

Nitric Oxide Synthase mRNA in Endothelial Cells: Synergistic Induction by Interferon- γ , Tumor Necrosis Factor- α and Lipopolysaccharide and Inhibition by Dexamethasone

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ABSTRACT—Regulation of nitric oxide synthase mRNA by interferon- γ , tumor necrosis factor- α , bacterial lipopolysaccharide (LPS) and dexamethasone in rat aortic endothelial cells was examined. The combination of interferon- γ (100 U/ml) and tumor necrosis factor- α (5000 U/ml) evoked a time-dependent increase in nitric oxide synthase mRNA and nitrite/nitrate production, both of which were inhibited by dexamethasone. Neither interferon- γ (100 U/ml), tumor necrosis factor- α (5000 U/ml) nor LPS (100 ng/ml) alone was capable of increasing nitric oxide synthase mRNA and nitrite/nitrate production in these cells. However, combinations of two of the three agents synergistically increased both nitric oxide synthase mRNA and nitrite/nitrate production. When the three agents were applied simultaneously, nitric oxide synthase mRNA and nitrite/nitrate production were both markedly increased. LPS contamination, which may affect the induction of nitric oxide synthase, was below 20 pg/ml in all experiments unless LPS was added exogenously, namely, the effects observed were those of the cytokines themselves. Our results suggest that in endothelial cells, these cytokines regulate the production of nitric oxide at the level of nitric oxide synthase mRNA induction.

Keywords: Nitric oxide synthase mRNA, Interferon- γ , Tumor necrosis factor- α , Dexamethasone, Lipopolysaccharide

Nitric oxide has been demonstrated to be a major regulatory substance in the vasculature mediating relaxation of vascular smooth muscle (1), modulation of platelet function (1) and inhibition of smooth muscle cell proliferation (2).

In addition to the immediate responses of vascular endothelial cells to various stimuli, cytokines and bacterial lipopolysaccharide (LPS), alone or combined, increase nitric oxide biosynthesis in endothelial cells after a lag time of several hours (3–6). However, it has not been determined whether the increase in nitric oxide synthase activity induced by these substances in endothelial cells is due to an increase in nitric oxide synthase mRNA. We investigated whether interferon- γ (IFN- γ), tumor necrosis factor- α (TNF- α) and LPS, alone or combined, induce nitric oxide synthase at the mRNA level.

All experiments were conducted under a low LPS condi-

tion, since it is known that contamination with small amounts of LPS in the experimental system evokes unexpected induction of nitric oxide synthase activity in macrophages (7), aortic tissue in vitro (8–10) and vascular smooth muscle cells (11). By maintaining LPS concentrations below 20 pg/ml in all culture media used, we were able to evaluate the effects of the cytokines themselves on induction of nitric oxide synthase in endothelial cells.

MATERIALS AND METHODS

Materials

Human recombinant TNF- α was a gift from Suntory (Osaka). Rat IFN- γ was purchased from Gibco BRL (Grand Island, NY, USA). LPS-free water (<1 pg/ml) was purchased from Otsuka Pharmaceutical Company, Limited (Tokyo). LPS-free pipette tips and LPS-free bovine serum albumin were from Seikagaku Corporation (Tokyo). Dexamethasone and *E. coli* LPS (serotype 055:

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B5) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dimethylsulfoxide (DMSO) was purchased from Kanto Chemical Co. (Tokyo). Concentrated TNF- α and IFN- γ were diluted in Dulbecco's modified essential medium without phenol red (DMEM) containing 0.1% LPS-free bovine serum albumin and aliquoted before storage at -20°C . The composition of a $20\times$ SSPE solution was as follows: 3.0 M NaCl, 0.2 M NaH_2PO_4 and 0.02 M ethylenediaminetetraacetic acid (EDTA) (pH 7.4). The composition of a $100\times$ Denhardt's solution was as follows: 2% Ficoll, 2% polyvinylpyrrolidone and 2% bovine serum albumin.

Endothelial cells

Rat aortic endothelial cells (RACE-1) were plated onto 75-cm^2 flasks (Corning, Corning, NY, USA) or onto 10-cm dishes (Costar, Cambridge, MA, USA) at an initial density of $1.5\text{--}2.6\times 10^5$ cells/flask or dish 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 (12). All RACE-1 cultures were used at passage 3 from stock cells, as described in the previous reports (12). 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 preparing culture media as described previously (10, 11). We used RACE-1 cloned from normal adult rats in order to assure that the nitric oxide synthase mRNA and nitric oxide originated from the endothelial cells rather than vascular smooth muscle cell contaminants.

Treatment of RACE-1 with cytokines

After the cells had reached confluence, they 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 then incubated in DMEM with SIT for the indicated periods, up to 48 hr, with or without reagents. The final volumes of DMEM with SIT in 75-cm^2 flasks and 10-cm dishes were 10 ml and 7 ml, respectively. We added IFN- γ (100 U/ml), TNF- α (5000 U/ml), or LPS (100 ng/ml), alone or in various combinations as indicated in the results. In the studies that assessed the effect of dexamethasone, the drug was added simultaneously with IFN- γ (100 U/ml) and TNF- α (5000 U/ml). Dexamethasone was dissolved in DMSO, the final concentration of which was 0.1% (v/v). Time-course and dexamethasone experiments were conducted simultaneously, and DMSO was added to achieve a final concentration of 0.1% (v/v) in all samples used; This concentration of DMSO had no effect on production of nitrite/nitrate, stable end products of nitric oxide (13–15) or the increase in

nitric oxide synthase mRNA. Aliquots of the culture medium were taken for LPS determination shortly after the reagents had been added and for measurement of nitrite/nitrate at the end of each incubation time. The samples for LPS measurement were stored until assay at -80°C , and those for nitrite/nitrate were stored at -20°C . LPS was quantified with a commercially available kit (Endospecy ES-6 set and Toxicolor DIA set, Seikagaku Corporation). LPS concentrations in the culture media were below 20 pg/ml in all experiments.

Measurement of nitrite/nitrate and mRNA

Nitrite/nitrate was measured on the basis of the Griess reaction, as described previously (11, 16, 17). Nitrite/nitrate values at 0 hr, all of which were below 4 μM , were subtracted from cumulative values obtained from the same flask or dish.

After aliquots of the culture medium for assay of nitrite/nitrate had been obtained, cells were collected, and the total RNA was extracted (18). RNA was applied to 1.2% formaldehyde-agarose gels, electrophoresed, and then transferred onto Biotrans B nylon membranes (Pall, East Hills, NY, USA), as previously described (19). The gel was stained by ethidium bromide after electrophoresis. A 700 base pair fragment of the 5' portion of cloned rat liver inducible nitric oxide synthase cDNA (20) was labelled as previously described (19). 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 then 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 then 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 then exposed to X-ray film.

Other dishes of cells, which were run in parallel with those for Northern blot analysis, were washed three times with 0.9% NaCl, and the protein content was determined with a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA).

Statistics

To test statistical significance, the paired *t*-test and *t*-test were used for Figs. 1 and 3, respectively.

RESULTS

Time-dependent nitrite/nitrate production and increased nitric oxide synthase mRNA

The combination of IFN- γ and TNF- α has been demonstrated to elicit nitrite/nitrate production from vascular endothelial cells (3, 6). Therefore, we used this combination to stimulate RACE-1 in these experiments. Unstimulated RACE-1 had produced no significant amounts of nitrite/nitrate by 48 hr (Fig. 1). The combination of IFN- γ (100 U/ml) and TNF- α (5000 U/ml) induced nitrite/nitrate production which was evident at 24 hr ($t=5.58$, $n=3$, $P<0.05$) and continued to increase for up to 48 hr.

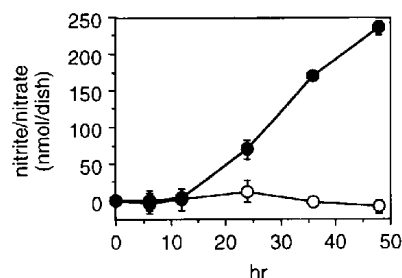


Fig. 1. Time-dependent accumulation of nitrite/nitrate for 48 hr in RACE-1 culture medium treated without cytokines (○) or with a combination of IFN- γ (100 U/ml) and TNF- α (5000 U/ml) (●). Cellular protein content was 1.2 mg/flask. Data are means \pm S.E. ($n=3$).

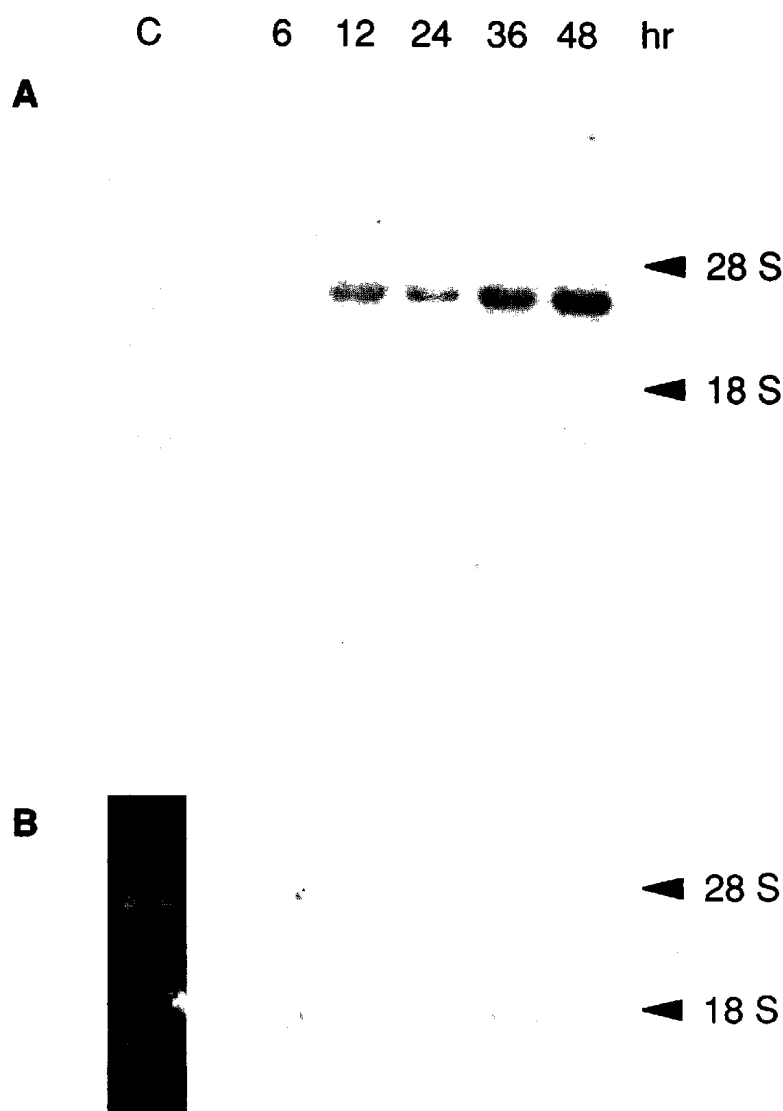


Fig. 2. Northern blot analysis of time-dependent increase in nitric oxide synthase mRNA. A: Total RNA was obtained from RACE-1 at the indicated times after the addition of both IFN- γ (100 U/ml) and TNF- α (5000 U/ml) to the culture media. The letter C indicates data from RACE-1 without the cytokines at 24 hr. B: Photograph of the gel stained with ethidium bromide. Cellular protein content was 0.9 mg/dish. The autoradiogram is representative of two experiments.

At these concentrations, IFN- γ has been shown to induce maximum nitrite production from endothelial cells in the presence of TNF- α and vice versa (3). Without stimulation, inducible nitric oxide synthase mRNA was undetectable in RACE-1 at 24 hr (Fig. 2A). However, when stimulated with the combination of IFN- γ (100 U/ml) and TNF- α (5000 U/ml), nitric oxide synthase mRNA was detected at 12 hr, and the level was markedly elevated at 48 hr (Fig. 2A). A trace amount of nitric oxide synthase mRNA was observed on the film at 6 hr with prolonged exposure (data not shown). The length of the nitric oxide synthase mRNA was estimated to be similar to those from mouse macrophages (21) and rat organs including the spleen and the liver (20).

Inhibition by dexamethasone of nitrite/nitrate production and increased nitric oxide synthase mRNA

Dexamethasone (1 μ M) inhibited the induction of nitrite/nitrate production by IFN- γ (100 U/ml) and TNF- α (5000 U/ml), but had no effect on nitrite/nitrate accumulation when applied alone (Fig. 3A). The increase in nitric oxide synthase mRNA induced by the cytokines was also inhibited by dexamethasone (1 μ M) at 48 hr (Fig. 3B).

Nitrite/nitrate production and increases in nitric oxide synthase mRNA induced by various combinations of cytokines and LPS

We next investigated the effects of various combinations of IFN- γ , TNF- α and LPS on the induction of nitric oxide synthase. We used LPS at the concentration which induces maximum production of nitrite/nitrate from vas-

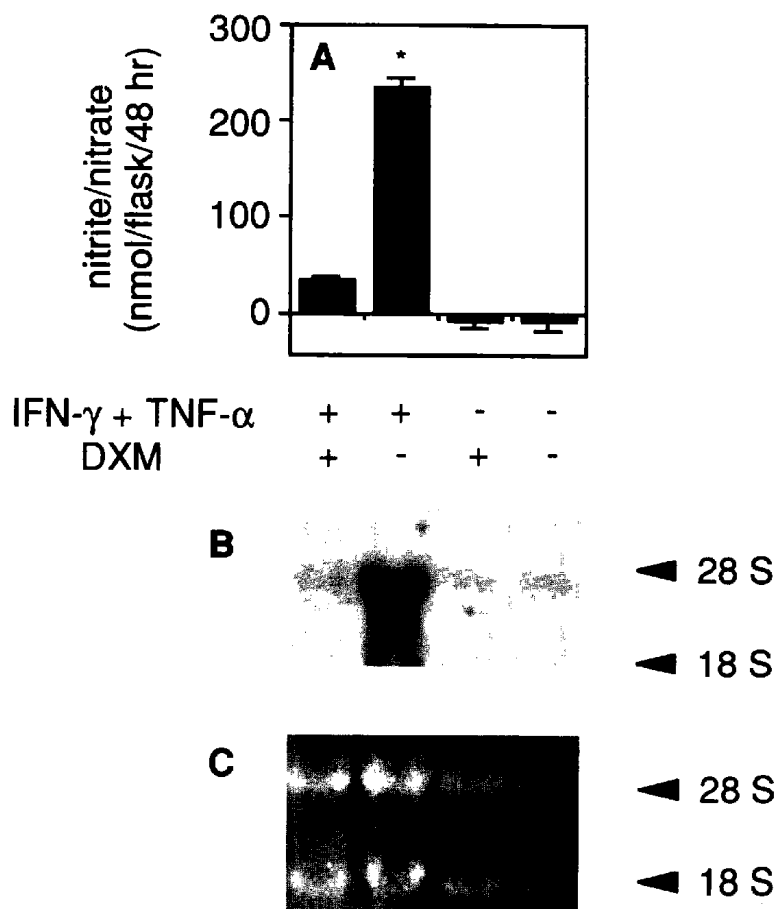


Fig. 3. Inhibitory effect of dexamethasone on induction of nitrite/nitrate production (panel A) and the increase in nitric oxide synthase mRNA (panel B). A: Nitrite/nitrate accumulation for 48 hr in RACE-1 culture medium treated with a combination of IFN- γ (100 U/ml), TNF- α (5000 U/ml) and dexamethasone (1 μ M), a combination of IFN- γ (100 U/ml) and TNF- α (5000 U/ml), with dexamethasone (1 μ M), and without reagent. Data are means \pm S.E. (n=3). *P<0.05 vs. other three columns. B: Northern blot analysis of nitric oxide synthase mRNA from a set of cells used in panel A. C: Photograph of the gel stained with ethidium bromide. Each lane of panels B and C corresponds to that of panel A. Cellular protein content was 1.2 mg/flask. The autoradiogram is representative of three experiments.

cular smooth muscle cells in the presence of $\text{TNF-}\alpha$ (11). As shown in Fig. 4A, unstimulated RACE-1 had not produced significant amounts of nitrite/nitrate by 48 hr. Neither $\text{IFN-}\gamma$ (100 U/ml), $\text{TNF-}\alpha$ (5000 U/ml) nor LPS (100 ng/ml) alone was capable of inducing significant nitrite/nitrate production by 48 hr. However, combinations of two of the three agents induced significant nitrite/nitrate production. Furthermore, when the three

were applied together, RACE-1 produced markedly elevated levels of nitrite/nitrate.

Northern blot analysis of the cells used for the nitrite/nitrate accumulation experiments in Fig. 4A revealed that neither $\text{IFN-}\gamma$ (100 U/ml), $\text{TNF-}\alpha$ (5000 U/ml) nor LPS (100 ng/ml) alone increased nitric oxide synthase mRNA at 48 hr (Fig. 4B). In contrast, the combination of $\text{IFN-}\gamma$ (100 U/ml) and $\text{TNF-}\alpha$ (5000 U/ml) and that of

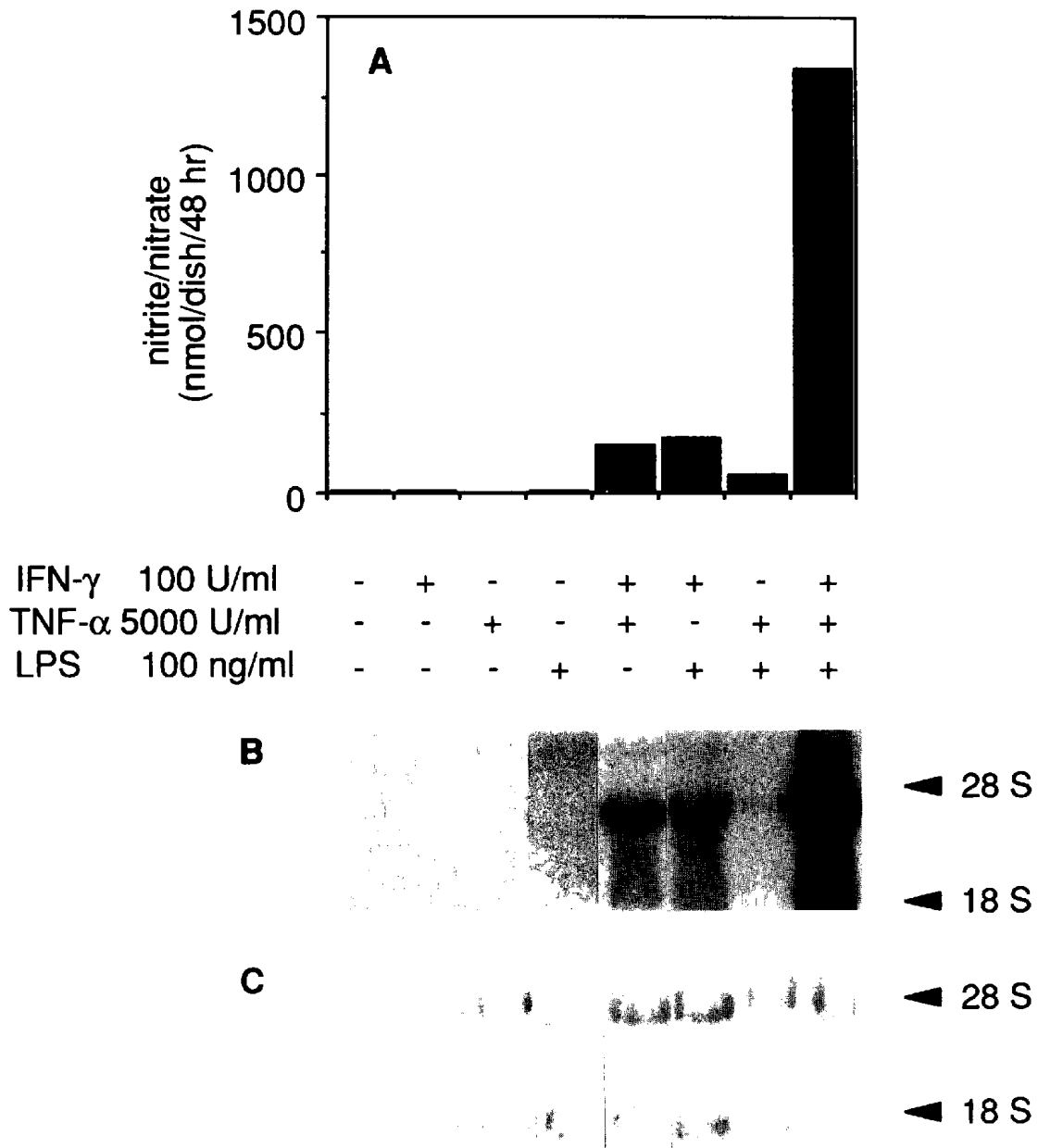


Fig. 4. Effects of various combinations of cytokines and LPS on the induction of nitric oxide synthase. **A:** Nitrite/nitrate accumulation in RACE-1 culture medium treated for 48 hr under the experimental conditions shown in the figure. **B:** Northern blot analysis of nitric oxide synthase mRNA from the cells used in panel A. **C:** Photograph of the gel stained with ethidium bromide. Each lane of panels B and C corresponds to that of panel A. Cellular protein content was 1.5 mg/dish. The result is representative of two experiments.

IFN- γ (100 U/ml) and LPS (100 ng/ml) induced significant increases in nitric oxide synthase mRNA. The combination of TNF- α (5000 U/ml) and LPS (100 ng/ml) elicited a small but significant increase in nitric oxide synthase mRNA at 48 hr. The combination of IFN- γ (100 U/ml), TNF- α (5000 U/ml) and LPS (100 ng/ml) induced a marked increase in nitric oxide synthase mRNA in RACE-1 at 48 hr. RACE-1 did not express detectable amounts of inducible nitric oxide synthase mRNA without either cytokines or LPS.

DISCUSSION

The increase in nitric oxide synthase activity, determined by nitrite/nitrate accumulation in the culture supernatant, was accompanied by an increase in nitric oxide synthase mRNA. This is consistent with the interpretation that the increases in nitric oxide biosynthesis induced by these cytokines and LPS are controlled at the mRNA level. Whether the increase in nitric oxide synthase mRNA observed in the present study is due to enhanced transcription or stabilization of mRNA remains to be investigated.

Though neither IFN- γ , TNF- α nor LPS was effective when applied alone, combinations of two of the three agents increased nitric oxide synthase mRNA and nitrite/nitrate production, and the combination of all three greatly enhanced this increase. These observations indicate that the increase is synergistic and that this synergism is regulated at the mRNA level. The mechanism underlying this synergistic increase in nitric oxide synthase mRNA is unknown; it is possible that these substances act in such a way that one induces the receptor for the other or that there are three distinct pathways leading to increased nitric oxide synthase mRNA.

Our results show that neither IFN- γ nor TNF- α alone is capable of inducing nitric oxide synthase, while combining IFN- γ with TNF- α is effective and these results are in accord with those of other investigators (3, 6). However, some studies indicate that TNF- α alone is capable of enhancing nitric oxide release from bovine aortic endothelial cells (5) and that IFN- γ inhibits this enhancement by TNF- α (22). The reason for these discrepancies is unknown, but possible explanations include: 1) species differences in endothelial cells or 2) differences in nitric oxide detection methods used; Gross et al. (6), Kilbourn and Belloni (3), and our group have measured nitrite/nitrate, whereas Lamas et al. (5, 22) used a bioassay system measuring cGMP content in mesangial cells, serving as detector cells, and also measured conversion of L-[14 C]-arginine to L-[14 C]citrulline in the culture plate, which is difficult to distinguish from urea cycle-derived L-citrulline.

Dexamethasone and cycloheximide have been demon-

strated to inhibit induction of nitric oxide synthase activity in endothelial cells (4). Cycloheximide is considered to inhibit induction of nitric oxide synthase activity by inhibiting protein synthesis. Our results show that dexamethasone exerts its inhibitory effect at the mRNA level.

In our experiments, unstimulated endothelial cells failed to produce detectable amounts of nitrite/nitrate by 48 hr, indicating the absence of basal nitric oxide release from these cells. Other investigators measuring nitrite released into culture media by vascular endothelial cells have also failed to detect basal production of nitric oxide (6, 23), which is in accord with our results. This may be because the amounts of basal nitric oxide release is below assay sensitivity limits. Malinski and Teha reported nitric oxide release from a single cell *in situ* by use of the porphyrinic-based microsensor method, which offers a detection limit 2–4 orders of magnitude lower than the estimated amounts of nitric oxide released per single cell (24). The basal release of nitric oxide from a single endothelial cell was undetectable in the absence of stimuli, even when a highly sensitive method was used (24). This may be partly due to lack of shear stress in an *in vitro* experimental system, since shear stress is considered to be a crucial physiological stimulus that causes the basal release of nitric oxide *in vivo* (1).

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

Inducible nitric oxide synthase cDNA has been cloned in macrophages (21, 25) and in hepatocytes (26). These inducible nitric oxide synthases are considered to be distinct gene products from constitutive nitric oxide synthases cloned in the brain (27) or aortic endothelial cells (28). Therefore, the induced nitric oxide synthase in RACE-1 seems to be different from that of the constitutive type.

Inhibitors of nitric oxide synthase have been shown to ameliorate hypotension in patients with septic shock (29). It has also been shown that pretreatment with either anti-TNF- α antibodies (30) or interleukin-1-receptor antagonist (31) prevents hypotension after intravenous *E. coli* infusion in animals. Since many substances are involved in the pathogenesis of septic shock, amelioration of hypotension by a single agent is difficult to interpret. However, considering our observation that cytokines and LPS 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 increased in endothelial cells during sepsis.

In vascular smooth muscle cells, interleukin-1 alone or in combination with TNF- α induces nitric oxide synthase activity (11, 32). However, in macrophages, interleukin-1 does not enhance nitric oxide synthase activity even in the presence of either IFN- γ or LPS (33, 34), which typically induce nitric oxide synthase when applied together (21). Therefore, cytokine induction of nitric oxide synthase appears to be cell type-specific. In situations in which nitric oxide synthase is induced in the vasculature, as in the case of sepsis, the major source of nitric oxide may depend on the combination of cytokines to which the vasculature is exposed.

In conclusion, we have demonstrated a synergistic induction of nitric oxide synthase in endothelial cells by IFN- γ , TNF- α and LPS, inhibited by dexamethasone, which is regulated at the mRNA level.

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