Mechanisms of Suppression of Macrophage Nitric Oxide Release by Transforming Growth Factor β

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Summary

Activated mouse peritoneal macrophages produce nitric oxide (NO) via a nitric oxide synthase that is inducible by interferon γ (IFN-γ): iNOS. We have studied the mechanisms by which transforming growth factor β1 (TGF-β) suppresses IFN-γ-stimulated NO production. TGF-β treatment reduced iNOS specific activity and iNOS protein in both cytosolic and particulate fractions as assessed by Western blot with monospecific anti-iNOS immunoglobulin G. TGF-β reduced iNOS mRNA without affecting the transcription of iNOS by decreasing iNOS mRNA stability. Even after iNOS was already expressed, TGF-β reduced the amount of iNOS protein. This was due to reduction of iNOS mRNA translation and increased degradation of iNOS protein. The potency of TGF-β as a deactivator of NO production (50% inhibitory concentration, 5.6 ± 2 pM) may reflect its ability to suppress iNOS expression by three distinct mechanisms: decreased stability and translation of iNOS mRNA, and increased degradation of iNOS protein. This is the first evidence that iNOS is subject to other than transcriptional regulation.

Recent appreciation of the role of endogenously generated nitric oxide (NO)[2] in the physiology and pathophysiology of every organ system raises interest in mechanisms by which NO production may be regulated (1). This question assumes additional significance in view of the ability of NO to kill or damage cells (2).

Constitutive isoforms of NO synthase (cNOS) are expressed in neurons and endothelium. These enzymes produce small amounts of NO over several minutes in response to agonists that elevate intracellular Ca^{2+} (1). Other cell types (macrophages [3], tumor cells [4], hepatocytes [5, 6], mesangial cells [7], endothelial cells [8], smooth muscle cells [9], cardiac myocytes [10–12], glial cells [13, 14], and keratinocytes [15]) express an inducible isoform of NO synthase (iNOS) that produces relatively large amounts of NO over many hours without dependence on secretagogues. Nothing is known to regulate iNOS after it has been induced (1).

Suppression of iNOS expression was first shown in macrophages exposed to TGF-β (16). These results were confirmed (17) and extended in mesangial cells (18), smooth muscle cells (19, 20), and cardiac myocytes (11). Subsequently, suppression of iNOS expression was observed with epidermal growth factor (EGF)-treated keratinocytes (15), platelet-derived growth factor (PDGF)-treated smooth muscle cells (19), IL-8-treated neutrophils (21), and IL-4-treated macrophages (22). In no case has the mechanism of suppression been analyzed.

The iNOS from the mouse macrophage-like cell line RAW 264.7 has been purified (23, 24) and cloned (25–27), and a monospecific antiserum to it raised (26), allowing us to study TGF-β-mediated suppression of NO production in macrophages at the molecular level.

Materials and Methods

Reagents. Nitroblue tetrazolium (NBT), 5-bromo-4-chloro-3-indolyl phosphate (BCIP), β-nicotinamide-adenine dinucleotide phosphate (reduced; NADPH), flavin adenine dinucleotide (FAD), and protease inhibitors were obtained from Sigma Chemical Co. (St. Louis, MO). Tetrahydrobiopterin was from Dr. B. Schirks Laboratories (Jona, Switzerland). Dithiothreitol was from GIBCO-BRL (Gaithersburg, MD). Recombinant mouse IFN-γ (protein concentration, 1.1 mg/ml; sp act, 5.2×10^6 U/mg; LPS content, <10 pg/ml) was kindly provided by Genentech (South San Francisco, CA). Recombinant human TGF-β1 (hereafter referred to as TGF-β) was a kind gift from Amgen (Thousand Oaks, CA). Rabbit anti-mouse iNOS IgG was raised against iNOS purified from the RAW 264.7 cell line (26). Mouse β-actin oligonucleotide and mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA were

[1] Yoram Vodovotz and Christian Bogdan contributed equally to the experiments described in this work.

[2] *Abbreviations used in this paper:* cNOS, constitutive isoforms of nitric oxide synthase; FAD, flavin adenine dinucleotide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; iNOS, inducible isoform of nitric oxide synthase; NADPH, β-nicotinamide-adenine dinucleotide phosphate; NO, nitric oxide.

from Clontech (Palo Alto, CA). Phagemid pBluescript SK(+) containing the mouse β-actin cDNA was a kind gift of Drs. M. Prystowsky and A. Orlofsky (University of Pennsylvania, Philadelphia, PA). All chemicals were of reagent or electrophoresis grade.

Cells Culture and Lysis. Female CD-1 mice, 8–12 wk old (Charles River Breeding Laboratories, Wilmington, MA), were injected intraperitoneally with 4% Brewer's thioglycollate broth (Difco Laboratories, Detroit, MI). 4 d after injection, peritoneal macrophages were harvested and processed as described (28). Macrophages made up 86 ± 9% of the total cell population by morphologic criteria (67 experiments). Cells were seeded either in 24-well plates (Costar, Cambridge, MA) or in 100-mm-diameter tissue culture dishes (Corning, Corning, NY) at 2×10^6 cells/ml in RPMI 1640 (JRH Biosciences, Lenexa, KS) with 10% heat-inactivated FCS (HyClone Laboratories, Logan, UT). IFN-γ was added with or without TGF-β at the indicated concentrations. The cells were incubated at 37°C, 5% CO_2 in humidified air. An aliquot of the conditioned medium was removed for assay of NO_2^- content (see below). Where noted, monolayers were washed in warm PBS and scraped into cold PBS. The cells were centrifuged at 500 *g* for 15 min at 4°C. The cell pellet was frozen at −70°C for later processing or resuspended in 500 μl of sonication buffer (100 mM sodium phosphate, pH 7.4, 25 mM NaCl, containing 5 μg/ml pepstatin A, 1 μg/ml chymostatin, 5 μg/ml aprotinin, 100 μM PMSF). The cell pellet was sonicated using a Sonifier 250 (Branson Ultrasonics Co., Danbury, CT), as follows: 50% duty cycle, 10 s each at intensities of 1, 2, and 3. An aliquot of the whole lysate was reserved at −70°C. Where noted, the remaining lysate was centrifuged at 100,000 *g* for 30 min at 4°C in order to separate cytosol and particulate fractions in a modification of the procedure of Pollock et al. (29). The cytosol was stored as for the lysate. The pellet was resuspended in buffer A (sonication buffer containing 1 M KCl) and centrifuged at 100,000 *g* for 30 min at 4°C. The pellet was resuspended in 0.2 mM 3-([3-cholamidopropyl]dimethylammonio)-1-propanesulfonate in sonication buffer and kept on ice for 20 min, with periodic resuspension. An aliquot of this suspension was used for protein assay, and the remainder stored at −70°C.

SDS-PAGE and Western Blot. SDS-PAGE was carried out according to the method of Laemmli (30) with prestained molecular weight markers (Bethesda Research Laboratories, Gaithersburg, MD). After completion of SDS-PAGE, proteins were transferred onto 0.2-μm pore-size nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, NH) using 20% methanol, 25 mM Tris, 192 mM glycine, pH 8.3. The membrane was blocked for 1 h at room temperature with 1% BSA in Tris-buffered saline (TBS; 25 mM Tris, pH 7.5, 150 mM NaCl, 0.02% NaN_3) + 0.2% Tween 20. The membrane was then washed once in TBS and incubated with rabbit anti-mouse iNOS IgG used at 1:2,000 or 1:3,000 dilution for 2–4 h. After washing, the membrane was incubated for 45 min with a 1:10,000 dilution of donkey anti-rabbit IgG, $F(ab')_2$ conjugated to alkaline phosphatase (Jackson Immunoresearch Laboratories, West Grove, PA). After washing, the membrane was equilibrated in alkaline phosphatase buffer (100 mM Tris, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) and developed in a solution of 16 μg/ml NBT and 167 μg/ml BCIP in alkaline phosphatase buffer.

Metabolic Labeling and Immunoprecipitation. 8×10^6 peritoneal cells were seeded in 4 ml of RPMI 1640 + 10% FCS in 60-mm-diameter tissue culture dishes (Corning). After adherence and washing as described above, the macrophages were stimulated with 20 ng/ml IFN-γ for 16–18 h. TGF-β (2.5 ng/ml) or medium was then added for the indicated times. The medium was aspirated and replaced with pulse medium (RPMI 1640 without L-cysteine [ICN Biomedicals, Costa Mesa, CA], containing 10 mM Hepes, pH 7.4). The macrophages were incubated at 37°C, 5% CO_2 for 45 min in order to deplete cellular stores of L-cysteine. [^{35}S]L-Cysteine (1,220 Ci/mmol, 300 μCi/dish [Amersham Corp., Arlington Heights, IL]) in pulse medium was added for 30 min. The medium was removed, the dishes were washed three times in warm PBS, and the cells were scraped into cold PBS. After centrifugation at 500 *g* for 15 min, the cell pellet was frozen at −70°C until further processed. The cell pellet was lysed in 150 mM NaCl, 10 mM Tris, pH 8.0, 1% Triton X-100, 5 μg/ml pepstatin A, 1 μg/ml chymostatin, 5 μg/ml aprotinin, 100 μM PMSF. The protein content of the lysate was determined by Bradford protein assay. Equal amounts of protein from each lysate (20–80 μg) were precleared overnight with protein A–Sepharose beads (Sigma Chemical Co.) at 4°C. These samples were then incubated overnight with 1 μl/sample of anti-iNOS antiserum or preimmune serum at 4°C. The antigen–antibody solution was then incubated with protein A–Sepharose beads for 1.5 h, washed, and the immunoprecipitated sample eluted from the beads using 4× Laemmli reducing buffer. The samples were subjected to SDS-PAGE as described above. The gel was fixed (30% methanol, 7% acetic acid, 1 h), treated with ENTENSIFY hydrofluor (New England Nuclear, Boston, MA) as directed by the manufacturer, dried, and subjected to autoradiography.

Pulse-Chase Analysis. Macrophage monolayers were prepared as in the section above and stimulated with 20 ng/ml IFN-γ for 16–18 h. TGF-β (2.5 ng/ml) or medium was then added for a further 45 min. The medium was aspirated and replaced with pulse medium; TGF-β diluted in pulse medium was added back to the appropriate dishes. The cells were starved of L-cysteine and pulsed with [^{35}S]L-cysteine as described above for 3 h. Chase medium (pulse medium containing 15 mg/liter cold L-cysteine) was then added for the indicated times. At the conclusion of the chase, the cells were processed as above for immunoprecipitation, SDS-PAGE, and autoradiography.

NO_2^- Accumulation. NO_2^- accumulation was used as an indicator of NO production in the medium and was assayed by the Griess reaction (16). 100 μl Griess reagent was added to 100 μl of each supernatant in triplicate. The plates were read using an ELISA plate reader (MR5000; Dynatech, Chantilly, VA) at 550 nm against a standard curve of $NaNO_2$.

NOS Enzyme Assay. NOS activity was measured as described (23) in 20 mM Tris-HCl, pH 7.9, containing 4 μM FAD, 4 μM tetrahydrobiopterin, 3 mM dithiothreitol, and 2 mM each of L-arginine and NADPH. The reaction was carried out in duplicate for 120 or 180 min at 37°C in 100 μl in 96-well plates (Corning). Residual NADPH was oxidized enzymatically as previously described (23), and the Griess assay was performed as above. As a control for turbidity or other interference with the assay, an additional well for each sample contained all reagents except L-arginine and NADPH, for which water was substituted. The absorbance value from this well (typically 0.05–0.1) was subtracted from that of the mean of the test wells.

DNA and Oligonucleotide Probes. DNA probes were random primer labeled with [^{32}P]dCTP according to standard protocols (31). Oligonucleotide probes were 5′ end radiolabeled with T4 polynucleotide kinase (Stratagene, La Jolla, CA) in the presence of 50 mCi of α-[^{32}P]ATP (~6,000 Ci/mmol; Amersham Corp.) to a specific activity of $>2 \times 10^8$ cpm/mg.

Northern Hybridization. 20×10^6 adherent macrophages were stimulated with medium only, IFN-γ only, or IFN-γ with TGF-β (5 ng/ml). In some experiments, the cells were stimulated with IFN-γ for 18 h before being treated with TGF-β. Total RNA was isolated by a modification of the rapid guanidinium isothiocyanate

method (28). Messenger RNA was obtained using the PolyATract kit (Promega, Madison, WI) and quantitated using the DNA Dipstick kit (Invitrogen, San Diego, CA). Equal amounts of RNA were denatured in formamide (50%), formaldehyde (6.5%) sample buffer (65°C, 15 min), run on formaldehyde (1.8%), agarose (0.8%) gels, and transferred to reinforced nitrocellulose membranes (Duralose-UV; Stratagene) using a positive pressure transfer apparatus (Posiblot; Stratagene) according to the manufacturer's protocol. The membranes were UV crosslinked, prehybridized for 3–4 h at 65°C in 6× SSPE (0.9 M NaCl, 60 mM sodium-phosphate, 3 mM EDTA, pH 7.4; 5× Denhardt's; 1% SDS; 100 μg/ml salmon sperm DNA), and hybridized with respective probes (see figure legends) for 16–24 h. After hybridization the filters were washed in 2× SSC, 0.1% SDS for 30 min at room temperature, followed by two washes (15 min each) in 6× SSC, 0.1% SDS at 65°C (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) and one wash (5 min) in 2× SSC, 0.1% SDS at 37°C, and exposed to autoradiography film. For reprobing, the filters were stripped of the oligonucleotide probe by two washes (15 min) in boiling elution buffer (0.05× SSC, 0.01 M EDTA, pH 8.0, 0.1% SDS), checked for complete removal of the probe by autoradiography, and rehybridized.

Nuclear Run-On Analysis. Nuclei were prepared from macrophage monolayers (20–40 × 10^6 cells) as described (28). For in vitro transcription, freshly thawed nuclei were incubated in transcription buffer (150 mM KCl, 10 mM Tris, pH 8, 5 mM $MgCl_2$, 0.5 mM ribonucleotide (adenosine, cytosine, guanosine) triphosphate, 5 mM DTT, 0.05 U/ml RNaseBlock II [Stratagene], 200 mCi α-[^{32}P]UTP [800 mCi/mmol; Amersham Corp.]) for 30 min at 30°C. The reaction was stopped by addition of 40 U RNase-free DNase I (2.2 × 10^6 U/mg; Stratagene). Elongated labeled transcripts were isolated by the method of Chomczynski and Sacchi (32), repeatedly precipitated with isopropanol, dissolved in 1% SDS, and counted. Equal amounts of [^{32}P]RNA probes were hybridized to an iNOS-specific cDNA fragment or to a fragment of mouse β-actin cDNA (see figure legends) that had been slot-blotted (Minifold II slot-blotter; Schleicher & Schuell, Inc.) and UV crosslinked onto nitrocellulose (200 ng/slot). Prehybridization (16 h, 65°C) and hybridization (48 h, 65°C) were performed in the same solution as for Northern blots. Posthybridization washes consisted of 2× SSC, 0.1% SDS (30 min, 25°C), 6× SSC, 0.1% SDS (twice for 20 min, 65°C), 2× SSC (30 min, 25°C), 2× SSC, 10 mg/ml RNAse A (30 min, 37°C), 2× SSC, 0.1% SDS (15 min, 65°C). The extent of hybridization to each slot was quantified by videodensitometry.

mRNA Stability Analysis. Macrophage monolayers were stimulated with IFN-γ (20 ng/ml) in the presence or absence of TGF-β (5 ng/ml) for 5 or 13 h. Actinomycin D (5 μg/ml) was added. Total RNA was prepared at the indicated time points and further processed for Northern hybridization as described above.

Results

Suppression of NO Release by TGF-β Is Due to Suppression of iNOS Enzyme Activity. In the course of the studies described below, we observed a mean suppression of NO release by TGF-β from IFN-γ-stimulated macrophages of 90.8 ± 0.4% at TGF-β concentrations of ≥1 ng/ml (mean ± SEM of 49 experiments), with 0.14 ± 0.05 ng/ml (5.6 ± 2 pM, 7 experiments) causing 50% inhibition, confirming the original observations (16).

Suppression of NO release by TGF-β could be due to a direct effect on iNOS, or instead, could reflect a decreased ability of the cells to take up L-arginine, provide NADPH, recycle oxidized biopterin, or preserve NO from other reactions before its encounter with dissolved O_2 and conversion to NO_2^-. To discriminate between these possibilities, we carried out enzyme activity assays on cell sonicates in the presence of optimal concentrations of L-arginine, NADPH, FAD, dithiothreitol, and tetrahydrobiopterin (23). As seen in Table 1, the activity of iNOS under these conditions was suppressed 97–100% when macrophages were treated with TGF-β.

TGF-β Blocks the Induction of iNOS Protein. The reduced activity of iNOS reflected a decrease of iNOS protein as revealed by immunoblot with a monospecific rabbit anti-iNOS antibody (26) (Fig. 1 *A*). No iNOS was detected in unstimulated macrophages after 14, 24, or 48 h of incubation (lanes *NS*). Upon stimulation with IFN-γ, iNOS was induced in increasing amounts from 14 to 48 h (lanes *I*). TGF-β blocked this induction (lanes *I+T*).

Endothelial cNOS (33) as well as mouse macrophage iNOS (34, 35) have been found in a particulate as well as a soluble fraction of cell lysates. The total absence of immunoreactive iNOS in lysates from TGF-β-treated cells suggested that both components were suppressed. This was confirmed by separately immunoblotting whole lysates (lanes *L*) as well as the cytosolic (lanes *C*) and the particulate (lanes *P*) fractions of whole lysates (Fig. 1 *B*).

TGF-β Treatment Reduces iNOS mRNA Levels. As shown in Fig. 2, IFN-γ induced iNOS mRNA (lanes *I*) within 4 h of treatment, with the signal persisting through 72 h. TGF-β, added at the same time as IFN-γ (lanes *I+T*), did not reduce the level of iNOS mRNA at 4 h, but caused a marked reduction of iNOS mRNA by 24 h. The complete suppression of iNOS induction seen at the protein level could therefore be explained partially, but not entirely, by the late decrease in iNOS mRNA.

TGF-β Does Not Affect iNOS Gene Transcription. As shown

Table 1. *Effect of TGF-β on IFN-γ-induced iNOS Enzyme Activity*

Exp.	IFN-γ	TGF-β	Specific activity	Suppression
			pmol/mg protein per min	%
1	−	−	0	N/A
	+	−	380	0
	+	+	10	97.4
2	−	−	0	N/A
	+	−	400	0
	+	+	0	100

12 × 10^6 thioglycollate broth–elicited mouse peritoneal cells were seeded in 100-mm tissue culture dishes, washed, and incubated with medium or TGF-β (2.5 ng/ml) for 0 (Exp. 1) or 16 h (Exp. 2) and then stimulated with IFN-γ (20 ng/ml). NOS enzyme activity was assayed 48 h later. The two experiments are representative of eight. N/A, not applicable.

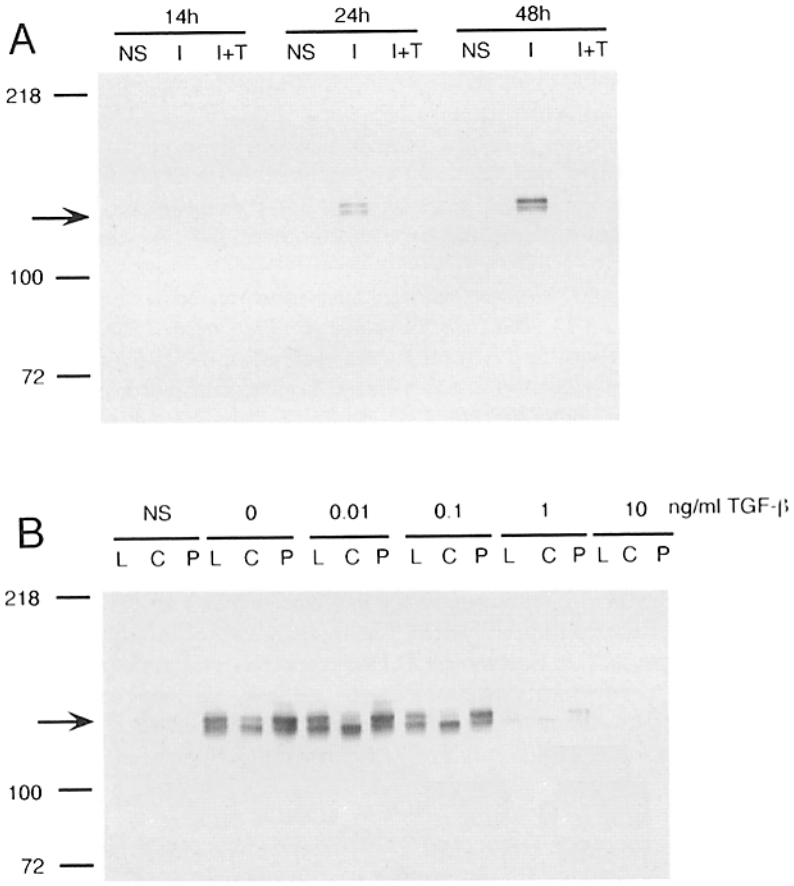

Figure 1. TGF-β suppresses iNOS induction in a dose-dependent manner in both soluble and particulate compartments. Thioglycollate broth–elicited mouse peritoneal cells were treated as follows. (*A*) Either no stimulus (lanes *NS*), 20 ng/ml IFN-γ (lanes *I*), or 20 ng/ml IFN-γ + 10 ng/ml TGF-β (lanes *I+T*) were added and the plates incubated for the periods of time indicated above the lanes before being lysed by sonication and subjected to SDS-PAGE followed by Western blot with anti-iNOS IgG. (*B*) Either no stimulus (lanes *NS*) or TGF-β at the indicated final concentrations (ng/ml) + IFN-γ (20 ng/ml) was added. The cells were incubated for 48 h before being lysed (lanes *L*), separated into cytosolic (lanes *C*) and particulate (lanes *P*) fractions, and subjected to SDS-PAGE followed by Western blot with anti-iNOS IgG. Relative molecular masses of prestained markers are shown at the left ($\times 10^3$). Arrows indicate the specific iNOS doublet ($\sim$130,000 M_r).

in Fig. 3, the level of transcription of iNOS was equal in macrophages stimulated with IFN-γ alone (lane *I*) or in combination with TGF-β (lane *I+T*). Transcription was also detected in unstimulated macrophages (lane *NS*). The results from three such nuclear run-on experiments were quantitated by videodensitometry. Setting the ratio of iNOS peak area/β-actin peak area to 1 for unstimulated macrophages, the ratio of iNOS/β-actin was 0.57 $\pm$ 0.25 and 0.56 $\pm$ 0.09 (mean $\pm$ SD) for the IFN-γ and the IFN-γ + TGF-β stimulation conditions, respectively, indicating that TGF-β did not affect iNOS gene transcription. Results were similar at 3.5 h of stimulation (Fig. 3) and at 24 h (data not shown).

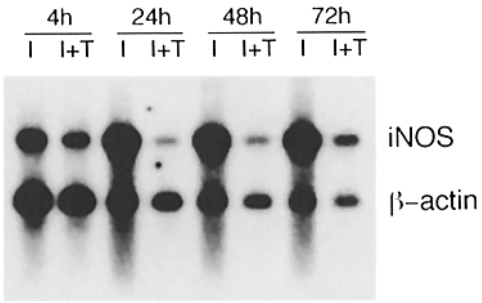

Figure 2. Exposure to TGF-β decreases iNOS mRNA. Macrophages were stimulated with 20 ng/ml IFN-γ alone (lanes *I*) or in combination with 5 ng/ml TGF-β (lanes *I+T*) for 4, 24, 48, or 72 h. The mRNA from each sample was blotted and hybridized with a DNA probe for iNOS, prepared from an EcoRI-AccI fragment of cDNA clone B2 (26). As a control, the blot was hybridized simultaneously with an oligonucleotide probe for β-actin (Clontech). The apparent increase in the β-actin region in lanes *I* reflects spillover from the iNOS band.

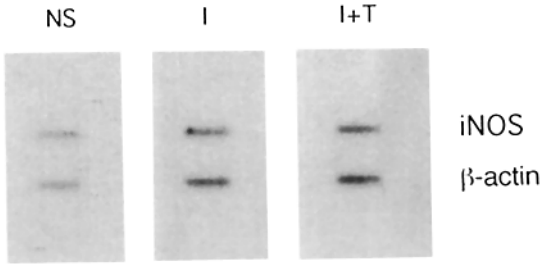

Figure 3. TGF-β treatment does not diminish iNOS gene transcription. Macrophages were unstimulated (lane *NS*) or stimulated with 20 ng/ml IFN-γ alone (lane *I*) or in combination with 5 ng/ml TGF-β (lane *I+T*) for 3.5 h. The rate of transcription of iNOS or of β-actin by isolated nuclei was determined by slot-blotting the elongated, labeled RNA transcripts and hybridizing them to the indicated gene fragments for autoradiography. The iNOS gene fragment was the same as in Fig. 2. The β-actin fragment was a 749-bp EcoRI fragment of phagemid pBluescript SK(+) containing the mouse β-actin cDNA. This result is representative of three experiments.

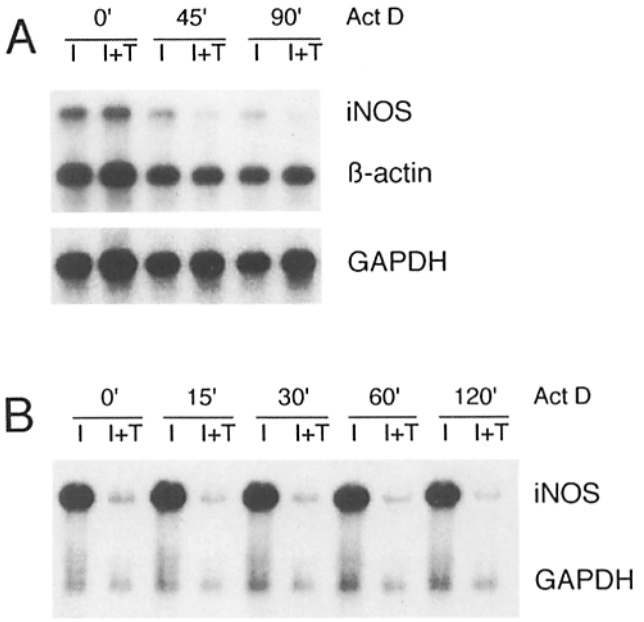

Figure 4. TGF-β treatment decreases iNOS mRNA stability. Macrophages were stimulated with 20 ng/ml IFN-γ alone (lanes *I*) or in combination 5 ng/ml with TGF-β (lanes *I+T*) for 5 (*A*) or 13 h (*B*). Actinomycin D (5 μg/ml) was added for 0, 45, or 90 (*A*) or for 0, 15, 30, 60, or 120 min (*B*). mRNA was prepared for Northern blot with an iNOS-specific probe (see Fig. 2). As a control, the blot was hybridized simultaneously with a probe for β-actin (see Fig. 2). In *A*, the blot was stripped and reprobed with a probe for GAPDH as an additional control.

TGF-β Treatment Reduces iNOS mRNA Stability. The foregoing results suggested that TGF-β treatment is likely to reduce iNOS mRNA stability. This possibility was tested through the use of the transcription inhibitor actinomycin D in two settings. In the first instance, macrophages were stimulated with IFN-γ in the presence or absence of TGF-β for 5 h. This time point was chosen so that the levels of iNOS mRNA would be identical in the TGF-β-treated macrophages (Fig. 4, lanes *I+T*) and in macrophages treated with IFN-γ alone (Fig. 4, lanes *I*; see also Fig. 2). Upon addition of actinomycin D, iNOS mRNA decreased faster in the TGF-β-treated macrophages than in the untreated cells (Fig. 4 *A*). In the second instance, macrophages were stimulated with IFN-γ in the presence or absence of TGF-β for 13 h (Fig. 4 *B*). This time point was chosen so that the level of iNOS mRNA in the TGF-β-treated macrophages (lanes *I+T*) was already greatly reduced compared to the level of iNOS mRNA in the cells treated with IFN-γ alone (lanes *I*). Strikingly, iNOS mRNA was now much more stable than at 5 h, and TGF-β no longer accelerated its degradation.

TGF-β also Acts at the Translational and Posttranslational Levels. Above, we showed that TGF-β reduced partially the level of iNOS mRNA (Fig. 2), while at the protein level the reduction of iNOS protein appeared complete (Fig. 1). This discrepancy suggested that TGF-β might also affect iNOS at the translational and/or posttranslational levels. Thus, we reexamined the notion that iNOS is no longer suppressible by TGF-β after iNOS is expressed (16). In the experiment shown in Fig. 5 *A*, macrophages were stimulated with IFN-γ for a total of 48 h. TGF-β or medium was added at 0, 3, 9, or 24 h after addition of IFN-γ. Cells to which TGF-β was added at 0, 3, 9, and even 24 h after IFN-γ contained little or no iNOS protein at 48 h, even though iNOS had already been well expressed by 24 h after exposure to IFN-γ (Fig. 1 *A*). Alternatively, macrophages were stimulated for 24 h with IFN-γ, at which time medium or TGF-β was added. Lysates were made at 0, 2, 6, 16, or 20 h after addition of medium or TGF-β. By 16 and 20 h of incubation with TGF-β, preexistent iNOS protein had been reduced. The apparent discrepancy with previous results (16) was explained by the observation that by 24–36 h after IFN-γ treatment, NO_2^- accumulation was maximal in the supernatants of cultured macrophages and did not further increase by 48 h (Fig. 5 *C*). This reflects factor accumulation in and/or depletion from the conditioned medium, since iNOS activity can be revived in non-TGF-β-treated cells by giving the cells fresh medium (Vodovotz, V., N. S. Kwon, C. Bogdan, J. Paik, Q.-w. Xie, and C. Nathan, manuscript in preparation).

After iNOS is expressed, reduction in iNOS protein by TGF-β out of proportion to the reduction in mRNA could be due to a decrease in iNOS mRNA translation and/or to enhanced degradation of iNOS protein. To test the first possibility, macrophages were stimulated for 17 h with IFN-γ, at which time they were incubated either with medium or with TGF-β for further periods. The macrophages were then pulsed with [^{35}S]L-cysteine for 30 min to label newly synthesized iNOS as detected in immunoprecipitates with anti-iNOS antiserum. No iNOS was immunoprecipitated by preimmune antiserum (data not shown). As seen in Fig. 6 *A*, no translation of iNOS was detected in unstimulated macrophages (lane *NS*). The rate of synthesis of iNOS was indistinguishable in medium- (lanes *M*) or TGF-β- (lanes *T*) treated cells up to 4 h of treatment. Thereafter, progressively less iNOS synthesis was sustained in TGF-β-treated cells; none could be detected by 8 h. As shown in Fig. 6 *B*, this effect could not be attributed to reduction of iNOS mRNA. Thus, when TGF-β was added 17 h after IFN-γ, the further accumulation of iNOS mRNA over the subsequent 24 h was blocked, but the level of mRNA that had been present at 17 h was only minimally reduced, consistent with the result in Fig. 4 *B*. We conclude that the results in Fig. 6 *A* are attributable primarily to decreased translation of iNOS mRNA.

We next examined whether TGF-β could also increase degradation of iNOS protein. Macrophages were stimulated for 18 h with IFN-γ, at which time they were incubated either with medium or with TGF-β, pulsed with [^{35}S]L-cysteine, and chased with an excess of nonradioactive L-cysteine. Immunoprecipitation with anti-iNOS antiserum was then carried out in order to examine the fate of iNOS (Fig. 6 *C*). By the end of the pulse period (a total of 4 h with TGF-β) the same amount of iNOS protein had been synthesized both by the medium-treated (lanes *M*) and the TGF-β-treated (lanes *T*) macrophages. However, during the chase period, the rate of degradation of labeled iNOS protein was greater in the

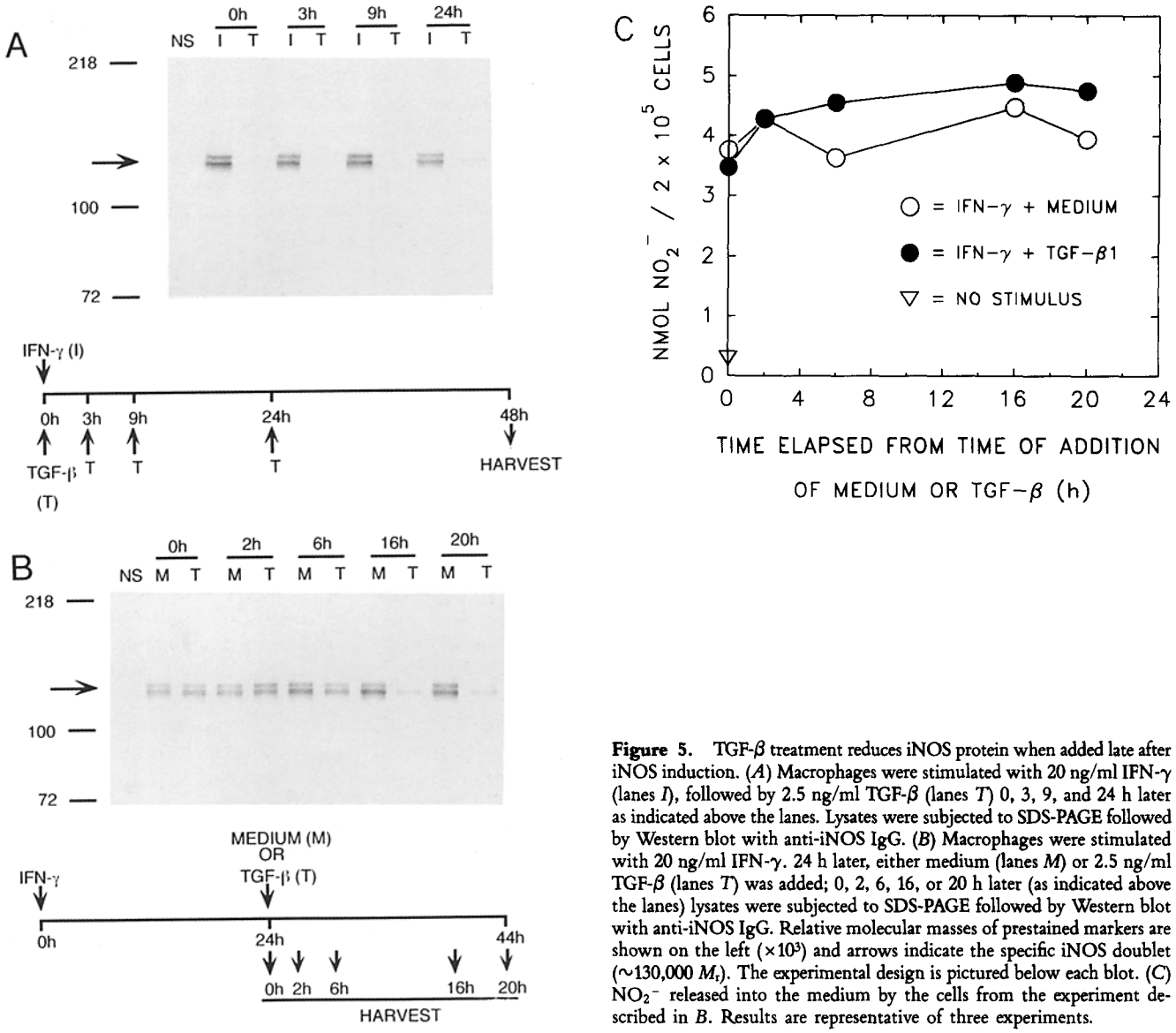

Figure 5. TGF-β treatment reduces iNOS protein when added late after iNOS induction. (*A*) Macrophages were stimulated with 20 ng/ml IFN-γ (lanes *I*), followed by 2.5 ng/ml TGF-β (lanes *T*) 0, 3, 9, and 24 h later as indicated above the lanes. Lysates were subjected to SDS-PAGE followed by Western blot with anti-iNOS IgG. (*B*) Macrophages were stimulated with 20 ng/ml IFN-γ. 24 h later, either medium (lanes *M*) or 2.5 ng/ml TGF-β (lanes *T*) was added; 0, 2, 6, 16, or 20 h later (as indicated above the lanes) lysates were subjected to SDS-PAGE followed by Western blot with anti-iNOS IgG. Relative molecular masses of prestained markers are shown on the left (×10³) and arrows indicate the specific iNOS doublet (~130,000 M_r). The experimental design is pictured below each blot. (*C*) NO_2^- released into the medium by the cells from the experiment described in *B*. Results are representative of three experiments.

TGF-β-treated cells (compare lanes *M* vs. lanes *T* at 8 h of chase and beyond).

Discussion

The suppression of iNOS expression is probably as important to its regulation as its induction. We have focused on the regulation of iNOS expression in macrophages by TGF-β because macrophages use NO to restrict the growth of tumor cells and pathogens; TGF-β is produced by many tumors (36) and induced by certain NO-sensitive pathogens (37), and TGF-β is produced by activated T cells and macrophages themselves (38, 39).

The complex biochemistry of NO production affords many potential sites for regulatory action. TGF-β might cause diversion of detectable NO away from NO_2^-, e.g., to nitrosothiols (40). TGF-β might act by limiting the availability or activity of cosubstrates, cofactors, prosthetic groups, or accessory proteins necessary for NO production, such as L-arginine, NADPH, glutathione, tetrahydrobiopterin, FAD, flavin mononucleotide, heme, calmodulin, and dihydropteridine reductase. In smooth muscle cells, for example, tetrahydrobiopterin availability appears to be a rate-limiting step in the induction of iNOS-like activity (41). Finally, TGF-β might induce the production of iNOS inhibitors, such as the N^G,N^G-dimethylarginine recently identified in human urine and plasma (42). While an effect of TGF-β on such factors was not excluded, the main action of TGF-β was to decrease the amount of iNOS protein in the cells through potentially synergistic action at three different levels: enhanced degrada-

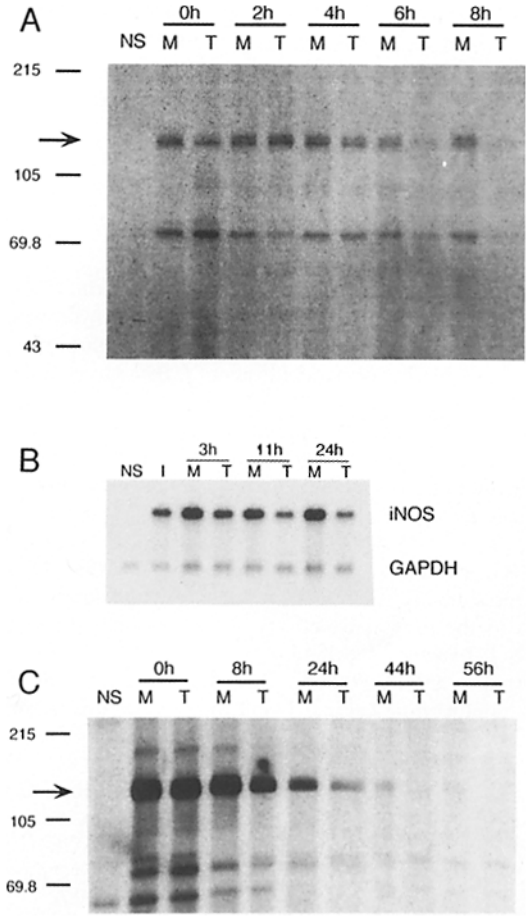

Figure 6. Late addition of TGF-β to already activated macrophages decreases translation of iNOS mRNA and increases degradation of iNOS protein. (*A*) Macrophages were unstimulated (lanes *NS*) or stimulated with 20 ng/ml IFN-γ (lanes *M* and *T*) for 17 h. Medium or 2.5 ng/ml TGF-β was then added for 0, 2, 4, 6, or 8 h, after which the cells were pulsed with [^{35}S]L-cysteine for 30 min. The cells were lysed, immunoprecipitated with anti-iNOS antiserum, and subjected to SDS-PAGE and autoradiography. (*B*) As a control for *A*, macrophages were either unstimulated (lane *NS*) or stimulated with 20 ng/ml IFN-γ alone (lane *I*) for 18 h. Medium (lanes *M*) or 5 ng/ml TGF-β (lanes *T*) was added for 3, 11, or 24 h and then mRNA blotted and hybridized with DNA probes specific for iNOS (see Fig. 2) and for GAPDH (as control). (*C*) Macrophages were unstimulated (lane *NS*) or stimulated with 20 ng/ml IFN-γ (lanes *M* and *T*) for 18 h. Medium or TGF-β was added for 45 min, the cells pulsed with [^{35}S]L-cysteine for 3 h, and then chased for a further 56 h. The cells were harvested at various times during the chase, lysates were immunoprecipitated with anti-iNOS antiserum, and subjected to SDS-PAGE and autoradiography. Numbers on the left side of each autoradiogram indicate the apparent relative molecular masses ($\times 10^3$) of prestained markers, and the horizontal arrow indicates the specific iNOS doublet ($\sim$130,000 M_r).

tion of iNOS mRNA and its impaired translation combined with acceleration of iNOS protein degradation.

The little that is known regarding the mechanisms of control of protein expression by TGF-β in other systems points to no single regulatory theme (36). In hepatocytes, TGF-β treatment reduces the levels of hepatocyte growth factor mRNA (43), and in melanoma cell lines and blood mononuclear cells, TGF-β treatment reduces MHC class II mRNA (44). In macrophages, TGF-β suppresses TNF-α production by reducing TNF-α mRNA translation (28), and the same mechanism underlies TGF-β-mediated suppression of the synthesis of milk caseins by lactogenic hormone-treated, pregnant mice (45). In human peripheral blood T lymphocytes, TGF-β blocks secretion of bioactive IL-1 while paradoxically increasing IL-1 mRNA (46). The foregoing effects of TGF-β each appear to be limited to one regulatory mechanism, in contrast to the multiple levels at which TGF-β affects iNOS.

No iNOS transcription was detected in unstimulated RAW 264.7 cells (26), and no accumulation of iNOS mRNA was detected in unstimulated, thioglycollate-elicited primary macrophages (Fig. 6 *B*). Thus, it was a surprise to find a low level of transcription of iNOS in primary macrophages without addition of IFN-γ (Fig. 3). This probably reflects a response to the thioglycollate broth used to inflame the peritoneum and elicit the cells. Since IFN-γ as a single stimulus appears to increase iNOS mRNA not by enhancing iNOS gene transcription (Fig. 3), it presumably suppresses iNOS mRNA degradation. In contrast, the synergistic combination of IFN-γ and LPS markedly enhances iNOS transcription both in RAW 264.7 cells (26) and in thioglycollate broth–elicited macrophages (data not shown). TGF-β appears powerless to diminish iNOS transcription; rather, it reduces iNOS expression post-transcriptionally. This may explain why TGF-β suppresses iNOS expression so effectively when iNOS is induced by IFN-γ alone, but so weakly when iNOS is induced by IFN-γ and LPS (16).

TGF-β has been implicated in diverse processes such as embryological development (36). In transgenic mice whose TGF-β1 gene had been knocked out, the main defect was lethal, multifocal inflammatory disease (47, 48). It would appear from these studies that the nonimmunological roles of TGF-β can be filled by other factors, while TGF-β's immunosuppressive role appears to be unique. The regulatory actions of TGF-β on iNOS may be critical for controlling inflammation.

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