The sphingomyelin signal transduction pathway is known to play a role in mediating the action of various cytokines. Here we examined the possible role of the sphingomyelin signaling pathway on lipopolysaccharide (LPS)- and cytokine-mediated production of NO and the expression of inducible nitric-oxide synthase (iNOS). Sphingomyelinase (SMase) treatment of astrocytes increased the cellular levels of ceramide without the induction of NO production. However, incubation of LPS or cytokine-stimulated astrocytes with SMase or by increasing intracellular ceramide by cell-permeable ceramide analogs (C2- or C6-ceramide) or inhibitor of ceramide phosphohydrase (N-oleoyl ethanolamine) led to a time- and dose-dependent increase in the production of NO. This increase in NO production was accompanied by an increase in iNOS activity, iNOS protein, and iNOS mRNA. Similar to astrocytes, SMase or ceramide analogs also stimulated the LPS- and cytokine-mediated expression of iNOS in the C6 glial cell line. Since activation of NF-κB is necessary for the induction of iNOS, we examined the effect of SMase and C2-ceramide on the activation of NF-κB. Although SMase or C2-ceramide alone was ineffective in activating NF-κB, both stimulated the LPS-mediated activation of NF-κB in LPS-activated astrocytes. Inhibition of ceramide and LPS-mediated induction of iNOS by antioxidant inhibitors of NF-κB (N-acetylcysteine and pyrrolidine dithiocarbamate) suggest that the stimulatory effect of ceramide on the induction of iNOS is due to the stimulation of NF-κB activation and that cellular redox plays a role in the activation of NF-κB and induction of iNOS. Inhibition of LPS-mediated as well as LPS and ceramide-mediated induction of iNOS and activation of NF-κB by PD98059, a specific inhibitor of activation of mitogen-activated protein (MAP) kinase kinase (MEK), and FPT inhibitor II, a selective inhibitor of Ras farnesyl protein transferase, indicate that the Ras-MAP kinase pathway is involved in LPS-ceramide induced activation of NF-κB and induction of iNOS, and that ceramide-mediated signaling events probably converge into the LPS-modulated MAP kinase signaling pathway resulting in greater activation of NF-κB and iNOS induction. This study illustrates a novel role of the sphingomyelin-ceramide signaling pathway in stimulating the expression of iNOS via LPS- or cytokine-mediated activation of NF-κB in astrocytes.

The sphingomyelin pathway is a newly described signal transduction pathway mediating the action of several extracellular stimuli and leading to important biochemical and cellular responses (1, 2). This pathway is initiated by the activation of neutral sphingomyelinase (SMase) which hydrolyzes membrane sphingomyelin to ceramide and phosphocholine. Ceramide, has emerged as a second messenger molecule which is considered to mimic most of the cellular effects of TNF-α, IL-1β, and LPS in terminal differentiation, apoptosis, and cell cycle arrest (3, 4). These conclusions are primarily based on the ability of exogenous, cell-permeable, ceramide analogs and endogenous ceramide generated by sphingomyelin-ceramide signaling events specifically to cause cell differentiation and growth inhibition. Although the ceramide dependent pathway for signal transduction is not very well established so far, present knowledge indicates that a ceramide-mediated signal transduction pathway includes activation of a specific proline-directed Ser/Thr protein kinase (5), a specific protein phosphatase (1, 2) and protein kinase Cζ (6), and inhibition of phospholipase D (7).

Since bacterial lipopolysaccharide (LPS) and cytokines (TNF-α, IL-1β, and IFN-γ) induce the production of ceramide and mediate the induction of inducible nitric-oxide synthase (iNOS) (1, 2, 8–10) it was of interest to examine the relationship between the sphingomyelin signaling pathway and the expression of iNOS. Nitric oxide (NO), a product of iNOS, is a diffusible gas that plays a part in many physiological and diverse pathological conditions. At low concentration NO has been shown to play a role in neurotransmission and vasodilatation while at higher concentration it is neurotoxic. This is of particular importance in demyelinating conditions (e.g. multiple sclerosis, experimental allergic encephalopathy, advanced HIV encephalitis, X-adrenoleukodystrophy) and in ischemia and traumatic injuries associated with infiltrating macrophages and the production of proinflammatory cytokines (11–13), where astrocyte and microglia-derived NO could contribute to oligodendrocyte degeneration and neuronal death.

In the present study we examined the possible involvement of the sphingomyelin pathway in the induction or stimulation of iNOS in rat primary astrocytes and C6 glial cells. We report that SMase or cell-permeable ceramide analogs by themselves were unable to activate NF-κB and induce iNOS, however, SMase and ceramide markedly enhanced the LPS- and cytokine-mediated activation of NF-κB and induction of iNOS. Antioxidant inhibitors of NF-κB activation (N-acetylcysteine and pyrrolidine dithiocarbamate) inhibited the LPS and ceramide-mediated induction of iNOS, indicating that ceramide

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stimulates the LPS-mediated induction of iNOS by stimulating the activation of NF-kB and that cellular redox plays a role in the activation of NF-kB and induction of iNOS. Inhibition of LPS- and ceramide-mediated activation of NF-kB and induction of iNOS by PD98059, an inhibitor of MAP kinase kinase, and FPT inhibitor II, an inhibitor of Ras farnesyl protein transferase, suggest that ceramide potentiates LPS-mediated MAP kinase signaling pathways to stimulate activation of NF-kB and iNOS expression. The ability of ceramides to potentiates the LPS- or cytokine-mediated activation of NF-kB activation and expression of iNOS and production of NO in astrocytes indicate that sphingomyelin-ceramide signaling events may play a role in inflammatory neuropathies associated with the induction of iNOS and production of NO. To our knowledge, this is the first example of a positive modulatory role in the regulation of iNOS expression by ceramides in a cell.

Materials and Methods

Reagents—Recombinant rat IFN-γ, DMEM/F-12, and fetal bovine serum were from Life Technologies, Inc. Human IL-1β was from Genzyme. Mouse recombinant TNF-α was obtained from Boehringer Mannheim, Germany. Sphingomyelinase (Staphylococcus aureus), LPS (Escherichia coli), NADPH, FAD, tetrahydrobiopterin, N-acetylcysteine, pyrrolidine dithiocarbamate, and Dowex 50W were from Sigma.

\[ N\text{pH 6.6, 50 mM NaCl, 12.5 mM MgCl}_2, 1 \text{mM EGTA, 2 mM dithiothreitol,} \]

Staphylococcus aureus

acetic acid (100:100:100:40:36:2). A standard sample of ceramide was produced from 1–5 \( \times 10^5 \) cpm/nmol) for 30 min at room temperature. The labeled ceramide 1-phosphate was resolved with a solvent system consisting of methyl acetate, n-propanol, chloroform, methanol, 0.25% KCl in water: acetic acid (100:100:100:40:36:2). A standard sample of ceramide was phosphorylated under identical conditions and developed in parallel. Both standard and samples had identical \( R_f \) value (0.46). Quantification of ceramide 1-phosphate was carried out by autoradiography and densitometric scanning using Imaging Densitometer (Model GS-670; Bio-Rad) and software provided with the instrument by the manufacturer. Values are expressed either as arbitrary units (absorbance) or as percent change.

Assay for NO Synthesis—Synthesis of NO was determined by assaying of culture supernatants for nitrite, a stable reaction product of NO with molecular oxygen. Briefly, 400 \( \mu l \) of culture supernatant was aliquot and mixed with 200 \( \mu l \) of Griess reagent (17, 18) at room temperature for 15 min. The optical density of the assay samples was calculated from a standard curve derived from the reaction of NaNO₂ in the assay.

Assay for NOS Activity—NOS activity was measured directly by production of 1\( -[\text{2,3,4,5-}^3\text{H}]\text{citrulline from} \ 1\text{[2,3,4,5-}^3\text{H}]\text{arginine (17, 18). In these experiments, 50 \( \mu l \) of astrocyte homogenate was incubated at 37 °C in the presence of 50 mm Tris-HCl, pH 7.8, 0.5 mm NADPH, 5 \( \mu M \) FAD, 5 \( \mu M \) tetrahydrobiopterin, and 12 \( \mu M \) 1\( -[\text{2,3,4,5-}^3\text{H}]\text{arginine (115 mCi/mmol) in a total volume of 200 \( \mu l \). The reaction was stopped by the addition of 500 \( \mu l \) of ice-cold 20 molar HepES, pH 5.5, followed by the addition of 2 ml of Dowex 50W equilibrated in the same buffer. The samples were then centrifuged and the concentration of 1\( -[\text{2,3,4,5-}^3\text{H}]\text{citrulline was determined in the supernatant by liquid scintillation counting. Protein was measured by the procedure of Bradford (19).}

Immunoblot Analysis for iNOS—Following 24-h incubations in the presence or absence of different stimuli, astrocytes were scraped off, washed with Hank's buffer, and homogenized in 50 mm Tris-HCl, pH 7.4, containing protease inhibitors. After electrophoresis, the proteins were transferred onto a nitrocellulose membrane and the iNOS band was visualized by immunoblottting with antibodies against mouse macrophage iNOS and \( ^{35} \)S-labeled protein A as described earlier (18).

Northern Blot Analysis—Stimulated primary astrocytes were taken out from culture dishes directly by adding UltraSpec-II RNA reagent (Biotec Laboratories Inc.) and total RNA was isolated according to the manufacturer's protocol. For Northern blot analyses, 20 \( \mu g \) of total RNA was electrophoresed on 1.2% denaturing formaldehyde-agarose gels, and transferred onto a nitrocellulose membrane. The membrane was hybridized with a probe specifically complementary to a 177 bp segment of the iNOS cDNA. The cDNA probe was made by polymerase chain reaction amplification using two primers (forward primer, 5\' -CTCTTCTCAAGAGGGCACAATAA-A-3\'; reverse primer, 5\' -CACCTCCCTCAGGATGTTGT-3\') (18, 20).

Hybridization was at 68 °C with \( ^{32} \)P-labeled cDNA probe using ExpressHyb hybridization solution (CLONTECH) as described by the manufacturer. The cDNA probe was made by polymerase chain reaction amplification using two primers (forward primer, 5\' -CTCTTCTCAAGAGGGCACAATAA-A-3\'; reverse primer, 5\' -CACCTCCCTCAGGATGTTGT-3\') (18, 20).

After hybridization, filters were washed two to three times in solution I (2 \( \times \) SSC, 0.05% SDS) for 1 h at room temperature followed by solution II (0.1 \( \times \) SSC, 0.1% SDS) at 50 °C for another hour. The membranes were then dried and exposed to x-ray film (Kodak). The same filters were stripped and rehybridized with probes for glyceraldehyde-3-phosphate dehydrogenase. The relative mRNA content for iNOS was measured after scanning the bands with a Bio-Rad (Model GS-670) imaging densitometer.

Nuclear Run-on Assay—For the measurement of gene transcription, nuclei were prepared and in vitro transcriptional activity was measured with nuclei (25 \( \times 10^6 \) nuclei per assay) using 30 \( \mu Ci \) of \( ^{32} \)P-UTP (400 Ci/mmol) as described by Caira et al. (21). Briefly, the arrays were hybridized in hybridization buffer (50% formamide, 5 \( \times \) SSC, 1% SDS, 15% dextran sulfate, 1 \( \times \) Denhardt's solution, 50 \( \mu g/ml \) heparin). Following 24 h of hybridization in the above buffer, hybridization was carried out with the labeled RNAs (1.3 \( \times 10^6 \) cpm) at 42 °C for 60 h to 3 \( \mu M \) of the immobilized plasmid pGEM-T as a control or to
plasmids containing inserts of rat glyceraldehyde-3-phosphate dehydrogenase, rat actin, and human iNOS cDNAs. The filters were washed twice in 2× SSC, 0.1% SDS for 15 min at 42 °C and twice in 0.5× SSC, 0.1% SDS for 15 min. Then the filters were treated with RNase buffer (300 mM NaCl, 10 mM Tris-HCl, pH 7.4, 40 mM EDTA, 10 μg/ml RNase A, and 350 units/ml RNase T1) at 37 °C for 30 min and then in the same buffer without RNase for another 30 min and autoradiographed.

**TABLE I**

**Effect of SMase on arginine-dependent nitrite accumulation in LPS-stimulated rat primary astrocytes**

Astrocytes were cultured for 24 h in serum-free DMEM/F-12 medium with the listed reagents; and nitrite concentration in the supernatants was measured as described under “Materials and Methods.” Arginase (100 units/ml), N-methyl-L-arginine (l-NMA) (0.1 mM), and different concentrations of SMase were added to the cells together with LPS (1.0 μg/ml). Data are mean ± S.D. of three different experiments.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Nitrite nmol/mg/24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.2 ± 0.5</td>
</tr>
<tr>
<td>LPS only</td>
<td>30.1 ± 3.2</td>
</tr>
<tr>
<td>LPS + arginase</td>
<td>5.8 ± 1.2</td>
</tr>
<tr>
<td>LPS + l-NMA</td>
<td>6.1 ± 0.8</td>
</tr>
<tr>
<td>SMase (200 milliunits/ml)</td>
<td>3.7 ± 0.7</td>
</tr>
<tr>
<td>LPS + SMase (50 milliunits/ml)</td>
<td>61.6 ± 5.9</td>
</tr>
<tr>
<td>LPS + SMase (100 milliunits/ml)</td>
<td>86.5 ± 7.3</td>
</tr>
<tr>
<td>LPS + SMase (200 milliunits/ml)</td>
<td>89.3 ± 6.9</td>
</tr>
</tbody>
</table>

**RESULTS**

**Bacterial SMase and LPS Are Capable of Inducing the Production of Ceramide in Primary Rat Astrocytes**—Rat primary

**FIG. 2.** Stimulation of LPS-induced expression of iNOS by SMase in rat primary astrocytes. Cells incubated in serum-free DMEM/F-12 received different concentrations of SMase along with 1.0 μg/ml LPS. A, after 30 min of incubation, lipid content was measured as described under “Materials and Methods.” Results are expressed as mean ± S.D. of three different experiments. B, after 24 h, activity of iNOS was assayed in cell homogenates as mentioned in the methods section. Data are mean ± S.D. of three different experiments. C, cell homogenates were electrophoresed, transferred on nitrocellulose membrane, and immunoblotted with antibodies against mouse macrophage iNOS as mentioned in under “Materials and Methods.” D, after 6 h of incubation, cells were taken out directly by adding UltraSPEC-II RNA reagent (Biotex Laboratories Inc.) to the plates for isolation of total RNA, and Northern blot analysis for iNOS mRNA was carried out as described under “Materials and Methods.”
astrocytes were cultured in serum-free media with LPS and SMase, alone or in combination, for different times and the amount of ceramide was quantitated by the diacylglycerol kinase assay (16). LPS alone induced a significant increase in the ceramide content within 10–60 min of exposure (Fig. 1). Almost 2.5-fold increase in ceramide was observed after 30 min of exposure. Bacterial neutral SMase, the enzyme capable of degrading sphingomyelin to ceramide, alone also induced a marked generation of ceramide (8.0-fold after 10 min) demonstrating that under these experimental conditions SMase alone can degrade sphingomyelin and increase the level of ceramide to the same level as the combined effect of LPS and SMase.

Stimulation of LPS- and Cytokine-induced Expression of iNOS and Production of Nitric Oxide by SMase in Rat Primary Astrocytes—Rat primary astrocytes were cultured in serum-free media in the presence of LPS and SMase. The activity of iNOS was measured as production of nitrite, a soluble product of NO in the culture medium (Table I) and as conversion of arginine to citrulline (Fig. 2B). It is evident from Table I that in astrocytes, LPS (1 μg/ml) induced the production of nitrite by more than 7-fold. Both Nω-methyl-L-arginine, a competitive inhibitor of NOS, and arginase, an enzyme that degrades the substrate (l-arginine) for NOS, suppressed the LPS-mediated nitrite secretion suggesting that LPS-induced nitrite release in primary astrocytes is dependent on NOS-mediated arginine metabolism. SMase by itself was neither stimulatory nor inhibitory to nitrite production in astrocytes. However, SMase, when added with LPS, potently stimulated the LPS-mediated induction of nitrite production in astrocytes. Almost 3-fold stimulation was observed when SMase was used at a concentration of 100 or 200 milliunits/ml (Table I). Similar effects of SMase were also observed on cytokine-induced nitrite production in astrocytes (Fig. 3A). Addition of SMase stimulated the TNF-α, IL-1β, or IFN-γ-induced nitrite production in astrocytes by 3–4-fold. Consistent with the production of nitrite, the formation of l-citrulline from l-arginine in an enzymatic assay with astrocyte homogenate was also stimulated approximately 3-fold by treatment of LPS- or cytokine-stimulated astrocytes with SMase (100 milliunits/ml) (Figs. 2B and 3A). To understand the mechanism of the stimulatory effect of SMase on LPS- and/or cytokine-induced activation of iNOS in astrocytes, we examined the effect of SMase on the expression of iNOS protein and iNOS mRNA. Immunoblot analysis with antibodies against murine macrophage iNOS and Northern blot analysis for iNOS mRNA of LPS-stimulated astrocytes clearly showed that SMase enhanced the LPS-mediated induction of iNOS protein (Fig. 2C) and mRNA (Fig. 2D). To correlate the expression of iNOS with ceramide, we measured ceramide levels under these conditions. LPS, capable of inducing only modest generation of ceramide (2.5-fold after 30 min of incubation) (Fig. 2A) induced the expression of iNOS and production of NO. In contrast, SMase (200 milliunits/ml) itself capable of inducing marked generation of ceramide (7.5-fold after 30 min of incubation) (Fig. 2A) was ineffective in inducing the expression of iNOS or production of NO. However, increased ceramide level produced by SMase markedly stimulated LPS-mediated induction of iNOS and production of NO (Fig. 2). Similar stimulatory effects of SMase were also found on cytokine-induced expression of iNOS (Fig. 3). In primary astrocytes, TNF-α, IL-1β, or IFN-γ alone were able to induce the expression of iNOS and the addition of SMase along with cytokines stimulated the induction of iNOS expression.

Exogenous Ceramide Stimulates the LPS- or Cytokine-induced iNOS Expression in Rat Primary Astrocytes—Ceramide, the breakdown product of sphingomyelin, serves as the second messenger in the sphingomyelin pathway. To evaluate its effect on the expression of iNOS, the experiments described above for SMase studies were repeated with the cell-permeable C2- and C6-ceramide analogs. Similar to SMase, ceramide analogs alone did not induce the production of nitrite but addition of C2- or C6-ceramide to astrocytes along with LPS increased expression of iNOS mRNA, iNOS protein as well as production of NO (Fig. 4). This stimulatory effect peaked at 10 μM for C2-ceramide, C6-ceramide. To examine the specificity of ceramide, we studied the effect of C2-dihydroceramide, the inactive analog of C2-ceramide, on LPS-mediated production of NO. In contrast to C2-ceramide, C2-dihydroceramide was ineffective in stimulat-

![FIG. 3. Effect of SMase on cytokine-induced production of NO and expression of iNOS in rat primary astrocytes. Cells incubated in serum-free DMEM/F-12 received 100 milliunits/ml SMase along with different cytokines (TNF-α, 100 ng/ml; IL-1β, 100 ng/ml; IFN-γ, 200 units/ml). After 24 h of incubation, concentration of nitrite was measured in cell homogenates (A) and activity of iNOS was assayed in cell homogenates (B) as mentioned under “Materials and Methods.” Data are mean ± S.D. of three different experiments. C, cell homogenates were electrophoresed, transferred on nitrocellulose membrane, and immunoblotted with antibodies against mouse macrophage iNOS as mentioned under “Materials and Methods.” D, after 6 h of incubation, cells were analyzed for iNOS mRNA using the Northern blotting technique as described earlier. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.](http://www.jbc.org/)


ing the LPS-induced production of nitrite (Fig. 5). A 10 μM concentration of ceramide produced a maximal effect on LPS-induced production of NO, however, a higher concentration of ceramide (25 μM) was ineffective in inducing the production of NO.

Oleylethanolamine (OE), an inhibitor of ceramidase, the enzyme responsible for the catabolism of ceramide to sphingosine and fatty acid, has been reported to induce apoptosis in neuroblastoma cells (23) because of the increased concentration of ceramide due to the inhibition of catabolism of ceramide (23). We examined the possible role of increased intracellular ceramide by the inhibition of ceramidase on the induction of iNOS mRNA, iNOS protein, and NO production. Treatment of astrocytes with OE 2 h before the addition of LPS stimulated the expression of iNOS mRNA, iNOS protein, and NO production. Treatment of astrocytes with OE 2 h before the addition of LPS stimulated the expression of iNOS mRNA, iNOS protein, and NO production. Treatment of astrocytes with OE 2 h before the addition of LPS stimulated the expression of iNOS mRNA, iNOS protein, and NO production. Treatment of astrocytes with OE 2 h before the addition of LPS stimulated the expression of iNOS mRNA, iNOS protein, and NO production. Treatment of astrocytes with OE 2 h before the addition of LPS stimulated the expression of iNOS mRNA, iNOS protein, and NO production.

SMase and Ceramide Stimulate the Transcription of the iNOS Gene in Rat Primary Astrocytes—To gain further insight into the mechanism of the stimulatory effect of SMase and ceramide on LPS- or cytokine-mediated expression of iNOS mRNA, we studied the influence of SMase and ceramide on the rate of iNOS gene transcription, as measured by nuclear run-on assays. Fig. 6 shows that LPS induced the transcription of the iNOS gene in astrocytes, and addition of SMase (100 milliunits/ml) or C2-ceramide (10 μM) along with LPS stimulated LPS-induced transcription of the iNOS gene. These results clearly suggest that SMase and ceramide stimulate LPS-induced expression of iNOS mRNA, protein, and activity by stimulating transcription of the iNOS gene.

SMase and Ceramide Stimulate the Expression of iNOS in Rat C6 Glial Cells—Similar to primary astrocytes, SMase also
stimulated LPS- and/or cytokine-induced production of nitrite as well as the expression of iNOS in rat C6 glial cells (Fig. 7). Unlike astrocytes, neither LPS nor cytokines alone was a sufficient inducer of NO production in rat C6 glial cells (17, 18, 24).

A combination of LPS and cytokines was required to induce the production of NO in C6 glial cells (17, 18, 24) (Fig. 7). However, the addition of neutral SMase along with LPS and cytokines to rat C6 glial cells stimulated the expression of iNOS protein and the production of NO by more than 2-fold. These observations suggest that both in primary astrocytes and C6 glial cells the sphingomyelin-ceramide signaling events up-regulate the cytokine-induced expression of iNOS and the production of NO.

SMase and Ceramide Stimulate LPS-mediated Activation of NF-κB in Rat Primary Astrocytes—Since the activation of NF-κB is necessary for the induction of iNOS (8, 18), the observed enhancement of iNOS expression by SMase or ceramides in rat primary astrocytes may be due to the enhanced activation of NF-κB. To examine this possibility, astrocytes were treated with either SMase or C2-ceramide, alone or along with LPS, and activation of NF-κB was examined as transcription of the transcription factor to the nucleus. NF-κB activation was evaluated by the formation of a distinct and specific complex in a gel-shift DNA-binding assay with nuclear proteins. Treatment of astrocytes with 1.0 μg/ml LPS resulted in the activation of NF-κB (Fig. 8). This gel-shift assay detected a specific band in response to LPS that was competed off by unlabeled probe. SMase or C2-ceramide alone at different concentrations failed to induce NF-κB. However, treatment of astrocytes with 1.0 μg/ml LPS in combination with variable concentrations of C2-ceramide or SMase resulted in enhancement of NF-κB activation, suggesting that the sphingomyelin-ceramide signaling pathway played a positive modulatory role in augmenting NF-κB activation. Moreover, the inability of ceramide to activate NF-κB and induce iNOS by itself and the augmentation of levels of LPS-induced NF-κB activation and iNOS expression by ceramide suggest that the sphingomyelin-ceramide signaling events instead of directly activating the NF-κB pathway, converge into the LPS-mediated cascade resulting in a greater signaling response. Consistent with this conclusion, N-acetylcysteine and pyrrolidine dithiocarbamate, antioxidant inhibitors of NF-κB activation (8, 25), inhibited the LPS- and ceramide-mediated production of NO (Fig. 9A) and expression of iNOS mRNA (Fig. 9B).

Inhibition of the LPS- and Ceramide-mediated Expression of iNOS and Activation of NF-κB by PD98059, an Inhibitor of MAP Kinase Kinase (MEK), and FPT Inhibitor II, an Inhibitor of Ras Farnesyl Protein Transferase—To understand the possible role of Ras and the MAP kinase pathway in LPS- and ceramide-mediated activation of NF-κB and induction of iNOS, we examined the effect of PD98059 and FPT inhibitor II on the LPS- as well as the LPS and ceramide-mediated activation of NF-κB and induction of iNOS. Preincubation of astrocytes with either PD98059 or FPT inhibitor II blocked the LPS-mediated as well as the LPS and ceramide-mediated activation of NF-κB and induction of iNOS. Preincubation of astrocytes with either PD98059 or FPT inhibitor II blocked the LPS-mediated as well as the LPS and ceramide-mediated production of NO (Fig. 10A), the expression of iNOS mRNA (Fig. 10B), and activation of NF-κB (Fig. 10C). These experiments suggest that the Ras-MAP kinase signaling pathway is involved in LPS-mediated induction of iNOS and that ceramide potentiates these signaling pathways in LPS-stimulated astrocytes for the stimulation of NF-κB activation and iNOS induction.

**Fig. 5.** Effect of C2-dihydroceramide on the LPS-mediated production of NO in rat primary astrocytes. Cells incubated in serum-free DMEM/F-12 received different concentrations of C2-ceramide and C2-dihydroceramide in the presence or absence of LPS (1.0 μg/ml). After 24 h of incubation, concentration of nitrite was measured in the supernatants as mentioned under “Materials and Methods.”
In the current work we provide evidence that the sphingomyelin signal transduction pathway has a stimulatory effect on the LPS- and cytokine-mediated expression of iNOS in astrocytes and C6 glial cells. This conclusion is based on the following observations. First, addition of SMase, the enzyme that hydrolyzes sphingomyelin in the plasma membrane to ceramide and phosphocholine, by itself has no effect on the production of NO both in astrocytes and C6 glial cells. Second, addition of SMase to LPS- or cytokine-stimulated astrocytes or C6 glial cells augments LPS- or cytokine-induced production of NO and expression of iNOS. Third, cell-permeable ceramide analogs and ceramide by itself was unable to induce iNOS even in the presence of neutral SMase, protein kinase C- and diacylglycerol in the insulin producing RINm5F cell line may involve activation of c-Jun NH2-terminal kinase and transcription factor ATF2 instead of activation of NF-κB and induction of iNOS, however, it up-regulates the LPS or cytokine-induced activation of NF-κB and induction of iNOS and that this up-regulation is blocked by inhibitors of Ras

FIG. 7. SMase and LPS stimulate the production of NO and the expression of iNOS in rat C6 glial cells. Cells incubated in serum-free media received SMase (100 milliunits/ml) along with LPS and cytokines. After 24 h of incubation, the production of nitrite was measured in supernatants (A), and cell homogenates were analyzed for iNOS protein by the immunoblotting technique (B) as described before. Concentration of different stimuli were: LPS, 0.5 μg/ml; TNF-α, 25 ng/ml; IL-1β, 25 ng/ml; IFN-γ, 50 units/ml.

FIG. 8. Enhancement of LPS-mediated activation of NF-κB by SMase and C2-ceramide in rat primary astrocytes. Cells incubated in serum-free DMEM/F-12 were treated with neutral SMase (100 milliunits/ml) or C2-ceramide (10 μM) alone or together with LPS (1.0 μg/ml). After a 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.” Lanes 1–8 represent nuclear extract of control cells, nuclear extract of LPS-treated cells, nuclear extract of LPS-treated cells incubated with 100-fold excess of unlabeled oligonucleotide, nuclear extract of cells treated with LPS and C2-ceramide, nuclear extract of cells treated with LPS and SMase, nuclear extract of LPS and SMase-treated cells incubated with 100-fold excess of unlabeled oligonucleotide, nuclear extract of C2-ceramide-treated cells, and nuclear extract of SMase-treated cells.

DISCUSSION

In the current work we provide evidence that the sphingomyelin signal transduction pathway has a stimulatory effect on the LPS- and cytokine-mediated expression of iNOS in astrocytes and C6 glial cells. This conclusion is based on the following observations. First, addition of SMase, the enzyme that hydrolyzes sphingomyelin in the plasma membrane to ceramide and phosphocholine, by itself has no effect on the production of NO both in astrocytes and C6 glial cells. Second, addition of SMase to LPS- or cytokine-stimulated astrocytes or C6 glial cells augments LPS- or cytokine-induced production of NO and expression of iNOS. Third, cell-permeable ceramide analogs (C2 and C6) as well as OE, an inhibitor of ceramidase, led to an increase in the production of NO and the expression of iNOS in LPS- or cytokine-stimulated rat primary astrocytes and in C6 glial cells.

The signaling events in cytokine-mediated induction of iNOS are not completely established so far. LPS, TNF-α, IL-β, and IFN-γ bind to their respective receptors and induce iNOS expression via activation of NF-κB (8, 25, 26). NF-κB, a pleiotropic transcription factor, is a heterodimer of 50- (p50, NF-κB 1) and 65-kDa (p65, rel A) subunits located in the cytoplasm in an inactive form, bound to the inhibitory protein IκB through the p65 molecule. Upon stimulation of cells, IκB is phosphorylated, proteolytically degraded, and dissociated from the complex (27). The dissociated NF-κB translocates into the nucleus and transactivates κB-dependent promoters (28). Identification of the binding site of NF-κB in the promoter region of the iNOS gene and the activation of NF-κB during cytokine-induced iNOS expression establishes the role of NF-κB activation in the induction of iNOS (8). In rat primary astrocytes, although SMase or ceramide by itself was unable to induce iNOS, SMase, or ceramide markedly stimulated cytokine-mediated activation of NF-κB.

Increase in the activation of NF-κB in cytokine-stimulated astrocytes by ceramide paralleled the increase in induction of iNOS. Moreover, inhibition of LPS- and ceramide-induced expression of iNOS by antioxidant inhibitors of NF-κB activation (e.g. N-acetylcysteine and pyrrolidine dithiocarbamate) in astrocytes suggests a role for cellular redox in the ceramide-LPS or proinflammatory cytokine induced activation of NF-κB and induction of iNOS. These observations also indicate that stimulation of iNOS expression in cytokine-activated rat primary astrocytes by cell-permeable ceramide analogs or neutral SMase is probably mediated via enhanced activation of NF-κB.

The potential immediate targets for ceramide signaling events identified so far include ceramide-activated protein kinase (5), protein kinase C-ζ (6), and ceramide-activated protein phosphatase (1, 2). However, the role of ceramide in the activation of NF-κB is not well understood thus far. Ceramide-mediated activation of NF-κB was observed in permeabilized Jurkat T cells (29), however, no such activation was observed in nonpermeabilized cells (30), suggesting that permeabilization of Jurkat T cells may activate another factor necessary for the activation of NF-κB in combination with ceramide. A recent study reports that the IL-1β-stimulated formation of ceramide and diacylglycerol in the insulin producing RINm5F cell line may involve activation of c-Jun NH2-terminal kinase and transcription factor ATF2 instead of activation of NF-κB and production of NO (15). Studies described in this paper clearly show that ceramide by itself has no effect on the activation of NF-κB and induction of iNOS, however, it up-regulates the LPS or cytokine-induced activation of NF-κB and induction of iNOS and that this up-regulation is blocked by inhibitors of Ras.
farnesyl protein transferase and MEK indicating that the Ras-MAP kinase pathway is involved in LPS- and ceramide-mediated activation of NF-κB and induction of iNOS in astrocytes. However, the precise mechanism of potentiation of a cytokine-induced MAP kinase cascade by ceramide is not understood at this time. The studies reported here suggest that sphingomyelin-ceramide signaling events may converge into cytokine-induced MAP kinase pathway leading to higher activation of NF-κB and higher induction of iNOS.

Since the discovery of the sphingomyelin cycle, several inducers (1α,25-dihydroxyvitamin D₃, radiation, antibody cross-linking, TNF-α, IFN-γ, IL-1, nerve growth factor, and brefeldin A) have been shown to be coupled to sphingomyelin-ceramide signaling events (31, 32). In the case of TNF-α, the pathway is initiated by the action of TNF-α on its 55-kDa receptor leading to phospholipase A₂ activation, generation of arachidonic acid, and subsequent activation of sphingomyelins (33). Several studies support a critical role for sphingomyelin hydrolysis as a stress-activated signaling mechanism with an important role for ceramide in growth suppression and apoptosis in various cell types including glial and neuronal cells (34, 35). Since NO can suppress growth and is an important candidate to induce apoptosis (36), the observed stimulation of NO production by ceramides in cytokine-activated astrocytes may be an important factor in NO-mediated cytotoxicity of neurons and oligodendrocytes in neurodegenerative diseases with neuroinflammatory conditions. The potentiation of LPS or cytokine-mediated induction of iNOS and production of NO by compounds that activate protein kinase A (18) and the compounds that inhibit the activation of Ras (37) suggest a positive mod-

![Fig. 9. N-Acetylcysteine and pyrrolidine dithiocarbamate inhibit LPS and ceramide-induced expression of iNOS in rat primary astrocytes. Cells preincubated in serum-free media with different concentrations of N-acetylcysteine (NAC) or pyrrolidine dithiocarbamate (PDTC) for 1 h received 1.0 μg/ml LPS and 10 μM C₂-ceramide. Concentration of nitrite (A) was measured in the supernatants after 24 h of incubation, however, after 6 h of incubation, cells were analyzed for iNOS mRNA (B) by the Northern blotting technique as described earlier. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.](image-url)
ulatory role for ceramide in the induction of iNOS in astrocytes.

In summary, we have demonstrated that the sphingomyelin-ceramide signaling events have a positive modulatory role on the expression of iNOS in rat astrocytes which are probably mediated via activation of NF-κB. The identification of a stimulatory effect of ceramides on LPS- and cytokine-induced expression of iNOS defines a novel role for the sphingomyelin signal transduction pathway in the regulation of glial cell proliferation, programmed cell death, and cytotoxicity in neurological disorders.

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REFERENCES
Ceramide Stimulates the Expression of iNOS in Astrocytes

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