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Prevention of nitric oxide synthase induction in vascular smooth muscle cells by microtubule depolymerizing agents

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We investigated the role of microtubules in the induction of nitric oxide synthase in cultured vascular smooth muscle cells. We found that like interleukin-1α, lipopolysaccharide elicited a time and concentration-dependent accumulation of cyclic GMP via induction of nitric oxide synthase. Nocodazole and colchicine, two chemically distinct microtubule depolymerizing agents, completely prevented lipopolysaccharide- and interleukin-induced (and nitric oxide-mediated) cyclic GMP generation. In contrast to lipopolysaccharide and interleukin-1α, cyclic GMP accumulation in response to sodium nitroprusside, an exogenous nitrovasodilator, was not altered by either nocodazole or colchicine. Our findings demonstrate that microtubule depolymerizing agents inhibit nitric oxide synthase induction and suggest a prominent role for microtubules in mediating the activation of the inducible nitric oxide pathway in smooth muscle cells.

Keywords: Colchicine: nocodazole; cytoskeleton; guanylate cyclase; cyclic GMP; sodium nitroprusside; endotoxin; interleukin-1α

Introduction Vascular smooth muscle cells are not only targets of endothelium-derived nitric oxide, but may also be the source of nitric oxide in pathological conditions. An endotoxin and cytokine-inducible pathway synthesizing large amounts of nitric oxide and increasing guanosine 3':5'-cyclic monophosphate (cyclic GMP) in the vasculature, appears to be responsible for the hypotension, reduced responsiveness to vasoconstrictors and cardiovascular collapse associated with septic shock (Fleming *et al.*, 1990; Busse & Mülsch, 1990; Kilbourne *et al.*, 1990; Beasley *et al.*, 1991). The mechanisms underlying the induction of nitric oxide synthase in the smooth muscle cells are not fully understood.

The cytoskeleton has been implicated in the regulation of cell shape, intracellular transport, secretion, signal transduction and gene activation (Penman *et al.*, 1983). Microtubules are involved in certain actions of endotoxin in macrophages, such as in the release of tumour necrosis factor α (TNF α) and in the inhibition of TNF α binding (Ding *et al.*, 1990). In light of this relationship between cytoskeleton and gene activation, we investigated the effects of microtubule depolymerizing agents on the induction of nitric oxide synthase in vascular smooth muscle cells.

Methods Rat aortic smooth muscle cells from Wistar rats were isolated by enzymatic dissociation and were identified by standard methods. Smooth muscle cells were subcultured at a seeding density of 5,000 cm^{-2}, were maintained in 50% F12 nutrient medium and 50% Dulbecco's Modified Eagle Medium (GIBCO Laboratories), supplemented with 10% foetal bovine serum, L-glutamine (200 mg l^{-1}), penicillin (Sigma, 10,000 u l^{-1}) and streptomycin (Sigma, 10,000 u l^{-1}) and reached confluence after 5-7 days.

Nitric oxide synthase activity was detected as L-arginine-sensitive intracellular accumulation of cyclic GMP. To monitor activation of the inducible pathway, smooth muscle cells were exposed to *E. coli* lipopolysaccharide (LPS, Sigma) in the presence or absence of the transcription inhibitor actinomycin D, the protein synthesis inhibitor cycloheximide, or various inhibitors of nitric oxide action. To depolymerize microtubules, smooth muscle cells were chilled to 4°C and incubated with dimethylsulphoxide (DMSO) as vehicle or were exposed to various concentrations of nocodazole (Calbiochem) or colchicine (Sigma) for 90 min at 4°C. Cells were then rewarmed to 37°C in the continued presence or absence of the agents for 30 min. LPS- or interleukin-1α (Boehringer Mannheim)-stimulation was then performed in the continued presence or absence of nocodazole or colchicine. After 3 h of exposure, generation of cyclic GMP was assessed by incubating the cells for 15 min in the presence of 3-isobutyl-1-methylxanthine (IBMX; 1 mM) to prevent the breakdown of cyclic GMP. Intracellularly accumulated cyclic GMP was then extracted into HCl and measured by radioimmunoassay (Marczin *et al.*, 1992).

Data are expressed as mean ± s.e.mean. Comparisons were performed by the one-way analysis of variance, followed by Dunnett's *t* test. Differences among means were considered significant when $P < 0.05$.

Results Preliminary experiments suggested that nitric oxide formation occurred with a delay of 2 h after LPS exposure, was maximal at 3 h (24 fold increase in cyclic GMP levels from baseline), and was maintained at least for 24 h. Polymyxin B, an antibiotic with antiendotoxin activity, completely prevented the stimulation of cyclic GMP generation by LPS (Figure 1a, PMB). Accumulation of cyclic GMP was also significantly reduced by inclusion, during the final 30 min of LPS incubation, of an inhibitor of nitric oxide synthase, N^G-monomethyl-L-arginine (Figure 1a, L-NMMA), whereas L-NMMA had no modulatory effects on baseline cyclic GMP levels in the absence of LPS (116 ± 11%). Methylene blue, an inhibitor of nitric-oxide-mediated guanylate cyclase activation, completely abolished the effect of LPS (Figure 1a, MB). The cyclic GMP increase elicited by LPS was also prevented by actinomycin D, an RNA transcription inhibitor, or cycloheximide, a protein synthesis inhibitor (Figure 1a). Similar results were obtained with dexamethasone, an inhibitor of nitric oxide synthase induction (Figure 1a, DM; Radomski *et al.*, 1990).

Although nocodazole treatment had no effect on basal cyclic GMP levels, it prevented LPS-induced cyclic GMP accumulation in a concentration-dependent manner (Figure 1b). Colchicine (10 μM) also inhibited LPS-stimulated cyclic GMP levels by 87%. Exposure of smooth muscle cells to interleukin-1α produced a 31 ± 3 fold increase in cyclic GMP levels. This effect of interleukin-1α was inhibited by both nocodazole (data not shown) and colchicine (Figure 1c).

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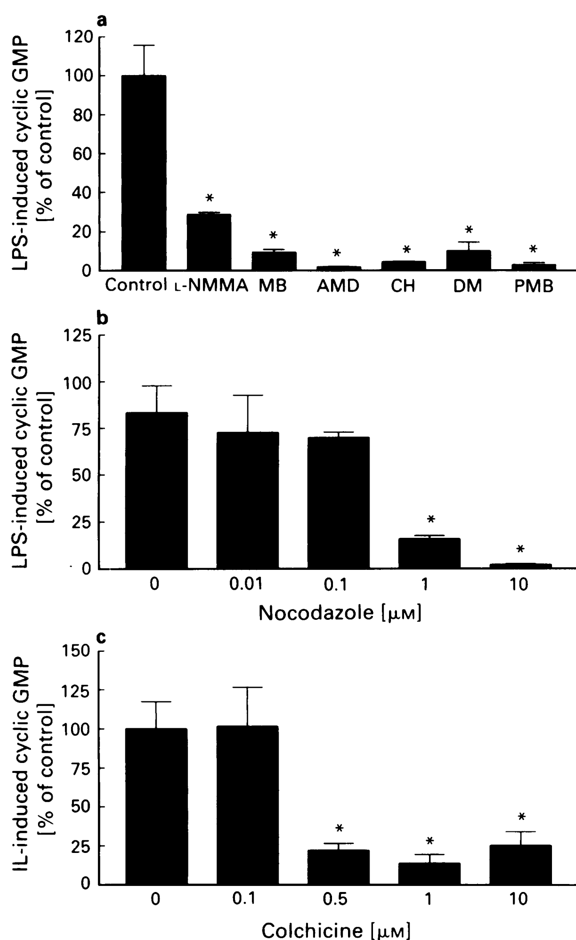

Figure 1 Modulation of lipopolysaccharide (LPS)- and interleukin-1α-induced cyclic GMP formation in cultured aortic smooth muscle cells of rat. (a) Effects of N^G-methyl-L-arginine (L-NMMA, 1 mM), methylene blue (MB, 20 μM), actinomycin D (AMD, 5 μg ml^{-1}), cycloheximide (CH, 20 μM), dexamethasone (DM, 10 μM) and polymyxin B (PMB, 2 μg ml^{-1}) on LPS (1 μg ml^{-1})-induced cyclic GMP levels. *$P<0.05$ from cyclic GMP levels in Control cultures. (b) Concentration effects of nocodazole on LPS (1 μg ml^{-1})-induced cyclic GMP levels. (c) Concentration effects of colchicine on interleukin-1α (10 u ml^{-1})-induced cyclic GMP levels. *$P<0.05$ from cyclic GMP levels in the absence of nocodazole or colchicine. Data shown are means ± s.e.mean in 4 cultures.

To investigate whether nocodazole and colchicine interfered with the activation of the endogenous nitric oxide generation pathway or inhibited guanylate cyclase activation, cyclic GMP formation in response to exogenous nitrovasodilator, or to co-culture of smooth muscle cells and bovine aortic endothelial cells (BAE), was determined. Sodium nitroprusside at 1 μM produced cyclic GMP levels comparable to those seen with LPS or interleukin (41 ± 5 fold increases). Microtubule depolymerization with either nocodazole or colchicine (10 μM) had virtually no effect on sodium nitroprusside-stimulated cyclic GMP formation in smooth muscle cells (89 ± 14% and 101 ± 9%, respectively) or on bradykinin-stimulated cyclic GMP formation in co-cultures of BAE and smooth muscle cells.

Discussion The major novel finding of our study is that microtubule depolymerizing agents prevented LPS- and cytokine-induced cyclic GMP formation in vascular smooth muscle cells, a proposed pathophysiological mechanism underlying septic shock and side-effects of antitumour therapy with cytokines.

Our characterization of LPS-induced cyclic GMP formation suggests the induction of a transcriptionally-regulated nitric oxide synthase as an underlying mechanism for guanylate cyclase stimulation. The slow onset, time-dependence and sensitivity of cyclic GMP responses to actinomycin D, cycloheximide and dexamethasone are all consistent with this assumption and with previous reports on cytokine-induced cyclic GMP formation in cultured vascular cells (Beasley *et al.*, 1991). Nocodazole and colchicine, two chemically distinct microtubule depolymerizing agents, prevented the stimulation of cyclic GMP generation by both LPS and interleukin-1α. This effect of nocodazole and colchicine is unlikely to involve a direct action on soluble guanylate cyclase, since cyclic GMP formation of comparable magnitude in response to sodium nitroprusside remained unaltered by these agents. Thus, we suggest that microtubule depolymerizing agents interfere with the induction of nitric oxide synthase, and furthermore, that the activation of this pathway depends on intact microtubule assembly.

There are other examples for similar involvement of microtubules in mediating expression of certain genes by immunomodulators. Disruption of microtubules reduces the expression of positive acute phase proteins in hepatocytes and tissue plasminogen activator in endothelial cells (Carter *et al.*, 1989; Santell *et al.*, 1992). Depolymerization of these filaments, on the other hand, stimulates expression of plasminogen activator inhibitor in endothelial cells (Santell *et al.*, 1992). The precise means by which cytoplasmic microtubules influence the activation of the inducible nitric oxide pathway remains to be determined. It 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 gene, transcription of mRNA and protein synthesis.

In conclusion, we have identified a new class of inhibitors of the activation of the nitric oxide pathway in vascular smooth muscle cells with therapeutic potential in immunologically based pathological conditions. We have also suggested a new mechanism potentially underlying some antiinflammatory properties of microtubule depolymerizing agents.

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