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# NITRIC OXIDE INHIBITS LPS-INDUCED TUMOR NECROSIS FACTOR SYNTHESIS *IN VITRO* AND *IN VIVO*

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Abstract. The effect of nitric oxide (NO) on LPS-stimulated TNF-α synthesis has been studied in vitro and in vivo. The synthesis of TNF-α in J774 macrophages stimulated with LPS (0.1μg/ml) was increased in concentration-related fashion by NO synthase inhibitor L-NMMA (3-30-300μM) and reduced by either L-arginine (3-30-300μM) or the NO donor SIN-1 (1-10-100μM). The level of TNF-α in the serum of LPS-challenged rats (6mg/kg/i.p.) was increased in animals pre-treated s.c. with L-NMMA (10 and 50mg/kg) and reduced in those given L-arginine (100 and 300mg/kg). These results show a negative feedback mechanism exhibited by NO on TNF-α synthesis suggesting an important regulatory link between NO and TNF-α in pathological processes.

*Key Words:* murine J774 macrophages, lipopolysaccharide, tumor necrosis factor-α, nitric oxide, $N^G$-monomethyl-L-arginine, 3-morpholinosydnonimine

## Introduction

Tumor necrosis factor alfa (TNF-α), a potent cytokine produced by activated monocyte/macrophage, is an endogenous mediator of inflammatory, immune and host defence functions (1). The TNF-α synthesis in response to bacterial lipopolysaccharide (LPS) is the initial and pivotal event in the pathogenesis of septic schock (2,3).

A growing body of evidence suggests that also nitric oxide (NO) is involved in the pathogenesis of LPS-induced shock (4). NO mediates several important biological functions such as regulation of vascular tone, neurotransmission and host defense mechanisms (5). NO is generated enzymatically from L-arginine by nitric oxide synthase (NOS) which is inhibited by some L-arginine analogues such as L-NMMA (6). The inducible NOS isoform (iNOS) is expressed to significant level in numerous cell types following immunological stimuli (7). The expression of the iNOS is tightly controlled by a large number of cytokines, including TNF-α (8).

Although the induction of iNOS by TNF-α has been extensively investigated, the reverse effect of NO on TNF-α synthesis is controversial. Some studies have shown that NO donors down-

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regulate TNF-α synthesis by human peripheral blood mononuclear cells (9) and RAW 264.7 cells (10). On the other hand immunologically-stimulated TNF-α synthesis appears to be reduced in mice (11) and J774 cells (12) by NOS inhibitors. In the present study we have investigated the effect of endogenous and exogenous NO on TNF-α synthesis in cultured J774 murine/macrophages stimulated with LPS. Furthermore, we have studied the effect of NO on TNF-α levels in the serum from LPS-treated rats.

## Methods

**In vitro studies**.The monocyte/macrophage cell-line J774 was grown in Dulbecco Modified Eagle's Medium (DMEM) supplemented with 10% foetal calf serum, 2mM glutamine, 100U/ml penicillin, 100μg/ml streptomycin at 37° C in 5% $CO_2$/95% air. The cells were plated in 24 well-culture plates at a density of 2.5x $10^5$ cells/ml/well and allowed to adhere for 2h at 37° C. Thereafter the medium was replaced with fresh medium and cells were activated by LPS (0.1μg/ml). L-NMMA (3-30-300μM), D-NMMA (300μM), L-arginine (3-30-300μM) and SIN-1 (1-10-100μM) were added to the cells 30 min before LPS challenge. The level of TNF-α in the cell medium, 3h after LPS challenge, was assessed on cells WEHI-164 by a biological assay using recombinant human TNF-α as reference standard (13). Rabbit antimurine TNF-α antiserum which cross-reacts with rat TNF-α was used in order to assess the specificity of TNF-α dependent cytototoxic activity. In some experiments NO was measured as nitrite ($NO_2^-$, nmol per $10^6$cells) accumulated in the incubation medium 24h after LPS challenge. A spectrophotometric assay based on the Griess reaction was used (14).

**In vivo studies**. Male Wistar rats (Nossan, Italy), weighing 250-300g, were used in all experiments. The animals were provided with food and water ad libitum. Groups of 4-6 rats were treated i.p. with LPS (6mg/kg) or saline. L-NMMA (10 and 50mg/kg) or L-arginine (100 and 300mg/kg) were injected subcutaneously 30 min before LPS challenge. After 2h the animals were anaesthetised with sodium pentobarbitone (60mg/kg i.p.) and blood samples were collected by intracardiac puncture. The TNF-α level in the serum was measured as previously reported.

**Materials**. All materials for the cell culture were from Gibco. LPS (from Salmonella Typhosa) was obtained from Difco. TNF-α, L-arginine, 3-morpholinosydnonimine (SIN-1), monomethyl-D-arginine (D-NMMA) and $N^G$-monomethyl-L-arginine (L-NMMA) were obtained from Sigma. Antimurine TNF-α antiserum was from Genzyme.

**Statisticals**. Data are expressed as percent of control (mean ± standard error of the mean). Comparisons were made by the unpaired two-tailed Student's *t*-test. The level of statistically significant difference was defined as $p< 0.05$.

## Results

**TNF-α synthesis from LPS-activated J774 cells**.

The level of TNF-α, measured in the cell medium 3h after LPS challenge, was 962.5 ± 180 U/ml (n = 10) as compared to the undetectable release by unstimulated cells (< 1U/ml; n = 10). The addition of L-NMMA (3-30-300μM) to the cells 30 min before LPS challenge

enhanced in a concentration-related manner TNF-α synthesis respectively by 14.0±1.4%, 47.0±8.0% and 139.0±24.0% (Fig.1A). L-NMMA, at the concentrations used, inhibited $NO_2^-$ accumulation at 24h respectively by 12.0±3.2%, 47.0±4.3% and 60.0±4.5%. D-NMMA had no effect on both TNF-α synthesis and $NO_2^-$ accumulation.. When the cells were stimulated with LPS in the presence of L-arginine (3-30-300μM) a concentration-dependent inhibition of TNF-α synthesis was observed (3.2±2.9%, 27.4±1.3% and 45.3±5.9%; Fig.1B) while the $NO_2^-$ accumulation at 24h was increased respectively by 10.0±3.1%, 16.3±4.2% and 35.0±5.2%. Also the NO donor SIN-1 (1-10-100μM) was able to suppress TNF-α synthesis in a concentration-dependent fashion respectively by 18.5±7.0%, 47.5±5.0%, 67.2±8.0% .

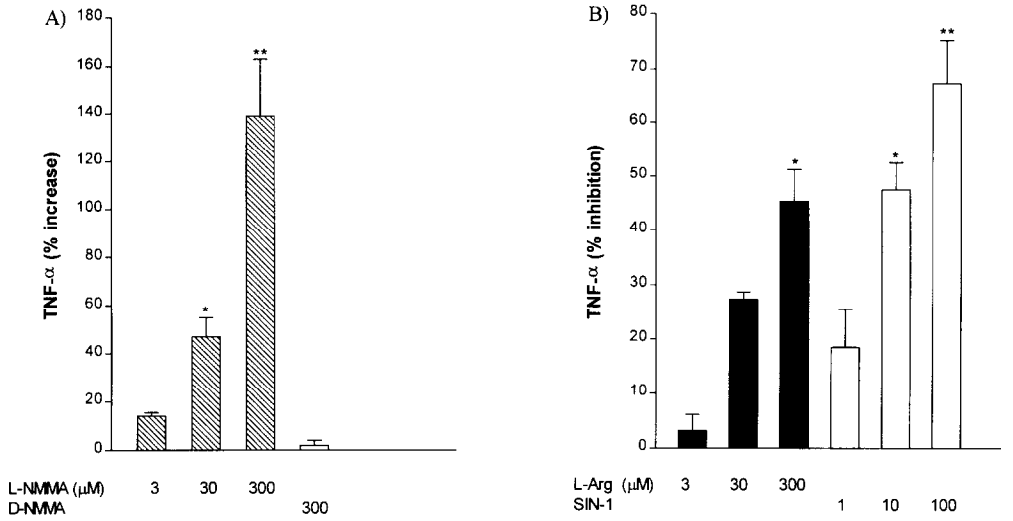


Fig. 1

Effect of L-NMMA and D-NMMA (A), L-arginine and SIN-1 (B) on TNF-α release by J774 murine macrophages 3 hours after LPS challenge (0.1μg/ml). Each column represents the mean ± S.E.M. of 8-10 separate experiments in tripliclate. *p< 0.05, **p< 0.01 vs control (LPS alone).

## TNF-α synthesis from LPS-treated rats

The effects of L-NMMA and L-arginine on TNF-α synthesis have been also studied in vivo. The TNF-α level in the serum collected from rats 2h after LPS-treatment (6mg/kg/i.p.) was 3424±253 U/ml (n = 6) as compared to the level from saline-treated controls (8.0±3.5U/ml; n = 6). The treatment of rats with L-NMMA (10 and 50mg/kg/s.c.) 30 min before LPS increased the serum TNF-α level by 160 ± 8.0% and 190 ± 8.0% respectively (Fig.2A). In contrast the TNF-α level in the serum from rats treated with L-arginine (100 and 300 mg/kg/s.c.) was reduced by 31.6±3.1% and 56.4 ±1.6% respectively ( Fig. 2B ).

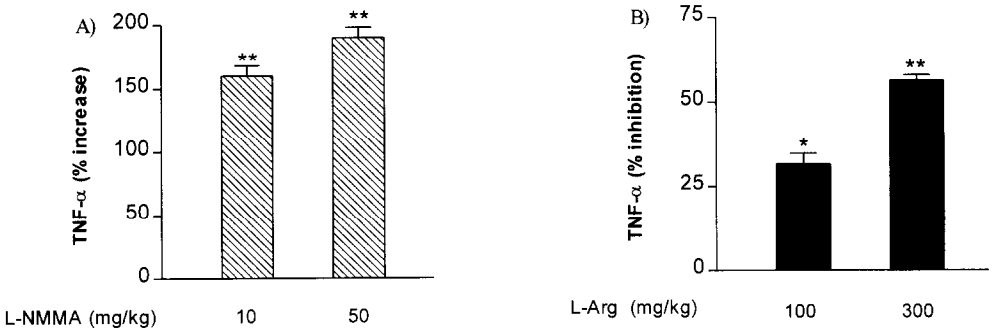


Fig. 2

Effect of L-NMMA (A) and L-arginine (B) on TNF-α release in the serum from rats 2 hours after LPS challenge (6mg/kg). Each column represents the mean ± S.E.M. of 4-6 rats. *p< 0.05, *p< 0.01 vs control (LPS alone).

## Discussion

The results of the present study show that LPS-stimulated TNF-α synthesis is modulated in vitro and in vivo by NO. Thus we have shown that NO synthase inhibitor L-NMMA caused a concentration-dependent increase of LPS-induced TNF-α release in J774 macrophages. In contrast D-NMMA, the biologically inactive enantiomer, had no effect. Both L-arginine and the NO donor SIN-1 inhibited in a concentration-dependent manner the LPS-induced TNF-α release by these cells. This finding is supported by the in vivo studies. In fact the level of TNF-α in the serum from LPS-challenged rats was significantly increased in animals pre-treated with L-NMMA and reduced in those given L-arginine. Our results are in agreement with previous experiments on human peripheral blood mononuclear cells (9) and RAW 264.7 macrophages (10) and seem to conflict with other studies in mice (11) and J774 cells (12). The apparent discrepancy between these last reports and our results might depend on differences in experimental setting such as type of stimulus, dose of LPS , animal species, time of TNF-α assay.

The mechanism of the NO-induced suppression of TNF-α synthesis is unclear. Several lines of evidence suggest that NO activates cyclooxygenase resulting in an increased production of prostaglandins (15,16,17). It has been also shown that $PGE_2$ inhibits TNF-α release through elevation of cAMP (18,19) and non steroidal antiinflammatory drugs significantly increase the serum level of TNF-α in murine endotoxic shock (20). Recently it has been reported that NO inhibits the activation of the transcription factor NF-κB (21). TNF-α mRNA formation is enhanced by activated transcription factor NF-κB (22). Thus the possibility that NO may regulate TNF-α synthesis through the modulation of NF-κB activation cannot be ruled out.

Although further studies are required to clarify the negative feedback mechanism exhibited by

NO on TNF-α synthesis, our data suggest an important regulatory link between NO and TNF-α in pathological processes, such as septic shock, where the concomitant formation of both TNF-α and NO occurs. In this light it would be interesting to establish whether the in vivo NO concentrations achieved in these pathological conditions might effectively inhibit TNF-α synthesis.

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