Inhibitors of Protein Phosphatase 1 and 2A Differentially Regulate the Expression of Inducible Nitric-oxide Synthase in Rat Astrocytes and Macrophages

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Nitric oxide 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 stroke and other neurodegenerative diseases. The present study underlines the importance of protein phosphatase (PP) 1 and 2A in the regulation of the differential expression of iNOS in rat primary astrocytes and macrophages. Compounds (calyculin A, microcystin, okadaic acid, and cantharidin) that inhibit PP 1 and 2A were found to stimulate the lipopolysaccharide (LPS)- and cytokine-mediated expression of iNOS and production of NO in rat primary astrocytes and C6 glial cells. However, these inhibitors inhibited the LPS- and cytokine-mediated expression of iNOS and production of NO in rat resident macrophages and RAW 264.7 cells. Similarly, okadaic acid, an inhibitor of PP 1/2A, stimulated the iNOS promoter-derived chloramphenicol acetyltransferase activity in astrocytes and inhibited the iNOS promoter-derived chloramphenicol acetyltransferase activity in macrophages, indicating that okadaic acid also differentially regulates the transcription of the iNOS gene in astrocytes and macrophages. The observed stimulation of the expression of iNOS in astrocytes and the inhibition of the expression of iNOS in macrophages with the inhibition of PP 1/2A activity clearly delineate a novel role of PP 1/2A in the differential regulation of iNOS in rat astrocytes and macrophages. Because the activation of NF-kB is necessary for the induction of iNOS and the expression of tumor necrosis factor (TNF)-α also depends on the activation of NF-kB, we examined the effect of okadaic acid on the LPS-mediated activation of NF-kB and production of TNF-α in rat primary astrocytes and macrophages. Interestingly, in both cell types, okadaic acid stimulated the LPS-mediated DNA binding as well as transcriptional activity of NF-kB and production of TNF-α. This study suggests that the stimulation of iNOS expression in astrocytes by inhibitors of PP 1/2A is possibly due to the stimulation of NF-kB activation; however, activation of NF-kB is not sufficient for the induction of iNOS in macrophages and that apart from NF-kB some other signaling pathway(s) sensitive to PP 1 and/or PP 2A is/are possibly involved in the regulation of iNOS in macrophages. This differential induction of iNOS as compared with similar activation of NF-kB by inhibitors of PP 1/2A indicates the involvement of different intracellular signaling events for the induction of iNOS in two cell types of the same animal species.

Nitric oxide (NO), a bioactive free radical, 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 and endothelial cells is calcium-dependent enzymes, whereas the inducible form present in macrophage and astrocytes is regulated at the transcriptional level in response to stimuli (e.g. cytokines/lipopolysaccharides) 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 pathophysiology of inflammatory diseases including demyelinating disorders (e.g. multiple sclerosis, experimental allergic encephalopathy, and 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 induction of iNOS by bacterial lipopolysaccharides (LPS) and a series of cytokines including interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ). Astrocytes in the healthy brain do not express iNOS but following ischemic, traumatic, neurotoxic, or inflammatory damage the reactive astrocytes express iNOS in 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 intracellular pathways evoked to transduce the signal from the cell surface to the nucleus for the induction of iNOS in macrophages and astrocytes is an active area of investigation. Identification of the DNA-binding site for NF-kB in the promoter region of iNOS (14), and inhibition of iNOS induction by inhibitors of NF-kB activation has established an essential role of NF-kB activation in the induction of iNOS (11–13, 15). Suppression of NF-kB 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-kB and the induction of iNOS. Recently we have observed that PD 98059, an inhibitor of MAP kinase kinase (MEK), the kinase responsible for the

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activation of MAP kinase, inhibits the LPS-induced activation of NF-κB 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). MAP kinases exhibit dual specificity, regulating both Ser/Thr phosphorylation and Tyr autophosphorylation (19–21). In addition, MAP kinases themselves require concurrent Thr and Tyr phosphorylation for activation and are in turn substrates for MEK (19–21). MEK is also a dual specificity kinase whose activation requires Ser/Thr phosphorylation (19–21). These observations suggest that cellular regulation of this signaling pathway may utilize Ser/Thr phosphorytases to modulate the phosphorylation state of critical phosphoproteins associated with the activation of NF-κB and the induction of iNOS.

Because PP 1 and PP 2A are the two most abundant Ser/Thr phosphorytases in the cell, the present study was undertaken to investigate the cellular regulation of the induction of iNOS by PP 1 and PP 2A in rat primary astrocytes and macrophages. Our results clearly demonstrate that calcyuin A, microcystin, cantharidin, and okadaic acid, inhibitors of PP 1 and 22A, stimulate the LPS- and cytokine-mediated expression of iNOS and production of NO in astrocytes and C6 glial cells, whereas the same inhibitors inhibit the LPS- and cytokine-mediated expression of iNOS and production of NO in macrophages and RAW 264.7 cells. This differential regulation of the induction of iNOS in astrocytes and macrophages by inhibitors of PP 1/2A suggests that different intracellular signaling events may be involved for the induction of iNOS in astrocytes and macrophages. However, despite this differential regulation of the induction of iNOS in astrocytes and macrophages, inhibitors of PP 1/2A stimulate the activation of NF-κB and the production of TNF-α in both astrocytes and macrophages.

MATERIALS AND METHODS

Reagents—Recombinant rat IFN-γ, DMEM/F-12 medium, fetal bovine serum, Hanks’ balanced salt solution, and NF-κB DNA-binding protein detection kit were from Life Technologies, Inc. Human IL-1β was from Genzyme. Mouse recombinant TNF-α was obtained from Boehringer Mannheim. LPS (Escherichia coli) was from Sigma. N3-(3-aminopropyl)aminopropyl methyamine (3-AMPA) was from Amersham Pharmacia Biotech. N-[32P]ATP (3000 Ci/mmol) was from Amersham. Poly(dI- dC)30 (10 mg/ml) was from Calbiochem. Biotin and fluorescein isothiocyanate (FITC) were from Sigma. N4, N6-dimethyl-L-arginine (L-NMA), okadaic acid, calcyuin C, cantharidin, and antibodies against mouse macrophage iNOS were obtained from Calbiochem. Deltamethrin and fenvalerate were obtained from Biomol. [γ-32P]ATP (3000 Ci/mmol) was from Amersham Pharmacia Biotech. Induction of NO Production in Astrocytes and C6 Glial Cells—Astrocytes and C6 glial cells were obtained from postnatal rat cerebral cortex as described by McCarty and Devellis (22). Cells were maintained in DMEM/F-12 medium containing 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 two more times after a gap of 1 or 2 weeks before subculturing to ensure the complete removal of all the oligodendrocytes and microglia. Cells were trypsinized, subcultured, and stimulated with LPS or different cytokines in serum-free DMEM/F-12. C6 glial cells obtained from ATCC were also maintained and induced with different stimuli as above.

Isolation of Rat Macrophages and Induction of NO Production—Resident macrophages were obtained from rat by peritoneal lavage with sterile RPMI 1640 medium containing 1% fetal bovine serum and 100 μg/ml gentamicin as reported earlier (13). Cells were washed three times with RPMI 1640 at 4 °C. All cells were cultured at 37 °C in a humidified incubator containing 5% CO2 in air. Macrophages at a concentration of 2 × 106/ml in RPMI 1640 medium containing L-glutamine and gentamicin were added in volumes of 800 μl to a 35-mm plate. After 1 h, nonadherent cells were removed by washing, and 800 μl of serum-free RPMI 1640 medium with various stimuli were added to the adherent cells. After incubation for 15–24 h at 37 °C, culture supernatants were transferred to measure NO production.

Assay for NO Synthesis—NO synthesis was determined by assay of culture supernatants for nitrite, a stable reaction product of NO with DAF-2, and the induction of iNOS, suggesting that different intracellular signaling events may be involved for the induction of iNOS in astrocytes, macrophages, or astrocytes and macrophages, inhibitors of PP 1 and PP 2A in rat primary astrocytes and macrophages. This differential regulation of the induction of iNOS in astrocytes and macrophages by inhibitors of PP 1/2A suggests that different intracellular signaling events may be involved for the induction of iNOS in astrocytes and macrophages. However, despite this differential regulation of the induction of iNOS in astrocytes and macrophages, inhibitors of PP 1/2A stimulate the activation of NF-κB and the production of TNF-α in both astrocytes and macrophages.

Inhibitors of PP 1 and 2A Regulate the Expression of iNOS

In Vitro PP 1/2A Assay—The extraction and assay for PP 1/2A were performed as described (24). Control and treated cells were scraped off the extraction buffer for containing 20 μM imidazole-HCl, 2 mM EDTA, 2 mM EGTA, pH 7.0, with protease inhibitors (1 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml antipain, 5 μg/ml leupeptin, and 5 μg/ml leupeptin). The cells were sonicated for 10 s and centrifuged at 2000 × g for 5 min, and the supernatants were used for the assay of phosphatase activities using the protein phosphatase assay kit (Life Technologies Inc.) according to the manufacturer’s protocol.

RNA Isolation and Northern Blot Analysis—Cells were taken from culture dishes directly after adding Ultratrace-II RNA reagent (Biotech Laboratories Inc.), and total RNA was isolated according to the manufacturer’s protocol. For Northern blot analyses, 20 μg of total RNA were denatured on 1% agarose formaldehyde gels, electrotransferred to Hybond-Nylon Membrane (Amerssharn), and hybridized at 68 °C with 32P-labeled cDNA probe using Express Hyb hybridization solution (CLONTECH) as described by the manufacturer. The cDNA probe was made by polymerase chain reaction amplification using two primers (forward primer, 5′-CTCT CCATA AAAAA AAC CTGGT CCA-3′; reverse primer, 5′-CAG CAC TTC CAG GAT GTT GTT-3′) (11, 12, 25). After hybridization, filters were washed two or three times in solution I (2× SSC, 0.05% SDS) for 1 h at room temperature followed by solution II (0.1× SSC, 0.1% SDS) at 50 °C for another hour. The membranes were then dried and exposed to x-ray films (Kodak). The same amount of RNA was hybridized with probe for glyceraldehyde-3-phosphate dehydrogenase. The relative mRNA content for iNOS (iNOS/ glyceraldehyde-3-phosphate dehydrogenase) was measured after scanning the bands with a Bio-Rad (model GS-670) imaging densitometer.

Construction of Reporter Plasmid, Transfection, and Assay of Chloramphenicol Acetyltransferase Activity—The chloramphenicol acetyltransferase (CAT) under the control of nitric-oxide synthase promoter (iNOS) was created by subcloning a 1.5-kilobase promoter from pGEM-NOS at SpI and SalI restriction site of pCAT-basic vector (Promega). Promoter was amplified by PCR, purified by using two primers (forward primer, 5′-GAG GKT GGT CAA GTA TTT GGA GAG G-3′; reverse primer, 5′-AAG GTG GCT GAG AAT TCA T-3′) from rat genomic DNA and cloned in pGEM-T vector (Promega) to produce pGEM-NOS. The clone was confirmed by restriction mapping and sequencing. The plasmids were transfected with 2 μg of reporter plasmid by using the lipotaxis (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. Radiolysis was used to assay CAT activity using a kit (Promega) as described by the manufacturer’s protocol.

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assay—Nuclear extracts from stimulated or unstimulated astrocytes (1 × 106 cells) were prepared using the method of Dignam et al. (27) 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 PMSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin A, and 5 μg/ml leupeptin) containing 0.1% Nonidet P-40 for 15 min on ice, vortexed vigorously for 15 s, and centrifuged at 14,000 rpm for 30 s. The pellet nuclei were resuspended in 40 μl of buffer B (20 mM HEPES, pH 7.9, 2% (v/v) glycerol, 0.42 m NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM dithiothreitol,1 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin A, and 5 μg/ml leupeptin). After 30 min on ice, lysates were centrifuged at 14,000 rpm for 10 min. Supernatants containing the nuclear proteins were diluted with 20 μl of modified buffer C (20 mM HEPES, pH 7.9, 2% (v/v) glycerol, 0.05 m KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.5 mM PMSF) and stored at −70 °C until use. Nuclear extracts for electrophoretic mobility shift assay using the NF-κB DNA-binding protein detection kit system kit (Life Technologies, Inc.), according to the manufacturer’s protocol.

Assay of Transcriptional Activity of NF-κB—To assay the transcriptional activity of NF-κB, cells were transfected with pNF-κB-Luc, an NF-κB-dependent reporter construct (obtained from Stratagene), using the lipotaxis method. 24 h after transfection, cells were treated with...
**TABLE I**

**Effect of inhibitors of different protein phosphatases on LPS-induced production of NO in rat primary astrocytes**

Astrocytes preincubated in serum-free DMEM/F-12 for 30 min with L-NMA and different inhibitors of protein phosphatases received LPS (1.0 μg/ml). After 24 h of incubation, nitrite concentration in the supernatants was measured as described under “Materials and Methods.” Data are expressed as the means ± S.D. of three different experiments.

| Stimuli                        | Nitrite (nmol/mg/24 h) |
|-------------------------------|------------------------|
| Control                       | 3.1 ± 0.3              |
| LPS only                      | 28.2 ± 3.1             |
| LPS + L-NMA (0.1 mM)          | 5.2 ± 0.4              |
| LPS + cypermethrin (1 mM)     | 27.6 ± 2.7             |
| LPS + deltamethrin (1 mM)     | 26.8 ± 2.9             |
| LPS + fenvalerate (20 mM)     | 27.1 ± 2.1             |
| LPS + calyculin A (2 mM)      | 67.8 ± 7.3             |
| LPS + microcystin (2 mM)      | 64.8 ± 7.2             |
| LPS + okadaic acid (10 mM)    | 59.5 ± 7.1             |

Different stimuli for 4 h. Total cell extracts were used to measure luciferase activity in a scintillation counter (Beckman LS 3801) (28) using an assay kit from Stratagene.

**Cell Viability—Cytotoxic effects of all the inhibitors were determined by measuring the metabolic activity of cells with the 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay.**

**RESULTS**

**Inhibitors of PP 1/2A Stimulate the LPS-induced Production of NO in Rat Primary Astrocytes**—Rat primary astrocytes were cultured in serum-free DMEM/F-12 in the presence of LPS and inhibitors of different protein phosphatases. The concentration of NO as nitrite (a stable reaction product of NO with molecular oxygen) was measured in culture supernatants after 24 h. It is evident from Table I that the bacterial LPS at a concentration of 1.0 μg/ml induced the production of NO as nitrite by about 9-fold. L-NMA, a competitive inhibitor of NO activity, suppressed the LPS-mediated nitrite secretion, suggesting that LPS-induced nitrite release in rat primary astrocytes is dependent on NOS-mediated arginine metabolism (Table I). Inhibitors of PP 1/2A (calyculin A, microcystin, and okadaic acid) or PP 2B (cypermethrin, deltamethrin, and fenvalerate) alone were neither stimulatory nor inhibitory to nitrite production in control astrocytes (data not shown). However, calyculin A, microcystin, and okadaic acid, when added with LPS, stimulated the LPS-mediated induction of nitrite production in astrocytes. In contrast, inhibitors of PP 2B (cypermethrin, deltamethrin, and fenvalerate) had no effect on LPS-induced nitrite production in astrocytes. These observations suggest that stimulation of LPS-induced production of NO in astrocytes is specific for the inhibitors of PP 1/2A. To understand the mechanism of stimulatory effect of inhibitors of PP 1/2A on the LPS-mediated nitrite production in astrocytes, we examined the effect of these inhibitors on the protein and mRNA level of iNOS. Consistent with the production of nitrite (Fig. 1A), Western blot analysis with antibodies against murine macrophage iNOS and Northern blot analysis for iNOS mRNA of LPS-stimulated astrocytes clearly show that inhibitors of PP 1/2A (calyculin A, microcystin, and cantharidin) enhanced the LPS-mediated induction of iNOS protein (Fig. 1B) and mRNA (Fig. 1C). Because the inhibitors of PP 1/2A stimulated the LPS-mediated induction of iNOS, we examined whether these inhibitors did in fact inhibit the activities of PP 1/2A in LPS-treated astrocytes under these conditions. The activities of PP 1/2A were measured in homogenates after 30 min of incubation. Fig. 2 shows that okadaic acid inhibited the activities of PP 1/2A and stimulated the LPS-mediated induction of iNOS protein and production of NO in astrocytes in a dose-dependent manner.

**Stimulation of LPS- and Cytokine-induced Production of NO by Calyculin A in C6 Glial Cells**—Similar to primary astrocytes, proinflammatory cytokines and the LPS induced the production of nitrite as well as the expression of iNOS in rat C6 glial cells (23, 29). Unlike astrocytes, LPS or cytokines alone were not a sufficient inducer of NO production in rat C6 glial cells. A combination of LPS and cytokines was required to induce the production of NO in C6 glial cells (Refs. 13, 23, and 29 and Fig. 3). Calyculin A (2 mM) stimulated the expression of iNOS protein and the production of NO by more than 3-fold in LPS- and cytokine-treated C6 cells (Fig. 3). The observed up-regulation of cytokine-induced expression of iNOS and production of NO in both rat primary astrocytes and C6 glial cells by inhibitors of PP 1/2A indicate that PP 1/2A may function as a negative regulator in these intercellular signaling pathways.

**Inhibition of LPS- and Cytokine-induced NO Production by**...
Inhibitors of PP 1 and 2A Regulate the Expression of iNOS

Inhibitors of PP 1/2A in Rat Peritoneal Macrophages—Because inhibitors of PP 1/2A stimulated the LPS- and cytokine-induced NO production in rat primary astrocytes and C6 glial cells, we examined the effect of these inhibitors on NO production and expression of iNOS in rat resident macrophages. Similar to astrocytes, inhibitors of PP 1/2A alone had no effect on the induction of NO production. However, in contrast to the stimulation of NO production in astrocytes (Fig. 1 and Table I), all of the three inhibitors of PP 1/2A (calyculin A, microcystin, and cantharidin) inhibited the LPS-induced NO production in macrophages (Fig. 4A). This decrease in NO production was accompanied by a decrease in iNOS protein (Fig. 4B) and iNOS mRNA (Fig. 4C). Okadaic acid, another very specific and potent inhibitor of PP 1/2A, also inhibited the LPS-mediated production of NO (Fig. 5A) and expression of iNOS protein (Fig. 5B) in macrophages in a dose-dependent manner. Similar to rat peritoneal macrophages, calyculin A was also found to inhibit the LPS- and cytokine-induced production of NO (Fig. 6A) and the expression of iNOS protein (Fig. 6B) in the murine macrophage cell line RAW 264.7. Taken together, these results indicate that PP 1/2A activities are required to induce iNOS gene expression in macrophages.

Differential Effect of Okadaic Acid on iNOS Promoter-derived CAT Activity in LPS-stimulated Rat Primary Astrocytes and Macrophages—Differential regulation of the induction of iNOS mRNA and protein in astrocytes and macrophages by the inhibitors of PP 1/2A suggests that these inhibitors may regulate the transcription of iNOS gene differentially in these two different cell lines. Therefore, to understand the effect of okadaic acid on the transcription of iNOS gene, astrocytes and macrophages were transfected with a construct containing the iNOS promoter fused to the CAT gene, and activation of this
promoter was measured after stimulating the cells with LPS in the presence or the absence of okadaic acid. Consistent with the effect of okadaic acid on the production of NO and the expression of endogenous iNOS in these two different cell types, okadaic acid stimulated the LPS-induced CAT activity in astrocytes but inhibited the LPS-induced CAT activity in macrophages, supporting the conclusion that okadaic acid differentially regulates the transcription of iNOS gene in astrocytes and macrophages (Fig. 7).

**Effect of Okadaic Acid on the Activation of NF-κB in Rat Primary Astrocytes and Macrophages**—Inhibitors of PP 1/2A stimulated the induction of iNOS in astrocytes but inhibited the induction of iNOS in macrophages, suggesting that PP 1/2A may transduce different signals in two different cell types for the differential regulation of iNOS. Because the activation of NF-κB is reported to be necessary for the induction of iNOS, we examined the effect of okadaic acid on the LPS-induced activation of NF-κB in astrocytes and macrophages to understand the basis of this differential regulation of induction of iNOS by inhibitors of PP 1/2A. Activation of NF-κB was monitored by both DNA binding and transcriptional activity of NF-κB. DNA binding activity of NF-κB was evaluated by the formation of a distinct and specific complex in a gel shift DNA binding assay. Consistent with previous reports (11, 12), treatment of astrocytes or macrophages with 1.0 μg/ml of LPS resulted in the induction of DNA binding activity of NF-κB (Fig. 8). This gel shift assay detected a specific band in response to LPS that was competed off by an unlabeled probe. Although okadaic acid alone at different concentrations failed to induce the DNA binding activity of NF-κB in astrocytes, okadaic acid alone induced the DNA binding activity of NF-κB in macrophages. However, in both astrocytes and macrophages, okadaic acid stimulated the LPS-induced DNA binding activity of NF-κB (Fig. 8). We then tested the effect of okadaic acid on NF-κB-dependent transcription of luciferase in astrocytes and macrophages in the presence or the absence of LPS, using the expression of luciferase from a reporter construct, pNF-κB Luc (Stratagene), as an assay. Consistent with the effect of okadaic acid on DNA binding activity of NF-κB, the okadaic acid induced NF-κB-dependent transcription in macrophages but not in astrocytes and stimulated the LPS-induced NF-κB-dependent transcription in both astrocytes and macrophages (Fig. 9) under the condition in which the LPS-mediated expression of iNOS was stimulated in astrocytes and inhibited in macro-

![Fig. 4. Inhibition of LPS-induced expression of iNOS by inhibitors of PP 1 and PP 2A in rat peritoneal macrophages.](image)

**Fig. 4.** Inhibition of LPS-induced expression of iNOS by inhibitors of PP 1 and PP 2A in rat peritoneal macrophages. Cells incubated in serum-free DMEM/F-12 received calyculin A (Cal. A), microcystin, or cantharidin along with 1.0 μg/ml of LPS. A, after 24 h, concentration of nitrite was measured in the supernatants as described earlier. Data are the means ± S.D. of three different experiments. B, cell homogenates were electrophoresed, transferred on nitrocellulose membrane, and immunoblotted with antibodies against mouse macrophage iNOS as described earlier. C, after 6 h of incubation, cells were analyzed for iNOS mRNA by Northern blotting technique as described earlier.

![Fig. 5. Effect of okadaic acid on LPS-mediated induction of iNOS in rat primary astrocytes.](image)

**Fig. 5.** Effect of okadaic acid on LPS-mediated induction of iNOS in rat primary astrocytes. A, cells incubated in serum-free DMEM/F-12 received different concentrations of okadaic acid (OA) in the presence or the absence of 1.0 μg/ml of LPS. After 24 h of incubation, nitrite concentrations were measured in supernatants. Data are the means ± S.D. of three different experiments. B, cells incubated in serum-free DMEM/F-12 received different concentrations of okadaic acid along with 1.0 μg/ml of LPS. After 24 h of incubation, cell homogenates were electrophoresed, transferred on nitrocellulose membrane, and immunoblotted with antibodies against mouse macrophage iNOS as described before.
Inhibitors of PP 1/2A Stimulate the LPS-Induced Production of TNF-α in Rat Primary Astrocytes and Macrophages—Okadaic acid stimulated the transcription of iNOS in astrocytes and attenuated the transcription of iNOS in macrophages. However, in contrast, okadaic acid stimulated the activation of NF-κB in both astrocytes and macrophages. Because the induction of TNF-α also depends on the activation of NF-κB (30), we examined the effect of okadaic acid on the LPS-induced production of TNF-α in astrocytes and macrophages. Consistent with the stimulatory effect of okadaic acid on the LPS-induced activation of NF-κB, okadaic acid stimulated the LPS-induced production of TNF-α in both astrocytes and macrophages (Table II).

Effect of Inhibitors of PP 1/2A on Cell Viability—Astrocytes or macrophages were incubated with different inhibitors of PP 1/2A 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 astrocytes and inhibition of the expression of iNOS in macrophages by inhibitors of PP 1/2A are not due to any change in viability of either astrocytes or macrophages.

DISCUSSION

Transient modulation of protein phosphorylation and dephosphorylation is a major mechanism of intracellular signal transduction pathways triggered by different cytokines (31–32). Therefore, it is reasonable to assume that inhibition of PP 1 and 2A activities will influence cytokine-induced signal transduction pathways for the induction of iNOS. The signaling events in cytokine-mediated induction of iNOS in astrocytes and macrophages are not well understood. A complete understanding of the cellular mechanisms involved in the induction of iNOS should identify novel targets for the therapeutic intervention in NO-mediated neuroinflammatory diseases. Several lines of evidence presented in this study support the conclusion that inhibition of PP 1/2A activity differentially modulates the LPS- and cytokine-induced expression of iNOS and production of NO in rat primary astrocytes and macrophages. Our conclusion is based on the following observations. First, compounds (calyculin A, microcystin, okadaic acid, and cantharidin) that inhibit PP 1/2A stimulated the LPS- and cytokine-mediated production of NO as well as expression of iNOS in astrocytes and C6 glial cells. However, in contrast, these inhibitors inhibited the LPS- and cytokine-mediated production of NO as well as expression of iNOS protein and mRNA in astrocytes and C6 glial cells. Therefore, it is reasonable to assume that inhibition of PP 1 and 2A activities will influence cytokine-induced signal transduction pathways for the induction of iNOS. The signal transduction events in cytokine-mediated induction of iNOS in astrocytes and macrophages are not well understood.

Cytokines (TNF-α, IL-1β, or IFN-γ) and LPS bind to their...
Okadaic acid and other inhibitors of PP 1/2A have also been shown to induce the activation of NF-κB in monocytes, Jurkat T cells, and HeLa cells (41, 42) as a result of phosphorylation of IkBα at protein phosphatase 2A-sensitive phosphorylation sites, and these sites are known to be different from cytokine-induced phosphorylation sites (43). In contrast to the effect of okadaic acid on the activation of NF-κB in other cell types (41, 42), okadaic acid by itself was unable to induce the activation of NF-κB in rat primary astrocytes. However, okadaic acid markedly stimulated the LPS- or cytokine-mediated activation of NF-κB in astrocytes (Figs. 8 and 9). The increase in the activation of NF-κB in LPS-stimulated astrocytes by okadaic acid was accompanied by a concomitant increase in the activation of iNOS, suggesting that okadaic acid paralleled the increase in induction of iNOS, consistent with the transcriptional activity of NF-κB in rat primary astrocytes and macrophages.

**Table II**

| Stimuli                        | Astrocytes | Macrophages |
|-------------------------------|------------|-------------|
| Control                       | 0.3 ± 0.03 | 0.5 ± 0.06  |
| LPS                           | 5.8 ± 0.7  | 18.9 ± 2.3  |
| LPS + calyculin A (1 nM)      | 12.5 ± 1.6 | 27.5 ± 3.1  |
| LPS + calyculin A (2 nM)      | 16.9 ± 2.1 | 31.2 ± 3.6  |
| LPS + okadaic acid (5 nM)     | 10.8 ± 1.2 | 24.3 ± 1.9  |
| LPS + okadaic acid (10 nM)    | 14.6 ± 1.8 | 28.9 ± 3.4  |

**Fig. 8.** Effect of okadaic acid on LPS-mediated DNA binding activity of NF-κB in rat primary astrocytes and macrophages. Astrocytes (A) and macrophages (B) incubated in serum-free DMEM/F-12 were treated with okadaic acid alone or together with LPS (1.0 μg/ml). After 1 h of 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.” Lane 1, nuclear extract of control cells; lane 2, nuclear extract of LPS-treated cells; lane 3, nuclear extract of LPS-treated cells incubated with 100-fold excess of unlabeled oligonucleotide; lane 4, nuclear extract of cells treated with okadaic acid (5 nM) alone; lane 5, nuclear extract of cells treated with okadaic acid (10 nM) alone; lane 6, nuclear extract of LPS and okadaic acid (5 nM)-treated cells; lane 7, nuclear extract of LPS and okadaic acid (10 nM)-treated cells. The upper arrow indicates the induced NF-κB band, whereas the lower arrow indicates the unbound probe.

**Fig. 9.** Effect of okadaic acid on LPS-mediated iNOS expression. Cells preincubated in serum-free DMEM/F-12 with different concentrations of okadaic acid for 30 min were stimulated with 1.0 μg/ml of LPS. After 24 h of incubation, concentration of TNF-α in the culture medium was measured in supernatants as described under “Materials and Methods.” Data are expressed as the means ± S.D. of three different experiments.
enhanced activation of NF-κB. However, consistent with the effect of okadaic acid on the activation of NF-κB in other cell types including monocytes, Jurkat T cells, and HeLa cells (41, 42), okadaic acid by itself induced the activation of NF-κB in macrophages. However, this activation of NF-κB by okadaic acid in macrophages did not result in the induction of iNOS, suggesting that activation of NF-κB by okadaic acid is not sufficient for the induction of iNOS in macrophages. Moreover, similar to astrocytes, the okadaic acid stimulated the LPS-mediated activation of NF-κB in rat peritoneal macrophages, but in sharp contrast to the effect of okadaic acid on the induction of iNOS in LPS-treated astrocytes, the stimulation of NF-κB by okadaic acid in LPS-treated macrophages did not parallel with the expression of iNOS. Instead, consistent with a previous report (44), okadaic acid and other inhibitors of PP 1/2A markedly inhibited the LPS- and cytokine-induced expression of iNOS in macrophages. The basis for this differential regulation of induction of iNOS in astrocytes and macrophages by inhibitors of PP 1/2A is not understood at the present time.

Earlier, we have observed that cAMP-dependent protein kinase (PKA) also differentially modulates the induction of iNOS in astrocytes and macrophages (12). The inhibition of cytokine-induced activation of NF-κB and the induction of iNOS with the increase in PKA activity in astrocytes (12) and the stimulation of the activation of NF-κB and the induction of iNOS with the decrease in PKA activities in astrocytes (Figs. 1 and 8) suggest that both PKA (a serine-threonine protein kinase) and PP 1/2A (serine-threonine phosphoprotein phosphatases) may provide inhibitory signals to LPS- and cytokine-induced signal transduction pathway for the induction of iNOS in astrocytes. In contrast, in macrophages, inhibitors of PKA inhibited the LPS-mediated activation of NF-κB and induction of iNOS (12), and inhibitors of PP 1/2A stimulated the LPS-mediated activation of NF-κB but inhibited the induction of iNOS, suggesting that both PKA and PP 1/2A are necessary components of the LPS-mediated signaling pathways for the induction of iNOS. However, the molecular basis for the differential regulation of activation of NF-κB and expression of iNOS gene by inhibitors of PP 1/2A in rat peritoneal macrophages is not known at the present time. In light of the fact that NF-κB is necessary but not sufficient for the expression of iNOS gene (45, 46) and that many of the signal transduction events are cell type specific (12, 47), the apparent stimulation of NF-κB and inhibition of iNOS gene expression by inhibitors of PP 1/2A clearly delineate that apart from the activation of NF-κB some other signaling pathway(s) sensitive to PP 1/2A is/are responsible for the expression of iNOS gene in macrophages.

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