JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2000, 51, 3, 411—419

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ENDOGENOUS NITRIC OXIDE PROVES NOT TO BE INVOLVED IN THE INHIBITION BY IL-1β OF TGF-α-STIMULATED PROLIFERATION OF RGM1 CELLS

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Combined treatment of isolated rat gastric mucosal (RGM1) cells with interleukin (IL)-1β and transforming growth factor (TGF)-α resulted in expression of inducible nitric oxide synthase (iNOS) mRNA and iNOS protein 24 hr after the treatment. Constitutive NOS (nNOS) protein was not proved in the cells and not activated by IL-1β + TGFα. Although IL-1β and TGF-α alone exerted little or no effect on NO_2^- production, their combination gradually increased NO_2^- production from 12 to 24 hr following treatment. NO_2^- production stimulated by IL-1β + TGFα was significantly reduced by N^G-nitro-L-arginine methyl ester (L-NAME) or aminoguanidine, yet not by D-NAME. S-nitroso-N-acetyl-D,L-penicillamine and sodium nitropruside significantly inhibited both spontaneous and TGF-α stimulated DNA synthesis. Nonetheless, L-NAME did not affect the inhibition by IL-1β of TGF-α-stimulated proliferation of RGM1 cells, eliminating the possibility of involvement of NO in the underlying mechanisms.

K e y w o r d s: *TGF-α, IL-1, iNOS, NO, RGM1 cells*

INTRODUCTION

Our previous studies demonstrated that both epidermal growth factor (EGF) and transforming growth factor (TGF)-α significantly enhance the healing of damage induced in isolated rat gastric mucosal (RGM1) cells by proliferation and migration (1,2). It is of particular interest that interleukin (IL)-1β markedly inhibits the enhanced healing stimulated by these growth factors. IL-1 is known to activate inducible nitric oxide synthase (iNOS) in several cells, such as chondrocytes, macrophages and mesengial cells (3—6). In addition, NO is also known to play an important role in the pathophysiology of the gastric mucosa, including the regulation of gastric epithelial cell proliferation (7, 8). Consequently, this study was performed to examine whether

or not endogenous NO is involved in the inhibitory mechanism induced by IL-1β on TGF-α-stimulated proliferation in RGM1 cells.

MATERIALS AND METHODS

Cell culture

RGM1 (RIKEN, Cell Bank, Tsukuba, Japan) was maintained in DMEM/F12 medium, as previously described (1). Cells (8×10^4 cells in 0.5 ml of medium) were incubated in 24 well plates or 6 cm dishes (Corning Coster, Corning, NY, USA), cultured for 24 hr, and then starved for 24 hr in a culture medium (DMEM/F12 containing 0.25% FBS and 25 mM HEPES; pH7.4) at 37 °C under 5% CO_2 in air.

Analysis of iNOS mRNA expression

To analyze iNOS mRNA by RT-PCR, RGM1 cells were treated with IL-1β (10 ng/ml) in the absence and presence of TGF-α (10 ng/ml) for 0, 1, 2, 6, 12 and 24 hr. Total cellular RNA was then isolated from RGM1 cells with TRIzol Reagent (Gibco/BRL, New York, USA.). First strand cDNA was prepared from 5 μg of the total RNA using Moloney murine leukemia virus reverse transcription (Gibco/BRL), according to the Gibco/BRL procedure. PCR was performed using rat GAPDH, with a PCR thermal cycler, and the following specific primer sequences (5'—3') for rat iNOS:

sense ACAACAGGAACCTACCAGCTCA

and anti-sense GATGTTGTAGCGCTGTGTGTCA;

the final PCR product size was 651 bp (9). After sample denaturation at 94°C for 1 min, PCR was performed for 30 cycles that consisted of denaturation at 94°C for 30 sec, annealing at 60°C for 45 sec, and extension at 72°C for 45 sec. The amplification was terminated by a 15 min final extension step at 72°C. After aliquots of the reactants were subjected to 2% agarose gel electrophoresis, the PCR products were visualized by ethidium bromide staining.

Immunoblot analysis of iNOS and nNOS protein

Expression of iNOS and constitutive NOS proteins, generally known as nNOS in the gastric mucosa (10), in RGM1 cells was examined by western blotting. Cells were treated with IL-1β (10 ng/ml) in the absence and presence of TGF-α (10 ng/ml) for 24 hr. After the treatment, cells were washed with ice-cold Ca^{2+} and Mg^{2+} free phosphate-buffered saline solution (PBS (−)) three times, then proteins were collected by adding 0.1% sodium dodecyl sulfate (SDS). They were boiled for 2 min and protein assay was performed using BioRad protein assay reagent (BioRad, Osaka, Japan). To analyze iNOS and nNOS, proteins (10 μg) was separated on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemli (10) and transferred to PVDF membrane (Immobilon, Millipore, Osaka, Japan). The membrane was incubated with monoclonal anti-iNOS antibody (1:1,000, Transduction Lab, KY USA) or polyclonal anti-nNOS antibody (1:1000, SC-1025, Santa Cruz, CA) over night at 4°C after nonspecific binding sites had been blocked. Subsequently, the membrane was reacted with horse radish peroxidase-conjugated rabbit anti mouse-IgG antibody (1:10,000, SC-2301, Santa Cruz, CA, USA) for iNOS detection or goat anti-rabbit-IgG antibody (1:10,000, 7071—1, New England Bio Labs, Beverly, MA) for nNOS detection for 30 min at room temperature. Western blots were visualized by enhanced chemiluminescence system (Western Blot Chemiluminescence Reagent Plus, NEN, Boston, USA).

Determination of nitrite concentration

Nitrite production by RGM1 cells was measured using a colorimetric assay based on the Griess reaction (11). After a 24-hr starvation, confluent cells were incubated in 0.5 ml of the medium in the presence of the indicated agents. The incubation medium (100 μl) was withdrawn for determination of nitrite concentration by addition of Griess agent containing 1% sulfanilamide and 0.1% N-1-napthylethylenediamine dihydro-chloride in 5% phosphoric acid. The absorbance was measured at 546 nm with the use of a spectrophotometer, and the nitrite concentration was calculated from a calibration curve using $NaNO_2$ as a standard.

Determination of cell proliferation

Cell proliferation was assessed as DNA synthesis; the incorporation of [^{3}H] thymidine into DNA was determined. In brief, RGM1 cells were incubated with the indicated agents and vehicles in the presence of [^{3}H] thymidine (7.4 kBq, 2.22—3.2 TBq/mmol; Amersham, Buckinghamshire, England) for the specified intervals. After washing the cells twice with 0.5 ml of 10% trichloroacetic acid, the cells were solubilized with 0.1 ml of 0.3 N NaOH at 37°C for 20 min. The radioactivity (25 μl) in the lysate was measured with a liquid scintillation counter (Beckman Instruments Inc.; Fullerton, CA, USA).

Drugs and reagents

rhIL-1β was kindly provided by Otsuka Pharmaceutical Co. (Tokushima, Japan). rhTGF-α (Pepro Tech, Rocky Hill, NJ, USA), N^G-nitro-L-arginine methyl ester (L-NAME, as a NOS inhibitor), D-NAME (used as an enantiomer of L-NAME), aminoguanidine (used as an iNOS-selective inhibitor) (Nakarai Tesque, Kyoto, Japan) and sodium nitroprusside (SNP) (used as a NO donor) (Cayman Chemical Co., MI, USA) were dissolved in PBS(−) containing 2.5 mg/ml of bovine serum albumin. S-nitroso-N-acetyl-D,L-penicillamine (SNAP) (used as a chemical NO donor) (Cayman Chemical Co., MI, USA) was dissolved in dimethylsulfoxide. All other chemicals used were of reagent grade.

Statistical analysis

The data are presented as means ± S.E. for the cultures. Statistical analysis was performed using the two-tailed Dunnett's multiple comparison test and the Student's t-test, with a P value of <0.05 regarded as significant.

RESULTS

Effects of IL-1β and TGF-α on mRNA and protein expression of iNOS and nNOS

Upon treatment of the RGM1 cells with IL-1β (10 ng/ml), expression of iNOS mRNA was found by RT-PCR from 1 hr up to 12 hr after the treatment (*Fig. 1*). TGF-α (10 ng/ml) treatment resulted in iNOS mRNA expression 6 hr after the treatment. In contrast, combined treatment of IL-1β and TGF-α induced a marked expression of iNOS mRNA from 2 hr up to 24 hr following

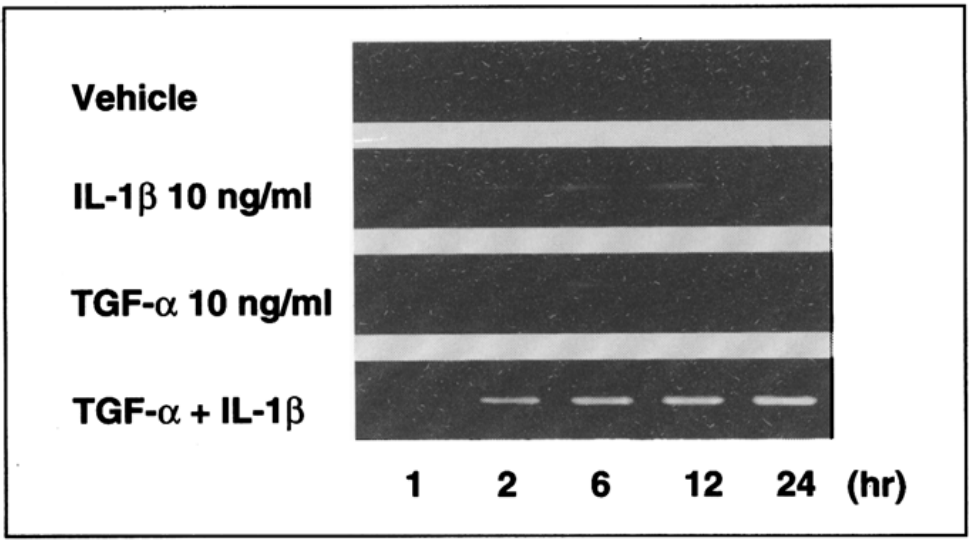

Fig. 1. RT-PCR analysis of iNOS mRNA expression in RGM1 cells treated with vehicle, IL-1β, TGF-α and IL-1β+TGF-α. Note that a clear expression of iNOS mRNA was observed 2 hr after the combined treatment with IL-1β+TGF-α for up to 24 hr.

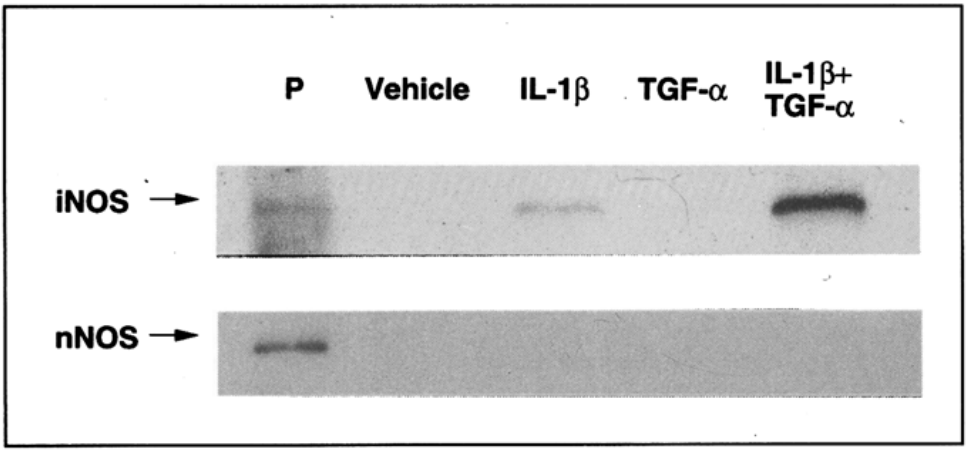

Fig. 2. Western blot analysis for iNOS and nNOS in RGM-1 cells treated with vehicle, IL1β, TGF-α and IL-1β+TGF-α. P indicates positive control. Note that iNOS protein was markedly expressed by the combination of IL-1β and TGF-α, but nNOS protein was not detected by the above treatment.

the treatment. In western blot analysis, iNOS protein was slightly expressed by IL-1β alone, yet not expressed by TGF-α alone. Nonetheless, their combination markedly expressed iNOS protein 24hr after the treatment (*Fig. 2*). It was of interest that nNOS protein was not detected in RGM-1 cells even with the combined treatment of IL-1β and TGF-α.

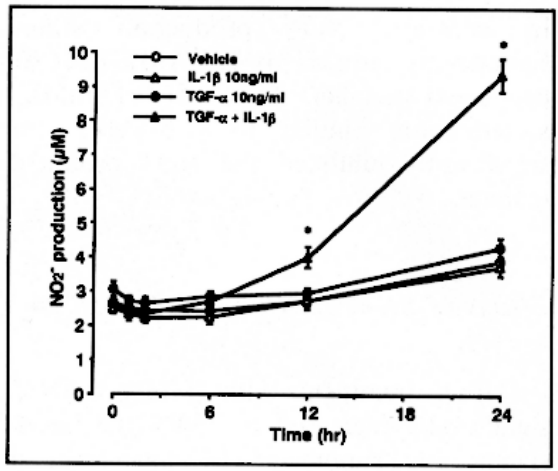

Fig. 3. Time course changes of NO_2^- production in RGM1 cells treated with vehicle, IL-1β, TGF-α and IL-1β + TGF-α for up to 24 hr. Ther are significant increase in NO_2^- production after the combined treatment. Data are presented as means × SE (n = 4). *Significantly different from the vehicle-treated group, with $P < 0.05$.

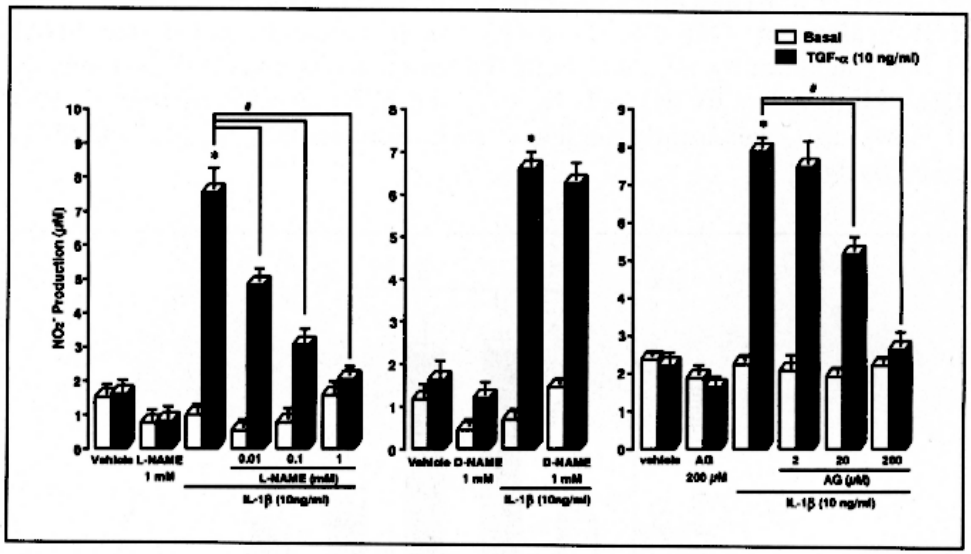

Fig. 4. Effects of L-NAME, D-NAME and aminoguanidine (AG) on the NO_2^- production of RGM1 cells stimulated by the combined treatment with IL-1β + TGF-α for 24 hr. Data are presented as means × S.E. (n = 4). *, ♯ Significantly different from the vehicle- and IL-1β + TGF-α-treated groups, respectively, with $P < 0.05$.

Effect s of IL-1β and TGF-α on NO^{2-} production

Both IL-1β and TGF-α did not result in a significant production of NO_2^- in RGM1 cells in comparison with the control value (*Fig. 3*). Nonetheless, the combined treatment of these substances significantly increased the NO_2^- production 12 and 24 hr after the treatment. Neither L-NAME nor D-NAME (1 mM) significantly affected NO_2^- production in RGM1 cells (*Fig. 4*).

In contrast, NO_2^- production stimulated by IL-1β+TGF-α was significantly reduced by L-NAME (0.01, 0.1 and 1 mM) in a concentration dependent manner. D-NAME (1 mM), however, did not exhibit such a reduction. Similar to L-NAME, aminoguanidine (20 and 200 μM) significantly inhibited the NO_2^- production in a concentration-dependent manner.

Effects of SNAP, SNP and L-NAME on TGF-α-stimulated cell proliferation

Upon treatment with TGF-α, DNA synthesis by RGM1 cells was significantly increased to 150% the basal value, but such an increase was significantly suppressed by concurrent treatment with IL-1β by 30.1%. L-NAME (1 mM) had little or no effect on DNA synthesis stimulated by either TGF-α alone or TGF-α+ IL-1β (*Fig. 5*). It should be noted that SNAP (1 mM) significantly inhibited both the spontaneous and TGF-α-stimulated DNA synthesis by RGM1 cells by 46% and 63%, respectively (*Fig. 6*). SNP (1 mM) also significantly inhibited such responses by 27.1% and 34%, respectively.

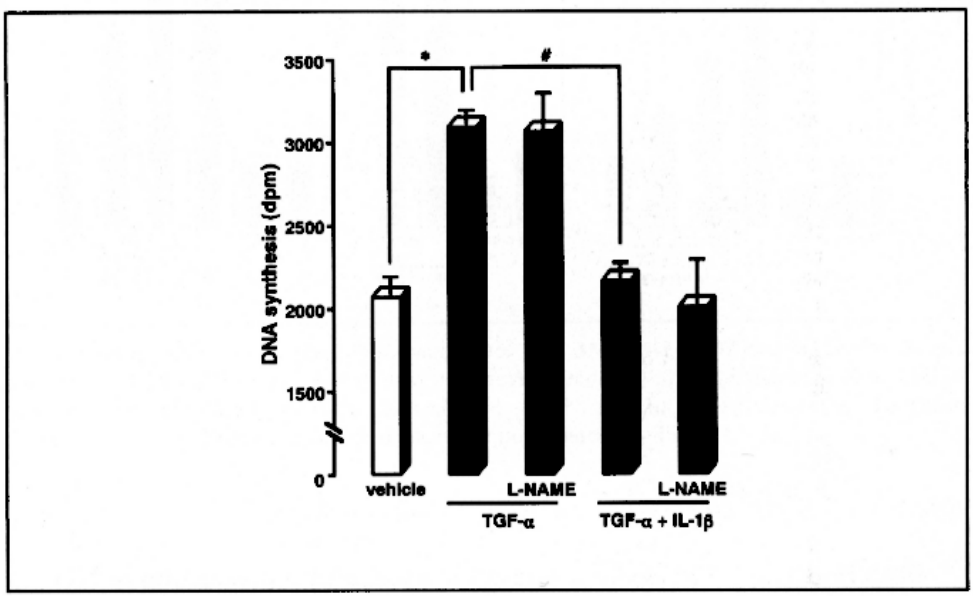

Fig. 5. Effect of L-NAME on DNA synthesis of RGM1 cells stimulated by TGF-α alone and on DNA synthesis in response to IL-1β+TGF-α. Data are presented as means × S.E. (n = 4). *, # Significantly different from the vehicle- and TGF-α-treated groups, respectively, with $P < 0.05$. N.S., non-significant.

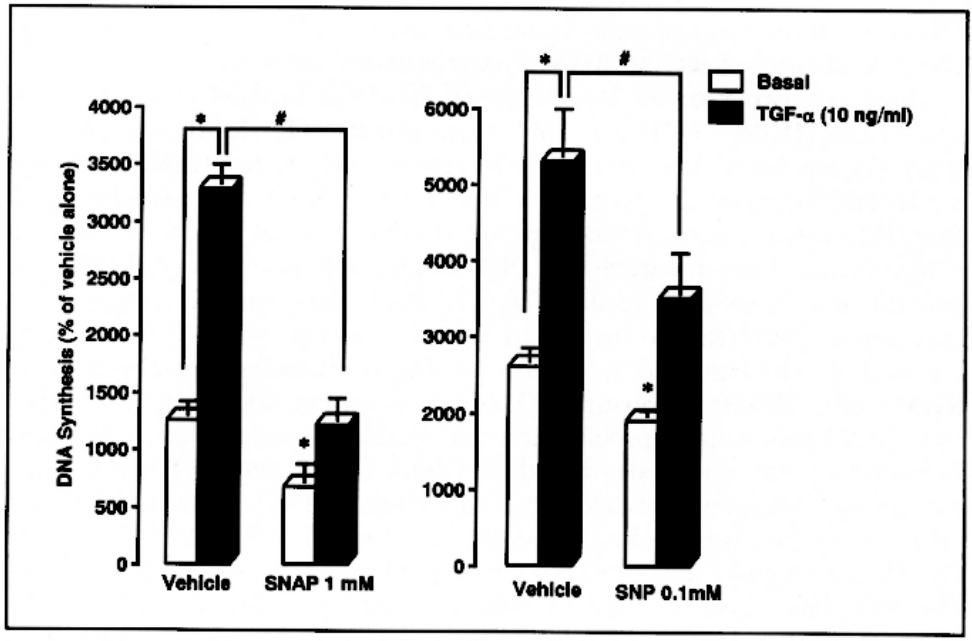

Fig. 6. Effects of SNAP and SNP on basal and TGF-α -stimulated DNA synthesis of RGM1 cells. Data are presented as means × S.E. (n = 4). *, # Significantly different from the vehicle- and TGF-α-treated groups, respectively, with $P < 0.05$.

DISCUSSION

The present study demonstrates that the treatment of RGM1 cells with IL-1β alone and IL-1β + TGF-α increased the expression of iNOS mRNA 2 hr after treatment; the enhancement of expression induced by the latter was more profound. In addition, the increase in iNOS protein in response to IL-1β alone and IL-1β + TGF-α was confirmed. It remains unknown how the combination of IL-1β and TGF-α induced potentiated iNOS expression both in mRNA and protein levels. Other groups have (13) reported that the combination of IL-1, TNF-α and interferon γ induces a potentiated expression of iNOS mRNA.

As expected, the combination of IL-1β + TGF-α gradually increased NO_2^- production in a time-dependent manner. It should be noted that L-NAME (but not D-NAME) and aminoguanidine significantly inhibited the NO_2^- production stimulated by TGF-α + IL-1β. Price *et al.* (13) reported that neuronal form of cNOS was localized in the rat gastric mucosa, especially in the surface epithelical cells. Therefore, it was possible that the NO_2^- production in response to IL-1β + TGF-α was resulted from the activated cNOS protein. In RGM-1 cells, however, we could not detect nNOS after the treatment with vehicle, IL-1β, TGF-α and IL-1β + TGF-α. Therefore, the involvement of

nNOS in the NO_2^- production could be ruled out, although the reason why nNOS could not detect in RGM-1 cells remains unknown.

There are two types of IL-1 receptors (IL-1R) , i.e, IL-1R1 in fibroblast and T-cells, IL-1R2 in B cells and macrophages (14, 15). In the previous study (1), we found that RGM1 cells express mRNA for IL-1R1, but not for IL-1R2. Therefore, it is possible that iNOS mRNA expressed by IL-1β after TGF-α-stimulation is mediated *via* IL-1R1 in RGM1 cells. In addition, it has already been reported that NO inhibits cell proliferation both in *in vivo* (8) and in *in vitro* studies (16, 17). Such data strongly suggest that endogenous NO released by IL-1β in the presence of TGF-α might be involved in IL1β-induced inhibition of TGF-α stimulated-proliferation of RGM1 cells. SNAP, a known NO donor, is known to inhibit both basal and bFGF-stimulated proliferation of vascular endothelial cells (16). Furthermore, this study also found that both SNAP and SNP significantly suppressed both spontaneous and TGF-α-stimulated DNA synthesis in RGM1 cells for 24 hr. This finding indicates, for the first time, that NO, even though exogenously generated, can inhibit DNA synthesis in RGM1 cells. The NO donor dosages used in this study had no effect on cell viability based on both the MTT assay and the crystal violet assay. Accordingly, it is clear that the inhibition of cell proliferation did not result from cytotoxicity induced by the NO donors. Upon consideration of the above findings, it is suggested that NO generated by IL-1β and TGF-α could inhibit TGF-α stimulated DNA synthesis in RGM1 cells.

In this study, we reconfirmed our previous findings that, although TGF-α significantly increases DNA synthesis in RGM1 cells 24 hr after treatment, IL-1β significantly suppresses such increased synthesis. It was unexpectedly revealed, however, that L-NAME, at a dosage that potently reduces NO_2^- production, could not alter the inhibition induced by IL-1β on TGF-α-stimulated proliferation of RGM1 cells. SNAP and SNP might have inhibited DNA synthesis due to a higher concentration of NO released by the cells. In conclusion, endogenous NO is not involved in the mechanism by which IL-1β inhibits the stimulatory effects of TGF-α.

Acknowledgement: We wish to thank CJ Hurt (John Hopkins University School of Medicine, USA) for critical reading of the manuscript, and N Kinoshita, A Tanaka, K Tomita for their technical assistance.

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Received: February 2, 2000
Accepted: July 6, 2000

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