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TRANSFORMING GROWTH FACTOR BETA DIFFERENTIALLY MODULATES THE INDUCIBLE NITRIC OXIDE SYNTHASE GENE IN DISTINCT CELL TYPES

Rebecca S. Gilbert and Harvey R. Herschman

Department of Biological Chemistry and Laboratory of Biomedical and Environmental Sciences, UCLA School of Medicine, Los Angeles, California 90024

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Nitric oxide is a mediator of paracrine cell signalling. An inducible form of nitric oxide synthase (iNOS) is expressed in macrophages and in Swiss 3T3 cells. Transforming growth factor beta (TGF-beta) is a cytokine that modulates many cellular functions. We find that TGF-beta cannot induce iNOS mRNA expression, either in macrophage cell lines or in Swiss 3T3 cells. However, TGF-beta *attenuates* lipopolysaccharide induction of iNOS mRNA in macrophages. In contrast, TGF-beta *enhances* iNOS induction by phorbol ester, serum or lipopolysaccharide in 3T3 cells. Thus TGF-beta can inhibit or augment iNOS mRNA induction in response to primary inducers, depending on the cell type in question.

Nitric oxide (NO) is both a cytotoxic product of activated macrophages and a mediator of intercellular communication (1,2). cDNAs for constitutively expressed nitric oxide synthase (NOS), the enzyme that synthesizes NO from arginine, have been cloned from brain (3) and endothelial cells (4-6). An inducible NOS, termed iNOS, was recently cloned from RAW 264.7 murine macrophage cells (7-9). iNOS message accumulation is induced in macrophages by bacterial endotoxin/ lipopolysaccharide (LPS) (7-9); interferon gamma augments LPS induction (10). Hepatocytes also express iNOS in response to a cocktail of inducing agents (11). Treatment with phorbol esters, serum, growth factors, or LPS rapidly and transiently induces iNOS mRNA in Swiss 3T3 cells (12).

Transforming growth factor beta (TGF-beta) is a potent cytokine that modulates embryologic development, wound healing, cellular proliferation, bone formation, and hematopoiesis (13-16). TGF-beta also has profound effects on the immune system and on inflammatory responses, negatively modulating many functions of activated macrophage (17-20). The critical role of TGF-beta$_1$ in inflammatory responses is emphasized in reports describing null mutations (21,22). Mice homozygous for the TGF-beta$_1$ null mutation can be carried to term, but inevitably die by one month of age. Autopsy demonstrates massive

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inflammatory infiltrations, composed primarily of macrophages, in many organs. Because of the pervasive action of TGF-beta and the key role NO plays in macrophage function and cell communication (1,2,23), we examined TGF-beta modulation of iNOS expression.

MATERIALS AND METHODS

Cell culture: RAW 264.7 cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum. Swiss 3T3 cells were grown as described previously (24).

Chemicals and reagents: RPMI medium was from ICN (Cleveland, OH). Dulbecco's' modified Eagle's medium was supplied by GIBCO (Grand Island, NY). Bacterial endotoxin/lipopolysaccharide (E. coli 0111:B4) and dexamethasone were from Sigma (St. Louis, MO). In some experiments human placental TGF-beta$_1$ from Upstate Biologicals (Lake Placid, NY) was used, in other experiments recombinant TGF-beta$_1$, the gift of A. Purchio of Bristol Myers/Squibb, was used. Results were similar for both preparations. TPA was purchased from Chemsyn (Lexena KN).

Northern analyses: RNA isolation and northern analysis were described previously (24). The iNOS probe was a gift from Dr. James Cunningham (Harvard Medical School). CHOB is a constitutively expressed gene used to normalize for message levels (24).

RESULTS AND DISCUSSION

TGF-beta inhibits iNOS induction in macrophage cell lines. Bacterial lipopolysaccharide (LPS) induces iNOS mRNA accumulation in RAW 264.7 macrophage cells (Fig. 1). Expression peaks at four hours, and returns to baseline by 24 hours. TGF-beta does not induce iNOS in RAW 264.7 cells. However, TGF-beta substantially attenuates LPS-induced iNOS expression. Similar results were observed for J774A.1 murine macrophage cells (data not shown). TGF-beta concentrations between 1-10 ng/ml ($4x10^{-11}$ to $4x10^{-10}$ M) are effective in attenuating iNOS message accumulation.

TGF-beta augments iNOS induction in Swiss 3T3 cells. TGF-beta directly modulates expression of some genes in Swiss 3T3 cells and can synergistically activate others. For example, TGF-beta enhances induction of GLUT1, the gene encoding the glucose transporter, in response either to tetradecanoyl phorbol acetate (TPA) or serum (25). Serum, platelet-derived growth factor, TPA, and LPS induce iNOS mRNA accumulation in Swiss 3T3 cells (12). TGF-beta cannot induce the iNOS gene in these cells (Fig. 2). However, TGF-beta substantially *augments* TPA-induced iNOS expression. Maximal synergy with TPA occurs between 0.1 and 1.0 ng/ml of TGF-beta.

LPS is a potent iNOS inducer in Swiss 3T3 cells (12). We could, therefore, compare the modulatory effects of TGF-beta on LPS-induced iNOS expression in 3T3 cells and macrophage. TGF-beta also augments LPS-induced iNOS expression in 3T3 cells (Fig. 3), in contrast to its inhibitory effect on LPS induction of iNOS mRNA in RAW 264.7 cells (Fig. 1). Modulatory effects of TGF-beta on iNOS expression are cell-type specific.

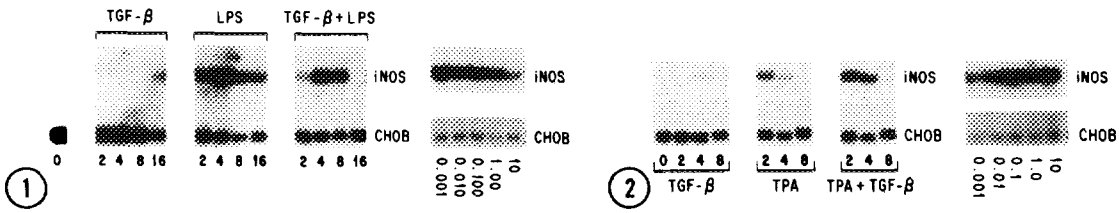

Figure 1. TGF-beta attenuates LPS-induced iNOS mRNA accumulation in RAW 264.7 cells. (Left panel) RAW 264.7 cells were treated with TGF-beta (10 ng/ml) or LPS (5 ng/ml). Alternatively, cells were preincubated with TGF-beta for one hour, then treated with LPS. Cells were harvested at the times (in hours) indicated. Total RNA was prepared and 10 μg was subjected to northern analysis. CHOB is a constitutive message used to normalize for the amount of mRNA. (Right panel) Cells were exposed for one hour to the concentrations (in ng/ml) of TGF-beta shown, then treated with LPS (5 ng/ml) for two additional hours. Total RNA was prepared and 10 μg was subjected to northern analysis.

Figure 2. TGF-beta augments TPA-induced iNOS mRNA accumulation in Swiss 3T3 cells. (Left panel) Confluent Swiss 3T3 cells were switched for 24 hours to medium containing 0.5% fetal calf serum. Cells were then treated with TGF-beta (10 ng/ml) or TPA (50 ng/ml). Alternatively, cells were preincubated with TGF-beta for one hour then treated with TPA. (Right panel) Cells were exposed for one hour to the concentrations (in ng/ml) of TGF-beta shown, then treated with TPA (50 ng/ml) for two additional hours.

Dexamethasone blocks TGF-beta enhanced iNOS induction in Swiss 3T3 cells. Dexamethasone (DEX) alone has no effect on iNOS expression in Swiss 3T3 cells. However, DEX suppresses iNOS induction by TPA, serum, or LPS (12). Because both glucocorticoids and TGF-beta have such pervasive effects on gene expression and cellular

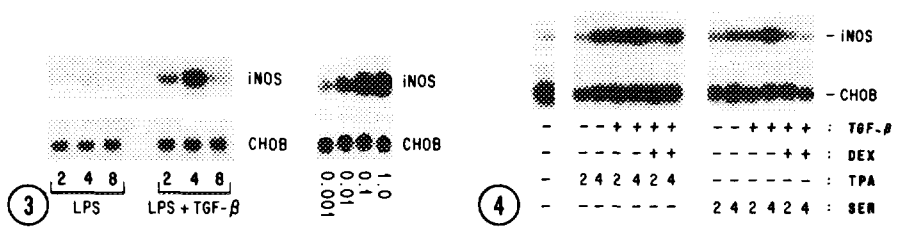

Figure 3. TGF-beta augments LPS-induced iNOS mRNA accumulation in Swiss 3T3 cells. (Left panel) Cells were treated with TGF-beta (10 ng/ml) or LPS (5 ng/ml). Alternatively, cells were preincubated with TGF-beta for one hour then treated with LPS. (Right panel) Cells were exposed for one hour to the concentrations (in ng/ml) of TGF-beta shown, then treated with LPS (5 ng/ml) for four additional hours.

Figure 4. Dexamethasone blocks TGF-beta augmented iNOS mRNA induction in Swiss 3T3 cells. Cultures were grown and prepared for ligand stimulation as described in the legend to Figure 2. A "+" in the DEX row indicates cells were preincubated with DEX (2 μM) for three hours prior to addition of TPA (50 ng/ml) or serum (20%). A "+" in the TGF-beta row indicates that cells were preincubated with TGF-beta (10 ng/ml) for one hour prior to addition of TPA or serum. Cells were exposed to TPA or serum for two and four hours, as indicated.

interactions, we asked whether the glucocorticoid (inhibitory) or TGF-beta (stimulatory) effects would dominate iNOS induction in Swiss 3T3 cells. TGF-beta enhances both TPA- and serum-induced iNOS mRNA accumulation (Fig. 4). However, DEX preincubation substantially attenuates not only TPA and serum induction of iNOS message, but also the synergistic interaction of these mitogens with TGF-beta (Fig. 4).

The induced forms of nitric oxide synthase and prostaglandin synthase share many regulatory features. NO has only recently been recognized as a paracrine cellular effector (1,2). In contrast, the eicosanoids have long been known as paracrine mediators of cellular interactions (26). We (24) and others (27-30) cloned the cDNA and gene (31) for TIS10/PGS-2, a primary response gene encoding an inducible prostaglandin synthase (PGS). Like iNOS, TIS10/PGS-2 is induced in activated macrophages (32-34). Both iNOS and TIS10/PGS-2 are also induced by mitogens in Swiss 3T3 cells (12,24). DEX blocks induction of both iNOS and TIS10/PGS-2, in Swiss 3T3 cells (12,35) and in macrophages (12,32,36). Moreover, TGF-beta also attenuates TIS10/PGS-2 induction in macrophages and augments TIS10/PGS-2 induction in Swiss 3T3 cells (S. Reddy, R. Gilbert, and H. Herschman, in preparation). Thus the TIS10/PGS-2 and iNOS genes share regulatory characteristics not common to most other primary response genes. Cloning of the regulatory regions of the TIS10/PGS-2 (31) and iNOS (37) genes should permit a molecular description of these mechanisms.

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