. Sci.*, **9,** 192–195.

BOTTERI, F.M., BALLMER-HOFER, K., RAJPUT, B. & NAGAMINE, Y. (1990). Disruption of cytoskeletal structures results in the induction of the urokinase type plasminogen activator gene expression. *J. Biol. Chem.*, **265,** 13327–13334.

BREDT, D.S., HWANG, P.M., GLATT, C.E., LOWENSTEIN, C., REED, R.R. & SNYDER, S.H. (1991). Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. *Nature*, **351,** 714–718.

BREITFELD, P.P., MCKINNON, W.C. & MOSTOV, K.W. (1990). Effect of nocodazole on vesicular traffic to the apical and basolateral surfaces of polarized MDCK cells. *J. Cell Biol.*, **111** (6 Pt 1), 2365–2373.

BUSSE, R. & MÜLSCH, A. (1990). Induction of nitric oxide synthase by cytokines in vascular smooth muscle cells. *FEBS Lett.*, **275,** 87–90.

CARTER, K.C., COOPER, R., PAPACONSTANTINOU, J. & RITCHIE, D.G. (1989). Microtubule depolymerization inhibits the regulation of α_1-acid glycoprotein mRNA by hepatocyte stimulating factor. *J. Biol. Chem.*, **264,** 515–519.

DINARELLO, C.A., WOLFF, S.M., GOLDFINGER, S.E., DALE, D.C. & ALLING, D.W. (1974). Colchicine therapy for Familial Mediterranean Fever. *N. Engl. J. Med.*, **291,** 934–937.

DING, A.H., PORTEU, F., SANCHEZ, E. & NATHAN, C.F. (1990). Down-regulation of tumor necrosis factor receptors on macrophages and endothelial cells by microtubule depolymerizing agents. *J. Exp. Med.*, **171,** 715–727.

DING, A.H., PORTEU, F., SANCHEZ, E. & NATHAN, C.F. (1992). Shared actions of endotoxin and taxol: induction of TNF release and downregulation of TNF receptors. *Science*, **248,** 370–372.

DUSTIN, P. (1984). *Microtubules*. Berlin: Springer-Verlag.

FLEMING, I., GRAY, G.A., JULOU-SCHAEFFER, G., PARRATT, J.R. & STOCLET, J. (1990). Incubation with endotoxin activates the L-arginine pathway in vascular tissue. *Biochem. Biophys. Res. Commun.*, **171,** 562–568.

FURCHGOTT, R.F. & ZAWADZKI, J.V. (1980). The obligatory role of the endothelial cells in the relaxation of arterial smooth muscle to acetylcholine. *Nature*, **288,** 373–376.

GARLAND, D.L. (1978). Kinetics and mechanism of colchicine binding to tubulin: evidence for ligand-induced conformational change. *Biochemistry*, **17,** 4266–4272.

GEINSTERFER, A.A.T., PEACH, M.J. & OWENS, G. (1988). Angiotensin II induces hypertrophy but not hyperplasia of cultured rat aortic smooth muscle cells. *Circ. Res.*, **62,** 749–756.

GEROULANOS, S., SCHILLING, J., CAKMAKCI, M., JUNG, H.H. & LARGIADER, F. (1992). Inhibition of NO synthesis in septic shock. *Lancet*, **339,** 435.

HIBBS, Jr, J.B., WESTENFELDER, C., TAINTOR, R., VAVRIN, Z., KABLITZ, C., BARANOWSKI, R.L., WARD, J.H., MENLOVE, R.L., MCMURRY, M.P., KUSHNER, J.P. & SAMLOWSKI, W.E.J. (1992). Evidence for cytokine inducible nitric oxide synthesis from L-arginine in patiens receiving interleukin-2 therapy. *J. Clin. Invest.*, **89,** 867–877.

HIROKAWA, K., O'SHAUGHNESSY, K., MOORE, K., RAMRAKHA, P. & WILKINS, M.R. (1994). Induction of nitric oxide synthase in cultured vascular smooth muscle cells: the role of cyclic AMP. *Br. J. Pharmacol.*, **112,** 396–402.

IGNARRO, L.J. (1990). Nitric oxide: a novel signal transduction mechanism for transcellular communication. *Hypertension*, **16,** 477–483.

IGNARRO, L.J., BUGA, G.M., WOOD, K.S., BYRNS, R.E. & CHAUDHURI, G. (1987). Endothelium derived relaxing factor produced from artery and vein is nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.*, **84,** 9265–9269.

ISHII, K., SHENG, H., WARNER, T.D., FÖRSTERMANN, U. & MURAD, F. (1991). A simple and sensitive bioassay method for detection of EDRF with RFL rat lung fibroblasts. *Am. J. Physiol.*, **261,** H598–H603.

KILBOURNE, R.G. & BELLONI, P. (1990). Endothelial cell production of nitrogen oxides in response to interferon in combination with tumor necrosis factor, interleukin-1, or endotoxin. *J. Natl. Cancer Inst.*, **82,** 772–776.

KILBOURNE, R.G., GROSS, S.S., JUBRAN, A., ADAMS, J., GRIFFITH, O.W., LEVI, R. & LODATO, R.F. (1990). N^G-methyl-L-arginine inhibits tumor necrosis factor-induced hypotension: Implications for the involvement of nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.*, **87,** 3629–3632.

KILBOURNE, R.G., JURBRAN, A., GROSS, S.S., GRIFFITH, O.W., LEVI, R., ADAMS, J. & LODATO, R.F. (1991). Reversal of endotoxin-mediated shock by N^G-methyl-L-arginine, an inhibitor of nitric oxide synthesis. *Biochem. Biophys. Res. Commun.*, **172,** 1132–1138.

KNOWLES, R.G., SALTER, M., BROOKS, S.L. & MONCADA, S. (1990). Anti-inflammatory glucocorticoids inhibit the induction by endotoxin of nitric oxide synthase in the lung, liver and aorta of the rat. *Biochem. Biophys. Res. Commun.*, **172,** 1042–1048.

LEE, J.C., FIELD, D.J. & LEE, L.L.Y. (1980). Effects of nocodazole on structures of calf brain tubulin. *Biochemistry*, **19,** 6209–6215.

LOEB, A.L., OWENS, G.K. & PEACH, M.J. (1985). Evidence for endothelium-derived relaxing factor in cultured cells. *Hypertension*, **7,** 804–807.

MALAWISTA, S.E. (1986). Colchicine: A common mechanism for its anti-inflammatory and anti-mitotic effects. *Arthritis Rheum.*, **11,** 191–197.

MALKINSON, F.D. (1982). Colchicine, New uses of an old drug. *Arch. Dermatol.*, **118,** 453–457.

MARCZIN, N., PAPAPETROPOULOS, A., JILLING, T. & CATRAVAS, J.D. (1993). Prevention of nitric oxide synthase induction in vascular smooth muscle cells by microtubule depolymerizing agents. *Br. J. Pharmacol.*, **109,** 603–605.

MARCZIN, N., RYAN, U.S. & CATRAVAS, J.D. (1992). Effects of oxidant stress on endothelium-derived relaxing factor-induced and nitrovasodilator-induced cyclic GMP accumulation in vascular cells, in culture. *Circ. Res.*, **70,** 326–340.

MONCADA, S., PALMER, R.M.J. & HIGGS, E.A. (1991). Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol. Rev.*, **43,** 109–142.

MORITZ, T., NIEDERLE, N., BAUMANN, J., MAY, D., KURSCHEL, E., OSIEKA, R., KEMPENI, J., SCHLICK, E. & SCHMIDT, C.G. (1989). Phase I study of recombinant human tumor necrosis factor alpha in advanced malignant disease. *Cancer Immunol. Immunother.*, **29,** 144–150.

MULLIGAN, M.S., HEVEL, J.M., MARLETTA, M.A. & WARD, P.A. (1991). Tissue injury caused by deposition of immune complexes is L-arginine dependent. *Proc. Natl. Acad. Sci. U.S.A.*, **88,** 6338–6342.

NAVA, E., PALMER, R.M. & MONCADA, S. (1991). Inhibition of nitric oxide synthesis in septic shock: how much is beneficial? *Lancet*, **338,** 1555–1557.

NUNOKAWA, Y., ISHIDA, N. & TANAKA, S. (1993). Cloning of inducible nitric oxide synthase in rat vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.*, **191,** 89–94.

OTTO, A.M., ZUMBE, A., GIBSON, L., KUBLER, A. & DE ASUA, L.J. (1979). Cytoskeleton-disrupting drugs enhance the effect of growth factors and hormones on initiation of DNA synthesis. *Proc. Natl. Acad. Sci. U.S.A.*, **76,** 6435–6438.

PALMER, R.M.J., ASHTON, D.S. & MONCADA, S. (1988). Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature*, **333,** 664–666.

PENMAN, S., CAPCO, D.G., FEY, E.G., CHATTERJEE, P., REITER, T., ERMISH, S. & WAN, K. (1983). The three-dimensional structural networks of cytoplasm and nucleus: Function in cells and tissue. *Modern Cell Biol.*, **2,** 385–415.

PETROS, A., BENNETT, D. & VALLANCE, P. (1991). Effect of nitric oxide synthase inhibitors on hypotension in patients with septic shock. *Lancet*, **338,** 1557–1558.

POLLOCK, J.S., FÖRSTERMANN, U., MITCHELL, J.A., WARNER, T.D., SCHMIDT, H.H.H.W., NAKANE, M. & MURAD, F. (1991). Purification and characterization of particular endothelium-derived relaxing factor synthase from cultured and native bovine aortic endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.*, **88,** 10480–10484.

RADOMSKI, M.W., PALMER, R.M.J. & MONCADA, S. (1990). Glucocorticoids inhibit the expression of an inducible, but not the constitutive, nitric oxide synthase in vascular endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.*, **87,** 10043–10047.

REES, D.D., CELLEK, S., PALMER, R.M.J. & MONCADA, S. (1990). Dexamethasone prevents the induction by endotoxin of a nitric oxide synthase and the associated effects on vascular tone. An insight into endotoxin shock. *Biochem. Biophys. Res. Commun.*, **173,** 541–547.

REES, D.D., PALMER, R.M.J. & MONCADA, S. (1989). Role of endothelium-derived nitric oxide in the regulation of blood pressure. *Proc. Natl. Acad. Sci. U.S.A.*, **86,** 3375–3378.

RYAN, U.S. (1984). Isolation and culture of pulmonary endothelial cells. *Environ. Health Perspect.*, **56,** 103–114.

SANTELL, L., MAROTTI, K., BARTFELD, N.S., BAYNHAM, P. & LEVIN, E.G. (1992). Disruption of microtubules inhibits the stimulation of tissue plasminogen activator expression and promotes plasminogen activator inhibitor type 1 expression in human endothelial cells. *Exp. Cell Res.*, **201,** 358–365.

SCHIFF, P.B., FANT, J. & HORWITZ, S.B. (1979). Promotion of microtubule assembly *in vitro* by taxol. *Nature*, **277,** 665–667.

SCHINI, V.B., CATOVSKI, S., DURANTE, W., SCOTT-BURDEN, T., SCHAFER, A.L. & VANHOUTTE, P.M. (1992). Thrombin inhibits induction of nitric oxide synthase in cultured vascular smooth muscle cells. *Am. J. Physiol.*, **264,** (Heart Circ. Physiol.), H611–H616.

STUEHR, D.J., CHO, H.J., KWON, N.S., WISE, M.F. & NATHAN, C.F. (1991). Purification and characterization of the cytokine-induced macrophage nitric oxide synthase, an FAD- an FMN-containing flavoprotein. *Proc. Natl. Acad. Sci. U.S.A.*, **88,** 7773–7777.

SUNDELL, C.L. & SINGER, R.H. (1991). Requirement of microfilaments in sorting of actin mRNA. *Science*, **253,** 1275–1277.

SZABÓ, C., MITCHELL, J.A., THIEMERMANN, C. & VANE, J.R. (1993). Nitric oxide-mediated hyporeactivity to noradrenaline precedes the induction of nitric oxide synthase in endotoxin shock. *Br. J. Pharmacol.*, **108,** 786–792.

UNEMORI, E.N. & WERB, Z.J. (1986). Reorganization of polymerized actin: a possible trigger for induction of procollagenase in fibroblasts cultured in and on collagen gels. *J. Cell Biol.*, **103,** 1021–1031.

VALLANCE, P., COLLIER, J. & MONCADA, S. (1989). Effects of endothelium-derived nitric oxide on peripheral arteriolar tone in man. *Lancet*, **ii,** 997–1000.

WALKER, P.R. & WHITFIELD, J.F. (1985). Cytoplasmic microtubules are essential for the formation of membrane bound polyribosomes. *J. Biol. Chem.*, **260,** 765–770.

XIE, Q., CHO, H.J., CALAYCAY, J., MUMFORD, R.A., SWIDEREK, K.M., LEE, T.D., DING, A., TROSO, T. & NATHAN, C. (1992). Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. *Science*, **256,** 225–228.

ZURIER, R.B., WEISSMANN, G., HOFFSTEIN, S., KAMMERMAN, S. & TAI, H.H. (1974). Mechanisms of lysosomal enzyme release from human leukocytes. II. Effects of cAMP and cyclic GMP, autonomic agonists, and agents which affect microtubule function. *J. Clin. Invest.*, **53,** 297–309.

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