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NITRIC OXIDE-RELEASING AGENTS ENHANCE CYTOKINE-INDUCED TUMOR NECROSIS FACTOR SYNTHESIS IN HUMAN MONONUCLEAR CELLS

Andreas Eigler, Bhanu Sinha, and Stefan Endres[1]

Medizinische Klinik, Klinikum Innenstadt der Ludwig-Maximilians-Universität München, FRG

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Summary. In septic shock tumor necrosis factor (TNF) leads to increased nitric oxide (NO) production by induction of NO synthase. An inverse regulatory effect, the influence of NO on cytokine synthesis, has rarely been investigated. The present study assessed the influence of NO-releasing agents on TNF production from interleukin-1α (IL-1α)-stimulated human peripheral blood mononuclear cells (PBMC). 3-Morpholino-sydnonimine (SIN-1) enhanced IL-1α-induced TNF synthesis to a maximum of 272% (mean of n = 5 donors), with 100% set as TNF production by stimulation with IL-1α alone. This finding was confirmed using another NO-donor, i. e., sodium nitroprusside (SNP). The effect was specific for TNF compared to the uninfluenced synthesis of IL-1β. Kinetic analysis showed the most pronounced increase in TNF synthesis when SIN-1 was added during the first 60 min after IL-1α addition. These data reveal an enhancing effect of NO on cytokine-induced TNF synthesis. It may contribute to the regulation of TNF synthesis in pathological processes such as microbicidal activitiy, tumor cell lysis or endothelium-mediated hypotension.

Tumor necrosis factor (TNF) is a pro-inflammatory polypeptide involved in a wide spectrum of pathological processes (1). Of particular interest are regulatory processes linking TNF and the reactive nitrogen mediator nitric oxide (NO). Several activities induced by TNF are, at least in part, mediated by NO. Synthesis of TNF is tightly controlled. Regulation occurs at the level of transcription: activated transcription factor NFκB enhancing TNF mRNA formation (2), and elevated cAMP concentration suppressing TNF mRNA synthesis (3, 4); and at the level of mRNA

[1]To whom correspondence and reprint requests should be addressed at Medizinische Klinik, Klinikum Innenstadt der Universität, Ziemssenstrasse 1, 80336 München, FRG.

Abbreviations: TNF, tumor necrosis factor; IL-1, Interleukin-1; NO, nitric oxide; SIN-1, 3-morpholino-sydnonimine; SNP, sodium nitroprusside; PBMC, peripheral blood mononuclear cells; NFκB, nuclear factor κ B; LPS, lipopolysaccaride; IFN-γ, interferon-γ.

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(uridine-adenosine in TNF mRNA, which render it sensitive to hydrolysis [1]). In septic shock high levels of LPS-induced TNF lead to the formation of inducible NO synthase in endothelial cells. Inducible NO synthase catalyses the release of NO from the guanidino nitrogen of L-arginine. NO, in turn, activates soluble guanylate cyclase generating cGMP that mediates vascular smooth muscle cell relaxation.
This pathway has been extensively studied *in vitro* (5) and the *in vivo* trials, using competitive arginine antagonists, resulted in a reversal of hypotension (6, 7).
Inverse regulatory relations – effects of NO *on* cytokine production – have rarely been investigated. Previous findings in our laboratory have shown a marked suppressive effect of NO on LPS-stimulated TNF synthesis from human peripheral blood mononuclear cells (PBMC) (8). In the present study we used the endogenous stimulus IL-1α, to investigate modulating effects of NO-donors on TNF production. Dose response and kinetic analysis of these effects were performed.

Materials and Methods

Stimulation of mononuclear cells. Blood was drawn from healthy, fasting volunteers who had been without medication for at least two weeks. The peripheral blood mononuclear cell (PBMC) fraction was obtained by gradient centrifugation over Ficoll Hypaque (Biochrom, Berlin, FRG) as described (9). The final concentration of the suspended cells was 2.5 x 10^6/ml. Lipopolysaccharide (LPS; *E. coli* 055:B5; Sigma, München, FRG) was freshly diluted from a frozen aliquot with supplemented RPMI medium containing 10 mg/ml endotoxin-free human albumin (Behringwerke, Marburg, FRG) to final LPS concentrations of 10 and 0.1 ng/ml. Frozen aliquots of human recombinant IL-1α (Hoffmann-La Roche, Nutley, NJ) were freshly diluted with supplemented medium containing 10 ng/ml human albumin to final concentrations of 10 and 1 ng/ml. 3-morpholino-sydnonimine (SIN-1, a gift from Dr. Grewe, Cassella-Riedel, Frankfurt/Main, FRG) was dissolved and diluted in supplemented RPMI medium containing 10 mg/ml endotoxin free human albumin within 10 min before the addition to the PBMC suspension. The final concentration ranged from 1000 to 10 μM SIN-1. Sodium nitroprusside (SNP; Schwarz Pharma, Monheim, FRG) provided as lyophilisate and inert solvent (excipient) was diluted by the same procedure. 250 μl of the various concentrations of SIN-1 or SNP and 250 μl LPS or IL-1α, respectively, were pipetted into wells of a 24-well culture plate (Falcon, Becton Dickinson, New Jersey, USA). Subsequent addition of 500 μl PBMC suspension gave a final volume of 1000 μl. A 20 h incubation period at 37°C in 5% CO_2, 90% humidified air was terminated by freezing the plates at -70°C to obtain combined cell lysate plus supernatant. Cell lysis was completed by two more freeze-thaw cycles.
Measurement of TNF and IL-1β. TNF and IL-1β were determined by specific RIA, as described elsewhere (10, 11).

Results

1. SIN-1 dose-dependently enhances TNF synthesis in IL-1α-stimulated PBMC.
Isolated PBMC from five healthy donors were stimulated with 10 ng/ml IL-1α alone or with simultaneous addition of SIN-1, at concentrations ranging from 10 to 1000 μM. The maximal increase in TNF production to a mean of 272 % of controls was

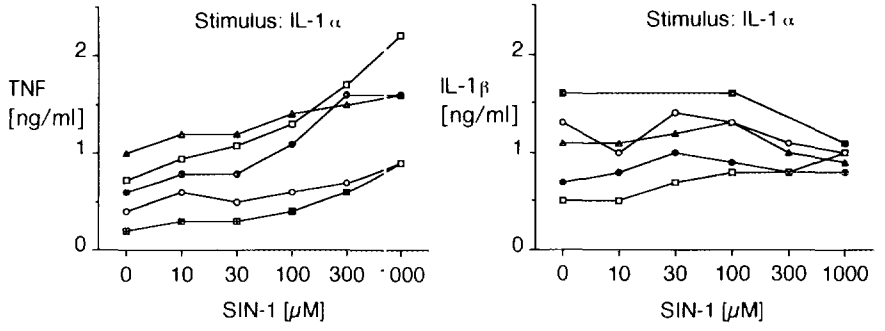

Figure 1.
Enhancement of IL-1α-induced TNF synthesis but not of IL-1β synthesis by the NO-releasing substance 3-morpholino-sydnonimine (SIN-1).
Freshly isolated peripheral blood mononuclear cells (PBMC; 2.5 x 10^6 cells/ml) from five healthy donors were stimulated with 10 ng/ml IL-1α alone or with increasing concentrations of SIN-1 (10 to 1000 μM). Total, i. e., cell-associated plus secreted TNF formed after 20 hrs was quantified by RIA. *Left panel*: Each symbol represents the values from one individual donor. Baseline stimulation with 10 ng/ml IL-1α alone ranged from 0.2 to 1.0 ng/ml TNF. 1000 μM SIN-1 addition increased TNF synthesis to values between 0.9 and 2.2 ng/ml (mean increase 272%). *Right panel*: Each symbol represents the values of one donor. Baseline stimulation with 10 ng/ml IL-1α resulted in 0.5 to 1.6 ng/ml IL-1β. Addition of 1000 μM SIN-1 yielded between 0.8 and 1.1 ng/ml IL-1β. In samples from one donor (top curve) only SIN-1 concentrations of 0, 100 and 1000 μM were tested.

seen with 1000 μM SIN-1. Control TNF synthesis was defined as the stimulation with IL-1α (10 ng/ml) alone. The inter-individual differences and the reproducible increase of TNF production in samples from five different donors are depicted in figure 1 (left panel). In cells from two other donors we examined the influence of SIN-1 on IL-1α-induced TNF production at concentrations above 1 mM. There was a plateau of maximal TNF production at the concentration range between 1 and 5 mM SIN-1 (mean 2.2 ng/ml TNF; compared to stimulation with 10 ng/ml IL-1α alone at 1.2 ng/ml TNF). Complete inhibition of TNF synthesis occurred at 10 mM SIN-1 (mean 0.1 ng/ml TNF), probably due to toxic effects. Using the identical samples we measured the effect of SIN-1 on IL-1α-induced synthesis of another cytokine, namely IL-1β. Depending on the donor, minor increases or decreases under the influence of SIN-1 could be observed (figure 1, right panel). The mean amounts of IL-1β induced, however, did not show any changes, even at the highest concentration of 1000 μM SIN-1 (1.0 ± 0.2 ng/ml IL-1β; mean ± SEM of 5 donors) compared to controls represented by stimulation with IL-1α alone (1.0 ± 0.04 ng/ml IL-1β).

The SIN-1 mediated *increase* of TNF synthesis induced by IL-1α was contrasted by the effect of SIN-1 when LPS was used as a stimulus. The addition of SIN-1 to LPS-stimulated PBMC resulted in a *decrease* of TNF production as we have previously reported (8). TNF synthesis was decreased to 61% of controls at a SIN-1 concentration of 1000 μM (mean of 5 donors). This inverse effect of SIN-1 on TNF

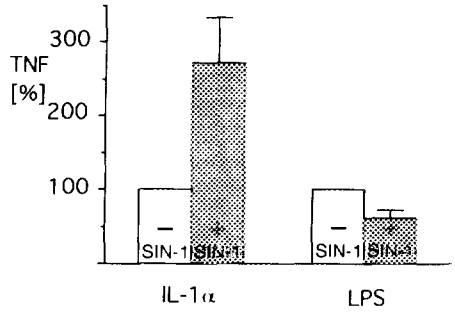

Figure 2.
Opposing effects of SIN-1 on TNF synthesis in LPS- versus IL-1α-stimulated PBMC.
Bars indicate the mean of samples from five donors in percent ± half range of the five percentages. 100% TNF synthesis represents baseline stimulation of 2.5×10^6 PBMC/ml with 10 ng/ml LPS or 10 ng/ml IL-1α alone. 1000 μM SIN-1 enhances TNF synthesis to 272% of control when PBMC were stimulated with 10 ng/ml IL-1α. 1000 μM SIN-1 suppresses TNF synthesis to 61% of control when PBMC were stimulated with 10 ng/ml LPS.

synthesis depending on the nature of the stimulus – IL-1α as a prototype endogenous stimulus, LPS as a prototype exogenous stimulus – is shown in figure 2. SIN-1 alone in concentrations ranging from 10 to 1000 μM, without addition of IL-1α or LPS, did not induce TNF concentrations higher than the cell-medium controls (range 0.05-0.13 ng/ml TNF).

2. Kinetic analysis reveals main effect of NO on TNF synthesis during the first hour.
To further investigate the mode of action of SIN-1, kinetic analysis was performed. SIN-1 (1000 μM) was added to PBMC simultaneously with IL-1α (10 ng/ml) or after time intervals ranging from 30 min to 3 hrs. Simultaneous addition of IL-1α and SIN-1 and addition of SIN-1 after 30 min or 60 min of stimulation with IL-1α resulted in markedly elevated TNF levels (figure 3, left panel). A notable reversal of this enhancement occured when SIN-1 was added 90 min or longer after IL-1α stimulation. In analogy the time dependence of the suppressive effect of SIN-1 on LPS-induced TNF synthesis was studied. Kinetic analysis revealed the main suppressive effect of SIN-1 on TNF synthesis after simultaneous addition of LPS and SIN-1 (figure 3, right panel). A small reversal of this suppression was noted when SIN-1 addition took place between 30 min and 3 hrs after LPS stimulation. Baseline TNF levels, represented by LPS stimulation alone, were not reached by the 3 hr values.

3. Enhancement of TNF synthesis in IL-1α-stimulated PBMC by another NO-donor, sodium nitroprusside.
The marked enhancement of IL-1α-induced TNF synthesis by SIN-1 raised the question, whether this effect might be a general property of NO-releasing

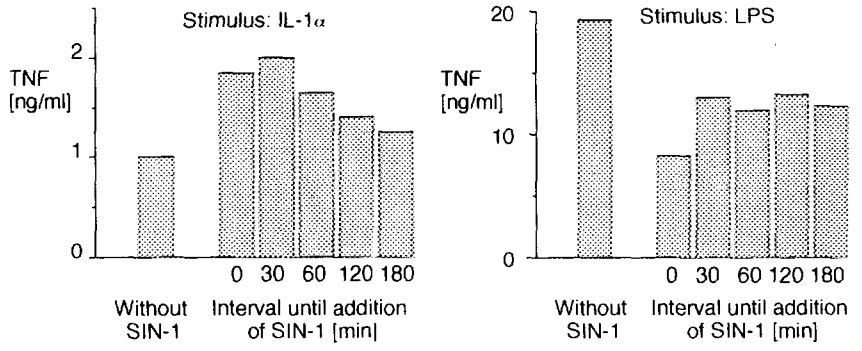

Figure 3.
The main effect of SIN-1 on TNF synthesis occurs during the first hour.
Each bar represents the mean of samples from two donors. The addition of 1000 μM SIN-1 took place in time intervals from 0 to 180 min. *Left panel*: TNF synthesis induced by IL1-α. Baseline TNF level, induced by 10 ng/ml IL-1α alone, is depicted as the isolated bar to the left. *Right panel*: TNF synthesis induced by LPS. Baseline TNF level, induced by 10 ng/ml LPS alone, is shown as the isolated bar to the left.

substances. Therefore we undertook follow-up experiments with the clinically used NO-releasing substance sodium nitroprusside (SNP). In the presence of 100 μM SNP IL-1α-stimulated PBMC synthesized 311% of TNF compared to controls (mean percent increase, n = 6 donors). Higher SNP doses lead to less elevated TNF production, with 1000 μM SNP resulting in 215% TNF production. The dose-dependent enhancement of IL-1α-induced TNF production by SNP is depicted in figure 4. Addition of SNP alone lead to a small, but consistent increase in TNF synthesis (mean ± SEM of n = 6 donors; 0.33 ng/ml ± 0,09 at 100 μM SNP) compared to cell-medium controls (range 0.06 to 0.16 ng/ml). The SNP excipient alone, in respective dilutions, did not induce TNF formation in PBMC (data not shown).

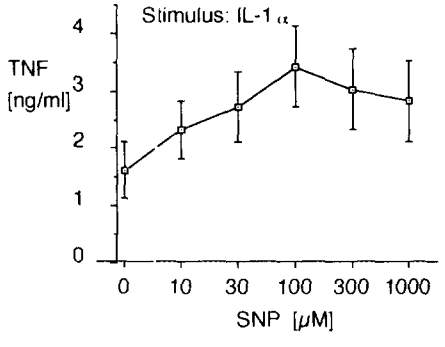

Figure 4.
Sodium nitroprusside (SNP) enhances IL-1α-induced TNF synthesis.
The mean TNF values ± SEM of 6 donor samples are depicted. 2.5 x 10^6 PBMC/ml were stimulated with 10 ng/ml IL-1α in the presence of 0 to 1000 μM SNP.

Discussion

The data presented demonstrate that TNF synthesis induced by a cytokine, i. e. IL-1α, is markedly enhanced by NO-releasing agents. The effect is sizeable, increasing TNF-synthesis to 272% of control levels. Kinetic analysis of the enhancing effect revealed a critical time window during the first hour of stimulation, suggesting a transcriptional influence of NO on TNF synthesis. An enhancing effect of NO on cytokine synthesis has been hypothesized (12), but to our knowledge there is only one publication in abstract form that describes enhancing effects of NO on cytokine production in leukemic cells (13).

SIN-1, the active metabolite of the antianginal drug molsidomine, has been used In a variety of experimental settings as a spontaneous NO-releasing agent (14, 15). With a half-life of about 35 min (16), freshly dissolved SIN-1 has to be exposed to cells in culture with as little delay as possible (10 min in this study). Sodium nitroprusside (SNP), another clinically used NO-releasing agent, increased TNF synthesis to 311% when SNP was added in a concentration of 100 μM. Higher concentrations of SNP lead to a decrease in TNF production. This may be due to toxic effects of *in vitro* accumulated cyanide, a known side effect of SNP administration. To assure endotoxin-free conditions for cell isolation and preparation a negative control was performed in each experiment, using PBMC suspended in medium. If appreciable TNF production (> 0.2 ng/ml) was detected in this sample the results of the experiment were discarded. This occurred in one out of 13 experiments. Stimulation with 10 ng/ml of LPS resulted in almost ten times higher TNF levels compared to the use of 10 ng/ml of IL-1α. LPS was also used at the lower concentration of 0.1 ng/ml. This induced TNF in concentrations similiar to those induced by 10 ng/ml of IL-1α (data not shown). These lower levels of LPS-induced TNF were also suppressed by addition of SIN-1. We therefore conclude that the effect of SIN-1 does not depend on the absolute levels of TNF induced.

To further investigate the regulatory role of NO on cytokine-induced TNF synthesis the underlying mechanisms have to be examined. A main pathway of signal transduction distal to intracellular NO is the soluble guanylate cyclase, which is activated by nitrosation of its heme group (17). This activation results in increased intracellular levels of cGMP. Elevated cGMP levels have been reported to induce TNF synthesis in rat peritoneal macrophages (18, 19) and human peripheral blood monocytes (20). High cGMP concentrations could therefore account for the NO-mediated enhancement of TNF synthesis. Another, only hypothesized, pathway for NO-mediated enhancement of TNF synthesis may involve the transcription factor NFκB (12) which increases TNF mRNA transcription when activated (2), as, for example, through reactive oxygen intermediates (21). Whether NO, as an oxidizing agent, is also able to activate NFκB remains to be elucidated.

There are few reports in the literature of the effects of NO or cGMP on cytokine synthesis. Elaborate studies on the relationship between cGMP and TNF have been published by the group of Gerrsa et. al. They were able to demonstrate that exogenous addition of cGMP as well as endogenous – SNP-induced – cGMP resulted in TNF synthesis in rat peritoneal macrophages (19) and in human monocytes (20). In our control experiments (without addition of LPS or IL-1α) we also noted a small increase in TNF production when SNP was added to PBMC alone. This could reflect a role of NO as an inducer of TNF synthesis in PBMC. However this interpretation has to be taken with caution, since TNF increases in our study were only marginal (mean ± SEM of n = 6 donors: control 0.13 ng/ml ± 0.01 ng/ml; addition of 100 μM SNP, 0.33 ng/ml ± 0.09 ng/ml). Furthermore, in our study the NO donor SIN-1 alone failed to induce any appreciable concentration of TNF.

In conclusion the data obtained in this study suggest a differential regulatory role of NO on cytokine synthesis. The enhancing effects of NO-releasing agents on IL-1α-induced TNF synthesis may reflect an important regulatory link between NO and TNF. It may contribute to the regulation of TNF synthesis in those pathological processes where formation of both TNF and NO has been demonstrated. These comprise for example: microbicidal activity (22), tumor cell lysis (23, 24) and endothelium-mediated hypotension (6).

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