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Regulation of Cytokine Production by Exogenous Nitric oxide in Murine Splenocyte and Peritoneal Macrophage

Jae-Soon Eun, Yong-Hoon Suh, Dae-Keun Kim, and Hoon Jeon

College of Pharmacy, Woosuk University, Samrye 565-701, Korea

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Nitric oxide (NO), products of activated macrophages, have a great impact on the regulation of cytokine production. The role of NO in non-specific host cells is commonly accepted. On the contrary, its role as an immuno-regulatory molecule is still controversial. In this study, we have investigated the effect of NO on the production of cytokines from murine splenocytes and macrophages. S-nitroso-L-glutathione inhibited the release of both interferone-γ and interleukin-2 produced by Th1 cells and tumor necrosis factor-α and interleukin-1β produced by macrophages, but did not affect the release of interleukin-4 and interleukin-10 produced by Th2 cells. These results suggest that NO exerts a down-regulatory effect on the secretion of cytokines from Th1 cells and macrophages which are implicated in immune response. Thus, NO may have an important role as an immuno-modulatory as well as effector molecule in the immune system.

Key words: S-nitroso-L-glutathione, Cytokines, Splenocyte, Macrophage

INTRODUCTION

Nitric oxide (NO) is formed when L-arginine is converted to L-citrulline by the action of NO synthetase (Nathan *et al.*, 1994). NO is a critical mediator of a variety of biological functions, including vascular and muscle relaxation, platelet aggregation, neural transmission and tumoricidal activity, and a range of immunopathologies (Xie *et al.*, 1994; Miller. *et al.*, 1995; Liew *et al.*, 1995).

NO is required in large amounts to combat infectious agents and tumors. However, production of excessive amounts of NO will lead to a different range of pathological outcomes and important pathologies. Therefore, the expression of inducible nitric oxide synthetase (iNOS) is necessarily under tight regulation. A number of cytokines are able to inhibit the expression of iNOS by murine macrophages (Ding *et al.*, 1990; Cenci *et al.*, 1993). NO can also downregulate its own synthesis by inactivating NOS in a feedback manner (Rogers *et al*, 1992) and inhibit the production of interferon-γ (IFN-γ) by Th1 cells, thereby effectively turning off one of the main sources of stimuli of NO synthesis (Tayler-Robinson *et al.*, 1994).

Correspondence to: Jae-Soon Eun, College of Pharmacy, Woosuk University, Samrye 565-701, Korea
E-mail: jseun@core.woosuk.ac.kr

Cytokines play a crucial role in the regulation of NO generation by iNOS. Murine macrophages activated with lipopolysaccharide (LPS) and Th1-like cytokines, especially with IFN-γ, produce high levels of NO. Conversely, the production of NO can be inhibited by Th2 cytokines, such as interleukin (IL)-4 and IL-10. A variety of macrophage secretory factors are involved in an autocrine regulation of NO production. Both IL-1 and tumor necrosis factor-α (TNF-α) increase, and TGF-β suppresses IFN-γ stimulated iNOS expression and, subsequently, NO release (Stuehr *et al.*, 1991; Cox *et al.*, 1992; Adler *et al.*, 1995; Taub *et al.*, 1995). In turn, NO may modulate the release of cytokines produced by both macrophages and T cells. Overproduction of NO results in an activation of a positive feed-back loop, leading to an increased release of proinflammatory cytokines such as IL-1 and TNF-α (Marcinkiewicz *et al.*, 1995).

We have also shown that NO can act as immunoregulator of macrophages during the phagocytosis (Suh *et al.*, 1999). A number of data indicate that NO plays a role as an immunoregulator and influences the cytokine production by murine macrophages. The regulation of lymphokine synthesis by NO is much more controversial (Liew 1995; Marcinkiewicz *et al.*, 1996). It is also unclear whether NO regulate cytokine production by the immune cells. The functional relationship between NO and Th cells and macrophages is presented here.

MATERIALS AND METHODS

Materials

S-nitroso-L-glutathione (GSNO), DME medium, lipopolysaccharide (026:B6), interferon-γ (Hu IFN-γ), zymosan and lucigenin were all purchased from Sigma (St. Louis, USA). RPMI1640 medium, FBS and thioglycollate were purchased from Gibco/BRL (Grand Island, USA). Mouse immunoassay kit was purchased form R&D Co. (Minneapolis, USA). The animals used were male BALB/c mice weighing about 18~20 g and obtained from Daehan Animal Center at Eumsung, Korea. The animals were maintained at constant temperature and environments with 12 h day/night cycle before use. They were fed lab. Chows (Cheil Jedang Co.) and tap water *ad lib.*

Cell preparation and culture

Murine splenocytes were prepared from spleen of mice by gently pressing the organs against fine stainless steel mesh submerged in RPMI1640 media. Single cell suspensions were washed twice in DME media and resuspended in cold RPMI1640 media. Murine macrophages were prepared from peritoneal exudate cells of mice injected intraperitoneally 3 days previously with 2.0 ml of 3% thioglycollate. Macrophages were purified by adherence to plastic for 2 h (37°C, 5% CO_2). The macrophages obtained after 2 h adherence period in RPMI1640 media were scraped. Adherent cell were >95% macrophages by Wright and nonspecific esterase staining (John *et al.*, 1991). The culture medium was supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin.

Cell proliferation

Murine splenocytes (1.2×10^6 cells/ml) were suspended in the culture medium and then seeded in an 0.2 ml/well in flat bottom microwells, and GSNO was added to the culture medium at four different doses (1, 10, 100 and 1,000 μM). The cells were then incubated in RPMI1640 media mixed with concanavalin A for 6, 12. 24 and 48 h. The cell proliferation was determined by MTT method.

NO assay

GSNO was used as a source of NO. GSNO was added to the culture medium at three different doses (1, 10 and 100 μM). The amount of NO released by GSNO in the medium was estimated by nitrite production, a stable oxidative metabolite of NO. Nitrite concentrations were measured using a Griess reagent (Rockett *et al.*, 1991).

Cytokine assay

Murine splenocytes (2×10^7 cells/ml) were cultured for 48 h in RPMI1640 media mixed with GSNO in the presence or absence of concanavalin A and the amounts of IFN-γ, IL-2, IL-4 and IL-10 were assayed by enzyme-linked immunosorbent assay. The peritoneal macrophages (2×10^7 cells/ml) were cultured for 48 h in RPMI1640 media mixed with GSNO in the presence or absence of lipopolysaccharide and the amounts of TNF-α and IL-1β were assayed by enzyme-linked immunosorbent assay.

Statistical analysis

The results obtained are expressed as means ± SE, n= 4. Statistical significance ($p<0.05$) of variance was analyzed followed by Student's *t-test.*

RESULTS AND DISCUSSION

Effect of GSNO on the cell proliferation

We first determined the maximum non-cytotoxic concentration of NO generating compounds using MTT assay. Our results showed that 100 μM GSNO did not affect on the cell proliferation of concanavalin A- or lipopolysaccharide-induced murine splenocytes, but 1,000 μM GSNO inhibited the cell proliferation to more than 50% (Fig. 1).

Accumulation of nitrite released from GSNO

Nitrite and nitrate are the stable metabolites of the short-lived molecule NO. In order to assess the quantities of nitrite liberated by GSNO, the compound was dissolved at various concentrations in RPMI1640 media. Nitrite was generated from GSNO in a dose-dependent manner. Identical results were obtained after 6, 12, 24 and 48 h of culture. After 48 h of culture, the amount of nitrite generated by 100 μM GSNO was 36.9 ± 0.4 μM (Table I).

Effect of GSNO on the cytokine production

We investigated the effects of NO on cytokine production of Th1 and Th2 cells. The culture supernatants of murine splenocyte were assayed for IFN-γ, IL-2, IL-4 and IL-10 after 48 h of culture in the presence or absence of concanavalin A. As shown Table II, GSNO inhibited the secretion of IFN-γ and IL-2 resulting in significant differences at the concentration of 100 μM GSNO, but did not affect the secretion of IL-4 and IL-10. When murine splenocytes were activated with concanavailn A, IL-10 prouduction was more susceptible to NO-mediated secretion than the other cytokines. Merryman *et al.* (1989) reported that 25 μM GSNO did not affect on IL-2 production by purified human peripheral blood mononuclear cells activated with PHA. Our data suggest a role for NO as a selective inhibitor of Th1-cytokine production in murine splenocytes.

Also, the purpose of this study was to determine whether NO downregulates inflammatory cytokine production

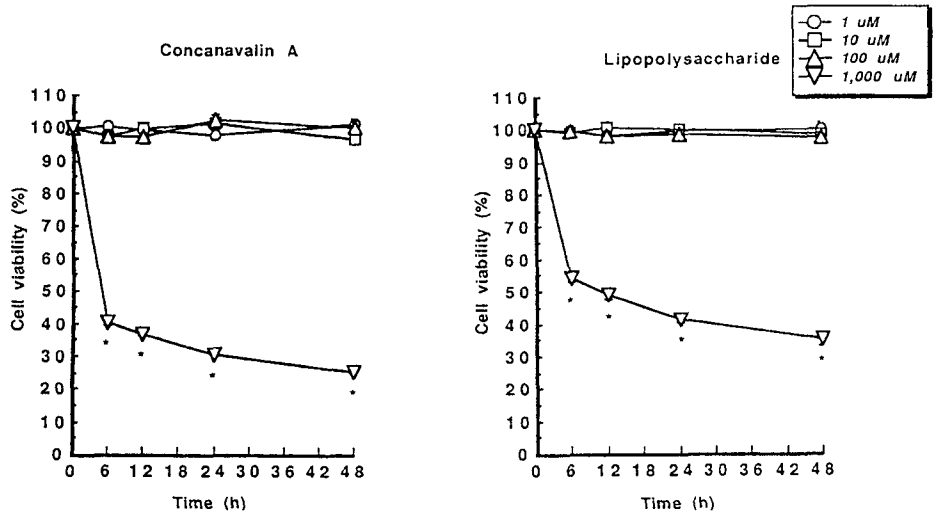

Fig. 1. Effect of S-nitroso-L-glutathione (GSNO) on the proliferation of concanavalin A- or lipopolysaccharide-induced murine splenocytes. GSNO was added 1, 10, 100 and 1,000 μM in RPMI1640 media and cultured for 6, 12, 24 and 48 h in CO_2 incubator at 37°C. The proliferation of splenocytes was determined by MTT method. Each bar represents the mean ± SE from 4 experiments. *; Significantly different from control group ($p<0.001$).

Table I. Generation of nitrite from S-nitroso-L-glutathione

Time (h)	GSNO Concentration (mM)				
	Medium	1	10	100	1,000
6	0.9 ± 0.1	1.3 ± 0.1	4.3 ± 0.1	18.0 ± 0.1	82.2 ± 1.2
12	1.0 ± 0.1	1.6 ± 0.1	4.9 ± 0.1	23.1 ± 0.2	111.9 ± 3.4
24	1.1 ± 0.1	1.7 ± 0.1	6.8 ± 0.1	27.7 ± 0.7	171.0 ± 3.2
48	1.6 ± 0.1	2.1 ± 0.1	8.1 ± 0.2	36.9 ± 0.4	223.0 ± 1.8

The concentration of nitric oxide was determined with a Griess reagents. The data represents the mean ± SE from 4 experiments.

Table II. Effect of GSNO on the secretion of Th-associated cytokines in concanavalin A-treated murine splenocytes

Samples	Th1 Cytokine (pg/ml)		Th2 Cytokine (pg/ml)	
	IFN-γ	IL-2	IL-4	IL-10
Control	505.6 ± 5.7	197.1 ± 1.4	48.5 ± 2.5	149.1 ± 1.9
GSNO 10	493.2 ± 2.3	182.5 ± 2.8*	45.8 ± 1.8	146.8 ± 3.9
GSNO 100	424.8 ± 5.7**	177.6 ± 2.7*	44.9 ± 2.7	148.9 ± 4.2
Con A	583.0 ± 6.2**	231.3 ± 4.3**	65.8 ± 1.8*	583.2 ± 5.4*
Con A+GSNO 10	562.6 ± 6.7	209.3 ± 3.5#	63.8 ± 3.5	585.2 ± 5.5
Con A+GSNO 100	520.2 ± 2.6#	198.0 ± 2.1#	62.7 ± 1.6	576.3 ± 7.5

The cells (2×10^7 cell/ml) were cultured for 48 h in RPMI1640 media mixed with GSNO 10 and 100 μM in the presence or absence of concanavalin A (Con A). The secretion of cytokines (pg/ml) was assayed by enzyme-linked immunosorbent assay in supernatants of cultures. The data represents the mean ± SE from 4 experi-ments. *; Significantly different from control group (*; $p<0.05$, **; $p<0.01$). #; Significantly different from Con A-treated group ($p<0.01$).

such as TNF-α and IL-1β from murine peritoneal macrophages. We found that NO decreased inflammatory cytokine production (Table III). Kim *et al.* (1998) tested the hypothesis is that NO regulates cytokine release by inhibiting IL-1β-converting enzyme or caspase-1 activity. The data indicated that NO suppressed IL-1β by inhibiting caspase-1 activity. These results suggest that NO's beneficial effects may be partially attributable to decreased production of inflammatory cytokines. In conclusion, NO exerted a down-regulatory effect on secretion of cytokines from Th1 cells and macrophages which are implicated in immune response. Thus, NO may have an important role as an immuno-modulatory as well as effector molecule in the immune system. On the

Table III. Effect of GSNO on the secretion of tumor necrosis factor-α and interleukin-1β in lipopolysaccharide-treated peritoneal marophages

Samples	Macrophage Cytokine (pg/ml)	
	TNF-α	IL-1β
Control	392.4 ± 5.1	115.5 ± 3.7
GSNO 10	385.9 ± 3.5	110.9 ± 4.1
GSNO 100	326.2 ± 4.3*	84.1 ± 3.2*
LPS	547.2 ± 6.3**	221.9 ± 4.2**
LPS+GSNO 10	540.1 ± 5.2	217.7 ± 3.6
LPS+GSNO 100	462.7 ± 5.3#	161.6 ± 4.8##

The cells (2×10^7 cell/ml) were cultured for 48 h in RPMI 1640 media mixed with GSNO 10 and 100 μM in the presence or absence of lipopolysaccharide (LPS). The secretion of cytokines (pg/ml) was assayed by enzyme-linked immunosorbent assay in supernatants of cultures. The data represents the mean ± SE from 4 experiments. *; Significantly different from control group (*; $p<0.01$, **; $p<0.001$). #; Significantly different from LPS-treated group (#; $p<0.01$, ##; $p< 0.001$).

other hand, the physiological role of NO as immunoregulatory molecule should be confirmed by further investigation in the human immune system.

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