Inhibition of Phosphatidylinositol 3-Kinase Induces Nitric-oxide Synthase in Lipopolysaccharide- or Cytokine-stimulated C6 Glial Cells*

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Nitric oxide (NO) produced by inducible nitric-oxide synthase (iNOS) in different cells including brain cells in response to proinflammatory cytokines plays an important role in the pathophysiology of demyelinating and neurodegenerative diseases. The present study underlines the importance of phosphatidylinositol 3-kinase (PI 3-kinase) in the expression of iNOS in C6 glial cells and rat primary astrocytes. Bacterial lipopolysaccharide (LPS) or interleukin-1β (IL-1β) was unable to induce the expression of iNOS and the production of NO in rat C6 glial cells. Similarly, wortmannin and LY294002, compounds that inhibit PI 3-kinase, were also unable to induce the expression of iNOS and the production of NO. However, a combination of wortmannin or LY294002 with LPS or IL-1β induced the expression of iNOS and the production of NO in C6 glial cells. Consistent with the induction of iNOS, wortmannin also induced iNOS promoter-derivered chimeraphenicol acetetyltransferase activity in LPS- or IL-1β-treated C6 glial cells. The expression of iNOS by LPS in C6 glial cells expressing a dominant-negative mutant of p85α, the regulatory subunit of PI 3-kinase, further supports the conclusion that inhibition of PI 3-kinase provides a necessary signal for the induction of iNOS. Next we examined the effect of wortmannin on the activation of mitogen-activated protein (MAP) kinase and nuclear factor NF-κB in LPS- or IL-1β-stimulated C6 glial cells. In contrast to the inability of LPS and IL-1β alone to induce the expression of iNOS, both LPS and IL-1β individually stimulated MAP kinase activity and induced DNA binding and transcriptional activity of NF-κB. Wortmannin alone was unable to activate MAP kinase and NF-κB. Moreover, wortmannin had no effect on LPS- or IL-1β-mediated activation of MAP kinase and NF-κB, suggesting that wortmannin induced the expression of iNOS in LPS- or IL-1β-stimulated C6 glial cells without modulating the activation of MAP kinase and NF-κB. Similar to C6 glial cells, wortmannin also stimulated LPS-mediated expression of iNOS and production of NO in astrocytes without affecting the LPS-mediated activation of NF-κB. Taken together, the results from specific chemical inhibitors and dominant-negative mutant expression studies demonstrate that apart from the activation of NF-κB, inhibition of PI 3-kinase is also necessary for the expression of iNOS and production of NO.

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Nitric oxide (NO), a vascular and neuronal messenger and a cytotoxic and cytostatic agent, is enzymatically formed from L-arginine by the enzyme nitric-oxide synthase (NOS). The NOS are basically divided into two forms. One constitutive form present in neurons (nNOS) and endothelial cells (eNOS) is a calcium-dependent enzyme, and the inducible form (iNOS) present in macrophages and astrocytes is regulated at the transcriptional level in response to stimuli (e.g. cytokine/lipopolysaccharide (LPS)) and does not require calcium for its activity (1, 2). Although the NO produced by iNOS accounts for the bactericidal and tumoricidal properties of macrophages, it is also of particular importance in the pathophysiology of inflammatory neurological diseases including demyelinating disorders (e.g. multiple sclerosis, experimental allergic encephalopathy, X-adrenoleukodystrophy) and in ischemia and traumatic injuries associated with infiltrating macrophages and the production of proinflammatory cytokines (3–8). It is now increasingly clear that glial cells in the central nervous system also produce NO in response to the induction of iNOS by bacterial LPS and a series of cytokines including interleukin-1β (IL-1β), tumor necrosis factor-α, and interferon-γ (IFN-γ). Astrocytes in the healthy brain do not express iNOS; however, after ischemic, traumatic, neurotoxic, or inflammatory damage the reactive astrocytes express iNOS in the mouse, rat, and human (9–13). NO derived from both astrocytes and macrophages is assumed to contribute to oligodendrocyte degeneration in demyelinating diseases and neuronal death during ischemia and trauma (3–5).

Characterization of the intracellular pathways required to transduce the signal from the cell surface to the nucleus for the induction of iNOS is an active area of investigation. Identification of the DNA binding site for nuclear factor (NF)-κB in the promoter region of iNOS (14) and inhibition of iNOS induction by inhibitors of NF-κB activation have established an essential role of NF-κB activation in the induction of iNOS (11–15). Suppression of NF-κB and inhibition of iNOS expression (16, 17) by inhibitors of tyrosine kinase in different cell types suggests the possible involvement of tyrosine phosphorylation in the activation of NF-κB and the induction of iNOS. Inhibition of LPS- and cytokine-induced activation of NF-κB and induction of iNOS by inhibitors of the mevalonate pathway and Ras farnesyl protein transferase also indicate that Ras may be involved in the activation of NF-κB and the induction of iNOS (11). Again, increasing cAMP and protein kinase A activity has

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The abbreviations used are: NO, nitric oxide; NOS, nitric-oxide synthase(s); iNOS, inducible NOS; LPS, lipopolysaccharide; IL-1β, interleukin-1β; IFN-γ, interferon-γ; NF-κB, nuclear factor κB; MAP kinase, mitogen-activated protein kinase; MEK, MAP kinase kinase; PI 3-kinase, phosphatidylinositol 3-kinase; DMEM, Dulbecco’s modified Eagle’s medium; CAT, chloramphenicol acetyltransferase; Erk, extracellular signal-regulated kinase; L-NMA, L-N-methylarginine.

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been shown to inhibit the activation of NF-xB and the induc-
ition of iNOS possibly because of the inhibition of Raf-1 (12).
Recently we have also observed that PD98059, an inhibitor of
mitogen-activated protein (MAP) kinase (MEK), the
kinase responsible for the activation of MAP kinase, inhibits
the LPS-induced activation of NF-xB and the induction of iNOS
in astrocytes, suggesting the possible involvement of the MAP
kinase pathway in the LPS- and proinflammatory cytokine-
mediated induction of iNOS (18). Taken together, these studies
suggest that any alteration of the Ras-Raf-MEK-MAP kinase
signal transduction pathway alters the activation of NF-xB and
so the induction of iNOS in astrocytes and C6 glial cells.

In this paper we present evidence that the signal mediated
by inhibition of phosphatidylinositol 3-kinase (PI 3-kinase)
induces/stimulates the expression of iNOS in LPS-
and cytokine-
stimulated C6 glial cells and rat primary astrocytes and that
the signal is not mediated via MAP kinase and NF-xB. Specif-
ic inhibitors of PI 3-kinase (wortmannin and LY294002) and
expression of the dominant-negative mutant of p85a, the
regulatory subunit of PI 3-kinase, induced the expression of iNOS
in LPS- and cytokine-stimulated C6 glial cells or stimulated the
expression of iNOS in rat primary astrocytes without modulating
the LPS- or cytokine-mediated activation of MAP kinase
and NF-xB, suggesting that apart from the activation of NF-xB
by LPS or cytokines, the inhibition of PI 3-kinase also provides
an essential signal for the expression of iNOS and production of
NO in C6 glial cells and astrocytes.

MATERIALS AND METHODS

Reagents—Recombinant rat IFN-γ, DMEM/F-12, fetal bovine serum,
Hanks’ balanced salt solution, and NF-xB DNA-binding protein detec-
tion kit were from Life Technologies, Inc. Human IL-1β was from
Genzyme, Escherichia coli and pyrrolidine dithiocarbamate were
purchased from Sigma. Phosphatidylinositol and phosphatidylserine
were purchased from Matreya Inc. Wortmannin, LY294002, and anti-
bodies against the regulatory subunit of PI 3-kinase (p85a) were ob-
tained from Calbiochem. Antibodies against mouse macrophage iNOS
were obtained from Transduction Laboratories, [γ^32P]ATP (3,000 Ci/
mmol) was from Amersham Pharmacia Biotech.

Induction of NO Production in Astrocytes and C6 Glial Cells—Astro-
cytes were prepared from rat cerebral tissue as described by McCarthy
and DeVellis (19). Cells were maintained in DMEM/F-12 medium con-
taining 10% fetal bovine serum. After 10 days of culture astrocytes were
separated from microglia and oligodendrocytes by shaking for 24 h in an
orbital shaker at 240 rpm. The shaking was repeated twice more after
a gap of 1 or 2 weeks before subculturing to ensure the complete
removal of all the oligodendrocytes and microglia. Cells were trypsin-
purchased from Invitrogen Corporation. Extracts of astrocytes were
obtained by using the Lipotaxi (Stratagene) method, as has been described in
manufacturer’s protocol. 24 h after transfection, cells were treated with
different stimuli for 14 h and harvested. The radiolabeled method was used to
assay CAT activity using a kit (Promega) as described in
manufacturer’s protocol.

Expression of the Dominant-negative Mutant of p85α in C6 Glial
Cells—The dominant-negative form of p85α, 35 amino acids in the
inter- SH2 region from residues 479–513 of wild type p85α, important
for binding the p110 subunit of PI 3-kinase, are deleted, and two other
amino acids (Ser-Arg) are inserted in this deleted position. The engi-
neering of the construct and description of the vector driving the
expression of the proteins have been published previously (23). C6 glial
cells were transfected with either the dominant-negative form of p85α
or an empty vector by Lipotaxi following manufacturer’s protocol. 24 h after
transfection, cells were treated with different stimuli.

Assay of PI 3-Kinase—After stimulation in serum-free DMEM/F-12
cells were lysed with ice-cold lysis buffer containing 1% w/v Nonidet
P-40, 100 mM NaCl, 20 mM Tris (pH 7.4), 10 mM iodoacetamide, 10 mM
NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride,
chloroform, and pyridoxal phosphatase were added, and the resulting mixture was further incubated for 1 h at
4 °C. Protein G-Sepharose beads were added, and the mixture was incubated for 1 h at
4 °C, bound proteins were washed twice with lysis buffer, once
with phosphate-buffered saline, once with 0.5 mM LiCl and 100 mM Tris
(pH 7.6), once in water, and once in kinase buffer (5 mM MgCl2, 0.25 mM
EDTA, 20 mM HEPES (pH 7.4)). PI 3-kinase activity was determined as
described (24) using a lipid mixture of 100 μl of 0.1 mg/ml PI and 0.1
mg/ml phosphatidylserine dispersed by sonication in 20 mM HEPES
(pH 7.0) and 1 mM EDTA. The reaction was initiated by the addition of
20 μl of [γ^{32}P]ATP (3,000 Ci/mmol; NEN Life Science Products) and
100 μM ATP and terminated after 15 min by the addition of 80 μl of
1 N HCl and 200 μl of chloroform:methanol (1:1). Phospholipids were
separated by TLC and visualized by exposure to iodine vapor and
autoradiography (24).

Assay of MAP Kinase—Cells were lysed directly with 2 × SDS
sample buffer, and the lysates were boiled, electrophoresed in 4–20% gradi-
et gels, transferred onto nitrocellulose membranes, and immuno-
blotted with phospho-specific MAP kinase antibody (New England
Biolabs). Phospho-specific p44/p42 MAP kinase antibody detects p42 and
p44 MAP kinase (Erk1 and Erk2) only when activated by phosphoryl-
ation at Tyr-204.

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift
Assay—Nuclear extracts from stimulated or unstimulated astrocytes
(1 × 10^6 cells) were prepared using the method of Dignam et al. (25)
with slight modification. Cells were harvested, washed twice with ice-
cold phosphate-buffered saline, and lysed in 400 μl of buffer A (10 mM
HEPES (pH 7.9), 10 mM KCl, 2 mM MgCl2, 0.5 mM dithiothreitol, 1 mM
phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml pepstatin A,
and 5 μg/ml leupeptin) containing 1% Nonidet P-40, 100 mM NaCl,
TABLE I

| Stimuli                  | Nitrite (nmol/mg/24 h) |
|-------------------------|------------------------|
| Control                 | 3.4 ± 0.3              |
| LPS only                | 3.7 ± 0.4              |
| Wort only               | 3.5 ± 0.35             |
| LYP29 only              | 3.3 ± 0.25             |
| LPS + Wort              | 32.6 ± 3.6             |
| LPS + LYP29             | 30.2 ± 2.8             |
| LPS + Wort + l-NMA      | 5.1 ± 0.6              |
| LPS + LYP29 + l-NMA     | 4.9 ± 0.5              |
| LPS + Wort + arginase   | 5.8 ± 0.4              |
| LPS + LYP29 + arginase  | 5.2 ± 0.7              |

**RESULTS**

**Inhibitors of PI 3-Kinase (Wortmannin and LY294002)**—Induce the Expression of iNOS and Production of NO in LPS-stimulated Rat C6 Glial Cells—We investigated the effect of specific inhibitors of PI 3-kinase (wortmannin and LY294002) on the induction of iNOS and production of NO in C6 glial cells. C6 glial cells were cultured in serum-free DMEM/F-12 in the presence of LPS and inhibitors of PI 3-kinase. Consistent with previous observations (12, 13, 16, 20, 28), the bacterial LPS or cytokines alone did not induce the production of NO in C6 glial cells (Table I). Wortmannin and LY294002 alone were also unable to induce the production of NO; however, addition of these inhibitors along with LPS induced the production of NO as nitrite by about 8–10-fold (Table I). Inhibition of this NO production by arginase, an enzyme that degrades the substrate (L-arginine) of NOS, and L-NMA, a competitive inhibitor of arginase, 100 units/ml.

FIG. 1. Wortmannin and LY294002 dose-dependently induced the production of NO. Cells incubated in serum-free DMEM/F-12 received different concentrations of wortmannin (Wort) or LY294002 (LY) along with 0.5 μg/ml LPS. Panel A, after 24 h, the concentration of nitrite was measured by the supernatants as described under “Materials and Methods.” Data are the mean ± S.D. of three different experiments. Panel B, cell homogenates were electrophoresed, transferred onto nitrocellulose membrane, and immunoblotted with antibodies against mouse macrophage iNOS as described under “Materials and Methods.”
Inhibition of PI 3-Kinase Induces iNOS

Expression of a Dominant-negative Mutant of p85α Induces the Expression of iNOS in LPS-stimulated C6 Glial Cells—Induction of NOS by wortmannin or LY294002, inhibitors of PI 3-kinase, in LPS- or cytokine-stimulated C6 glial cells suggests that inhibition of PI 3-kinase activity may provide an essential signal for the expression of iNOS. To confirm this observation that inhibition of PI 3-kinase is able to induce the expression of iNOS in LPS-treated C6 glial cells, we transfected C6 glial cells with a dominant-negative mutant of p85α. PI 3-kinase is a heterodimer consisting of 85-kDa (p85) and 110-kDa (p110) subunits where p85 is the regulatory subunit that links PI 3-kinase activity in the catalytic subunit (p110) to the tyrosine-phosphorylated proteins. Expression of a dominant-negative mutant of p85α, in which the inter-SH2 region required for binding of the p110 subunit is disrupted, results in the inhibition of PI 3-kinase activity in different cell types including adipocytes and Chinese hamster ovary cells (23, 29). We have also found that expression of the same dominant-negative mutant of p85α in C6 glial cells inhibited the lipid kinase activity of PI 3-kinase, but expression of the control empty vector had no effect (Fig. 3), indicating that the overexpressed dominant-negative mutant protein of p85α did not associate with the catalytic subunit of PI 3-kinase. In control C6 cells as well as in vector-transfected cells, LPS was unable to induce the production of NO and the expression of iNOS protein (Fig. 4). However, LPS induced the production of NO and the expression of iNOS protein in C6 cells transfected with the dominant-negative mutant of p85α (Fig. 4), suggesting that inhibition of PI 3-kinase activity is sufficient to induce the expression of iNOS in LPS-treated C6 glial cells.

Wortmannin Induces the Expression of iNOS in LPS- or IL-1β-treated C6 Glial Cells without Modulating the Activation of MAP Kinase and NF-κB—Because the activation of NF-κB is necessary for the expression of iNOS (11–18), and PD98059, an inhibitor of MEK, inhibits the LPS-induced expression of iNOS in astrocytes (18), to understand the basis of wortmannin-induced expression of iNOS in LPS- or IL-1β-treated C6 glial cells, we examined the effect of wortmannin on the activation of MAP kinase and NF-κB. Treatment of C6 glial cells with LPS alone resulted in the time-dependent activation of both Erk1 and Erk2 as evident from the Western blot analysis of stimulated C6 glial cells with antibodies against tyrosine-phosphorylated MAP kinase (Fig. 5). This activation was maximum after 10 min of treatment; however, with the increase in time of

Fig. 2. Effect of wortmannin on the expression of iNOS in LPS- or cytokine-treated C6 glial cells. Panel A, cells incubated in serum-free DMEM/F-12 received LPS and cytokines in the presence or absence of wortmannin. After a 24-h incubation, nitrite concentrations were measured in the supernatants. Data are the mean ± S.D. of three different experiments. Panel B, cell homogenates were electrophoresed, transferred onto nitrocellulose membrane, and immunoblotted with antibodies against mouse macrophage iNOS as described before. Panel C, after a 6-h incubation, cells were taken out directly from the plates for isolation of total RNA, and Northern blot analysis for iNOS mRNA was carried out as described under “Materials and Methods.” GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Panel D, cells were transfected with the construct containing the iNOS promoter fused to the CAT gene using Lipotaxi. 24 h after transfection, cells received LPS and cytokines in the presence or absence of wortmannin (Wort); after 14 h of stimulation, CAT activity was measured. Data are the mean ± S.D. of three different experiments. The concentrations of the different stimuli were as follows: LPS, 0.5 μg/ml; IL-1β, 50 ng/ml; IFN-γ, 50 units/ml; wortmannin, 300 nM.

Fig. 3. Expression of a dominant-negative mutant of p85α inhibits the lipid kinase activity of PI 3-kinase in C6 glial cells. Cells were transfected with various concentrations of a dominant-negative mutant of p85α and the control empty vector using Lipotaxi as described under “Materials and Methods.” After 24 h of transfection, cells were maintained in serum-free media for 24 h, and the lipid kinase activity of PI 3-kinase of was determined in cell lysates as described under “Materials and Methods.”

to induce the CAT activity in IFN-γ-treated cells. These results indicate that inhibition of PI 3-kinase by wortmannin is able to provide a necessary signal for the transcription of the iNOS gene in LPS- or IL-1β-stimulated C6 cells.

Expression of a Dominant-negative Mutant of p85α Induces the Expression of iNOS in LPS-stimulated C6 Glial Cells—Induction of NOS by wortmannin or LY294002, inhibitors of PI 3-kinase, in LPS- or cytokine-stimulated C6 glial cells suggests that inhibition of PI 3-kinase activity may provide an essential signal for the expression of iNOS. To confirm this observation that inhibition of PI 3-kinase is able to induce the expression of iNOS in LPS-treated C6 glial cells, we transfected C6 glial cells with a dominant-negative mutant of p85α. PI 3-kinase is a heterodimer consisting of 85-kDa (p85) and 110-kDa (p110) subunits where p85 is the regulatory subunit that links PI 3-kinase activity in the catalytic subunit (p110) to the tyrosine-phosphorylated proteins. Expression of a dominant-negative mutant of p85α, in which the inter-SH2 region required for binding of the p110 subunit is disrupted, results in the inhibition of PI 3-kinase activity in different cell types including adipocytes and Chinese hamster ovary cells (23, 29). We have also found that expression of the same dominant-negative mutant of p85α in C6 glial cells inhibited the lipid kinase activity of PI 3-kinase, but expression of the control empty vector had no effect (Fig. 3), indicating that the overexpressed dominant-negative mutant protein of p85α did not associate with the catalytic subunit of PI 3-kinase. In control C6 cells as well as in vector-transfected cells, LPS was unable to induce the production of NO and the expression of iNOS protein (Fig. 4). However, LPS induced the production of NO and the expression of iNOS protein in C6 cells transfected with the dominant-negative mutant of p85α (Fig. 4), suggesting that inhibition of PI 3-kinase activity is sufficient to induce the expression of iNOS in LPS-treated C6 glial cells.

Wortmannin Induces the Expression of iNOS in LPS- or IL-1β-treated C6 Glial Cells without Modulating the Activation of MAP Kinase and NF-κB—Because the activation of NF-κB is necessary for the expression of iNOS (11–18), and PD98059, an inhibitor of MEK, inhibits the LPS-induced expression of iNOS in astrocytes (18), to understand the basis of wortmannin-induced expression of iNOS in LPS- or IL-1β-treated C6 glial cells, we examined the effect of wortmannin on the activation of MAP kinase and NF-κB. Treatment of C6 glial cells with LPS alone resulted in the time-dependent activation of both Erk1 and Erk2 as evident from the Western blot analysis of stimulated C6 glial cells with antibodies against tyrosine-phosphorylated MAP kinase (Fig. 5). This activation was maximum after 10 min of treatment; however, with the increase in time of
were transfected with various concentrations of a dominant-negative mutant of p85α and the control empty vector using Lipotaxi as described. After 24 h of transfection, cells were stimulated with LPS (0.5 μg/ml) for 24 h, and nitrite concentrations (panel A) were measured in the supernatants as described. Data are the mean ± S.D. of three different experiments. Panel B, cell homogenates were electrophoresed, transferred onto nitrocellulose membrane, and immunoblotted with antibodies against tyrosine-phosphorylated MAP kinase as described under "Materials and Methods." Lanes 1–4 represent nuclear extract of control cells, nuclear extract of LPS-treated cells, nuclear extract of LPS-treated cells incubated with a 50-fold excess of unlabeled oligonucleotide, and nuclear extract of LPS-treated cells incubated with a 100-fold excess of unlabeled oligonucleotide. The upper arrow indicates the induced NF-κB band, and the lower arrow indicates the unbound probe.

**FIG. 4.** Expression of a dominant-negative mutant of p85α induces the expression of iNOS in LPS-treated C6 glial cells. Cells were transfected with various concentrations of a dominant-negative mutant of p85α and the control empty vector using Lipotaxi as described. After 24 h of transfection, cells were stimulated with LPS (0.5 μg/ml) and quantitated by densitometry (see Fig. 6). Wortmannin, capable of inducing the expression of iNOS in LPS- and IL−1β-stimulated C6 cells, had no effect on LPS- and IL−1β-mediated phosphorylation of MAP kinase (see Fig. 6), suggesting that wortmannin induced the expression of iNOS in LPS- or IL−1β-treated C6 cells without modulating the MAP kinase pathway. Next we examined the effect of wortmannin on the activation of NF-κB. Activation of NF-κB was monitored by both DNA binding as well as transcriptional activity of NF-κB. The DNA binding activity of NF-κB was evaluated by the formation of a distinct and specific complex in a gel shift DNA binding assay. Treatment of C6 glial cells with 0.5 μg/ml LPS resulted in the induction of DNA binding activity of NF-κB (Fig. 6). This gel shift assay detected a specific band in response to LPS which was competed off by an unlabeled probe (Fig. 6). In contrast to the inability of LPS or IL−1β to induce the expression of iNOS, both of these stimuli induced the DNA binding activity of NF-κB (Fig. 6). Wortmannin alone neither induced the DNA binding activity of NF-κB nor modulated the LPS- and IL−1β-mediated DNA binding activity of NF-κB (Fig. 6). We then tested the effect of wortmannin on NF-κB-dependent transcription of luciferase in C6 glial cells in the presence or absence of LPS and cytokines, using the expression of luciferase from a reporter construct, pNF-κB-Luc (Stratagene), as an assay. Consistent with the effect of wortmannin on DNA binding activity of NF-κB, wortmannin alone did not induce the NF-κB-dependent transcription of luciferase, and it also had no effect on the magnitude of LPS- and IL−1β-induced transcriptional activity of NF-κB (Fig. 7C). On the other hand, consistent with the inability of IFN-γ to induce the expression of iNOS in C6 glial cells, IFN-γ did not induce the DNA binding or transcriptional activity of NF-κB, whereas the combination of LPS and IFN-γ was able to induce the DNA binding as well as transcriptional activity of NF-κB (Fig. 7B and C) and the induction of iNOS (Fig. 2). To examine whether wortmannin-induced expression of iNOS in LPS- or cytokine-treated C6 cells requires the activation of NF-κB, we studies the effect of pyrrolidine dithiocarbamate, an antioxidant inhibitor of NF-κB activation, on the induction of iNOS and the activation of NF-κB in cells treated with the combination of LPS and wortmannin. Pyrrolidine dithiocarbamate inhibited
the activation of NF-κB and the induction of NO production in LPS- and wortmannin-treated C₆ glial cells (Fig. 8). Taken together, these studies indicate that activation of NF-κB is necessary but not sufficient for the induction of iNOS, and the signal induced by inhibition of PI 3-kinase by wortmannin for the induction of iNOS is not mediated via activation of MAP kinase and NF-κB.

Inhibition of PI 3-Kinase Is Necessary for the Expression of iNOS in C₆ Glial Cells—Because inhibitors of PI 3-kinase induced the expression of iNOS in LPS- or IL-1β-treated C₆ cells, we sought to examine whether inhibition of PI 3-kinase is necessary for the expression of iNOS in C₆ cells. Cells treated with LPS and IL-1β alone or in combination, for different time intervals were assayed for the lipid kinase activity of PI 3-kinase. Although LPS or IL-1β alone had no effect on PI 3-kinase activity (Fig. 9, A and B), the combination of LPS and IL-1β inhibited the activity of PI 3-kinase within 5–10 min of incubation (Fig. 9C). Consistent with the inhibitory effect of wortmannin on PI 3-kinase activity in other cell types (30, 31), wortmannin inhibited the lipid kinase activity of PI 3-kinase in LPS-treated C₆ cells (Fig. 9D). These results indicate that inhibition of PI 3-kinase activity may be necessary to induce the expression of the iNOS gene in C₆ glial cells.

Wortmannin Stimulates the LPS-induced Production of NO in Rat Primary Astrocytes without Modulating the Activation of NF-κB—Because wortmannin induces the production of NO and the expression of iNOS in LPS- or cytokine-treated C₆ glial cells without modulating the activation of NF-κB, we investigated the effect of wortmannin on LPS-induced production of NO and the activation of NF-κB in rat primary astrocytes. In sharp contrast to the inability of LPS to induce the expression
of iNOS and the production of NO in C6 glial cells, LPS alone was able to induce the expression of iNOS and the production of NO in rat primary astrocytes as reported previously (12, 13, 16, 20, 26). Fig. 10 shows that LPS alone induced the production of NO, and the activation of NF-κB in rat primary astrocytes and wortmannin alone was unable to induce the production of NO and the activation of NF-κB. However, wortmannin markedly stimulated the LPS-induced production of NO (Fig. 10A) without modulating the degree of activation of NF-κB (Fig. 10B), suggesting that similar to C6 glial cells the wortmannin-induced stimulation of NO production in primary astrocytes is also not caused by the stimulation of NF-κB activation.

Effect of Inhibitors of PI 3-Kinase on Cell Viability—C6 glial cells or astrocytes were incubated with different concentrations of wortmannin and LY294002 for 24 h, and their viability was determined as measured by the 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. None of the inhibitors at the concentrations used in this study decreased or increased the viability of the cells (data not shown). Therefore, stimulation of the expression of iNOS in C6 and astrocytes by inhibitors of PI 3-kinase is not caused by any change in viability of the cells.

DISCUSSION

The signaling events transduced by proinflammatory cytokines and LPS for the induction of iNOS are poorly understood. A complete understanding of the cellular signaling mecha-
nisms involved in the induction of iNOS should identify novel targets for the therapeutic intervention in NO-mediated neuroinflammatory diseases. Recently PI 3-kinase-associated signaling events have been shown to prevent apoptosis in a number of cell types including cerebellar granule neurons (30) and hematopoietic cells (31). Several lines of evidence presented in this study support the conclusion that the inhibition of PI 3-kinase activity, independent of the activation of MAP kinase, phosphorylates the NH2-terminal regulatory domain of IκB, causing a transient inhibition of PI 3-kinase, and induced the expression of iNOS in C6 glial cells. Our hypothetical model depicting the signals for the biosynthesis of iNOS in C6 glial cells is summarized in Fig. 11. Consistent with the apoptotic activity of NO (32, 33) and the antiapoptotic activity of activated PI 3-kinase (30, 31), the observed up-regulation of LPS- or cytokine-induced expression of iNOS and production of NO in both C6 glial cells and rat primary astrocytes by inhibitors of PI 3-kinase indicates that PI 3-kinase may function as a negative regulator in the induction of iNOS and that this property of PI 3-kinase may contribute to its antiapoptotic activity.

Proinflammatory cytokines (tumor necrosis factor-α, IL-1β, or IFN-γ) and LPS bind to their respective receptors and induce iNOS expression via activation of NF-κB (11–15, 34, 35). The presence of a consensus sequence in the promoter region of iNOS for the binding of NF-κB (14) and the inhibition of iNOS expression with the inhibition of NF-κB activation establishes an essential role of NF-κB activation in the induction of iNOS (11, 12, 15). Activation of NF-κB by various cellular stimuli involves the proteolytic degradation of IκBα and the concomitant nuclear translocation of the liberated NF-κB heterodimer (36, 37). Although the biochemical mechanism underlying the degradation of IκBα remains unclear, it appears that degradation of IκBα induced by various mitogens and cytokines occurs in association with the transient phosphorylation of IκBα on serines 32 and 36 (38). Upon phosphorylation, IκBα that is still bound to NF-κB apparently becomes a high affinity substrate for an ubiquitin-conjugating enzyme (39). After phosphorylation-controlled ubiquitination, the IκBα is rapidly and completely degraded by the 20S or 26S proteosome, and the NF-κB heterodimer is targeted to the nucleus (40). Recently, it has been reported that 90-kDa ribosomal S6 kinase (p90 RSK), a downstream candidate of the well characterized Ras-Raf-MEK-MAPK kinase pathway, phosphorylates the NHR-terminal regulatory domain of IκBα on serine 32 (41), suggesting the possible involvement of the MAP kinase pathway in the phosphoryla-

FIG. 10. Effect of wortmannin on the LPS-induced production of NO and activation of NF-κB in rat primary astrocytes. Cells incubated in serum-free DMEM/F-12 received different concentrations of wortmannin (Wort) in the presence or absence of 0.5 μg/ml of LPS. Panel A, after a 24-h incubation, nitrite concentrations were measured in supernatants. Data are the mean ± S.D. of three different experiments. Panel B, after 1-h incubation, cells were taken out to prepare nuclear extracts, and nuclear proteins were used for the electrophoretic mobility shift assay as described under "Materials and Methods."

FIG. 11. Hypothetical model describing the signaling pathways for the expression of iNOS in C6 glial cells. MAPK, MAP kinase.
Inhibition of PI 3-Kinase Induces iNOS

In the absence of inhibitors of protein phosphate 1/2A stimulate the expression of iNOS, induced by the combination of LPS and IFN-γ (19). On the other hand, cell-permeable ceramides analogs and lipoxigenase inhibitors that inhibit the p21^-activated protein kinase A, mevalonate inhibitors that inhibit primary astrocytes (18).

In summary, studies reported in this manuscript underscore the necessity of inhibition of PI 3-kinase in the LPS- or cytokine-mediated induction of iNOS. Moreover, the signal induced by inhibition of PI 3-kinase for the induction of iNOS is not mediated by MAP kinase or NF-κB.

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