**Introduction**

Nitric oxide (NO), a bioactive free radical, is involved in various physiological and pathological processes in many organ systems, including the brain (1, 2). At low concentrations, NO plays a role in neurotransmission and vasodilation; and these physiological events are exerted by an increase in the guanosine-3',5'-cyclic monophosphate (cGMP) concentration via activation of soluble guanylate cyclase (sGC) (3–7). NO is enzymatically formed from arginine by the enzyme NO synthase (NOS). This enzyme exists in 2 forms. One is the constitutive form, which is present in neurons (nNOS) and endothelial cells (eNOS) and is a calcium-dependent enzyme (4, 8). The other is the inducible form (iNOS), which is expressed in various cell types including microglial cells in response to a wide variety of stimuli, is regulated mainly at the transcriptional level, and does not require calcium for its activity (9). Microglial cells are the resident macrophage-like cells in the central nervous system (CNS). They play a pivotal role in the innate immune response of the CNS and are the first line of defense against invasion by microorganisms and against injury (10). Microglial cells in the healthy brain do not express iNOS; but following ischemic, traumatic, neurotoxic or inflammatory damage, they become activated to produce iNOS and to release a large amount of NO (11–14). The excess production of NO causes neuronal apoptosis after acute traumatic spinal cord injury, provokes delayed neuronal death following acute injury in the striatum, and induces delayed neurotoxicity following brain ischemia (13, 15–17). It appears that NO production may be regulated by NO, but the mechanism is poorly understood.

In the present study, we examined the effect of an NO donor on NO production induced by lipopolysaccharide...

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**Abstract.** The present study examined the effect of the nitric oxide (NO) donor NOC18 on lipopolysaccharide (LPS)-induced NO production to investigate a regulation mechanism of NO production by microglial cells. LPS increased the levels of NO and inducible NO synthase (iNOS) protein in BV-2 murine microglial cells in a concentration-dependent manner. Pretreatment with NOC18 for 24 h concentration-dependently attenuated the LPS-induced iNOS protein expression and NO production. The inhibitory effect of NOC18 on LPS-induced NO production was partially blocked by LY83583, a soluble guanylate cyclase inhibitor. Pretreatment with dibutyryl guanosine-3',5'-cyclic monophosphate (DBcGMP), a cell-permeable cGMP analogue, for 24 h attenuated partially LPS-induced iNOS protein expression and NO production. Furthermore, the effects of LPS on iNOS and NO production were inhibited by the c-Jun N-terminal kinase (JNK) inhibitor SP600125, and LPS-induced phosphorylation of JNK and c-Jun was inhibited by NOC18 and DBcGMP. These results suggest that NO production by microglial cells is controlled by a negative feedback mechanism via the NO/cGMP signaling pathway.

**Keywords:** nitric oxide (NO), guanosine-3',5'-cyclic monophosphate (cGMP), microglia, inducible nitric oxide synthase (iNOS), negative-feedback regulation
(LPS) to investigate a possible negative-feedback regulation of NO production in microglial cells. We found that NO attenuated the LPS-induced NO production in BV-2 cells, a murine microglial cell line, by inhibiting the iNOS protein induction via cGMP-dependent mechanisms and that the NO/cGMP signaling pathway attenuated LPS-induced c-Jun N-terminal kinase (JNK) activation, which was important for the iNOS protein induction.

Materials and Methods

Antibodies and chemicals

Anti-iNOS polyclonal antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho-JNK, anti-JNK, and anti-phospho-c-Jun polyclonal antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). LPS, the NOS inhibitor N\(^\text{G}\)-nitro-L-arginine methyl ester (L-NAME), and the cell-permeable cGMP analog dibutyryl-cGMP (DBcGMP) were obtained from Sigma (St. Louis, MO, USA). The sGC inhibitor LY83583, the mitogen-activated protein kinase (MEK)/extracellular-signal-regulated kinase (ERK) pathway inhibitor PD98059, and the p38 MAP kinase inhibitor SB203580 were purchased from Calbiochem (San Diego, CA, USA). The JNK inhibitor SP600125 was obtained from Tocris Bioscience (Ellisville, MO, USA). All other chemicals were purchased from Wako Pure Chemical (Osaka).

Cell culture and treatment with drugs

Clonal murine microglial cell line BV-2 was generously donated by Dr. E. Blasi (18). BV-2 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal calf serum (Gibco-BRL, Gaithersburg, MD, USA) containing 100 \(\mu\)g/mL streptomycin, 100 IU/mL penicillin, and 1 mg/mL fungizone in a humidified atmosphere of 95% air – 5% CO\(_2\) at 37°C. The cells were plated at a density of \(1 \times 10^5\)/well in 24-well tissue culture plates for the Griess assay or at \(6 \times 10^5\) in 60 mm-diameter dishes for Western blotting. LY83583 was added 1 h before exposure to NOC18; and PD98059, SB203580, or SP600125 was added 1 h before exposure to 0.1 \(\mu\)g/mL LPS.

Griess assay

For verification of NO production, nitrite, a stable end product of NO metabolism, was measured in cell-free supernatants by using the Griess reagent (Model 680; Bio-Rad, Hercules, CA, USA), and nitrite concentrations were determined with reference to a calibration curve prepared with sodium nitrite standards.

Western blot analysis

To prepare cell lysates for immunodetection of iNOS, we lysed BV-2 cells in a buffer consisting of 50 mM Tris-HCl (pH 7.4), 150 mM sodium chloride, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate (SDS), 10 \(\mu\)g/mL aprotinin, and 100 \(\mu\)M PMSF. For preparation of cell lysates for immunodetection of phospho-JNK, JNK, and phospho-c-Jun, BV-2 cells were lysed in a lysis buffer consisting of 20 mM Tris-HCl (pH 7.5), 2% SDS, 10 mM sodium fluoride, 1 mM sodium vanadate, 10 \(\mu\)g/mL aprotinin, and 100 \(\mu\)M PMSF; and the cell lysate was then heat-denatured. Depending on the experiment, 10 – 30 \(\mu\)g of the cell lysate was blotted onto a polyvinylidene difluoride membrane after separation of the protein by SDS-PAGE. The membrane was then rinsed 3 times with Tris-buffered saline (TBS) and blocked for 30 min with 1% non-fat dry milk / TBS / 0.1% Tween-20. It was next incubated for 2 h at room temperature with the desired primary antibody (1:1000 dilution). Next, the membrane was rinsed 5 times with TBS / 0.1% Tween-20, and subsequently incubated for 1 h with horseradish peroxidase–conjugated anti-rabbit IgG polyclonal antibody (Cappel, West Chester, PA, USA). Signals were detected with a chemiluminescence detection kit (NEN Life Science Products, Boston, MA, USA).

Statistical evaluation

The results were expressed as the means ± S.E.M. obtained from 3 – 4 independent experiments. One-way ANOVA was used to test for differences between group means. When appropriate, post hoc multiple comparisons were performed to test for differences between experimental groups (Scheffe’s test or Dunnett’s test). When the \(P\) value was less than 0.05, the difference was considered to be significant.

Results

Exogenous NO attenuated LPS-induced NO production and iNOS protein expression

We estimated LPS-induced NO production of BV-2 cells by using the Griess assay. Treatment of the cells with LPS for 24 h increased NO production in a concentration-dependent manner (Fig. 1A). The iNOS protein level also increased concentration-dependently by LPS (Fig. 1; B and C). Next, we examined the effect of pretreatment of the cells with NOC18, an NO donor, which releases NO with a half-life of approximately 21
h in aqueous solution (19), on the LPS-induced NO production. NOC18 at a concentration up to 100 μM did not have any cytotoxicity towards the BV-2 cells (data not shown). Pretreatment of the cells with NOC18 for 24 h attenuated the LPS-induced NO production in a concentration-dependent manner (Fig. 1D). In addition, the LPS-induced increase in the level of iNOS protein also was attenuated concentration-dependent by pretreatment with NOC18 (Fig. 1: E and F). Pretreatment with NOC18 did not affect the basal levels of NO production and iNOS protein (data not shown). These results indicate that NO attenuated LPS-induced NO production by inhibiting the induction of iNOS protein in these microglial cells.

Endogenous NO inhibited the expression of iNOS protein

To determine whether endogenously produced NO would regulate the expression of iNOS protein, we exposed BV-2 cells to LPS in the absence or presence of a NOS inhibitor, L-NAME. The LPS-induced NO production was markedly inhibited by 3 mM L-NAME (Fig. 2A). L-NAME at 3 mM obviously enhanced the induction of iNOS protein by LPS (Fig. 2: B and C). These results indicate that NO production by LPS-activated microglia was negatively regulated via endogenously produced NO that inhibited the induction of iNOS protein.

NO inhibited LPS-induced NO production via cGMP signaling pathway

It has been demonstrated that many of the physiological actions of NO are mediated by an increase in the cGMP concentration in cells via the activation of sGC. Therefore, we investigated the involvement of the cGMP signaling pathway in the attenuation of LPS-induced NO production by NOC18. The sGC inhibitor LY83583 (1 μM) inhibited the attenuation of LPS-induced NO pro-

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**Fig. 1.** Pretreatment with NOC18 attenuated LPS-induced increase in the levels of NO and iNOS protein in microglial BV-2 cells. A – C) BV-2 cells were treated with LPS at the indicated concentrations for 24 h. The levels of nitrite (A) and iNOS protein (B) were then determined by performing the Griess assay and Western blotting, respectively. C) The level of iNOS protein was quantitated by densitometry and expressed as relative to the maximum expression level, which was arbitrarily set as 1.0. D – F) BV-2 cells were treated with NOC18 at the indicated concentrations for the 24 h. Then the cells were treated with 0.1 μg/mL LPS for 24 h. The levels of nitrite (D) and iNOS protein (E) were subsequently determined by performing the Griess assay and Western blotting, respectively. E) The level of iNOS protein was quantitated by densitometry and expressed as relative to the maximum expression level, which was arbitrarily set as 1.0. Results show the mean ± S.E.M. obtained from 4 independent experiments (A, C, D, and F) and blots representative of 4 independent experiments (B and E). *P < 0.05, significantly different from values without NOC18 pretreatment.
Inhibition of NO production increased the level of iNOS protein in microglia. BV-2 cells were treated for 24 h with 0.1 μg/mL LPS in the absence or presence of 0.3 – 3 mM L-NAME. A) The nitrite levels were determined by using the Griess assay. B) The level of iNOS protein was determined by Western blotting. C) The level of iNOS protein was quantitated by densitometry and expressed as relative to the maximum expression level, which was arbitrarily set as 1.0. Results show the mean ± S.E.M. obtained from 3 independent experiments (A and C) and a blot representative of 3 independent experiments (B). *P < 0.05, significantly different from values without L-NAME treatment.

Pretreatment with NO attenuated the LPS-induced increase in the levels of NO and iNOS protein in microglial BV-2 cells via the cGMP signaling pathway. A) BV-2 cells were left untreated or were treated for 24 h with 100 μM NOC18 in the absence or presence of 1 μM LY83583. Then the cells were treated or not with 0.1 μg/mL LPS for 24 h. The nitrite levels were determined by performing the Griess assay. Results show the mean ± S.E.M. obtained from 3 independent experiments. *P < 0.05, significant difference between bracketed values. B – D) BV-2 cells were treated with DBcGMP at the indicated concentrations for 24 h. Then the cells were treated with 0.1 μg/mL LPS for 24 h. The levels of nitrite (B) and iNOS protein (C) were determined by performing the Griess assay and Western blotting, respectively. D) The level of iNOS protein was quantitated by densitometry and expressed as relative to the maximum expression level, which was arbitrarily set as 1.0. Results show the mean ± S.E.M. obtained from 4 independent experiments (B and D) and a blot representative of 4 independent experiments (C). *P < 0.05, significantly different from value obtained with LPS but without DBcGMP pretreatment.
LPS-induced NO production via the cGMP signaling pathway.

**NO/cGMP signaling pathway attenuated LPS-induced NO production via inhibition of JNK activation**

MAP kinases, such as ERK, p38 MAP kinase, and JNK, have been shown to play important roles in the induction of iNOS in many types of cells (20 – 23). Therefore, we examined the effect of MAP kinase inhibitors on the LPS-induced NO production in BV-2 cells. Neither the ERK inhibitor PD98059 (20 μM) nor the p38 MAP kinase inhibitor SB203580 (20 μM) affected LPS-induced NO production.

**Fig. 4.** Effect of MAP kinase inhibitors on LPS-induced NO production and iNOS protein expression. BV-2 cells were left untreated (control) or were treated for 24 h with 0.1 μg/mL LPS in the absence or presence of PD98059 (PD), SB203580 (SB), or SP600125 (SP). The levels of nitrite (A) and iNOS protein (B) were determined by performing the Griess assay and Western blotting, respectively. C) The level of iNOS protein was quantitated by densitometry and expressed as relative to the maximum expression level, which was arbitrarily set as 1.0. Results show the mean ± S.E.M. obtained from 4 independent experiments (A and C) and a blot representative of 4 independent experiments (B). *P < 0.05, significantly different from 0.1 μg/mL LPS.

**Fig. 5.** Effect of pretreatment with NOC18 and DBcGMP on LPS-induced activation of JNK. Cells were treated with 100 μM NOC18 (A) or 1 mM DBcGMP (B) for 24 h. Then the cells were treated with 0.1 μg/mL LPS for the indicated times. The cells were then harvested, and the levels of phosphorylated JNK and c-jun were determined by Western blotting. C and D) The phosphorylation levels of JNK and c-jun were quantitated by densitometry and expressed as relative to the maximum increase, which was arbitrarily set as 1.0. Results show the mean ± S.E.M. obtained from 3 of 4 independent experiments (C and D) and a blot representative of 3 or 4 independent experiments (A and B). **P < 0.01, significantly different from values without NOC18 or DBcGMP pretreatment.
NO production (Fig. 4A). In contrast, SP600125 (20 μM), a JNK inhibitor, did reduce the LPS-induced NO production. The increase in the level of iNOS protein induced by LPS was also attenuated by SP600125 (20 μM), but not by PD98059 (20 μM) or SB203580 (20 μM, Fig. 4: B and C). These results indicate that the LPS-induced NO production and iNOS protein expression were mediated by the activation of JNK.

Next, we examined the effect of pretreatment with NOC18 and DBcGMP on the LPS-induced activation of JNK. Treatment of BV-2 cells with 0.1 μg/mL LPS resulted in the transient phosphorylation of JNK and its substrate c-Jun (Fig. 5). The phosphorylation of both JNK and c-Jun was maximal at 30 – 60 min and then returned to the basal level. Pretreatment with 100 μM NOC18 for 24 h significantly attenuated the LPS-induced phosphorylation of JNK and c-Jun (Fig. 5: A, C, and D). Pretreatment with 1 mM DBcGMP also attenuated the LPS-induced phosphorylation of JNK and c-Jun (Fig. 5: B – D). These results indicate that the NO/cGMP signaling pathway attenuated LPS-induced NO production via inhibition of JNK activation.

Discussion

The major findings of the present study are that NO attenuates NO production of LPS-stimulated BV-2 cells by reducing the induction of iNOS protein via cGMP-dependent mechanisms and that the NO/cGMP signaling pathway attenuates the activation of JNK, which is important for LPS-induced iNOS protein production.

Since the overproduction of NO in the CNS is assumed to contribute to neuronal injury, NO production of microglial cells may be regulated properly by certain mechanisms to prevent the overproduction of NO. In this study, we demonstrated that pretreatment of BV-2 cells with NO attenuated NO production and iNOS protein expression induced by LPS (Fig. 1: D – F). In addition, l-NAME increased iNOS protein expression, while it inhibited LPS-induced NO production (Fig. 2). This finding suggests that l-NAME-induced decrease in NO levels may attenuate the inhibitory mechanism of iNOS expression by NO. Altogether, our results indicate that endogenously produced NO negatively regulated LPS-induced NO production by inhibiting iNOS protein expression in microglial BV-2 cells.

A number of physiological events induced by NO are elicited by cGMP-dependent mechanisms (3 – 5), while the inhibitory effects of NO on cytokine-induced iNOS protein expression in primary rat hepatocyte and rat vascular smooth muscle cells are through cGMP-independent mechanisms (24, 25). In this study, we demonstrated that the attenuation of NO production by NOC18 was negated in the presence of LY83583 (Fig. 3A). In addition, pretreatment with DBcGMP reduced both NO production and iNOS protein expression induced by LPS (Fig. 3: B – D), and pretreatment with YC-1, a sGC activator, also reduced both NO production and iNOS protein expression induced by LPS (data not shown). These results indicate that NO attenuates NO production and iNOS protein expression through cGMP-dependent mechanisms. The difference in cGMP dependency of the attenuation of iNOS protein expression by NO between the previous reports and our study could be explained by the differences in types of cells, NO donors, and cGMP analogues used in the experiments. In the present study, pretreatment with NOC18 or DBcGMP markedly attenuated LPS-induced iNOS protein expression (Figs. 1F and 3D), while the pretreatment only slightly decreased LPS-induced NO production. This discrepancy may be explained by the post-transcriptional modification of iNOS, since LPS induces the phosphorylation of iNOS in murine macrophages and the phosphorylation of iNOS is found to be associated with increased iNOS activity (26, 27). The NO/cGMP signaling pathway may not affect the LPS-induced post-transcriptional modification of iNOS. Further studies will be needed to elucidate the discrepancy between the iNOS protein level and the NO production.

MAP kinase has been shown to play important roles in LPS-induced iNOS expression in many types of cells (20 – 23). It also has been reported that LPS-induced iNOS expression is mediated by p38 MAP kinase in BV-2 cells (28). In contrast, our results demonstrated that the LPS-induced NO production and iNOS protein expression of BV-2 cells were inhibited by SP600125, but not by PD98059 or SB203580 (Fig. 4), indicating that LPS-induced NO production and iNOS protein expression were mediated by JNK, not by ERK or p38 MAP kinase. These differences may have been due to the difference in experimental conditions such as the concentrations of LPS and MAP kinase inhibitors. In this study, NOC18 and DBcGMP attenuated LPS-induced JNK activation in BV-2 cells (Fig. 5: A – C). Overall, these results indicate that the NO/cGMP signaling pathway attenuated NO production and iNOS protein expression by inhibiting JNK activation. It has been demonstrated that the transcription of iNOS is regulated by transcriptional factors AP-1 and NF-κB in microglial cells (29 – 33). JNK has been shown to phosphorylate c-Jun, resulting in an activation of AP-1 (34, 35). These reports and our findings suggest that the NO/cGMP signaling pathway attenuated LPS-induced iNOS expression at the transcriptional level by inhibiting AP-1 activation. In agreement with this idea, NO donors and/or cGMP analogues have been reported to attenuate the activation...
of AP-1 induced by endothelin-1 in murine macrophages, elicited by ischemia/reperfusion in rat livers, and stimulated by strain in rat mesangial cells (36 – 38). Furthermore, NO inhibits cytokine-induced NF-κB activation via cGMP-independent mechanisms in human endothelial cells and rat vascular smooth muscle cells (25, 39), suggesting that NO attenuates LPS-induced iNOS expression by inhibiting the NF-κB activation via cGMP-independent mechanisms in microglial cells. In this study, the sGC inhibitor LY83583 inhibited the attenuation of LPS-induced NO production by NOC18 (Fig. 3A). In addition, NOC18 and DBcGMP prevented LPS-induced phosphorylation of c-jun in BV-2 cells (Fig. 5: A, B, and D). Although we cannot exclude the possibility that the inhibition of NF-κB activation caused NO-induced attenuation of LPS-induced iNOS expression, our findings indicate that NO attenuated LPS-induced iNOS expression, in part, by inhibiting the AP-1 activation via cGMP-dependent mechanisms. Further studies will need to assess whether the inhibition of iNOS induction by the NO/cGMP signaling pathway in microglial cells was due to the inhibition of NF-κB activation. Previous reports have shown that AP-1 mediates the expression of proinflammatory cytokines in microglial cells stimulated by LPS (33, 34). Therefore, the attenuation of JNK activation by the NO/cGMP signaling pathway may play a significant role not only in the negative-feedback regulation of NO production but also in the regulation of gene expression of cytokines. In this context, it has been reported that NO donors and/or cGMP analogues reduce LPS-induced production of cytokines in human peripheral blood mononuclear cells, inhibit LPS-induced TNF-α production in mouse bone marrow macrophages, inhibit CpG-oligodeoxy nucleotide AAC-30–induced IFN-α production in human plasmacytid dendritic cells, and inhibit production of cytokines in anti-CD3–stimulated human T cells (40 – 43). The precise mechanism of attenuation of JNK activation by the NO/cGMP signaling pathway is now under investigation in our laboratory. One possibility is that activated JNK may be dephosphorylated/inactivated by phosphatases. In this context, it has been demonstrated that NO induces mitogen-activated protein kinase phosphatase-1 (MKP-1), a MAP kinase phosphatase that dephosphorylates/inactivates JNK, via a cGMP signaling pathway in rat primary vascular smooth muscle cells (44). The NO/cGMP signaling pathway may attenuate JNK activation of BV-2 cells via the induction of MAP kinase phosphatases such as MKP-1.

In summary, this study reveals that the NO/cGMP signaling pathway acted as a negative-feedback regulator of NO production in LPS-activated microglia. This signaling pathway operating in microglial cells may thus play an important role in the regulation of NO production during inflammation of the CNS.

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