Expression of Nitric Oxide Synthase by Cytokines in Vascular Smooth Muscle Cells

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Abstract In cultured vascular smooth muscle cells, the baseline mRNA and protein levels of an inducible type of nitric oxide synthase were barely detectable. Interferon gamma, tumor necrosis factor-alpha, and interleukin-1beta each markedly increased mRNA and protein levels of this enzyme in parallel with the production of nitrite, a stable oxidative metabolite of nitric oxide. Actinomycin D abolished the cytokine-induced increases in mRNA levels and nitrite production. Cycloheximide, which abolished the cytokine-induced increase in nitrite production, had no effect on the interferon-gamma-induced increase in mRNA levels but partially inhibited that induced by interleukin-1beta and markedly inhibited that induced by tumor necrosis factor-alpha. Transforming growth factor-beta, which inhibited the interferon gamma-, interleukin-1beta-, and tumor necrosis factor-alpha-induced nitrite production, did not affect the increases in mRNA levels caused by these cytokines. Transforming growth factor-beta, however, significantly inhibited the increase in protein levels caused by these cytokines. These findings suggest that interferon gamma directly induces the expression of the inducible nitric oxide synthase gene, whereas tumor necrosis factor-alpha and interleukin-1beta induce it, at least in part, via the induction of intermediary protein(s), and that transforming growth factor-beta inhibits cytokine-induced nitric oxide production by blocking the posttranscriptional synthesis of inducible nitric oxide synthase.

Key Words • nitric oxide • interferons • tumor necrosis factor • transforming growth factor beta • muscle, smooth, vascular

In addition to its powerful vasodilating action, nitric oxide (NO) is thought to have diverse effects in blood vessels, including inhibition of adhesion and aggregation of platelets, superoxide anion production, leukocyte adhesion, endothelin generation, and the proliferation of vascular smooth muscle cells (VSMCs). Recent studies have clearly shown that VSMCs express a system that generates NO from L-arginine only after treatment with endotoxin or inflammatory cytokines such as interleukin-1beta (IL-1beta) and tumor necrosis factor-alpha (TNF-alpha). NO thus produced has been implicated not only in the pathogenesis of atherosclerosis and hypertensive vascular lesions in which infiltration of inflammatory cells occurs. Recently, it has been shown that transforming growth factor-beta (TGF-beta) inhibits the cytokine-induced NO production by macrophages, renal mesangial cells, and VSMCs, suggesting that TGF-beta may be an endogenous modulator of NO production. Although the findings of previous biochemical and pharmacological studies strongly suggest that IL-1beta and TNF-alpha induce the expression of an inducible type of NO synthase (iNOS) gene and that TGF-beta inhibits the expression of that gene in VSMCs, the mechanisms by which these cytokines regulate iNOS expression remain unclear.

Recently, cDNA encoding iNOS has been cloned from a macrophage-derived cell line that has been activated by interferon gamma (IFN-gamma) and endotoxin, and it has been shown that IFN-gamma and endotoxin stimulate the transcription of the iNOS gene in this same cell line. In a preceding report, we demonstrated that IFN-gamma and TNF-alpha induced the increase in iNOS mRNA in cultured VSMCs. We also showed that cycloheximide had no effect on the IFN-gamma-stimulated increase in iNOS mRNA but partially inhibited that induced by interleukin-1beta and markedly inhibited that induced by tumor necrosis factor-alpha. Transforming growth factor-beta inhibits cytokine-induced nitric oxide production by blocking the posttranscriptional synthesis of inducible nitric oxide synthase.

Methods VSMCs were isolated from rat thoracic aorta by enzymatic dissociation as described previously and used at passage levels 9 through 16. Although VSMCs from one isolate were used for the experiments reported here, almost identical results were obtained using VSMCs from another isolate. The mouse monococyte-macrophage cell line J774A.1 was obtained from Japanese Cancer Research Resources Bank, Tokyo, Japan. Recombinamouse IFN-gamma (1X10^7 U/mg), TNF-alpha (4X10^7 U/mg), and human platelet TGF-beta (1X10^7 U/mg) were purchased from Genzyme, Cambridge, Mass. Recombinant human IL-1beta (2X10^7 U/mg) was a gift from Otsuka Pharmaceutical Co, Ltd, Tokushima, Japan. Lipopolysaccharide (LPS) (from Escherichia coli 055:B5) and nitrate reductase (10 U/mL) were from Sigma Chemical Co, St Louis, Mo, and Boehringer Mannheim Yamanouchi, Tokyo, Japan, respectively. [alpha-32P]Deoxyctydine triphosphate (3000 Ci/mmol) was from Amersham Japan, Tokyo, Japan. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was a gift from Dr Kimihiko Sano, Kobe University School of Medicine, Kobe, Japan. Other materials and chemicals were obtained from commercial sources.
A cDNA fragment of iNOS was prepared by a reverse transcription polymerase chain reaction using total RNA from IFN-γ plus LPS-stimulated J774A.1 cells as described,12 Northern blot analysis for iNOS and GAPDH mRNA levels was performed as described,12

Nitrite was measured by mixing 500 μL of the medium with an equal volume of Griess reagent.16 Data were normalized to the protein content of VSMCs in each dish. In pilot experiments, nitrate was also measured as described.17 Because the ratio of nitrite to nitrate was approximately 1:1 and remained similar for VSMCs treated with IFN-γ, IL-1β, or TNF-α in the presence and absence of TGF-β1, nitrite concentration in the media was used as an index of NO production for the experiments reported here. Western blot analysis of VSMC extracts with the anti-iNOS antibody was performed as described.14 Where applicable, results are expressed as mean±SEM. Differences between means were evaluated by Student’s t test where appropriate. A value of P<.05 was taken to be significant.

Results and Discussion

Treatment of VSMCs with each of IFN-γ, IL-1β, and TNF-α resulted in marked nitrite production (Fig 1A). Under identical conditions, these cytokines also increased iNOS mRNA levels (Fig 1B). Protein levels of iNOS were also increased after exposure to each of IFN-γ, IL-1β, and TNF-α (Fig 4). The dose dependencies and time courses of the IFN-γ, IL-1β, and TNF-α–induced increases in iNOS mRNA were similar to those of the increases in iNOS protein caused by the corresponding cytokines. They also correlated well with those of the nitrite production (data not shown). These results clearly indicate that IFN-γ, IL-1β, and TNF-α induce iNOS mRNA and protein expression in VSMCs. We then made use of two types of metabolic inhibitors, actinomycin D, a DNA-dependent RNA transcription inhibitor, and cycloheximide, a protein synthesis inhib-
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**Fig 2.** Graph shows inhibition by transforming growth factor-β (TGFβ) of interferon gamma-, interleukin-1β-, and tumor necrosis factor-α-induced nitrite production. Vascular smooth muscle cells were stimulated with interferon gamma (100 U/mL) (●), interleukin-1β (100 ng/mL) (▲), tumor necrosis factor-α (100 ng/mL) (○), or vehicle (□) in the presence of various concentrations of TGFβ1 for 24 hours. Nitrite accumulated in the culture medium was measured (n=3). *Significantly lower than nitrite production in the absence of TGFβ1.*

... cytokine-induced nitrite production. These results suggest that IFN-γ directly stimulates the expression of iNOS mRNA, whereas IL-1β stimulates this expression both directly and via the induction of intermediary protein(s), and TNF-α stimulates it principally via the induction of intermediary protein(s). If this is the case, the induction and action of intermediary protein(s) must be fairly rapid, because time courses of iNOS induction by these three cytokines were similar (data not shown). However, the exact reason for the different sensitivities to cycloheximide of iNOS mRNA induction by IFN-γ, IL-1β, and TNF-α is not clear at present, and further studies are obviously needed for clarification.

Inhibition of NO production by TGF-β was first described for macrophages activated by IFN-γ7 and then described for renal mesangial cells stimulated by IL-1β or TNF-α.8 In these initial studies, TGF-β was thought to act at the early stages of the induction of iNOS, because the presence of TGF-β during the early induction phase was required for the inhibition and TGF-β was inactive once cells had been stimulated by the cytokines. Recently, Junquero et al9 have shown that TGF-β1 inhibits IL-1β-induced NO production in human cultured VSMCs. Consistent with the previous observations,9,10 TGF-β1 dose-dependently inhibited the nitrite production stimulated by IFN-γ, IL-1β, and TNF-α (Fig 2). TGF-β1 appeared to be less effective when IFN-γ was used for stimulation. To locate the inhibitory effect of TGF-β1 in the pathway leading to NO production, we examined whether TGF-β1 inhibits the induction of iNOS mRNA and protein by IFN-γ, IL-1β, and TNF-α. Fig 3 shows the effect of 10 ng/mL TGF-β1, a concentration at which potent inhibition of IFN-γ-, IL-1β-, and TNF-α-stimulated nitrite production occurs, on the increase in iNOS mRNA levels induced by the cytokines. Although TGF-β1 by itself induced a small increase in iNOS mRNA levels, it had no effect on the increase in iNOS mRNA levels resulting from administration of IFN-γ, IL-1β, and TNF-α. As shown in Fig 4,
however, the increases in iNOS protein stimulated by IFN-γ, IL-1β, and TNF-α were significantly inhibited by TGF-β1. These results indicate that TGF-β1 inhibits the cytokine-induced NO production by blocking the post-transcriptional synthesis of iNOS.

The functional consequences of the formation of NO by iNOS in VSMCs have not been clearly determined. Because massive infiltration of inflammatory cells was observed in vascular lesions in hypertensive humans and animals, it seems possible that NO synthesized in VSMCs by iNOS may modulate the formation of hypertensive vascular lesions. NO may decrease the severity of such vascular lesions via its inhibitory effects on adhesion and aggregation of platelets, superoxide anion production, leukocyte adhesion, endothelin generation, and VSMC proliferation. On the other hand, NO may function as a potentially damaging molecule because it reacts with superoxide anions, resulting in the formation of peroxynitrite, which decays to nitrogen dioxide and hydroxyl radical, the strongest oxidant in biologic systems.1

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References
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