Volume 318, number 3, 213–217 FEBS 12158 March 1993

Cytokine-induced expression of an inducible type of nitric oxide synthase gene in cultured vascular smooth muscle cells

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Received 14 December 1992; revised version received 17 January 1993

In unstimulated cultured vascular smooth muscle cells (VSMC), mRNA of an inducible macrophage-type of nitric oxide synthase (iNOS) was barely detectable. Interferon γ (IFNγ) and tumor necrosis factor α (TNFα) markedly increased iNOS mRNA levels in time- and dose-dependent manners. The induction of iNOS mRNA paralleled the cytokine-induced nitrite production. Actinomycin D abolished the IFNγ- and TNFα-induced increases in iNOS mRNA and nitrite production. Cycloheximide, which abolished both the IFNγ- and TNFα-induced increases in nitrite production, had no effect on the IFNγ-induced increase in iNOS mRNA but markedly inhibited the TNFα-induced one. These results suggest that IFNγ directly induces the expression of the iNOS gene whereas TNFα mainly induces it via the induction of an intermediary protein in cultured VSMC.

Nitric oxide; Nitric oxide synthase; Cytokine; Interferon γ; Tumor necrosis factor α; Vascular smooth muscle cell

1. INTRODUCTION

NO accounts for many of the biological properties of endothelium-derived relaxing factor such as relaxation of vascular smooth muscle and inhibition of platelet adhesion and aggregation via the activation of soluble guanylate cyclase [1,2]. NO is synthesized by oxidation of the terminal guanidino-nitrogen atom(s) of L-arginine by a family of enzymes called the NOS [3]. At least three distinct types of NOS have been identified: a constitutive Ca^{2+}- and calmodulin-dependent cytosolic form which is present in the brain, platelets and adrenal glands, a constitutive Ca^{2+}- and calmodulin-dependent membrane-bound form which is present in endothelial cells and an inducible Ca^{2+}-independent form which is present in macrophages, hepatocytes, mesangial cells and endothelial cells only after the treatment with endotoxin or certain cytokines such as IL1, TNFα or IFNγ [1,2,4]. The inducible form of NOS activity is also induced in rat aortic rings and in cultured VSMC by cytokines and endotoxin [5,6], which is suggested to be responsible for the loss of vascular responsiveness observed in septic shock and during antitumor therapy with TNFα [7]. Moreover, it has recently been demonstrated that in vivo balloon injury locally induces NOS activity in rat carotid arteries [8]. Therefore, it is possible that the NOS induction in VSMC plays some role not only in the pathogenesis of septic shock but also in local vascular inflammatory lesions such as vasculitis, atherosclerosis and arterial injury following ballooning angioplasty where macrophages and T lymphocytes accumulate and release the cytokines.

The biochemical and pharmacological experiments described above strongly suggest that the cytokines induce the expression of the iNOS gene. In these experiments, however, the expression of iNOS is demonstrated only by the generation of cyclic GMP, the second messenger of NO, and nitrite, a stable oxidative metabolite of NO. Thus, it is not obvious whether these cytokines directly induce the expression of the iNOS gene or act via the induction of an intermediary protein.

In the present study, we demonstrate for the first time that IFNγ and TNFα increase iNOS mRNA levels in VSMC. We also provide evidence that IFNγ directly induces the increase in iNOS mRNA but that TNFα induces it mainly through a newly synthesized protein intermediate.

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Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase; IL1, interleukin 1; TNFα, tumor necrosis factor α; IFNγ, interferon γ; VSMC, vascular smooth muscle cells; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

2. MATERIALS AND METHODS

2.1. *Materials*

VSMC were isolated from rat thoracic aorta by enzymatic dissociation as described previously [9] and used at passage levels 9–16. The mouse monocyte-macrophage cell line J774A.1 was obtained from the Japanese Cancer Research Resources Bank. Recombinant mouse IFNγ and TNFα were purchased from Genzyme, Cambridge, MA, USA. LPS (from *E. coli* 055:B5) was from Sigma, St. Louis, MO, USA. [α-^{32}P]dCTP (3,000 Ci/mmol) was from Amersham Japan,

Tokyo, Japan. GAPDH cDNA was gifted from Dr. K. Sano, Kobe University School of Medicine, Kobe, Japan. cDNA for a constitutive brain-type of NOS was a generous gift of Drs. D.S. Bredt and S.H. Snyder, Johns Hopkins University School of Medicine, Baltimore, MD, USA. Other materials and chemicals were obtained from commercial sources.

2.2. *Preparation of cDNA probe for iNOS*

RNA prepared from IFNγ plus LPS-stimulated J774A.1 cells was reverse transcribed into cDNA. A polymerase chain reaction was used to amplify a 1,033-base pair iNOS cDNA fragment. The sequences of the forward (ACAGGGAAGTCTGAAGCACTAG) and reverse (CATGCAAGGAAGGGAACTCTTC) primers were based on the published iNOS cDNA sequence (nucleotides 1,621–2,653) [10,11]. Authenticity of the polymerase chain reaction product was confirmed by sequencing by the dye primer method using an Applied Biosystems DNA sequencer model 373A (Applied Biosystems Japan, Inc., Tokyo, Japan).

2.3. *RNA extraction and Northern blotting*

Total RNA was extracted by the method of Chomczynski and Sacchi [12]. Total RNA (30 μg per lane) was subjected to electrophoresis on 1% agarose gels containing formaldehyde and transferred to nitrocellulose filters. The filters were hybridized with a random primed, ^{32}P-labelled iNOS cDNA probe for 20 h. The hybridized filters were then washed twice with 150 mM NaCl, 15 mM sodium citrate, and 0.1% sodium dodecyl sulfate at 65°C and analyzed using a FUJIX bio-imaging analyzer BAS2000 (Fuji Photo Film Co. Ltd., Tokyo, Japan). The filters were subsequently stripped and rehybridized with a ^{32}P-labelled GAPDH cDNA probe.

2.4. *Measurement of nitrite*

Nitrite was measured by mixing 500 μl of the medium with an equal volume of Griess reagent (1 part 0.1% naphthylethylenediamine dihydrochloride to 1 part 1% sulfanilamide in 5% phosphoric acid) [13]. The absorbance at 550 nm was measured and nitrite concentration was determined using a curve calibrated from sodium nitrite standards. Data were normalized to the protein content of VSMC in each dish.

3. RESULTS

To examine whether IFNγ and TNFα cause the induction of iNOS mRNA, total RNA prepared from VSMC treated with IFNγ or TNFα was analyzed by Northern blotting. iNOS mRNA was barely detectable in total RNA prepared from control VSMC. Treatment of the cells with IFNγ and TNFα induced marked increases in iNOS mRNA in similar time courses (Fig. 1A). Levels of iNOS mRNA significantly increased within 6 h and continued to increase for at least 30 h after exposure to either IFNγ or TNFα. The most prominent transcript is about 5.0 kb in length and two larger transcripts were also detected after prolonged exposure of autoradiography. The pattern of these transcripts was similar to that reported in activated macrophages [10]. When Northern blots were probed with GAPDH cDNA, a single message was detected at about 1.4 kb. The intensities of hybridization of the GAPDH cDNA to RNA were equal among all lanes, verifying that equal amounts of RNA were loaded per lane and that the observed induction of iNOS mRNA was not due to a generalized effect on the mRNA content in the cells. Time courses of nitrite accumulation induced by IFNγ and TNFα correlated well with those of the induction of iNOS mRNA (Fig. 1B). Levels of nitrite accumulated in the medium significantly increased within 6 h and continued to increase for at least 30 h after exposure to either IFNγ or TNFα. In another set of experiments, we also investigated mRNA levels of the constitutive brain type of NOS in VSMC as well as in various rat tissues. We observed a clear expression of

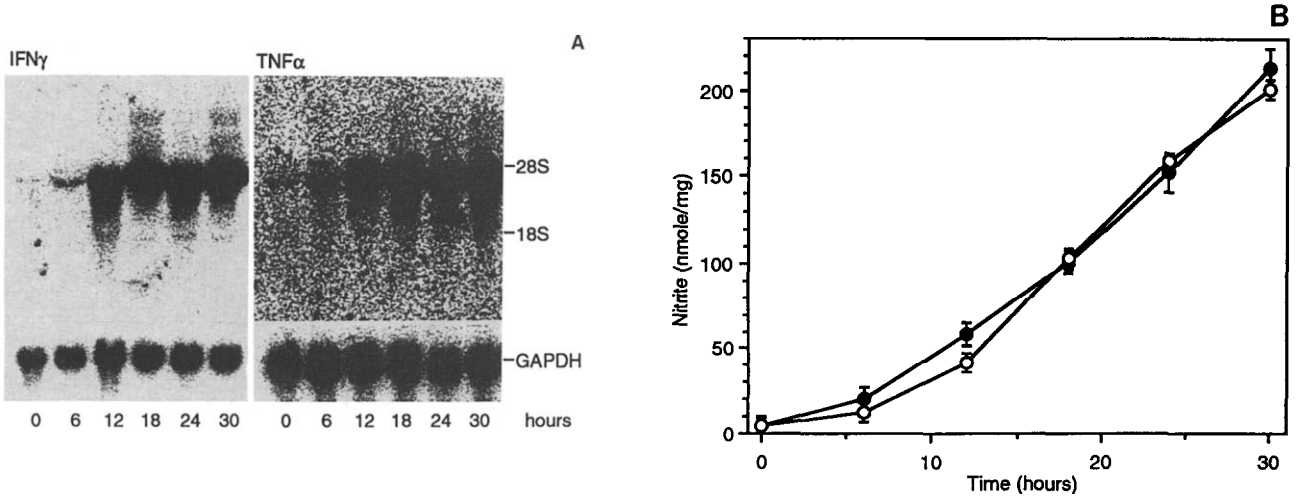

Fig. 1. (A) Time courses of the iNOS mRNA induction by IFNγ and TNFα. VSMC were stimulated with 100 units/ml IFNγ (left panel) or 100 ng/ml TNFα (right panel) for various periods of time as indicated. Total RNA was isolated and analyzed by Northern blotting with iNOS (upper panel) and GAPDH (lower panel) cDNA probes. The positions of the 28 S and 18 S ribosomal RNA subunits are indicated at the right. The experiment shown represents one of three independent trials which gave nearly identical results. (B) Time courses of the nitrite production by IFNγ and TNFα. VSMC were stimulated with 100 units/ml IFNγ (○) or 100 ng/ml TNFα (●) for various periods of time as indicated. Nitrite concentration in the culture medium was measured. Points and bars represent the mean ± S.E.M. (n = 3).

its mRNA in the cerebellum and adrenal glands but could not detect it in VSMC before or after exposure to the cytokines (data not shown), suggesting that IFNγ and TNFα specifically induce the expression of the iNOS gene in VSMC. Fig. 2A shows dose–response relationships of the IFNγ- and TNFα-induced increases in iNOS mRNA. The dose dependencies of the IFNγ- and TNFα-stimulated nitrite accumulation were similar to those of the IFNγ- and TNFα-stimulated induction of iNOS mRNA, respectively (Fig. 2B).

We next investigated the effects of actinomycin D, an inhibitor of DNA-dependent RNA transcription, or cycloheximide, an inhibitor of protein synthesis on the IFNγ- and TNFα-induced increases in iNOS mRNA and nitrite accumulation. As shown in Fig. 3, actinomycin D had no effect on the basal levels of iNOS mRNA and nitrite production. When actinomycin D was given in concert with IFNγ or TNFα, the IFNγ- and TNFα-stimulated increases in iNOS mRNA levels as well as nitrite accumulation were abolished, suggesting that these cytokines regulate iNOS activity at the level of iNOS gene transcription. Although the basal level of iNOS mRNA was slightly elevated by cycloheximide, the induction of iNOS mRNA by IFNγ was virtually unaffected by cycloheximide, indicating that the increase in iNOS mRNA is a primary response to IFNγ. Under the same conditions, cycloheximide markedly inhibited the TNFα-induced increase in iNOS mRNA, suggesting that the TNFα-induced increase in iNOS mRNA requires de novo protein synthesis. Cycloheximide completely inhibited the IFNγ- and TNFα-induced nitrite accumulation, confirming the effectiveness of our preparation of cycloheximide.

4. DISCUSSION

In the present study, we demonstrated for the first time that IFNγ and TNFα increase iNOS mRNA levels in VSMC. Since the induction of iNOS mRNA by IFNγ was unaffected by cycloheximide, expression of the

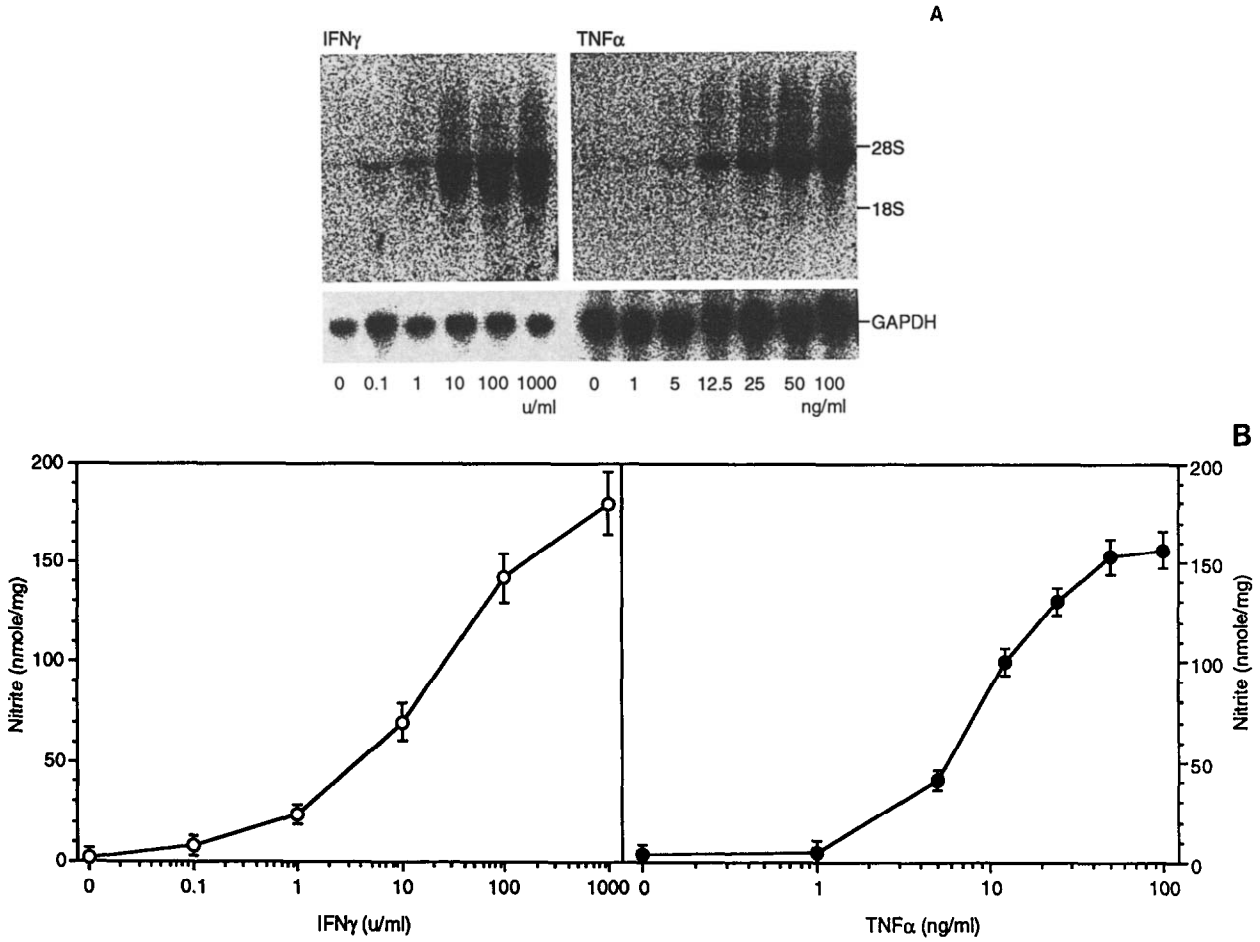

Fig. 2. (A) Dose–response relationships of the IFNγ- and TNFα-stimulated iNOS mRNA induction. VSMC were stimulated for 24 h with various concentrations of IFNγ (left panel) and TNFα (right panel) as indicated. Total RNA was isolated and analyzed by Northern blotting with iNOS (upper panel) and GAPDH (lower panel) cDNA probes. The positions of the 28 S and 18 S ribosomal RNA subunits are indicated at the right. The experiment shown represents one of three independent trials which gave nearly identical results. (B) Dose–response curves of the IFNγ- and TNFα-stimulated nitrite production. VSMC were stimulated for 24 h with various concentrations of IFNγ (left panel) and TNFα (right panel) as indicated. Nitrite concentration in the culture medium was measured. Points and bars represent the mean ± S.E.M. (n = 3).

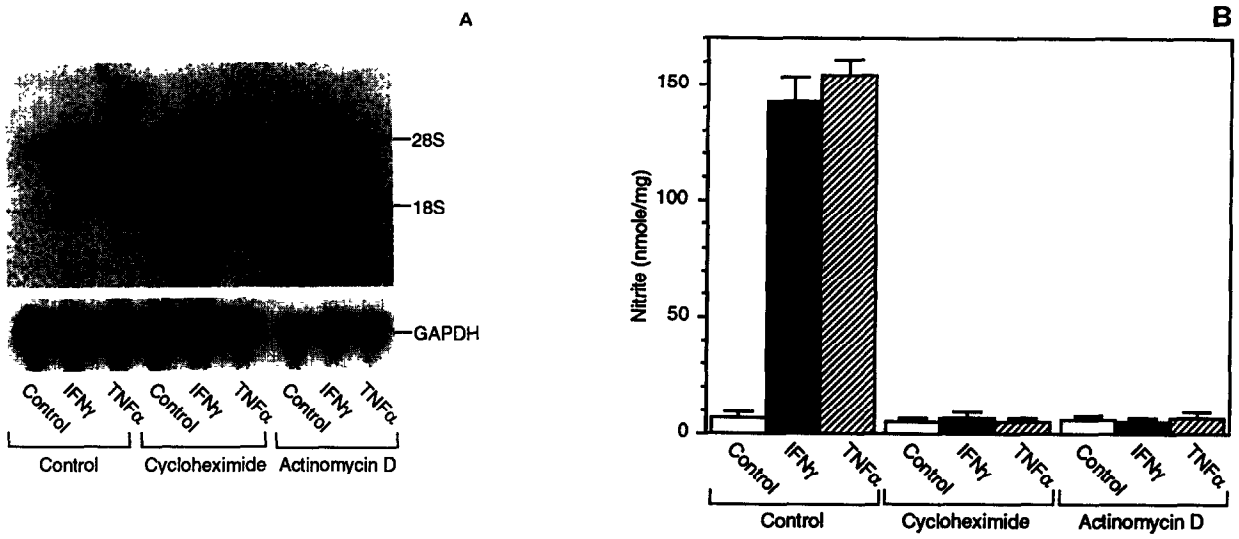

Fig. 3. (A) Effects of cycloheximide and actinomycin D on the cytokine-induced increases in iNOS mRNA. VSMC were preincubated with 20 μg/ml cycloheximide, 2 μg/ml actinomycin D or their vehicle for 1 h and incubated with 100 units/ml IFNγ, 100 ng/ml TNFα or their vehicle for an additional 24 h in the presence of the metabolic inhibitors. Total RNA was isolated and analyzed by Northern blotting with iNOS (upper panel) and GAPDH (lower panel) cDNA probes. The positions of the 28 S and 18 S ribosomal RNA subunits are indicated at the right. The experiment shown represents one of four independent trials which gave nearly identical results. (B) Effects of cycloheximide and actinomycin D on the cytokine-induced nitrite production. VSMC were preincubated with 20 μg/ml cycloheximide, 2 μg/ml actinomycin D or their vehicle for 1 h and incubated with 100 units/ml IFNγ, 100 ng/ml TNFα or their vehicle for an additional 24 h in the presence of the metabolic inhibitors. Nitrite concentration in the culture medium was measured. Points and bars represent the mean ± S.E.M. ($n = 4$).

iNOS gene appears to be directly regulated by IFNγ. Interferons including IFNγ directly stimulate the expression of a number of genes and many of them have been shown to possess a common DNA element, interferon response sequence, which is usually present in the 5′-upstream region of these genes [14]. It is possible that the iNOS gene is one of the members of an interferon-inducible gene family. It remains to be clarified whether interferon response sequence is also present in the 5′-upstream region of the iNOS gene. On the other hand, the marked inhibition by cycloheximide of the TNFα-induced increase in iNOS mRNA suggests that TNFα induces a protein which in turn induces the increase in iNOS mRNA. TNFα has been shown to cause accumulation of IL1 mRNA in human VSMC and subsequent release of IL1 from them [15]. Since IL1 has been shown to induce NO production in VSMC [6], it is possible that TNFα increases iNOS mRNA levels via the induction of IL1. However, we cannot completely rule out the possibility that the inhibitory effect of cycloheximide on the TNFα-induced increase in iNOS mRNA is due to a potentiative effect of cycloheximide on a cytotoxic effect of TNFα. But this possibility seems unlikely based on the following observations. Although the cells appeared to be damaged to some extent after the prolonged exposure to cycloheximide, there was no difference in the extent of cellular damage between the cells treated with TNFα plus cycloheximide and IFNγ plus cycloheximide as assessed by trypan blue exclusion. Moreover, there was no difference in the amounts of total RNA and GAPDH mRNA per dish between those cells. Further studies are obviously needed to deny this possibility and identify the intermediary protein involved in the TNFα-induced increase in iNOS mRNA.

IFNγ is produced by activated T lymphocytes and exerts antiviral, immunomodulatory and antiproliferative activities for various cells [16]. TNFα is produced by activated macrophages and VSMC and exerts tumoricidal, immunomodulatory and proliferative activities for various cells [16,17]. For VSMC, IFNγ inhibits cell proliferation in a cell culture system [16] and suppresses the development of intimal thickening of rat carotid arteries induced by in vivo balloon injury [18]. TNFα also acts on VSMC and induces prostaglandin E2 release [15] as well as IL1 production. Immunohistochemical studies have clearly shown that activated T lymphocytes and macrophages are present in atherosclerotic lesions and lesions after vascular injury [16,19,20]. Immunoreactive IFNγ and TNFα proteins have also been detected within human atherosclerotic lesions [19]. Recently, Joly et al. have reported that in vivo balloon injury induces NOS activity in rat carotid arteries [8]. Therefore, it is very likely that, via the induction of iNOS in VSMC, IFNγ and TNFα locally released within atherosclerotic or vascular injured lesions may play some roles such as a vasorelaxing activity which counteracts the vasoconstrictive properties of atherosclerotic arteries. They might also provide an anti-thrombogenic activity for exposed VSMC after endothelial denudation by ballooning angioplasty. Previ-

ously, we and others have reported that NO-generating vasodilators inhibit the proliferation of VSMC [21,22]. Very recently, Nunokawa and Tanaka have provided evidence suggesting that IFNγ inhibits the proliferation of VSMC via NO production by iNOS [23]. Thus, it is also possible that, via the induction of iNOS in VSMC, IFNγ and TNFα inhibit the proliferation of VSMC within atherosclerotic and vascular injured lesions.

Acknowledgements: This investigation was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan (1991), and by grants from the Yamanouchi Foundation for Research on Metabolic Disease (1991) and the Mochida Memorial Foundation for Medical and Pharmaceutical Research (1992). We are grateful to Yoshie Agehara and Noriko Sasaki for their skillful technical assistance.

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