Involvement of Phospholipase A2 and Lipoxygenase in Lipopolysaccharide-Induced Inducible Nitric Oxide Synthase Expression in Glial Cells

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KEY WORDS
PLA2; COX; 5-LO; 12-LO; iNOS; NFκB; nitric oxide; glial cells

ABSTRACT
The present study underlines the importance of phospholipase A2 (PLA2)- and lipoxygenase (LO)-mediated signaling processes in the regulation of inducible nitric oxide synthase (iNOS) gene expression. In glial cells, lipopolysaccharide (LPS) induced the activities of PLA2 (calcium-independent PLA2; iPLA2 and cytosolic PLA2; cPLA2) as well as gene expression of iNOS. The inhibition of cPLA2 by methyl arachidonyl fluorophosphates (MAFP) or antisense oligomer against cPLA2 and inhibition of iPLA2 by bromoelol lactone reduced the LPS-induced iNOS gene expression and NFκB activation. In addition, the inhibition of LO by nordihydroguaiaretic acid (NDGA; general LO inhibitor) or MK886 (5-LO inhibitor), but not baicalein (12-LO inhibitor), completely abrogated the LPS-induced iNOS expression. Because NDGA could abrogate the LPS-induced activation of NFκB, while MK886 had no effect on it, LO-mediated inhibition of iNOS gene induction by LPS may involve an NFκB-dependent or -independent (by 5-LO) pathway. In contrast to LO, however, the cyclooxygenase (COX) may not be involved in the regulation of LO-mediated induction of iNOS gene because COX inhibition by indomethacin (general COX inhibitor), SC560 (COX-1 inhibitor), and NS398 (COX-2 inhibitor) affected neither the LPS-induced iNOS expression nor activation of NFκB. These results indicate a role for cPLA2 and iPLA2 in LPS-mediated iNOS gene induction in glial cells and the involvement of LO in these reactions. © 2005 Wiley-Liss, Inc.

INTRODUCTION

Recent research has focused on the regulation of the inducible nitric oxide (NO) synthase (iNOS) isoform. The iNOS produces large quantities of NO over prolonged periods of time. In central nervous system, NO produced by iNOS is a major pathophysiological mediator in many inflammatory neurodegenerative diseases, such as ischemia, Alzheimer’s disease, and multiple sclerosis, in which astrocyte- and microglia-derived NO contribute to oligodendrocyte degeneration and neuronal death (Iadecola et al., 1997; Liu et al., 2002; Minagar et al., 2002).

In addition to iNOS gene induction, the activation of phospholipase A2 (PLA2) and accumulation of arachidonic acid (AA) in those disease conditions are another hallmark of neuroinflammation (Huterer et al., 1995; Stephenson et al., 1996; Arai et al., 2001). Three groups of PLA2 are involved in AA generation, depending on their cellular localization and functional regulation: secretory PLA2 (sPLA2), cytosolic PLA2 (cPLA2), and calcium-independent PLA2 (iPLA2). AA produced by these PLA2 have been known to regulate various signal transduction pathways directly via activation of protein kinase C (PKC) (Murakami and Routtenberg, 1985), intracellular Ca2+ level increase (Ferrante et al., 1999), neutral sphingomyelinase (Robinson et al., 1997), and mitogen-activated protein kinases (ERK1/2, p38, and JNK) (Cui and Douglas, 1997; Rizzo and Carlo-Stella, 1996; Hii et al., 1995, 1998) or indirectly via generation of various eicosanoids.

In mammalian cells, the release of AA constitutes the rate-limiting step in the biosynthesis of eicosanoids including prostaglandins (PGs), leukotrienes (LTs), and platelet-activating factor (PAF), all of which act as potent inflammatory mediators. Among these, PGs that play a role as an intracellular or intracellular messenger (Bjorkman, 1998; Dubois et al., 1998; Vane et al., 1998) are implicated in various physiological or pathophysiological processes, such as smooth muscle tone, vascular permeability, cellular proliferation, and inflammatory/immune function (Harris et al., 2002). Currently, two isoforms of enzymes responsible for generation of PGs have been identified; cyclooxygeanse-1 (COX-1) is constitutively expressed in most tissue, COX-2 is primarily considered an inducible gene product whose synthesis can be upregulated by mitogenic or inflammatory stimuli (Dubois et al., 1998). In addition to PGs, leukotrienes (LTs) are potent lipid mediators that have

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important roles in the regulation of both normal cell function and the inflammatory process. The synthesis of LTs is initiated by the enzymes belong to the family of lipooxygenases (LO). Currently, three isofoms of LO have been identified: 5-LO, 15-LO, and 12-LO (Nathoo et al., 2004). Recent studies have documented the involvement of 5-LO in the biosynthesis of leukotrienes, which constitute mediators of anaphylactic and inflammatory disorders (Engels and Nijkamp, 1998). In addition, an involvement of 12/15-LO in atherogenesis has been reported (Feinmark and Cornicelli, 1997; Kuhn and Chan, 1997). However, the role of PLA2, COX, and 5-LO in lipid-mediated signaling in inflammatory disease is not well understood.

The involvement of PLA2-dependent pathway in the regulation of iNOS gene expression has been studied recently. AA generation by group IIA secretary PLA2 (Baek et al., 2001) and the production of prostaglandins (PGs) by COX-2 (Chen et al., 1999) are reported to be positive regulator for iNOS gene expression in macrophages. However, even if the inductions of PLA2 activity and AA accumulation are observed in various neuroinflammatory disease conditions (Huterer et al., 1995; Stephenson et al., 1996; Arai et al., 2001), less attention has been paid to the their involvement, as well as AA metabolism, in the regulation of gene expression of inflammatory mediators including iNOS in the CNS. In the present study, we examined the effect of AA metabolism on the induction of iNOS gene expression in glial cells and also sought to determine the involvement of NFkB in AA-metabolism-mediated regulation of iNOS gene expression. We report that activation of cPLA2 and iPLA2, and AA metabolites of 5-LO pathway are involved in LPS-induced iNOS gene expression via both activation of NFkB-dependent and -independent pathways in glial cells.

**MATERIALS AND METHODS**

**Isolation and Maintenance of Primary Rat Astrocytes and C6 Rat Glioma Cells**

Astrocytes were prepared from 1–2-day-old rat cerebral tissue as described by McCarthy and DeVellis (1980). Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 3 g/L of glucose, 5% fetal bovine serum (FBS), and 10 µg/ml gentamicin on a 75-cm² polystyrene flask (Costar®, Corning, NY). After 10 days of culture, astrocytes were separated from microglia and oligodendrocytes by shaking for 24 h in an orbital shaker at 240 rpm. To ensure the complete removal of all the oligodendrocytes and microglia before subculturing, the shaking was repeated twice after a gap of 1 or 2 days. Cells were trypsinized and subcultured and further incubated for 2 days for stabilization. C6 rat glioma cells (obtained from American Type Cell Culture [ATCC]) were maintained in DMEM containing 3 g/L of glucose, 10% FBS, and 10 µg/ml gentamicin. All cultured cells were maintained at 37°C in 5% CO₂/95% air. At 80% confluence, the cells were incubated with serum-free DMEM medium for 24 h before incubation with LPS and other agents.

**Assay of cPLA2 and iPLA2 Activity**

To assess cPLA2 activity, the cells were washed once with phosphate-buffered saline (PBS), suspended in buffer composed of 10 mM Tris-HCl (pH 7.4) and 150 mM NaCl (Tris-buffered saline [TBS]), and lysed by sonication. The resulting lysates were incubated in 250 µl of buffer composed comprising 100 mM Tris-HCl (pH 9.0), 4 mM CaCl₂, and 2 µM 1-palmitoyl-2-[14C]arachidionyl-glycerophosphoethanolamine (NEN Life Science Products, Boston, MA) as the substrate at 37°C for 30 min. To assess iPLA2 activity, the lysates (prepared in 10 mM HEPES (pH 7.5) containing 1 mM EDTA, 1 mM dithiothreitol (DTT), and 0.34 M sucrose), were incubated in 250 μl of buffer composed of 100 mM HEPES (pH 7.5), 5 mM EDTA, 0.4 mM Triton X-100, 0.1 mM ATP, and 10 μM 1-palmitoyl-2-[14C]arachidionyl-glycerophosphoethanolamine at 40°C for 30 min (Ackermann et al., 1995; Balsinde et al., 1997). The [14C]-AA released was extracted and the associated radioactivity was counted.

**Assay for NO Production and Induction of iNOS Enzyme**

The cells were cultured in 12-well plastic tissue culture plates; 24 h after the LPS treatment, production of NO was determined by an assay of the culture supernatant for nitrite by using Griess reagent and NaNO₂ as a standard (Green et al., 1982). For iNOS protein, cells stimulated with LPS or other drugs for 12 h washed with cold TBS (20 mM Trizma base, and 137 mM NaCl, pH 7.5), lysed in 1× sodium dodecyl sulfate (SDS) sample loading buffer (62.5 mM Trizma base, 2% w/v SDS, 10% glycerol), sonicated briefly, and centrifuged at 15,000g for 5 min. The resulting supernatant was used for the iNOS Western immunoblot assay. All samples were boiled for 3 min with 0.1 vol of 10% β-mercaptoethanol and 0.5% bromophenol blue mix; 50 µg of total cellular protein was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After blotting to polyvinylidene difluoride (PVDF) filter, the iNOS bands were detected according to standard protocol by using anti-iNOS rabbit polyclonal antibody (BD Transduction Laboratories, cat. no: 610332 Franklin Lakes, NJ).

**Isolation of Total RNA and Northern Blot Assay**

The isolation and Northern blot assay was performed as described previously (Won et al., 2003). Five µg of total RNA was electrophoresed on 1% agarose-formaldehyde gels and transferred onto nylon Hybond-N hybridization membrane sheets (Amersham Pharmacia Biotech, Piscataway, NJ). After ultraviolet (UV)-cross linking, the membranes were prehybridized at 68°C in prehybridization
buffer (5× SSC, 50% formamide, 0.02% SDS, 0.1% sodium N-lauroyl sarcosine, and 2% blocking reagent), and then digoxigenin (DIG)-labeled iNOS antisense-cRNA (1–893 bp) probes were added to prehybridization buffer and incubated overnight. The membranes were washed three times in 2× SSC and 0.1% SDS at room temperature (RT), and in 0.1× SSC and 0.1% SDS at 68 °C. After equilibrating the membranes in maleic acid buffer (100 mM maleic acid and 150 mM NaCl, pH 7.5), the membranes were blocked in maleic acid buffer containing 1% blocking reagent (Roche Molecular Biochemicals, Indianapolis, IN). Chemiluminescent autoradiography detection was performed as suggested by the manufacturer (Roche Molecular Biochemicals) using alkaline phosphatase-conjugated anti-DIG F(ab)2 fragment (Roche Molecular Biochemicals) and CSPD (disodium 3-(4-methoxyphenyl)2-dioxetane-3,2-cyclopropene-1,2-dioxetane-3,2-(5′-chlorotricyclo[3.3.1.17,7]decane-4-yl)phenyl phosphate; Roche Molecular Biochemicals).

**Knockdown of cPLA₂ Using Antisense DNA Oligomer**

For the transfection, C6 rat glioma cells were cultured on 24-well plates. At 80% of confluence, phosphorothioated cPLA₂ antisense DNA oligomer (GsTsGCTGATAAGATCTCAsT) directed against codons 4–9 of the rat cytosolic, calcium-dependent PLA₂ (GeneBank ID: 19424223) and its sequence scramble mixture (GsAsTAGAAGCTGTTTCAsCsT) were transfected using Oligofectamin™ (Invitrogen, Carlsbad, CA) according to manufacturer’s instructions.

**Gel Shift Analysis**

Nuclear extract from C6 cells (1 × 10⁶ cells) were prepared using previously published method (Dignam et al., 1983) with slight modification. Cells were harvested, washed twice with ice-cold TBS, and lysed in 1 ml of buffer A [10 mM KCl, 2 mM MgCl₂, 0.5 mM DTT, protease inhibitor cocktail (Sigma Chemical Co., St. Louis, MO), and 0.1% Nonidet P-40 (NP-40) in 10 mM HEPES, pH 7.9] for 10 min on ice. After centrifugation at 1,000 g, the pelleted nuclei were washed with buffer A without NP-40, and resuspended in 40 μl of buffer B [25% (v/v) glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, and protease inhibitor cocktail (Sigma) in 20 mM HEPES, pH 7.9] for 30 min on ice. The lysates were centrifuged at 15,000 g for 15 min and the supernatants containing the nuclear proteins were stored at −70 °C until use; 10 μg of nuclear proteins was used for the electrophoretic mobility shift assay for detection of NFkB DNA binding activity. The DNA-protein binding reactions were performed at room temperature for 20 min in 10 mM Trizma base (pH 7.9), 50 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 1 μg poly (di-dc), 5% (v/v) glycerol, and ~0.3 pmol of NFkB (Santa Cruz Biotechnology, Santa Cruz, CA) labeled with DIG-ddUTP, using terminal deoxynucleotidyl transferase (Roche Molecular Biochemicals). Protein–DNA complexes were resolved from protein-free DNA in 5% polyacrylamide gels at room temperature in 50 mM Tris, pH 8.3, 0.38 M glycine, and 2 mM EDTA, and electroblotted onto positively charged nylon membranes. Chemiluminescence detection for DIG-labeled probes was performed as described in Northern blot analysis.

**Nuclear Run-On Assay**

The cells (5 × 10⁶) were pelleted at 500g for 5 min at 4°C, and then washed in 10 ml of ice-cold TBS. The cells were placed in 2 ml of lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 0.1% NP-40) for 3 min on ice, and the nuclei were obtained by centrifugation at 1,000g for 5 min at 4°C. The nuclei were washed twice in 5 ml lysis buffer without NP-40. The nuclear pellet was suspended in 120 μl nuclear freezing buffer (50 mM Tris-HCl, pH 8.3, 40% glycerol, 5 mM MgCl₂, 0.1 mM EDTA). The nuclei were either used directly or stored at −70°C. If frozen, nuclei were thawed on ice before use. The nuclei were mixed with 30 μl of 5× run-on buffer (25 mM Tris-HCl, pH 8, 12.5 mM MgCl₂, 750 mM KCl, 5 mM DTT, 5 mM EDTA, 2 mM each of ATP, GTP, and CTP and UTP, and 20 mM DIG-11-UTP). The mixture was incubated for 30 min at 30°C, and then 15 μl RNase-free DNase 1 (Promega, Madison, WI; 1 U/μl) was added and incubated for another 15 min. The nuclear RNA was extracted by using Trizol (Invitrogen). After washing RNA pellet twice with 75% ethanol, it was dissolved in 50 μl of formamide and further diluted with prehybridization buffer. For slot blotting, linearized iNOS (Won et al., 2003) and cyclophilin (Takahashi et al., 1989) plasmid DNA (5 μg per sample) were dissolved 20 μl with TE and further dilute with 180 μl of 6× SSC. After denaturation by boiling for 10 min, the DNA solution was applied to N+ nylon membrane (Amersham-Pharmacia) using a slot blot apparatus (Bio-Rad, Hercules, CA). After UV cross-linking, each membrane was prehybridized with prehybridization buffer for 2 h at 60°C and hybridized with sample probe solution overnight at 60°C. The washing and chemiluminescence detection for DIG-labeled probes was performed as described in Northern blot analysis.

**Transient Transfection and Reporter Gene Assay**

The cells (3 × 10⁵ cells/well) were cultured in 6-well plates for 36 h before the transfection. Transfection was performed with 2 μg of kb repeat SEAP reporter construct (pNFkB-SEAP, BD Bioscience, CA) and 0.2 μg of pCMV-β-Gal (Stratagene, La Jolla, CA). One day after transfection, the cells were placed in serum-free media overnight. After appropriate treatment, the media was used for the detection of level of SEAP, and the cells were washed with PBS, scraped, and then resuspended with 100 μl of lysis
buffer (40 mM of Tricine pH 7.8, 50 mM of NaCl, 2 mM of EDTA, 1 mM of MgSO4, 5 mM of DTT, and 1% of Triton X-100) for the detection of β-galactosidase. Detection of SEAP and β-galactosidase was performed using Great EscAPE™ SEAP chemiluminescence detection kit and β-gal Detection Kit (BD Bioscience). The emitted light and optical absorbance was measured using Spectra Max/ Gemini XG (Molecular Devices, Sunnyvale, CA) and SpectraMax 190 (Molecular Devices), respectively.

Statistical Analysis

All values shown in the figures are expressed as means ± standard error of mean (SEM) of n determinations, obtained on at least three independent experiments. The results were examined by one- and two-way analysis of variance (ANOVA). Individual group means were then compared with the Bonferroni test. A P-value of <0.05 was considered significant.

RESULTS

LPS Induces Enzymatic Activities of cPLA2 and iPLA2 in Glial Cells

The involvement of cPLA2 and iPLA2 in various inflammatory conditions has been suggested in various cell types. To address the role of these enzymes in the regulation of iNOS gene expression, we examined the effect of LPS (1 μg/ml) in the regulation of activation of cPLA2 and iPLA2 in C6 rat glioma cells. As depicted in Figure 1, LPS increased enzyme activities of cPLA2 and iPLA2 within 30 min. These activities continued to increase until 3 h followed by a slow decline. Similar results were observed in three sets of independent experiments by using arachidonyl thiophosphatidylcholine as a colorimetric substrate (data not shown).

LPS-Induced iNOS Gene Expression Is Mediated by cPLA2 and iPLA2

To examine the involvement of LPS-mediated induction of cPLA2 and iPLA2 activities in iNOS gene regulation, the cells were preincubated with methyl arachidonyl fluorophosphonate (MAFP, a cPLA2 inhibitor) or bromoeno lactone (BEL, iPLA2 specific inhibitor) before LPS treatment. As shown in Figure 2, the LPS-induced NO production and iNOS mRNA and protein expression were inhibited by MAFP (Fig. 2A,C) and BEL (Fig. 2B,C) in C6 rat glia cells as well as in primary cultured astrocytes (Fig. 2D).

Since MAFP is known to inhibit the activity of iPLA2 as well as cPLA2 (Lio et al., 1996), we confirmed the role of cPLA2 in the regulation of iNOS gene expression by using antisense DNA oligomer against cPLA2 mRNA. As shown in Figure 3A, the knock-down of cPLA2 protein by antisense DNA inhibited LPS-mediated increases in NO pro-
Production via inhibition of iPLA2 rather than PAP. This result indicates that BEL inhibits iNOS enhanced LPS-induced NO production and iNOS protein expression. As shown in Figure 3B, 150 μM propranolol, a PAP inhibitor, further enhanced LPS-induced NO production and iNOS protein expression. This result indicates that BEL inhibits iNOS production via inhibition of iPLA2 rather than PAP.

Cyclooxygenases Are Not Involved in LPS-Induced iNOS Gene Expression

Possible involvement of COX in the upregulation of iNOS gene expression has been suggested in various cell types (Minghetti et al., 1997; Chen et al., 1999; Hori et al., 2001). To address the involvement of COX in the regulation of iNOS expression in glial cells, the cells were preincubated with indomethacin (INDO, a general inhibitor of COX), NS389 (a COX-2 specific inhibitor), or SC560 (a COX-1-specific inhibitor) before LPS treatment. As shown in Figure 4, the LPS-induced NO production and iNOS mRNA and protein expression were not affected by any of the COX inhibitors (INDO, NS389, or SC560) in C6 rat glioma cells and in primary cultured astrocytes, suggesting that COX are not involved in the regulation of iNOS expression in glial cells.

Lipoxygenases Mediate LPS-Induced iNOS Gene Expression

Growing evidence suggests certain leukotrienes have a critical role in the signaling cascade of inflammatory gene expression (Sala et al., 1998). To address the involvement of LO in the regulation of iNOS expression in glial cells, the cells were preincubated with nordihydroguaiaretic acid (NDGA, a general LO inhibitor), MK886 (5-LO specific inhibitor), or baicalein (BCL, a 12-LO specific inhibitor, 5,6,7-trihydroxyflavone) before LPS treatment. As shown in Figure 5, the LPS-induced NO production and iNOS mRNA and protein expression were inhibited by NDGA at a dose-dependent manner in C6 rat glioma cells and primary cultured astrocytes. Furthermore, MK886, but not BCL, inhibited LPS-induced iNOS expression, suggesting that 5-LO is one of LO which mediates LPS signaling to iNOS gene expression.

Inhibitors of PLA2 or LO Inhibit iNOS Expression Via Inhibition of Transcriptional Activity

To identify whether the inhibition of iNOS expression by the inhibitors of PLA2 or LO is mediated by the inhibition of transcription activity of iNOS mRNA, nuclei run on assay was performed. As shown in Figure 6, the activation of transcription of iNOS mRNA by LPS is downregulated by the preincubation with MAFP, BEL, NDGA, and MK886 similar to the level of mRNA accumulation depicted in Figures 2 and 5, suggesting that the inhibition of iNOS mRNA level by those inhibitors resulted from the inhibition of its transcriptional activity.

PLA2/LO-Mediated LPS-Induced iNOS Expression via NFκB-Dependent or -Independent Pathways

NFκB is an essential transcription factor in the activation of iNOS gene in rodents as well as humans (Lowenstein et al., 1993; Beck and Sterzel, 1996; Marks-Konczalik et al., 1998). To define the role of NFκB in PLA2- or LO-mediated inhibition of iNOS expression, the effects of inhibitors of those enzymes on LPS-induced increase in NFκB DNA binding activity and its transactivity were examined. As shown in Figure 7, MAFP, BEL, and NDGA inhibited LPS-induced activation of NFκB DNA binding activity and its transactivity (κB-luciferase reporter gene activity), suggesting that PLA2 and LO are involved in NFκB-mediated iNOS gene regulation. Although, MK886 inhibited the iNOS expression (Fig. 5) but it had no effect on NFκB DNA binding activity as well as its transactivity, suggesting that 5-LO may mediate iNOS gene expression via NFκB-indepenent pathways.

DISCUSSION

These studies document the involvement of cPLA2 and iPLA2-mediated signaling pathways in the induction of iNOS expression by LPS, and indicate LO as one of the mediators that may regulate PLA2-mediated iNOS gene induction by activation of NFκB-dependent or independent pathways in glial cells. These conclusions are based on the following observations: (1) addition of cPLA2 or iPLA2 inhibitor and decoy of cPLA2 mRNA inhibited the LPS-induced iNOS gene expression; and (2) addition of
general inhibitor of LO (NDGA) and 5-LO specific inhibitor, but none of inhibitors for COX (INDO, NS398, or SC560) and 12-LO used, inhibited LPS-induced iNOS gene expression. Finally, LPS-induced activation of NFκB was inhibited by the inhibitor of cPLA2, iPLA2 and general inhibitor of LO, but not by specific inhibitor of 5-LO.

Previously, the possible involvement of cPLA2 in TNF-α-mediated signal transduction pathway leading to nuclear translocation of NFκB and to NFκB-activated gene expression in a keratinocyte cell line has been documented by using cPLA2 inhibitor, AACOCF3 (Thommesen et al., 1998). Consistent with this study, we also observed that cPLA2-inhibitor (MAFP) inhibited NFκB activation and iNOS gene expression in glial cells. However, since both inhibitors (AACOCF3 and MAFP) also inhibit the activity of iPLA2 (but not sPLA2) (Balsinde and Dennis, 1996; Lio et al., 1996), it is unclear whether LPS-induced iNOS gene expression is mediated by cPLA2 or iPLA2. Therefore, the observed inhibition of NO production and iNOS protein expression by antisense DNA oligomer against cPLA2 mRNA in this study documents the role of cPLA2 in LPS-mediated iNOS expression in glial cells.

Recently, the role of iPLA2 in pathophysiological signaling cascades has been described. In rat cardiac ventricular myocytes, iPLA2 is activated during exposure to interleukin-1β (IL-1β) (McHowat and Liu, 1997). In macrophages, iPLA2 is upregulated by reactive oxygen species (Martinez and Moreno, 2001). In addition, iPLA2 inhibitor prevents double strand RNA- and virus-induced iNOS and COX-2 expression in macrophages (Maggi et al., 2002; Steer et al., 2003). In the present study, we demonstrated the possible involvement of iPLA2 in LPS-mediated NFκB activation and iNOS gene expression in glial cells by using bromoenol lactone (BEL). Although BEL is a potent (1,000-fold more than cPLA2), selective inhibitor for iPLA2, it is also known to inhibit magnesium-dependent PAP in mouse P388D1 macrophages (Balsinde and Dennis, 1996). However, enhancement of LPS-induced NO production and iNOS protein expression by 150 μM propranolol, a PAP inhibitor at high concentration (>100 μM) (Billah et al., 1989; Bourgoin et al., 1990; Kanaho et al., 1991; Thompson et al., 1991), indicates that BEL-mediated inhibition of iNOS gene expression may be mediated by the inhibition of iPLA2 rather than PAP. The mechanism by which LPS stimulates iPLA2 activation has yet to be determined. Although iPLA2 contains consensus sites for a number of serine kinases (Ma and Turk, 2001), LPS-mediated phosphorylation and activation of iPLA2 has not been described so far. Moreover, the mechanism of iPLA2-mediated regulation of NFκB path-
ways in response to LPS should be examined in future study.

It has been reported that cytokine induces COX-2 expression and followed PGE2 production mediate upregulation of iNOS gene expression in macrophages via activating cAMP pathway (Chen et al., 1999). However, no involvement of COX in iNOS gene regulation was observed in glial cells in the present study. The differences between these cell types for the role of COX-2 in iNOS gene expression remain unclear, but the undetectable COX-2 protein level in resting glial cells and the opposite role of cAMP in the regulation of iNOS expression between in macrophages and glial cells may account, at least in part, for these differences (Minghetti et al., 1997; Pahan et al., 1997; Chen et al., 1999; Won et al., 2001).

It has been shown that a significant amount of cPLA2 resides within the nucleus (Sierra-Honigmann et al., 1996). Moreover, 5-LO translocates to a perinuclear site in stimulated cells (Peters-Golden and McNish, 1993). Therefore, in activated cells cPLA2 may be ideally positioned to provide free arachidonic acid to 5-LO in the nucleus. In this study, we observed that the MK886 (an inhibitor of 5-LO) inhibited the LPS-induced iNOS gene expression without altering NF-kB activation. However, general inhibitor of LO (NDGA) inhibited LPS-induced activation of NF-kB as well as iNOS gene expression. Since NDGA can also interfere weakly with PLA2 and COX activities (Aktan et al., 1993; Meade et al., 1993) and act as an antioxidant (Ramasamy et al., 1999), the inhibitory effect of NDGA on iNOS gene expression and NF-kB activation induced by LPS may be via inhibiting PLA2 activities or its anti-oxidative properties. Moreover, these data indicate the possible involvement of other NDGA-sensitive enzymes that regulate of iNOS expression via regulation of NF-kB activation. The involvement of leukotrienes in the regulation of iNOS gene expression has been reported previously in various cell types. In rat vascular smooth muscle cells, the 12-LO product, 12-HETE, dose-dependently enhanced nitrite production induced by IL-1β (Shimpo et al., 2000). Furthermore, BCL (12-LO inhibitor) inhibits iNOS gene expression in macrophages (Chen et al., 2001; Hashimoto et al., 2003). However, we did not observe the involvement of 12-LO in iNOS gene expression or in NF-kB activation in C6 rat glioma cells as well as primary astrocytes. Therefore, the involvement of 12-LO in iNOS gene expression is believed to be a cell type specific event.

In conclusion, our results document that PLA2 and LO play an essential role in the activation of iNOS gene

Fig. 5. Lipopolysaccharide (LPS)-induced iNOS gene expression is mediated by LO. To examine the involvement of lipoygenases (LO) in iNOS gene expression, C6 rat glioma cells (A–C) and primary astrocytes (D) were treated with vehicle (VHC; dimethylsulfoxide), NDGA (nordihydroguaiaretic acid, general LO inhibitor), MK886 (5-LO inhibitor), and BCL (baicalein, 12-LO inhibitor) for 30 min before lipopolysaccharide (LPS; 1 μg/ml) treatment. To quantify NO production (A,D) and iNOS protein (C,D), nitrite level in media and iNOS proteins were determined 24 h after LPS treatment as described in Materials and Methods. For the detection of iNOS mRNA, total RNA was extracted 6 h after LPS treatment, and 5 μg of total RNA was used for detection of iNOS mRNA. The equal amounts of CPN (cyclophilin) mRNA verify that equal amounts of RNA were loaded. Vertical bars indicate standard error of mean (*P < 0.05,**P < 0.01, ***P < 0.001 compared to control group; ++P < 0.05, +++P < 0.01, ++++P < 0.001 compared to LPS-treated group).
expression via NFκB-dependent or -independent pathways. To define the role of NFκB in PL2α- (A) or LO- (B) mediated inhibition of iNOS expression, the effects of inhibitors of those enzymes on LPS-induced increase in NFκB DNA binding activity and its transactacy were examined. For the reporter gene assay, pNFκB-SEAP (secreted alkaline phosphatase) and pCMV-βGal (β-galactosidase) were co-transfected to the cells. The cells were treated with vehicle (VHC; dimethylsulfoxide), MAFF (methyl arachidonyl fluorophosphonate; cPLA2 and iPLA2 inhibitor, 10 μM) and BEL (bromoenol lactone; iPLA2 specific inhibitor, 1 μM), NDGA (nordihydroguaiaretic acid, general LO inhibitor, 20 μM), and MK886 (5-LO inhibitor, 10 μM) for 30 min before lipopolysaccharide (LPS; 1 μg/ml) treatment. At 3 h after LPS treatment, the nuclei were isolated from cells and nuclear run-on assay was performed as described in Materials and Methods. The equal amounts of CPN (cyclophilin) mRNA verify that equal amounts of mRNA were loaded.

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