Augmentation of Nitric Oxide Production by Gamma Interferon in a Mouse Vascular Endothelial Cell Line and Its Modulation by Tumor Necrosis Factor Alpha and Lipopolysaccharide

AKIKO MORIKAWA, NAOKI KOIDE, YUTAKA KATO, TSUYOSHI SUGIYAMA,
DIPSHIKHA CHAKRAVORTTY, TOMOAKI YOSHIDA, AND TAKASHI YOKOCHI*

Department of Microbiology and Immunology and Division of Bacterial Toxins, Research Center for Infectious Diseases, Aichi Medical University, Nagakute, Aichi 480-1195, Japan

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Nitric oxide (NO) exhibits a wide range of important functions in vivo, acting as a releasing factor mediating vasodilatation, a neuronal messenger molecule, and a major regulatory molecule associated with the immune system (3, 9, 17). NO is synthesized by constitutively expressed NO synthase (cNOS) for short periods of time. On the other hand, it is also synthesized by an inducible isoform of NOS (iNOS) that, once expressed, produces NO for long periods of time (17, 22). NO production with cNOS and iNOS is regulated in a complicated fashion by various stimuli. The best-studied example of the regulation of NO production almost certainly involves murine macrophages (21). It has been established that NO production is enhanced by gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), and lipopolysaccharide (LPS) in vitro (20, 26). Further, their combination augments NO production markedly in murine macrophages (20, 26). Several cytokines (interleukin 4, interleukin 10, and transforming growth factor β) are also known to modulate NO production directly (10, 22, 31). The extent of their effect, however, seems to vary dramatically under various experimental conditions.

With the discovery of endothelial cell-derived NO, it was found that vascular endothelial cells are capable of producing NO and play an active role in the regulation of vascular tone (11, 16). In addition to its role in the regulation of vasomotor function, NO is also important in the progression of a wide variety of diseases. The activity of cNOS allows for constitutive low-level production of NO by unstimulated vascular endothelial cells and is thought to be fundamental for the maintenance of a nonthrombogenic surface and the inhibition of cell adhesion to the endothelium (22). Vascular endothelial cells stimulated with various agents (LPS, cytokines, and growth factors) begin to accumulate mRNA encoding iNOS several hours following agonist stimulation (4, 25, 29, 34, 35). NO production by both cNOS and iNOS increases endothelial cell permeability, and the increased permeability allows the accumulation of growth factors necessary for stimulation of mitogenesis and tissue repair (22). However, we have recently used endotoxin-induced hepatic injury as an experimental endotoxic shock model (23) to demonstrate that the expression of iNOS and peroxynitrite-induced nitrotyrosine is detected mainly around blood vessels. NO produced by iNOS in vascular endothelial cells might play a critical role in endotoxin-induced tissue injury. The regulation of NO production by cNOS and iNOS in vascular endothelial cells remains a complex issue. In the present study, we examined the effect of IFN-γ, TNF-α, and LPS on NO production by using the mouse vascular aortic endothelial cell line END-D (24) because of the difficulty of obtaining normal vascular endothelial cells from mice and demonstrating the expression of iNOS in human cells.

MATERIALS AND METHODS

Reagents. Murine recombinant IFN-γ and TNF-α were purchased from R&D Systems (Minneapolis, Minn.) and Wako Pure Chemicals (Osaka, Japan), respectively. LPS from Escherichia coli O55:B5 was purchased from Difco Laboratories, Detroit, Mich. Polyclonal rabbit antibody against iNOS was obtained from Affinity Bioreagents, Neshanic Station, N.J. L-N6-(1-L-argininoethyl)lysine (L-NIL) and NG-monomethyl-L-arginine (L-NMMA) were obtained from Alexis, San Diego, Calif., and Dojindo, Kamamoto, Japan, respectively. Hydrocortisone and SB203580 were purchased from Sigma, St. Louis, Mo., and Calbiochem, San Diego, Calif., respectively.

Cell culture. The murine aortic endothelial cell line END-D, kindly provided by K. Kimata, Institute for Molecular Science of Medicine, Aichi Medical University, was maintained in Dulbecco’s minimal essential medium (Sigma) containing 10% heat-inactivated fetal calf serum and antibiotics. Heat inactivation was performed at 60°C for 30 min. END-D cells were positive for vascular cell adhesion molecule 1 and intercellular adhesion molecule 1 and negative for E-selectin. They were reported to be suitable for vascular development and...
RESULTS

NO production in END-D cells stimulated with IFN-γ, TNF-α, and LPS alone or in combination. Nitrite was measured in the culture supernatants from cells stimulated with LPS (10 μg/ml), IFN-γ (20 ng/ml), and TNF-α (10 ng/ml) alone or in combination for 2 or 4 days (Fig. 1). LPS and TNF-α inhibited NO production of END-D cells at 2 days. Treatment with combined LPS and TNF-α also inhibited NO production of END-D cells. The possibility that reduced NO production was due to injury of END-D cells was excluded, since there was no significant difference in the morphology and growth rates of untreated END-D cells and those stimulated with LPS and/or TNF-α. On the other hand, stimulation with combined IFN-γ and LPS or IFN-γ and TNF-α markedly enhanced NO production of END-D cells at 2 and 4 days, although IFN-γ alone did not affect NO production.

NO production in END-D cells stimulated with various concentrations of IFN-γ, TNF-α, and LPS. NO production was determined for cultures of END-D cells stimulated with various concentrations of LPS, IFN-γ, and TNF-α for 4 days (Fig. 2). LPS and TNF-α definitely reduced NO production of END-D cells at all concentrations tested. On the other hand, IFN-γ exhibited contrary effects on NO production, depending on its concentration. A relatively low concentration (0.1 or 1 ng/ml) of IFN-γ significantly reduced NO production of END-D cells, whereas a relatively high concentration (50 or 100 ng/ml) of IFN-γ enhanced NO production. IFN-γ at the intermediate concentration (10 ng/ml) did not significantly alter NO production. This was consistent with the result shown in Fig. 1.
Time course of NO production in END-D cells stimulated with IFN-γ, TNF-α, and LPS. A time course of NO production in END-D cells stimulated with LPS (10 μg/ml), IFN-γ (1 and 100 ng/ml), or TNF-α (10 ng/ml) was followed for 6 days (Fig. 3). Treatment of END-D cells with a high concentration of IFN-γ (100 ng/ml) induced an increase in NO production in a time-dependent fashion. NO production in untreated END-D cells also increased gradually, although its intensity was much lower than that of END-D cells stimulated with IFN-γ. END-D cells stimulated with LPS, TNF-α, and a low concentration of IFN-γ (1 ng/ml) produced less NO than untreated END-D cells did, and there was no significant difference in reduced NO production among them.

NO production in END-D cells stimulated by sequential incubation with LPS and IFN-γ. We demonstrated above that IFN-γ and LPS synergistically enhanced NO production in END-D cells. To verify the synergism between LPS and IFN-γ, the effect of sequential incubations with LPS (10 μg/ml) and a low (1-ng/ml) or high (100-ng/ml) concentration of IFN-γ on NO production was examined (Fig. 4). Exposure of END-D cells to LPS for 1 or 2 days followed by the addition of a high concentration of IFN-γ did not exhibit a synergistic effect on NO production (Fig. 4A). Rather, it gave lower values than treatment with IFN-γ alone. The pretreatment with LPS seemed to counteract the enhancement of NO production by IFN-γ. In addition, the sequential treatment with LPS and a low concentration of IFN-γ suppressed NO production in END-D cells, like the individual treatments did.

Next, the effect of the reversed order of sequential incubations with a low or high concentration of IFN-γ and LPS on NO production was examined (Fig. 4B). The exposure of END-D cells to IFN-γ (100 ng/ml) for 1, 2, or 3 days followed by the addition of LPS further augmented IFN-γ-triggered NO production. LPS, which by itself exhibited an inhibitory action, definitely enhanced NO production in IFN-γ-pretreated END-D cells. However, the enhancing effect of LPS was not seen in END-D cells pretreated with a low concentration (1 ng/ml) of IFN-γ. It was concluded that a high concentration of IFN-γ provided a critical signal to trigger NO production to END-D cells, and LPS exhibited an enhancing action on the cells once NO production was triggered by IFN-γ.

Detection of iNOS expression in END-D cells stimulated with IFN-γ, TNF-α, and LPS. Two different NOSs, cNOS and iNOS, are known to participate in the NO production of vascular endothelial cells (22). Therefore, we studied the expression of iNOS in END-D cells stimulated with IFN-γ, TNF-α, and LPS alone or in combination by an immunoblotting method in order to verify the participation of iNOS in the augmentation of NO production. As shown in Fig. 5, the immunoblotting analysis clearly demonstrated the expression of iNOS (with a molecular mass of 130 kDa) in END-D cells stimulated with a high concentration (100 ng/ml) of IFN-γ or combined IFN-γ and LPS or IFN-γ and TNF-α. On the other hand, no expression of iNOS was detected in untreated END-D cells or those treated with LPS, TNF-α, or a low concentration of IFN-γ (1 ng/ml). Therefore, we concluded that a high concentration of IFN-γ induced the expression of iNOS and enhanced NO production through iNOS and that LPS and TNF-α augmented NO production via IFN-γ-induced iNOS expression.

Inhibitory effect of 1-NIL and 1-NMMA on NO production in END-D cells stimulated with IFN-γ, TNF-α, and LPS. It was suggested that a high concentration of IFN-γ triggered the expression of iNOS and that LPS and TNF-α further enhanced NO production through IFN-γ-induced iNOS expression. It was of interest to determine whether or not the specific inhibitors of iNOS were present NO production in END-D cells treated with IFN-γ alone or with combined IFN-γ and LPS or IFN-γ and TNF-α. The addition of either 1-NIL (500 μM) or 1-NMMA (100 μM) definitely reduced NO production in stimulated END-D cells but did not inhibit NO production in untreated END-D cells (Fig. 6). Once again, the finding strongly suggested that the enhanced NO production in stimulated END-D cells was due to iNOS expression triggered by IFN-γ.

Inhibitory effect of hydrocortisone on NO production in END-D cells stimulated with IFN-γ alone or with combined IFN-γ and LPS. Based on the inhibitory effect of hydrocortisone on the activity of iNOS (2, 28), the effect of hydrocortisone on NO production in END-D cells stimulated with IFN-γ alone or with combined IFN-γ and LPS was studied (Fig. 7). Hydrocortisone markedly reduced NO production of END-D cells in a dose-dependent manner. The addition of hydrocortisone at 100 ng/ml completely blocked the enhancement of NO production in stimulated END-D cells.

Inhibitory effect of SB203580, an inhibitor of p38 MAPK, on NO production in END-D cells stimulated with IFN-γ. We studied the signaling pathway involved in NO production of END-D cells by IFN-γ. SB203580, PD98059, and genistein were used as the inhibitors of p38 MAPK, extracellular signal-regulated kinase 1/2 MAPK, and tyrosine kinase, respectively. As shown in Fig. 8, the addition of SB203580 to END-D cells stimulated with IFN-γ or with combined IFN-γ and LPS completely blocked the enhancement of NO production. SB203580 exhibited no toxic effect on the cell viability of END-D cells. However, the effect of PD98059 and genistein on NO production was not determined since their treatment was deleterious to END-D cells.

DISCUSSION

The present study demonstrated that high concentrations of IFN-γ triggered high-level production of NO in END-D cells through the induction of iNOS and that LPS and TNF-α fur-
ther augmented NO production once iNOS was induced by IFN-γ. Therefore, we concluded that iNOS might play a pivotal role in high NO production of vascular endothelial cells stimulated by high concentrations of IFN-γ alone, combined IFN-γ and LPS, or combined IFN-γ and TNF-α. This conclusion was suggested by several lines of evidence: first, the immunoblotting analysis demonstrated the expression of iNOS only in END-D cells stimulated by high concentrations of IFN-γ alone, combined IFN-γ and LPS, or combined IFN-γ and TNF-α; second, L-NIL and L-NMMA, inhibitors specific for iNOS, completely blocked the augmentation of NO production in stimulated END-D cells; and third, hydrocortisone, which is also known to inhibit the activity of iNOS, reduced NO production in a dose-dependent fashion. It was strongly suggested that the enhanced NO production in END-D cells stimulated with high concentrations of IFN-γ alone or in combination with LPS and TNF-α was caused by the activity of iNOS but not cNOS. In addition, iNOS from vascular endothelial cells appeared to produce a lower level of NO than that from macrophages.

It has been reported that IFN-γ in combination with LPS or TNF-α enhanced NO production in mouse vascular endothelial cells (4, 25, 29, 34, 35). However, the individual actions of IFN-γ, LPS, and TNF-α are not well-documented. Walter et al. (34) reported that treatment with IFN-γ or LPS alone suppresses cNOS-mediated NO production in a mouse vascular endothelial cell line and does not induce the expression of iNOS. This indicates that IFN-γ and LPS, which are unable to trigger iNOS by themselves, can activate iNOS only when they are combined. This is inconsistent with our finding in the present study. We demonstrated for the first time that IFN-γ at a high concentration might trigger the expression of iNOS in vascular endothelial cells by itself. Considering that IFN-γ itself initiates the activation of iNOS in vascular endothelial cells, IFN-γ might play a central role in switching from cNOS to iNOS induction of NO production in vascular endothelial
cells. LPS and TNF-α exclusively modulate the activity of iNOS as the amplifier.

Treatment with LPS or TNF-α alone reduced NO production in END-D cells. The reduction in NO production was not due to damages to END-D cells by LPS and TNF-α. Therefore, we suggested that LPS and TNF-α down-regulated the activity of cNOS and reduced NO production, because unstimulated END-D cells did not express iNOS. Furthermore, LPS appeared to inhibit the induction of iNOS by IFN-γ, since prior treatment of END-D cells with LPS abolished IFN-γ-mediated enhancement of NO production. Normal endothelium secretes a low level of NO that may inhibit cellular adhesion and contribute to the maintenance of an antithrombogenic surface (22). Reduced NO production by LPS, TNF-α, and low concentrations of IFN-γ might result in the adhesion of inflammatory cells, including monocytes and neutrophils, to vascular endothelial cells. On the other hand, LPS and TNF-α can induce the expression of iNOS in those inflammatory cells and enhance NO production markedly (20, 26). It is possible that LPS shifts NO production from vascular endothelial cells to inflammatory cells in such conditions.

SB203580, a highly specific p38 MAPK inhibitor, completely blocked the iNOS-mediated NO production by IFN-γ and combined IFN-γ and LPS. This indicated that p38 MAPK might be involved in the induction of iNOS by IFN-γ. The involvement of ERK1/2 MAPK or tyrosine kinase was unclear since treatment with PD98059 or genistein suspended in dimethyl sulfoxide was harmful to END-D cells. Recently, SB203580 has been reported to inhibit the induction of iNOS by LPS in a macrophage cell line (1, 5, 6, 8). The p38 MAPK and c-Jun NH2-terminal kinase/stress-activated protein kinase are known to be key molecules in the signaling of LPS (13, 14, 18) and IFN-γ (7, 15, 30), respectively. In the present study, we demonstrated for the first time that p38 MAPK might be involved in the signaling of IFN-γ-mediated iNOS expression in vascular endothelial cells. Further studies are needed to clarify the exact relationship between IFN-γ-induced expression of iNOS and p38 MAPK.

The present study suggested the presence of a complicated regulation of iNOS expression in vascular endothelial cells by...
proinflammatory cytokines and LPS. However, it was unclear whether the in vitro findings in the present study could be applied to the in vivo phenomenon or not. Killer (cytotoxic) NO is synthesized by iNOS, whereas signal (messenger) NO is synthesized by cNOS. Recently, we have reported the participation of NO and peroxynitrite in LPS-induced hepatic injury in d-galactosamine-sensitized mice (23). The expression of iNOS was detected in vascular endothelial cells and hepatocytes of the livers. Further, NO also plays an important role in the killing of pathogenic microorganisms (12, 19, 27). The exact role of NO produced in vivo by iNOS in vascular endothelial cells stimulated with IFN-γ, TNF-α, and LPS alone or in combination is still a matter for speculation.

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REFERENCES


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