Induction of nitric oxide synthase by cytokines in vascular smooth muscle cells

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We investigated the mechanisms by which cytokines lead to a diminished responsiveness of vascular smooth muscle to vasoconstrictors. The attenuation of noradrenaline-induced contraction by 6 to 24 h incubations with the cytokines, tumor necrosis factor and interleukin-1, in endothelium-denuded rabbit aorta was associated with an increase in intracellular cyclic GMP level. This increase was abolished by the stereoselective inhibitor of nitric oxide-synthase, N^G-nitro-L-arginine and by cycloheximide. Formation of nitric oxide was detected in the cytosol of cytokine-treated native and cultured smooth muscle cells by activation of purified soluble guanylate cyclase, and depended on tetrahydrobiopterin, but not on Ca^{++}-calmodulin. The results indicate that cytokines induce a nitric oxide-synthase of the macrophage-type in vascular smooth muscle.

Nitric oxide synthase; Vascular smooth muscle; Cyclic GMP; Tetrahydrobiopterin; N^G-nitro-L-arginine; Cytokine

1. INTRODUCTION

Cytokines, such as tumor necrosis factor α (TNF) and interleukin-1 (IL-1), are secreted by macrophages in response to endotoxin, and may be responsible for the severe hypotension and circulatory failure in septic shock. This view is supported by the findings that, (i) administration of anti-TNF monoclonal antibodies to animals protected completely against hypotension in bacteraemia [1], and (ii) cytokines inhibited the responses of vascular smooth muscle to vasoconstrictors [2–4]. However, the mechanism by which cytokines result in diminished responsiveness of vascular smooth muscle is still unknown [5–7]. Recent reports have presented indirect evidence that L-arginine-derived nitric oxide (NO) may be a principal mediator of TNF-induced hypotension [6]. In the present study we describe the induction of NO synthase in native and cultured vascular smooth muscle cells from the rabbit aorta after 12 to 24 h exposure to TNF and IL-1. The results imply that cytokine-induced NO formation in the vasculature is a major factor responsible for the loss of vascular responsiveness, as observed in septic shock and during antitumor therapy with cytokines [8].

2. MATERIALS AND METHODS

2.1. *Measurement of vasomotor responses*

Endothelium-denuded segments from rabbit thoracic aorta were incubated for 20 h (37°C) in M 199 medium containing 10% fetal calf serum, in the absence (control) and presence of either IL-1 (200 U·ml^{-1}), IL-1 plus cycloheximide (20 μg·ml^{-1}), or TNF/interferon γ (TNF/IF; 3000/500 U·ml^{-1}). Cumulative dose-response curves to noradrenaline (NA) were obtained by perfusion (40 ml·h^{-1}) of the segments with oxygenated Tyrode's solution in an organ bath (37°C). The outer diameters of the segments were recorded photoelectrically [9].

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2.2. *Culture of smooth muscle cells*

The medium was prepared from rabbit aortic segments by careful removal of the endothelium and the adventitia. Smooth muscle cells isolated from proteolytic digests (elastase/collagenase) of medial strips were cultured for 7 days in DMEM/Ham's F-12 medium (1:1) with 10% fetal calf serum as described [10]. Thereafter they were incubated (20 h) in the absence (control) and in the presence of IL-1 (200 U/ml).

2.3. *Preparation of cytosols and detection of NO synthase*

Following incubation with cytokines, segments and cultured cells were homogenized by means of a potter-elvejehm or sonication, respectively. The cytosol was prepared by centrifugation (1 h 100 000 × *g*). NO formation in isolated cytosols was measured by its activating effect on a homogenously purified soluble guanylate cyclase [11]. Cytosols (0.13 mg·ml^{-1} protein) were incubated (30 min; 37°C) in 15 mM HEPES (pH 7.5) with the ingredients required for cell-free *formation* (1 μM (6R)-tetrahydrobiopterin/0.3mM L-arginine/0.1 mM NADPH) and *detection* (1μg·ml^{-1} guanylate cyclase/0.2 mM, 0.1 μCi[α-^{32}P]GTP/0.1 mM cyclic GMP/0.3 μM superoxide dismutase/4 mM $MgCl_2$/2 mM glutathione/3.5 mM creatine phosphate/4.8 U creatine phosphokinase/1 mM 3-isobutyl-1-methylxanthine/0.1 mg.ml^{-1} bovine γ-globulin) (final volume of the incubates 100 μl) of NO. Guanylate cyclase activity was calculated from the amount of cyclic [^{32}P]GMP formed. The basal guanylate cyclase activity in the absence of cytosol was 105 ± 9 nmol·mg^{-1}·min^{-1}.

2.4. *Determination of cGMP content*

Segments from rabbit thoracic aorta, denuded of endothelium were incubated in the presence of zaprinast (M&B 22948;0.1 mM). Thereafter, segments were homogenized in icecold trichloroacetic

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acid (6%) and the intracellular cyclic GMP was measured by a specific radioimmunoassay with a commercially available kit.

2.5. *Materials*

Substances were supplied as follows: recombinant human interferon γ (Sigma, München); superoxide dismutase (3000 U/mg) (Boehringer, Mannheim); recombinant human tumor necrosis factor (Knoll-BASF, Mannheim); (6R)-5,6,7,8-tetrahydrobiopterin (Dr Schircks Laboratories, Jona, Switzerland); L-arginine, NADPH, N^G-nitro-L-arginine (Serva, Heidelberg). Tetrahydrobiopterin was stored as a 10 mM stock solution in 1 mM HCl in the dark under nitrogen at −30°C.

2.6. *Data evaluation*

Data represent means ± SEM of at least 3 independent determinations performed in triplicate. Significance of differences was tested by Student's *t*-test, with the Bonferroni-correction for the comparison of multiple means [12]. $P < 0.05$ was considered significant.

3. RESULTS AND DISCUSSION

Noradrenaline-induced contractions were measured in pairs of cytokine-treated and untreated (control) de-endothelialized segments of rabbit aorta. TNF/IF and IL-1 markedly reduced agonist-induced contractions (Fig. 1a,b). The attenuation by cytokines of vascular contractions was probably caused by activation of the soluble guanylate cyclase effector system, since it was associated with an increase in intracellular cGMP content (Fg. 2a). These effects of cytokines were abolished by the presence of cycloheximide during the incubation (Figs. 1a and 2a), indicating the requirement for protein biosynthesis for both cellular responses. Since cytokines are known to induce NO-synthase in other cells [13], we tested whether NO generated from L-arginine accounted for the guanylate cyclase activation by using the stereospecific inhibitor of NO-synthase, N^G-nitro-L-arginine (L-NNA) [14]. Administration of L-NNA to the organ bath restored the contractile response to noradrenaline (Fig. 1b).

Accordingly, incubation of the segments with L-NNA prevented the cGMP increase elicited by cytokines (Fig. 2a). Expression of NO-synthase started within 12 h after exposure to cytokines as indicated by the time course of cGMP elevation, which can be taken as an index for endogenous NO-formation (Fig. 2b) [15].

To detect NO-synthase in the cytosol prepared from the medial layer of rabbit aorta we measured activation of a purified soluble guanylate cyclase (GC) by the cytosol in the presence of L-arginine and NADPH. Cytosol obtained from TNF/IF-treated segments induced a marked increase in GC activity (Fig. 3a), which was not observed with cytosol from control or cycloheximide/cytokine-treated segments. In contrast to cytosol from native smooth muscle cells (segments), cytosol from cultured vascular smooth muscle cells induced a two-fold stimulation of GC activity (control), which was increased 7-10-fold by cytokine treatment (Fig. 3b). Also, cultured cells incubated with cytokines exhibited a more than 11-fold elevation in cGMP con-

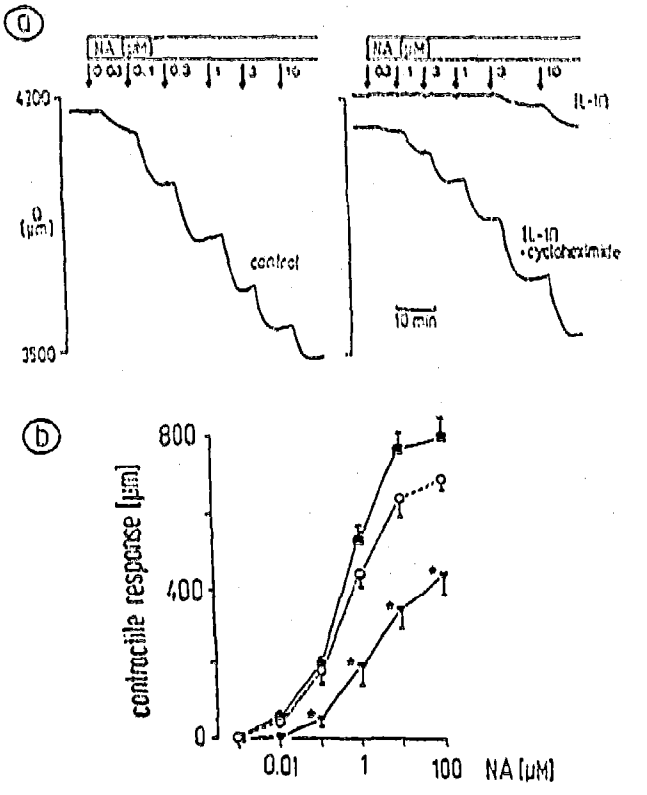

Fig. 1. Cytokines attenuate the contractile response to noradrenaline of endothelium-denuded rabbit aorta. (a) Cycloheximide (20 $\mu g \cdot ml^{-1}$) in the incubate prevents the attenuation by interleukin-1β (IL-1β;200 $U \cdot ml^{-1}$ of contractile responses to noradrenaline. Representative recording from $n = 5$ (b) N^G-nitro-L-arginine (■, 30 μM) in the perfusate restores the attenuation of noradrenaline-induced contraction in tumor necrosis factor α/interferonγ (3000/500 $U \cdot ml^{-1}$)-treated segments (▼). *significantly different ($P < 0.05$) from control (o, $n = 7$, mean ± SEM).

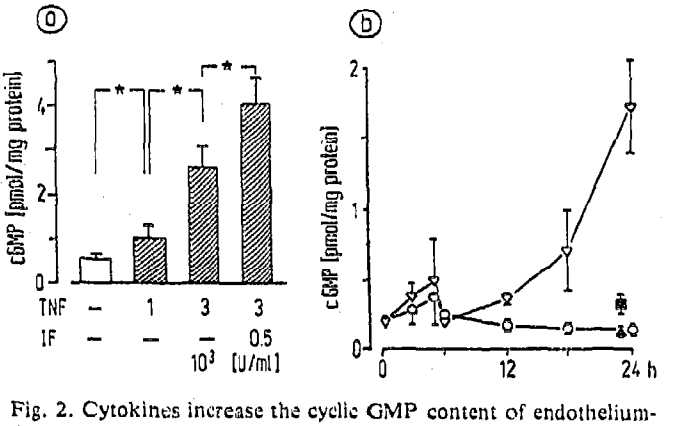

Fig. 2. Cytokines increase the cyclic GMP content of endothelium-denuded rabbit aortae. (a) The dose-dependent cyclic GMP increase to tumor necrosis factor α (TNF) was enhanced by interferonγ (IF). Data from 4-7 preparations (mean ± SEM; *$P < 0.05$). (b) Time course of cyclic GMP response to cytokines. Segments were incubated without (o, control) and with TNF/IF (▽;3000/500 $U \cdot ml^{-1}$) for the time indicated (mean ± SEM, $n = 3$). Cycloheximide (▲, 20$\mu g \cdot ml^{-1}$) in the incubate abolished, and N^G-nitro-L-arginine (L-NNA; ■, 30 μM) attenuated the increase in cyclic GMP.

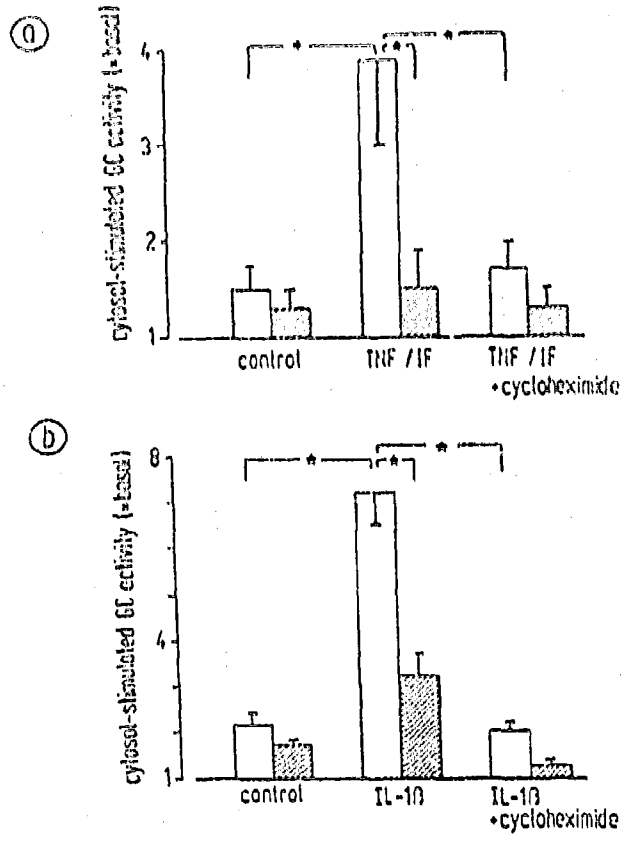

Fig. 3. Cytokines induce cytosolic NO synthase in native (a) and cultured (b) vascular smooth muscle cells. Incubation (20 h) was performed in the absence (control) and in the presence of cytokines (segments: tumor necrosis factor α/interferon γ (TNF^{α}/IF^{γ}; 3000/500 $U \cdot ml^{-1}$); cultured cells: IL-1,200 U/ml). NO formation in isolated cytosols was measured by its activating effect on soluble guanylate cyclase. Induction by TNF/IF of cytosolic NO synthase was completely prevented by cycloheximide (20 $\mu g \cdot ml^{-1}$) and its activity was inhibited by N^{G}-nitro-L-arginine (0.3 mM, hatched columns). Data from 3 different cytosols with activity determinations performed in triplicate ($^{*}P < 0.05$).

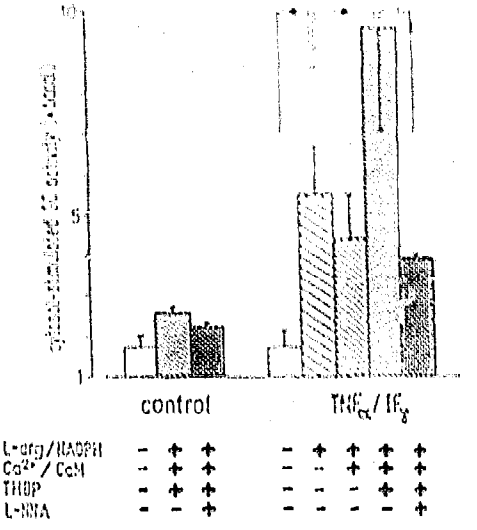

Fig. 4. Cofactors required for catalytic activity of NO synthase in cytosol from cultured smooth muscle cells. NO synthase activity, induced by tumor necrosis factor α/interferon γ (3000/500 $U \cdot ml^{-1}$) was measured as described, but with variable additives as indicated (-: absence; +: presence). L-arginine (L-arg, 0.3 mM), NADPH (0.1 mM), (6R)-tetrahydrobiopterin (THBP, 1 μM), Ca^{2+} (2 μM), calmodulin (CaM, 1 μM), N^{G}-nitro-L-arginine (L-NNA, 0.3 mM). Data from 3–4 different cytosols ($^{*}P < 0.05$).

tent (from 7.2 ± 0.7 to 79.3 ± 15.6 pmol/mg protein) and a significant increase in production of NO_2^- [16], a stable metabolite of the oxidative L-arginine pathway [17] (from 9 ± 1.5 to 20.3 ± 3.1 nmol/mg protein/24 h). All effects of cytokines in cultured cells were inhibited by either L-NNA or cycloheximide (Fig. 3b; NO_2^--data not shown).

We obtained strong evidence that the NO-synthase expressed in smooth muscle cells in response to cytokines resembles the macrophage-type enzyme [5,18]: activation was completely dependent on the presence of L-arginine and NADPH and was enhanced by tetrahydrobiopterin. In contrast to the constitutively expressed NO-synthase of endothelial cytosol [19,20], NO-synthase in smooth muscle cytosol was independent of Ca^{2+}-calmodulin and was less potently inhibited by L-NNA (Fig. 4) than the endothelial enzyme [4,21]. In support of our findings, two recent reports provide also indirect evidence (bioassay, cGMP and nitrite accumulation) for the existence of both a constitutive [22] and an inducible [23] NO synthase pathway in endothelium-denuded arterial rings.

A cytokine-induced production of nitrogen oxides has recently been described in cultured endothelial cells [7]. We also found a slight, but significant increase in the Ca^{2+}/calmodulin-dependent NO-synthase activity in cultured porcine aortic endothelial cells incubated for 24 h with TNF and IL-1 (Mülsch and Busse, unpublished observations).

However, the presence of endothelium did not affect the cytokine-induced inhibition of contractile responses of rabbit aorta to noradrenaline (data not shown). Similar findings have been reported in rat aorta after 3 h exposure to endotoxin [24]. Therefore the severe hypotension and circulatory failure observed under conditions of elevated cytokine levels in vivo, such as septic shock and anti-cancer therapy with cytokines, may be mainly caused by NO, formed in the vascular smooth muscle, and exerting a critical autocrine function.

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REFERENCES

[1] Tracey, K.J., Fong, Y., Hesse, D.G., Manogue, K.R., Lee, A.T., Kuo, G.C., Lowry, S.F. and Cerami, A. (1987) Nature 330, 662–664.
[2] Kutsky, P. (1990) (abstract) Circ. Shock 27, 296.
[3] Beasley, D., Cohen, R.A. and Levinsky, N.G. (1989) J. Clin. Invest. 83, 331–335.
[4] McKenna, T.M., Reusch, D.W. and Simpkins, C.O. (1988) Circ. Shock 25, 187–196.
[5] Nathan, C.F. and Stuehr, D.J. (1990) J. Natl. Cancer Institute 82, 726–728.
[6] Kilbourn, R.G., Gross, S.S., Jubran, A., Adams, J., Griffith, O.W., Levi, R. and Lodato, R.F. (1990) Proc. Natl. Acad. Sci. USA 87, 3629–3632.
[7] Kilbourn, R.G. and Belloni, P. (1990) J. Natl. Cancer Institute 82, 772–776.
[8] Moritz, T., Niederle, N., Baumann, J., May, D., Kurschel, E., Osieka, R., Kempeni, J., Schlick, E. and Schmidt, C.G. (1989) Cancer Immunol. Immunother. 29, 144–150.
[9] Busse, R., Förstermann, U., Matsuda, H. and Pohl, U. (1984) Pflügers Arch. 401, 77–83.
[10] Mülsch, A., Busse, R., Winter, J. and Bassenge, E. (1989) Naunyn-Schmiedebergs Arch. Pharmacol. 339, 568–574.
[11] Mülsch, A., Bassenge, E. and Busse, R. (1989) Naunyn-Schmiedebergs Arch. Pharmacol. 340, 767–770.
[12] Waltenstein, S., Zucker, C.L. and Hess, J.L. (1980) Circ. Res. 47, 1–9.
[13] Marletta, M.A. (1989) Trends Biochem. Sci. 14, 488–492.
[14] Mülsch, A. and Busse, R. (1990) Naunyn-Schmiedebergs Arch. Pharmacol. 341, 143–147.
[15] Moncada, S., Palmer, R.M.J. and Higgs, E.A. (1989) Biochem. Pharmacol. 38, 1709–1715.
[16] Green, L.C., Wagner, D.A., Glogowski, L., Skipper, P.L., Wishnok, J.S. and Tannenbaum, S.R. (1982) Anal. Biochem. 126, 131–138.
[17] Marletta, M.A., Yoon, P.S., Iyengar, R., Leaf, C.D. and Wishnok, J.S. (1988) Biochemistry 27, 8706–8711.
[18] Hauschildt, S., Luckhoff, A., Mülsch, A., Kohler, L., Bessler, W. and Busse, R. (1990) Biochem. J. 270, 351–356.
[19] Busse, R. and Mülsch, A. (1990) FEBS Letters 265, 133–136.
[20] Mayer, B., Schmidt, K., Humbert, P. and Böhme, E. (1989) Biochem. Biophys. Res. Commun. 164, 678–685.
[21] Mülsch, A., Hauschildt, S., Bessler, W.G., Bassenge, E. and Busse, R. (1990) in: Nitric Oxide from L-arginine: a Bioregulatory System (Moncada, S. and Higgs, E.A., eds), pp 235–242, Elsevier, Amsterdam.
[22] Beasley, D. (1990) Am. J. Physiol. 259, R38–R44.
[23] Wood, K.S., Buga, G.M., Byrns, R.E. and Ignarro, L.J. (1990) Biochem. Biophys. Res. Commun. 170, 80–88.
[24] Beasley, D., Cohen, R.A. and Levinsky, N.G. (1990) Am.J.Physiol. 258, H1187–H1192.