Dexamethasone Inhibits Nitric Oxide Synthase mRNA Induction by Interleukin-1 α and Tumor Necrosis Factor- α in Vascular Smooth Muscle Cells

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ABSTRACT—The effects of interleukin-1 α , tumor necrosis factor- α and dexamethasone on the induction of nitric oxide synthase mRNA in rat aortic smooth muscle cells were studied. Neither interleukin-1 α (up to 100 U/ml) nor tumor necrosis factor- α (up to 5000 U/ml) was capable of inducing nitrite/nitrate production and nitric oxide synthase mRNA in smooth muscle cells. In contrast, treatment for 12 hr or longer with a combination of the two synergistically induced nitrite/nitrate and cyclic GMP production in cell culture media and nitric oxide synthase mRNA, both of which were prevented by dexamethasone. Contamination with bacterial lipopolysaccharide, which may affect the induction of nitric oxide synthase, was below 30 pg/ml in all experiments. Our findings show that dexamethasone and these cytokines regulate the induction of nitric oxide synthase at the mRNA level in vascular smooth muscle cells.

Keywords: Nitric oxide synthase mRNA, Interleukin-1 α , Tumor necrosis factor- α , Dexamethasone, Lipopolysaccharide

In the vasculature, nitric oxide is known to exhibit diverse biological functions, such as vasodilation, inhibition of adhesion and aggregation of platelets (1) and inhibition of smooth muscle cell growth (2). Nitric oxide generated by constitutive nitric oxide synthase in endothelial cells mediates endothelial dependent relaxation by stimulating soluble guanylate cyclase in adjacent vascular smooth muscle cells (1).

Under some in vivo conditions, such as sepsis (1) and post-balloon catheterization (3), nitric oxide synthase activity is induced in the vasculature. Many cell types such as endothelium, vascular smooth muscle cells, monocytes, neutrophils and macrophages exhibit inducible nitric oxide synthase activity after stimulation by cytokines and bacterial lipopolysaccharide (LPS) (1).

We previously reported that LPS (more than 100 pg/ml) induces nitric oxide synthase activity in vascular smooth muscle cells treated with tumor necrosis factor- α (TNF- α), which by itself does not evoke nitrite/nitrate production (4). Therefore, it is essential to keep LPS con-

tamination below 100 pg/ml when investigating the induction of nitric oxide synthase. Although induction of nitric oxide synthase has been reported in vascular smooth muscle cells (1), this induction under known LPS concentrations in culture media has not been investigated. Therefore, we maintained LPS concentrations below 30 pg/ml in all culture media used in the present study and evaluated the effects of cytokines on induction of nitric oxide synthase mRNA.

Dexamethasone has been shown to inhibit the induction of nitric oxide synthase activity in rat deendothelialized aorta (1). However, it has not yet been determined whether the inhibitory effect is exerted at the nitric oxide synthase mRNA.

In this study, measuring nitric oxide synthase mRNA, nitrite/nitrate, stable end products of nitric oxide (5–7), and cyclic GMP (cGMP) in culture media, we have demonstrated that the combination of interleukin-1 α (IL-1 α) and TNF- α induces nitric oxide synthase at the mRNA level, and that this induction is inhibited by dexamethasone in cultured vascular smooth muscle cells.

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MATERIALS AND METHODS

Materials

Human recombinant IL-1 α was a gift from Dainippon Pharmaceutical Co., Ltd. (Osaka). Human recombinant TNF- α was a gift from Suntory (Osaka). LPS-free water (<1 pg/ml) was purchased from Otsuka Pharmaceutical Co., Ltd. (Tokyo). LPS-free pipette tips and LPS-free bovine serum albumin were from Seikagaku Corporation (Tokyo). Dexamethasone was from Sigma Chemical Co. (St. Louis, MO, USA). Dimethylsulfoxide (DMSO) was from Kanto Chemical Co. (Tokyo). Concentrated IL-1 α and TNF- α were diluted in Dulbecco's modified essential medium without phenol red (DMEM) containing 1% and 0.1% LPS-free bovine serum albumin and aliquoted before storage at -80°C and -20°C , respectively. The composition of $20\times$ SSPE solution was as follows: 3.6 M NaCl, 0.2 M NaH_2PO_4 and 0.02 M ethylenediaminetetraacetic acid (EDTA) (pH 7.4). The composition of $100\times$ Denhardt's solution was as follows: 2% Ficoll, 2% polyvinylpyrrolidone and 2% bovine serum albumin.

Smooth muscle cells

Rat aortic smooth muscle cells (RACS-1) were plated onto 10-cm dishes (Costar, Cambridge, MA, USA) or 9-cm² flasks at an initial density of approximately 2.1×10^5 cells/dish or 4.6×10^4 cells/flask and grown in Earle's M199 medium supplemented with 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 U/ml) until the cells reached confluence, as previously described (8, 9). All RACS-1 cultures were used at passage 5 from stock cells, as described in the previous reports (8, 9). Glassware was used after heating at 250°C for 2 hr, and LPS-free pipette tips were used throughout the experiments. LPS-free water was used for culture media as described previously (10). We used RACS-1 cloned from normal adult rats in order to assure that the nitric oxide synthase mRNA and nitric oxide originated from the smooth muscle cells rather than endothelial cell contaminants. We were also able to eliminate the influence of cytokines which can be released from endothelial cells in response to exogenous cytokines.

Treatment of RACS-1 with IL-1 α and TNF- α

Cells were washed once with DMEM supplemented with sodium selenite (5 ng/ml), insulin (5 $\mu\text{g}/\text{ml}$), transferrin (5 $\mu\text{g}/\text{ml}$), penicillin (100 U/ml) and streptomycin (100 U/ml) (SIT) and were incubated in DMEM with SIT for the indicated periods, up to 48 hr, with or without reagents. Cells were stimulated by the addition of IL-1 α , TNF- α , or a combination of IL-1 α and TNF- α . In the studies that assessed the effect of dexamethasone, dexamethasone was added simultaneously with the cytokines.

Dexamethasone was dissolved in DMSO, the final concentration of which was 0.1% (v/v). Aliquots of the culture medium were taken for LPS determination soon after the reagents had been added and for nitrite/nitrate and cGMP measurement at the end of each incubation time. For the cGMP assays, EDTA (pH 7.6) was added in the collected media so as to achieve a final concentration of 5 mM. The samples for LPS were stored until assay at -80°C and those for nitrite/nitrate and cGMP at -20°C .

Assays

LPS was quantified with a commercially available kit (Endospecy ES-6 set and Toxicolor DIA set, Seikagaku Corporation). Nitrate was reduced to nitrite by passing the samples through a Cd column and the total amounts of nitrite were measured based on the Griess reaction as described previously (4, 11, 12). cGMP was measured with a commercially available radioimmunoassay kit (Yamasa Shoyu Co., Chohshi).

After aliquots of the culture medium had been obtained for assays of nitrite/nitrate and cGMP, cells were collected, and their total RNA was extracted (13). RNA was applied to 1.2% formaldehyde-agarose gels and after electrophoresis, transferred onto Biotrans B nylon membranes (Pall, East Hills, NY, USA), as previously described (14). The gel stained by ethidium bromide after electrophoresis revealed that equal amounts of RNA had been loaded (data not shown). A 700-bp fragment of the 5' portion of cloned rat liver inducible nitric oxide synthase cDNA (15) was labelled as previously described (14). The specific activity of the radiolabelled probe was $>5.0\times 10^5$ cpm/ng. The blots were prehybridized in 50% formamide, $5\times$ SSPE, $5\times$ Denhardt's solution, 0.1% sodium dodecyl sulfate (SDS) and 100 $\mu\text{g}/\text{ml}$ salmon sperm DNA for 4–6 hr at 42°C and hybridized with the probe in 50% formamide, $5\times$ SSPE, $2\times$ Denhardt's solution, 0.1% SDS and 100 $\mu\text{g}/\text{ml}$ salmon sperm DNA overnight at 42°C . The blots were washed in $6\times$ SSPE and 0.1% SDS at room temperature for 30 min and in $1\times$ SSPE and 0.1% SDS at 37°C for 30 min and exposed to X-ray film at -80°C with intensifying screens for 1–5 days. When the background radioactivities were high, the blots were further washed in $1\times$ SSPE and 0.1% SDS at 55°C for 30 min and exposed to X-ray film.

Cells which ran parallel with cells for Northern blot analysis were washed three times with 0.9% NaCl, and their protein content was determined by a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA).

LPS concentrations in the culture media were below 30 pg/ml in all experiments.

RESULTS

As shown in Fig. 1a, neither IL-1 α (10 U/ml) nor

TNF- α (500 U/ml) was capable of inducing significant nitrite/nitrate production by 48 hr. Even at higher concentrations, neither IL-1 α (100 U/ml) nor TNF- α

Fig. 1. Effects of interleukin-1 α and tumor necrosis factor- α on the induction of nitric oxide synthase. **a:** Nitrite/nitrate accumulation in RACS-1 culture medium. The protein content was 1.0 mg/dish. The last column shows nitrite/nitrate accumulation in culture medium in the absence of RACS-1 which ran in parallel with dishes containing RACS-1 treated for 48 hr under the experimental conditions shown in the figure. **b:** Northern blot of nitric oxide synthase mRNA from the cells used in panel **a**. Along with the major band of nitric oxide synthase mRNA, an unknown minor band of smaller size was observed in cells treated with 100 U/ml interleukin-1 α and 5000 U/ml tumor necrosis factor- α . The autoradiogram is representative of two experiments.

(5000 U/ml) alone induced nitrite/nitrate production. However, when applied together, the cytokines induced concentration-dependent production of nitrite/nitrate. Without cytokines, RACS-1 did not produce significant amounts of nitrite/nitrate, which is consistent with our previous report (4). After a 48-hr incubation, a small amount of nitrite/nitrate accumulation was observed in culture media without cells which were run in parallel with dishes containing RACS-1 (Fig. 1a).

Northern blot analysis of the same cells which were used for the nitrite/nitrate accumulation experiments in Fig. 1a revealed that neither IL-1 α (10 U/ml) nor TNF- α (500 U/ml) had induced nitric oxide synthase mRNA in RACS-1 at 48 hr (Fig. 1b). In contrast, the combination of IL-1 α (10 U/ml) and TNF- α (500 U/ml) had induced a trace amount of nitric oxide synthase mRNA at 48 hr (Fig. 1b). Though neither IL-1 α (100 U/ml) nor TNF- α (5000 U/ml) alone in higher concentrations was capable of inducing nitric oxide synthase mRNA, the combination of the two had induced significant amounts of nitric oxide synthase mRNA in RACS-1 at 48 hr. Therefore, we

used the combination of IL-1 α (100 U/ml) and TNF- α (5000 U/ml) to stimulate RACS-1 in all subsequent experiments. The length of the nitric oxide synthase mRNA was estimated to be similar to those from mouse macrophages (16) and rat organs (15). Nitric oxide synthase mRNA was not detected in RACS-1 without cytokine (Fig. 1b).

A reproducible increase in nitrite/nitrate accumulation in the culture media of RACS-1 treated with IL-1 α (100 U/ml) and TNF- α (5000 U/ml) was detectable from 24 hr and continued up to 48 hr (Fig. 2). Nitric oxide synthase mRNA of these cells was evident at 12 hr, and the peak intensity was seen at 36 hr and slightly decreased at 48 hr (Fig. 3).

Dexamethasone concentration-dependently inhibited nitrite/nitrate production by RACS-1 treated with the combination of IL-1 α (100 U/ml) and TNF- α (5000 U/ml) (Fig. 4).

Fig. 2. Time-dependent accumulation of nitrite/nitrate in RACS-1 culture media treated with the combination of 100 U/ml interleukin-1 α and 5000 U/ml tumor necrosis factor- α (●). Protein content was 1.0 mg/dish. The same cells were used for the Northern blot in Fig. 3.

Fig. 4. Concentration-response curve for dexamethasone (DXM) on inhibition of production of nitrite/nitrate in vascular smooth muscle cells treated with a combination of 100 U/ml interleukin-1 α and 5000 U/ml tumor necrosis factor- α for 48 hr. Data are means $\pm$ S.E. ($n=3$). Symbols without bars indicate S.E. within the symbols. The final concentrations of DMSO in all samples were 0.1% (v/v). The protein content was 0.25 mg/flask.

Fig. 3. Northern blot of nitric oxide synthase mRNA in RACS-1 treated with the combination of 100 U/ml interleukin-1 α and 5000 U/ml tumor necrosis factor- α at 0.5 hr (lanes 1 and 2), 1 hr (3), 2.5 hr (4), 3 hr (5), 6 hr (6 and 7), 12 hr (8), 24 hr (9 and 10), 36 hr (11) and 48 hr (12). The Northern blotting data of Fig. 3 are derived from the cells shown in Fig. 2. The autoradiogram is representative of three experiments.

The combination of IL-1 α (100 U/ml) and TNF- α (5000 U/ml) induced nitrite/nitrate and cGMP accumulation in the culture media at 24 hr, which was prevented by dexamethasone (1 μ M) (Table 1). This drug (1 μ M) also prevented the induction of nitric oxide mRNA by these cytokines (Fig. 5).

Table 1. Effect of dexamethasone on nitrite/nitrate and cGMP accumulation

Treatment	Accumulation of	
	nitrite/nitrate (nmol/dish)	cGMP (pmol/dish)
No cytokine (1)	4.5	0.27
IL-1 α + TNF- α (2)	60.3	2.31
IL-1 α + TNF- α (3)	65.1	2.04
IL-1 α + TNF- α + DXM (4)	6.0	0.27

Effect of 1 μ M dexamethasone (DXM) on 24-hr nitrite/nitrate and cyclic GMP (cGMP) accumulation in culture media of vascular smooth muscle cells treated with a combination of 100 U/ml interleukin-1 α (IL-1 α) and 5000 U/ml tumor necrosis factor- α (TNF- α). Samples were obtained from the culture media. The same cells were used for Northern blot in Fig. 5. Numbers (1) through (4) correspond to lanes (1) through (4) in Fig. 5. The final concentrations of DMSO in samples treated with cytokines, with or without dexamethasone, were 0.1% (v/v). Protein content was 1.3 mg/dish.

Fig. 5. Inhibitory effect of dexamethasone on induction of nitric oxide synthase mRNA. Data of lanes (1) through (4) were obtained from the same cells as those shown in Table 1 in numerical order: (1) no cytokine added; (2) and (3) (duplicate), treated with 100 U/ml interleukin-1 α (IL-1 α) and 5000 U/ml tumor necrosis factor- α (TNF- α); (4), treated with 100 U/ml IL-1 α , 5000 U/ml TNF- α and 1 μ M dexamethasone. The final concentrations of DMSO in samples treated with cytokines, with or without dexamethasone, were 0.1% (v/v).

DISCUSSION

It has been recently reported that interferon- γ and TNF- α stimulate the activity of constitutive nitric oxide synthase in human umbilical vein endothelial cells by up-regulating the production of the cofactor tetrahydrobiopterin without induction of the enzyme (17). In contrast to these cells, our results demonstrate that the combination of IL-1 α and TNF- α induces nitric oxide synthase at the mRNA level in RACS-1.

Furthermore, the 700-bp fragment of the 5' portion of cDNA encoding rat liver inducible nitric oxide synthase hybridizes with rat inducible nitric oxide synthase mRNA in aortic smooth muscle cells. This suggests a high degree of homology in this region between the nitric oxide synthases obtained from two distinct cell types.

The induction of nitric oxide synthase by IL-1 α and TNF- α is synergistic since neither is effective when applied alone. Moreover, this synergism was observed at the mRNA level. The synergistic effects of IL-1 α and TNF- α observed in the present study also suggest, that in smooth muscle cells, a distinct signal transduction pathway may be used by these cytokines to induce nitric oxide synthase mRNA.

Both IL-1 and TNF- α have been shown to induce a wide variety of genes by either induction of new transcription or prolongation of mRNA half-life (18). Whether the observed increase in nitric oxide synthase mRNA is due to enhanced gene transcription, stabilization of mRNA or both remains to be elucidated.

A number of reports indicating that nitric oxide synthase can be induced in vascular smooth muscle cells by TNF- α alone have been published recently (19–21). However, since the LPS concentrations in the culture media used in these studies were not reported, it is difficult to compare the results with ours. The observation that TNF- α alone does not induce nitric oxide synthase mRNA is consistent with our previously reported results (4).

Our results show that IL-1 α (up to 100 U/ml) does not induce nitric oxide synthase mRNA, although interleukin-1 β (IL-1 β) has been demonstrated to induce nitric oxide activity in smooth muscle cells (4, 22). It has been shown that IL-1 α and IL-1 β do not always share the same biological activities (23). Whether IL-1 α and IL-1 β differ in the induction of nitric oxide synthase mRNA in vascular smooth muscle cells awaits further study.

Overproduction of nitric oxide by induced nitric oxide synthase in the vascular wall is at least partially responsible for the hypotension seen in patients with septic shock (1). It has also been shown that pretreatment with either anti TNF- α antibodies (24) or interleukin-1 receptor antagonist (25) prevents hypotension after intravenous *E. coli* infusion in animals. Considering our

results that these cytokines act synergistically, induction of nitric oxide synthase may be efficiently inhibited by antagonizing one of these agents, leading to effective prevention of hypotension in the septic state. We can also speculate that nitric oxide synthase mRNA is induced in smooth muscle cells during sepsis. Moreover, IL-1 (26) and TNF- α (27) are present in atherosclerotic lesions. We can speculate that nitric oxide synthase mRNA is induced in vascular smooth muscle cells in such lesions.

Dexamethasone concentration-dependently inhibited the induction of nitric oxide synthase, and this inhibition was controlled at the mRNA level of the enzyme. Beneficial effects of administering dexamethasone to patients with severe typhoid fever have been reported (28). Dexamethasone may prevent septic shock via inhibition of nitric oxide synthase induction.

In conclusion, we have demonstrated, under low LPS conditions, that IL-1 α and TNF- α synergistically induce nitric oxide synthase mRNA and that this increase in mRNA is prevented by dexamethasone.

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