啓明醫大論文集 第17卷 第4號
Keimyung Med J
Vol. 17, No. 4, December, 1998

Effect of Serum on Nitric Oxide Synthesis in Mouse Embryonic Liver Cells

Hun Taeg Chung*, M.D., Hyun Ock Pae, M.D. and Ji Chang Yoo, M.D.

Department of Microbiology, Immunology,*
Wonkwang University School of Medicine,
Iksan, Korea

= Abstract =

Serum that is commonly used as a culture medium supplement contains biologically active factors, including growth factors and cytokines. Nitric oxide (NO) is an important mediator of various biochemical and physiological processes. Its synthesis by various cells is controlled by various stimuli including cytokines or growth factors. To study the control of NO synthesis in the liver cells, the effects of serum on NO synthesis were investigated in this study. Mouse embryonic liver cell line BNL CL.2 cells were cultured 6hr in 20%, 10%, 5%, 2% serum-supplied or serum-free DMEM, respectively, and stimulated with interferon-γ (IFN-γ), lipopolysaccharide (LPS), or combination of two stimuli. Cells cultured in 2% serum-supplied or serum-free DMED synthesized NO in response to IFN-γ alone in a dose- and time-dependent manner, while cells cultured in 20%, 10% or 5 % serum-supplied DMEM did not. When cells were stimulated with IFN-γ plus LPS in 2% serum-supplied or serum-free DMEM, a synergistic increase in NO synthesis was observed, whereas decrease in NO synthesis was observed in uncreasing serum concentration. These findings evidence that in vitro NO synthesis by mouse liver cells may be dependent on concentrations of serum in culture.

Key Words: Serum, Nitric oxide, BNL CL.2 cells, Liver

Introduction

Many cells have the ability to synthesize NO from L-arginine (Palmer *et al* 1988; Rees 1989; Amezcua *et al* 1989; Drexler *et al* 1991). They include macrophages, endothelial cells, smooth muscle cells, liver cells, and neurons (Palmer *et al* 1988; Rees 1989; Amezcua *et al* 1989; Drexler *et al* 1991). NO is an important mediator of various biochemical and physiological processes (Palmer *et al* 1988; Rees 1989; Amezcua *et al* 1989; Drexler *et al* 1991). Two distinct but functionally and structurally related genes involved in the biosynthesis of NO have been identified in mammalian cells (Knowles & Moncada, 1994; Nathan & Xie, 1994). They are referred to as cNOS, constitutionally expressed in cells of

neuronal origin or endothelial origin (Knowles & Moncada, 1994) and to as iNOS, the activity of which is controlled at the transcriptional level in response to wide array of pro-inflammatory cytokines, bacterial cell wall products such as LPS, and through the engagement of signal pathways involved in host defense against pathogens and tumor cells (Nathan & Xie, 1994).

Maximal induction of iNOS depends upon synergistic combinations of stimuli (Michel & Feron, 1997), and the most effective stimuli vary with the cell type. These stimulatory compounds include cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interferon-gamma (IFN-γ), lipopolysaccharide (LPS), and cellular signal activating agents (Vane & Botting, 1995).

Serum such as fetal bovine serum (FBS) contains many biologically active factors, including growth factors, and thus is used as a culture medium supplement (Morita *et al*, 1995). Several studies evidenced that serum significa-ntly potentiates the ability of LPS to activate macrophages to produce NO (Chen *et al*, 1994) and that TNF-α secretion by macrophages exposed to LPS was markedly potentiate by serum (Kilikae *et al*, 1997).

The aim of this work is first to examine if serum in the medium is capable of affecting NO synthesis in BNL CL.2 cells. BNL CL.2 cells are normal embryonic liver cells derived from the BALB/c mouse. Once stimulated with stimuli including IFN-γ and LPS, they synthesize NO. In this study, we found that serum deprivation from cell culture medium rendered the BNL CL.2 cells responsive to IFN-γ for the production of NO.

Materials and Methods

Materials : Dulbecos modified Eagle medium (DMEM) and trypsin-ethylen-ediami- netetraacetic acid (EDTA) were purchased from GIBCO Laboratories (Grand Island, NY). Fetal bovine serum (FBS) was obtained from Hyclone Laboratories (Logan, UT). Murine recombinant interferon-g (IFN-γ) was purchased from Genzyme (Cambridge, MA). Lipopolysaccharide (LPS) (phenol extracted Salmonella entritidis) were purchased from Sigma Chemical Co. (St. Louis, MO). Ninety-six well tissue culture plates and other tissue culture dish were purchased from Nunc, Inc. (North Aurora Road, IL). Other tissue culture reagents were purchased from Life Technologies (Gaithersburg, MD). All reagents and media for tissue culture experiments were tested for their LPS content with the use of a colorimetric Limulus amoebocyte lysate assay (detection limit, 10 pg/ml (Whittaker Bioproducts, Walkersville, MD)).

Cell Culture in the Presence or Absence of Serum and Treatment of Cells with Serum or Stimuli : BNL CL.2 cells, mouse embryonic liver cell line, was obtained from American Type Culture Collection (Rockville, MD) and routinely

grown in culture flask in DMEM (4.5g/L glucose) supplemented with 10% fetal bovine serum (FBS). The cells were harvested from the culture flask by treatment with trypsin-EDTA and maintained by subculture every 3 to 4 days at a density of 2 x 105 cells / ml. Before experiments were carried out, cells were plated overnight in DMEM containing 10% FBS at 5 x 103 cells / well. Approximately 12 hr later, the medium was replaced with 20%, 10%, 5%, 2% serum-supplied or serum-free DMEM. After further incubation for 6 hr, cells were stimulated with stimuli. The medium was not changed for 3 days.

Assay for Nitrite Concentration :

Accumulated nitrite (NO2-) in culture medium was measured using an automated colorimertic assay based on the Griess reaction (8). Briefly, 100 ml of samples was reacted with the Griess reagent (1% sulfanilamide, 0.1% napthylethylenediamine dihydrochloride / 2.5% H3PO4) at room temperature for 10 minutes, and the nitrite concentration was determined by measuring the absorbance at 540 nm in a Titertek Multiscan MC340 (Flow Laboratories, North Ryde, Australia). The standard curve was obtained using the known concentration of sodium nitrite. In all experiments, nitrite concentration in wells containing medium only was also measured as a blank control.

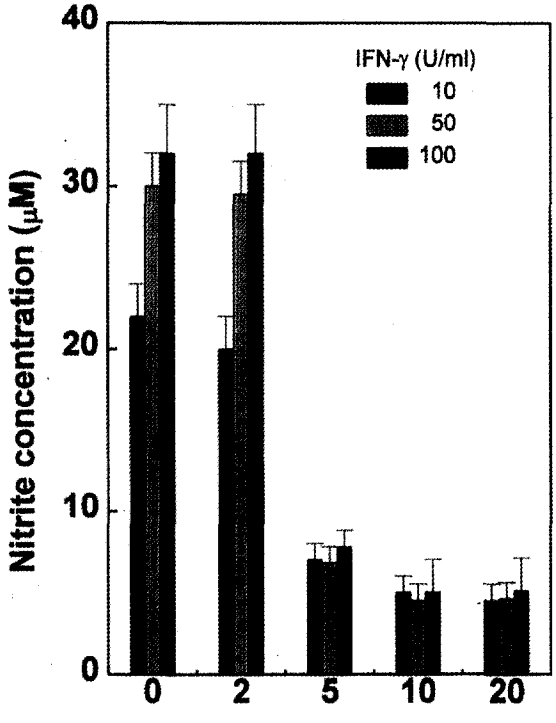

Figure 1. Inhibitory effects of serum on NO synthesis by BNL CL.2 cells stimulated with IFN-γ alone. BNL CL.2 cells were cultured in serum-free medium or medium supplemented with 2%, 5%, 10%, or 20% serum for 6hr and then stimulated with 10U/ml, 50U/ml, or 100U/ml IFN-γ alone. After incubation for 72 hr, the culture supernatants were collected and measured for nitrite concentration by Griess reaction (Nitrite is a stable oxidation product of NO.). Results are mean ± standard error of five separate experiments.

Results

1. Effect of Serum on NO Synthesis by the Cells Stimulated with IFN-γ.

When cells were cultured with serum-insufficient DMEM, the cell viability did not significantly decreased until day 3 of culture (>85%) (data not shown). However, the viability precipitously dropped after day 4 of culture, suggesting that the viability of cells is dependent on serum concentration in culture (data not shown). Because IFN-γ is recognized as one of agents (besides LPS) capable of synthesizing NO (Lee *et al*, 1997), the stimulatory effect of IFN-γ on NO synthesis was first examined. BNL CL.2 cells were cultured 6hr in 20%, 10%, 5%, 2% serum, or serum-free DMEM, and then stimulated with 10U/ml, 50U/ml, and 100U/ml IFN-γ. As shown in Fig.1, NO synthesis was induced by cells cultured in 2% serum-supplied or serum-free DMEM in response to IFN-γ alone, but not by cells cultured in 20%, 10%, or 5% serum-supplied DMEM. NO secretion by IFN-γ stimulation was time-dependent (Figure 2).

2. Effect of Serum on NO Synthesis by the Cells Stimulated with LPS.

It has been studied that serum significantly potentates the ability of LPS to activate macrophages to produce NO (Lee *et al*, 1997). To determine whether

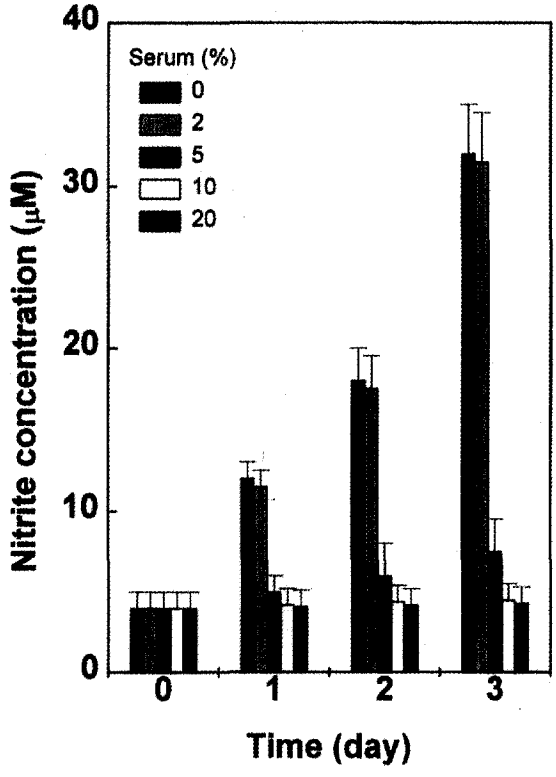

Figure 2. Time-dependent effect of IFN-γ on NO synthesis. BNL CL.2 cells were cultured in serum-free medium or medium supplemented with 2%, 5%, 10%, or 20% serum for 6hr and then stimulated with 100U/ml IFN-γ. The culture supernatants were collected at indicated times and measured for nitrite concentration by Griess reaction. Results are mean ± standard error of five separate experiments.

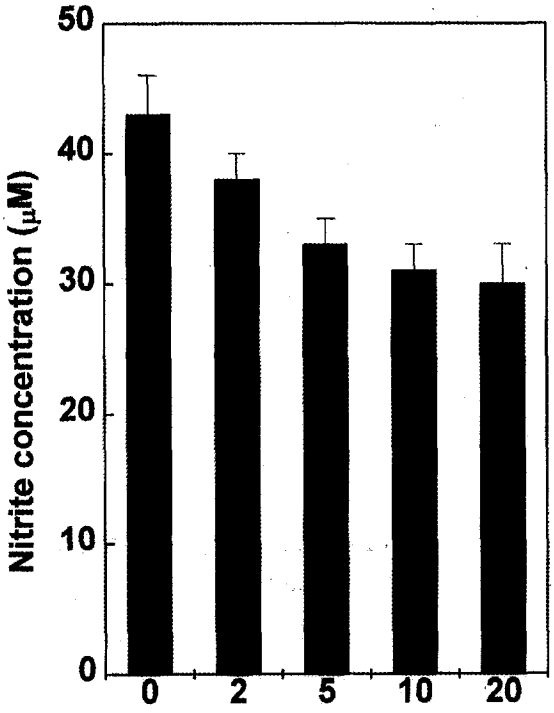

Figure 3. Inhibitory effects of serum on NO synthesis by BNL CL.2 cells stimulated with IFN-γ plus LPS. BNL CL.2 cells were cultured in serum-free medium or medium supplemented with 2%, 5%, 10%, or 20% serum for 6hr and then stimulated with 10U/ml IFN-γ plus 1mg/ml LPS. The culture supernatants were collected at indicated times and measured for nitrite concentration by Griess reaction. Results are mean ± standard error of five separate experiments.

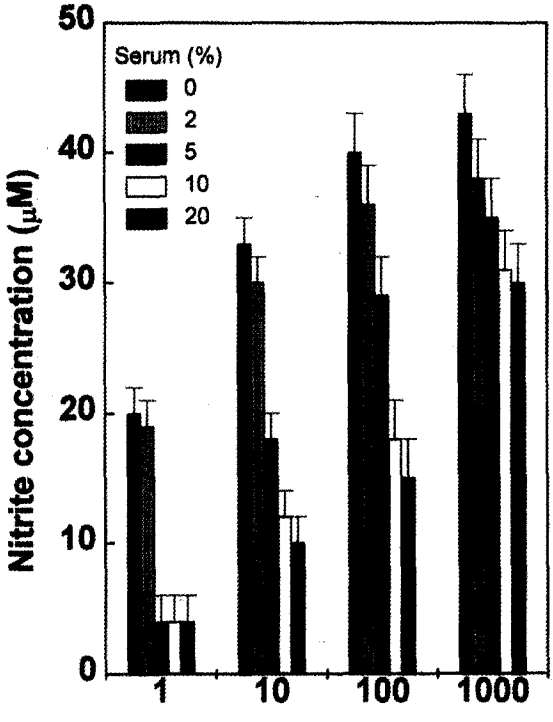

Figure 4. Effects of serum on the sensitivity of IFN-γ-primed cells to LPS. BNL CL.2 cells were cultured in serumfree medium or medium supplemented with 2%, 5%, 10%, or 20% serum for 6hr and then stimulated with 10U/ml IFN-γ in the absence or presence of indicated doses of LPS. After incubation for 72 hr, the culture supernatants were collected and measured for nitrite concentration by Griess reaction. Results are mean ± standard error of five separate experiments.

LPS alone could induce NO secretion by the cells cultured in serum-free medium or medium supplemented with 2%, 5%, 10%, or 20% serum, we examined effects of LPS on NO secretion. LPS even at a high concentration itself did not stimulate cells cultured in various serum conditions to produce NO (data not shown).

3. Effect of Serum on NO Synthesis by the Cells Stimulated with IFN-γ plus LPS

It has been reported that the combination of IFN-γ and LPS synergistically increased NO synthesis in BNL CL.2 cells cultured in 10% serum-supplied DMEM (Lee *et al*, 1997). Thus, we examined whether serum could inhibit NO secretion by the cells stimulated with IFN-γ plus LPS. BNL CL.2 cells were cultured in serum-free medium or medium supplemented with 2%, 5%, 10%, or 20% serum for 6hr and then stimulated with 10U/ml IFN-γ plus 1μg/ml LPS. As shown in Figure 3, NO secretion was significantly decreased with an increase in serum and proven to be dependent on serum containing in culture.

4. Effect of Serum on the Response of IFN-γ-Primed Cells to LPS

Although LPS itself did not stimulate cells to produce NO, it enhanced NO secretion by IFN-γ-primed cells cultured in various serum condition (Figure 3). Thus, we asked whether serum could decrease LPS-dose sensitivity of IFN-γ-primed cells. Treatment of 20%, 10%, or 5% serum-supplied cells with 10U/ml IFN-γ plus 1mg/ml LPS induced maximal NO secretion, whereas treatment of serum-starved or 2% serum-supplied cells with 10U/ml IFN-γ plus 100ng/ml LPS resulted in reaching maximal NO secretion (Figure 4).

Discussion

NO synthesis by normal hepatocytes and by LPS-treated hepatocytes has been reported to require multiple cytokines (Spitzer, 1994). Although the functional role of NO synthesis is not well defined, cytokine-mediated NO synthesis in the liver may modulate liver injury in disease states inducing sepsis (Lee *et al*, 1997). The knowledge of the mechanism involved in the control of NO synthesis by liver cells is a subject of current interest because of the possible protective effect of NO on the liver injury (Lee *et al*, 1997). To more effectively study the control of NO synthesis, the effects of serum on NO synthesis were first investigated in this study. We showed that NO synthesis by mouse liver BNL CL.2 cells in response to IFN-γ alone or IFN-γ plus LPS is dependent on concentrations of serum in culture. A previous study demonstrated that NO production by BNL CL.2 cells cultured in 10% serum-supplied DMEM was induced only by IFN-γ plus LPS (Lee *et al*, 1997). In our study, BNL CL.2 cells cultured in serum-

insufficient DMEM induced NO synthesis by the treatment with IFN-γ alone, but not with LPS (Figure 1 and 2). In addition, serum inhibited NO synthesis by combination of IFN-γ and LPS and decreased the response of IFN-γ-primed cells to LPS (Figure 3 and 4).

Serum that is used as a culture med-ium supplement contains many biologically active factors, including growth factors, cytokines, and antioxidants (Morita *et al*, 1995). Thus, it influences cell growth and biological functions, possibly including NO or cytokine production. In fact, several studies showed that it significantly potentiates the ability of LPS to activate macrophages to synthesize NO (Chen*et al*, 1994) and that TNF-α and IL-6 secretion by macrophages exposed to LPS was markedly potentiate by serum (Kirikae *et al*, 1997). In addition, it has been reported that cytokines, growth factors, antioxidants, cell density, and metabolic states regulate the secretion of NO by hepatocytes. Serum contains above regulators and thus may influence directly or indirectly NO synthesis.

Taken the above-mentioned studies together with our results, it is likely that NO synthesis and iNOS expression by the liver cells could be regulated by serum. However, the mechanism underlying serum-dependent suppression of NO synthesis in BNL CL.2 cells remains unknown and is under investigation.

References

Amezcua JL, Palmer RMJ, Souza BM, Moncada S: Nitric oxide synthesized from L-arginine regulates vascular tone in the coronary circulation of the rabbit. *Br J Pharmacol* 1989;97:1119-1124.

Augustijns P, Geusens P, Verbeke N: Chloroquine levels in blood during chronic treatment of patients with rheumatoid arthritis. *Eur J Clin Pharmacol* 1992;42:429-433.

Coffey RG, Snella E, Johnson K, Pross S: Inhibition of macrophage nitric oxide production by tetrahydrocannabinol in vivo and in vitro. *Int J Immunopharmacol* 1996;18:749-752.

Chen T, Scott E, Morrison DC: Differential effects of serum on lipopolysaccharide receptor-directed macrophage activation for nitric oxide production. *Immunol Lett* 1994;40:179-187.

Drexler H, Zeiher AM, Meinzer K, Just H: Correction of endothelial dysfunction in the coronary microcirculation of hypercholesterolemic patients by L-arginine. *Lancet* 1991;338:1546-1550.

Edwards G, Looareesuwan S, Davies AJ, Wattanagoon Y, Phillips RE, Warrell DA: Pharmacokinetics of chloroquine in Thais: plasma and red-cell concentrations following an intravenous infusion to healthy subjects and patients with Plasmodium vivax malaria. *Br J Clin Pharmacol* 1988;25:477-485.

Kirikae T, Tamura H, Hashizume M, *et al* Endotoxin contamination in fetal bovine serum and its influence on tumor necrosis factor production by macrophage-like cells J774.1 cultured in the presence of the serum. *Int J*

Immunopharmacol 1997;19:255-62.

Knowles RG, Moncada S: Nitric oxide synthases in mammals. *Biochem J* 1994;298:249-258.

Lee B-S, Kang HS, Pyun, K-H, Choi I: Roles of tyrosine kinases in the regulation of nitric oxide. *Hepatology* 1997;25:913-919.

Michel T, Feron O: Nitric oxide synthases: which, where, how, and why? *J Clin Invest* 1997;100:2146-2152.

Morita M, Watanabe Y, Akaike T: Protective effect of hepatocyte growth factor on interferon-gamma-induced cytotoxicity in mouse hepatocytes. *Hepatology* 1997;21:1585-1593.

Nathan C, Xie Q-W: Nitric oxide synthases: roles, tolls, and controls. *Cell* 1994;78:915-918.

Palmer RMJ, Ashton DS, Moncada S: Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature* 1988;333:664-666.

Rees DO, Palmer RM, Hodson HF, Moncada S: A specific inhibitor of nitric oxide formation from L-arginine attenuates endothelium-dependent relaxation. *Br J Pharmacol* 1989;96:418-424.

Spitzer JA: Cytokine stimulation of nitric oxide formation and differential regulation in hepatocytes and nonparenchymal cells of endotoxemic rates. *Hepatology* 1994;19:217-228.

Vane JR, Botting RM: New insights into the mode of action of anti-inflammatory drugs. *Inflamm Res* 1995;44:1-10.