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# Lipoteichoic acid induces prostaglandin $E_2$ release and cyclooxygenase-2 synthesis in rat cortical neuronal cells: Involvement of PKCε and ERK activation

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**Abstract**

Inflammatory processes occur in the central nervous system (CNS) through mechanisms that differ from other inflammation, and with distinct cellular effects. Neuronal injury in bacterial meningitis is not a monocausal event, but is mediated by several factors. One is possible direct toxicity of bacterial compounds. Lipoteichoic acid (LTA) is a cell wall component unique to Gram-positive bacteria. In a previous report, LTA could interact with CD14 to induce NF-κB activation, which is involved in transcriptional regulation of adhesion molecules, enzymes and cytokines. Although there are many aspects to neuroinflammation, the pathways involving the cyclooxygenase (COX)-2 and subsequent generation of prostaglandin clearly play a role. LTA has been shown to stimulate inflammatory responses in a number of in vivo and in vitro experimental models. However, little was known about the molecular mechanisms of LTA implicated in inflammatory responses in neurons. In this study, we characterized the mechanisms underlying signaling transduction in rat cortical neuronal cells challenged by LTA. Here, we first showed that in rat cortical neuronal cells, LTA might activate protein tyrosine kinase (PTK), phosphatidylcholine-specific phospholipase C (PC-PLC), and phosphatidylinositol-specific phospholipase C (PI-PLC) to induce protein kinase Cε activation, which in turn induces extracellular signal-regulated kinase (ERK) activation, finally inducing $PGE_2$ release and COX-2 synthesis.


*Keywords:* LTA; $PGE_2$; COX-2; PKCε; ERK

## Introduction

In bacterial meningitis, bacteria enter the central nervous system (CNS) via the blood stream or focal infection in the vicinity of the CNS. Within the cerebrospinal fluid (CSF), bacteria multiply, lyse spontaneously and release proinflammatory and toxic compounds by autolysis and secretion (Stuertz et al., 1998, 1999). More than half of invasive bacterial infections are Gram-positive in origin. The Gram-positive bacterium *Staphylococcus aureus* is one of the bacteria most commonly isolated from patients with sepsis. Lipoteichoic acid (LTA) is a cell wall component unique to Gram-positive bacteria, which contains an acyl group anchored to the cell membrane. LTA functions as an adhesion molecule to facilitate the binding of bacteria to cells, colonization, and invasion (Alkan and Beachey, 1978). LTA could interact with CD14 to induce NF-κB activation (Opitz et al., 2001), which is involved in inflammatory cytokine production. LTA can induce the secretion of proinflammatory cytokines, including IL-1β, IL-6 and TNF-α from macrophages and monocytes (Keller et al., 1992). LTA also has capacity to activate leukocytes and leads to inflammatory responses (Ginsburg, 2002). LTA induced the expression of inducible nitric oxide synthase (iNOS) in vascular smooth muscle cells (Auguet et al., 1992) and in the mouse macrophage cell line J774 (Hattor et al., 1997). LTA induced the expression of cyclooxygenase (COX)-2 in human pulmonary epithelial cells (Lin et al., 2001).

Although there are many aspects to neuroinflammation, the pathways involving the COX and subsequent generation of

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prostaglandin (PG) clearly play a role. In the CNS, COX is localized to neurons, astrocytes, and microglia and can be induced under various conditions. There are two distinct COX isozymes known as COX-1 and COX-2 that are 65% homologous. COX-1 is constitutively expressed in most tissues and produces prostaglandin that generally serves a housekeeping function. Conversely, COX-2 was initially characterized as an inducible enzyme that is expressed in response to inflammatory stimuli, cytokines, and mitogens (O'Banion, 1999). The regulation and function of COX-2 and prostaglandin synthesis in neurons are not completely understood. There appears to be a dual role for the products of the COX-2 in CNS. Some prostaglandins promote the survival of neurons, while others promote apoptosis (Hoffmann, 2000).

LTA has been shown to stimulate inflammatory responses in a number of in vivo and in vitro experimental models. However, little was known about the molecular mechanisms of LTA implicated in inflammatory responses in neurons. In the present study, we were interested in determining whether LTA treatment can induce $PGE_2$ release and COX-2 expression in the rat cortical neuronal cells, and further to elucidate the underlying signaling pathway.

## Materials and methods

### *Materials*

Modified Eagle medium (MEM), fetal calf serum (FCS), and horse serum (HS) were purchased from Life Technologies (Gaithersburg, MD). SB203580, PD98059, Go 6983, R0 31-8220, and PKCε translocation inhibitor peptide (EAVSLKPT) were purchased from Calbiochem (San Diego, CA). Lipoteichoic acid (LTA) derived from *S. aureus*, actinomycin D, and cycloheximide were purchased from Sigma (St. Louis, MO). U-73122 and D-609 were purchased from RBI (Natick, MA). Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) unless otherwise indicated. Reagents for SDS-polyacrylamide gel electrophoresis were from Bio-Rad (Hercules, CA).

### *Rat cortical neuronal cell culture*

As previously described (Yeh et al., 2002), rat neocortices obtained from fetal Sprague–Dawley rat brains at embryonic day 16–18 (Animals Center of National Science Council, Taiwan) were dissected and freed from the meninges. The tissues were then incubated with 0.25% trypsin at 37 °C for 1 h. Cells were mechanically dissociated by repetitive pipetting with a fire-polished glass pipette. After low-speed centrifugation, the cell-containing pellet was suspended in Minimum Essential Medium supplemented with 5% fetal calf serum and 5% horse serum. The cells were plated in poly-D-lysine coated 12-well culture plates at a final cell density of $1\times10^5$ cells/ml and maintained in a humidified $CO_2$ incubator. The cells were incubated for 24 h, following which the medium was removed and the same medium without serum was added. Cell cultures were incubated for up to 7 days without changing the medium. At DIV (day in vitro) 7–9, 10 μM cytosine arabinofuranoside was added to cultures to minimize glial growth, following which one-half of the medium was replaced. Rat cortical neuronal cell cultures at DIV 13–15 were used for experiments.

### *Immunofluorescent staining*

Primary rat cortical neuronal cell cultures at DIV 13–15 were fixed with 4% paraformaldehyde for 15 min at room temperature, treated with 0.1% Triton X-100 and 3% normal goat serum in phosphate-buffered saline for 1 h, and then stained with specific primary antibodies (neuron-specific enolase for neuronal cells, glial fibrillary acidic protein for astrocytes, ED 8 for microglia/macrophages, and CD 31 for endothelial cells). The primary antibodies were then detected with fluorescein conjugated secondary antibody. The cells were counterstained with PI to identify all nuclei.

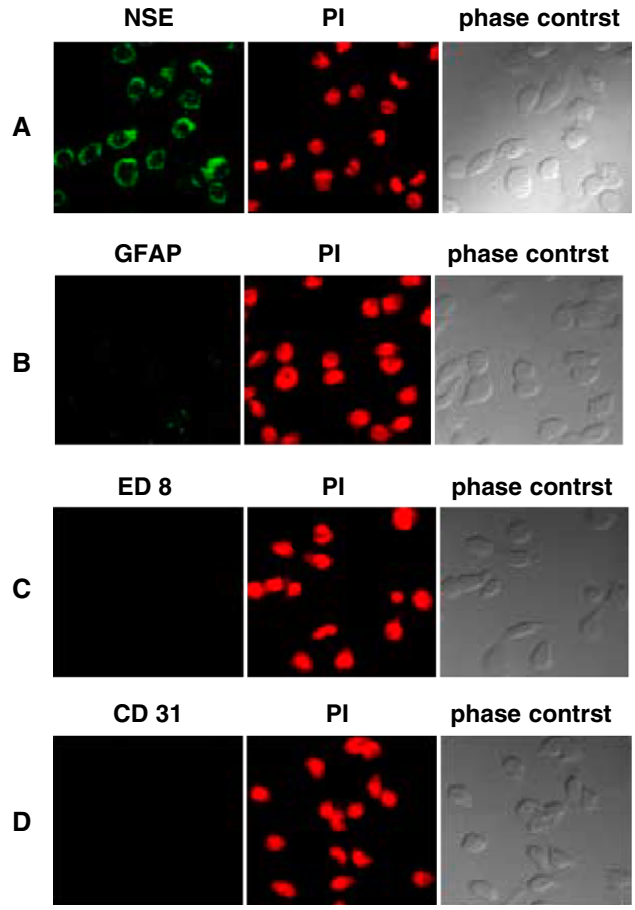


Fig. 1. Primary rat cortical neuronal cell cultures contain at least 99% neuronal cells. Cell cultures at DIV 13–15 were immunolabeled for NSE, GFAP, ED 8 and CD 31 (A to D). Immunolabel-positive cells were shown in green (left column). Nuclei were stained with PI and shown in red (middle column). Light microscopy photomicrographs were also shown (right column). Cell cultures contain at least 99% neuronal cells, less than 1% astrocytes. There is no contamination with microglia/macrophages and endothelial cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

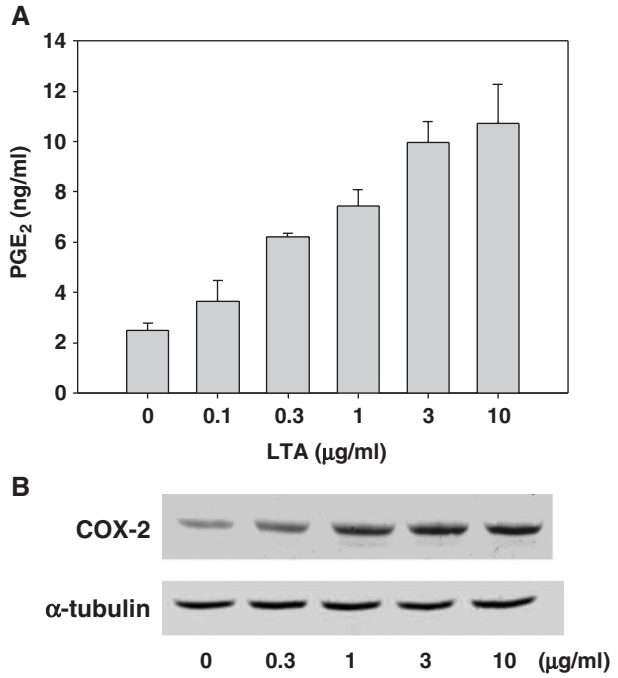


Fig. 2. LTA stimulated dose-dependent increase in $PGE_2$ release and COX-2 expression in rat cortical neuronal cells. A. Rat cortical neuronal cells were treated with various concentration of LTA for 24 h. The supernatant was collected and analyzed for $PGE_2$ release. The results are expressed as the mean±SD of three independent experiments. B. Rat cortical neuronal cells were treated with various concentration of LTA for 24 h. Whole cell protein was obtained, and 30 μg/sample were run on a 10% SDS-PAGE gel. Western analysis was performed for COX-2 protein and α-tubulin. Immunoreactive bands were visualized using chemiluminescence and Hyperfilm.

*Electrophoresis and immunoblotting*

Rat cortical neuronal cell cultures at DIV 13–15 were exposed to LTA (0.1–10 μg/ml) for 24 h or LTA (3 μg/ml) for indicated time intervals. In some experiments, cells were exposed to LTA (3 μg/ml), or pretreatment with specific inhibitors as indicated followed by LTA (3 μg/ml). After incubation, cells were harvested. Protein was extracted with lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM $Na_3VO_4$, 1 μg/ml leupeptin, 1 mM PMSF). Electrophoresis was ordinarily carried out on different percentages of SDS-polyacrylamide electrophoresis (SDS-PAGE) according to the method of Laemmli. Following electrophoresis, proteins on the gel were electrotransferred onto a nitrocellulose membrane according to the method of Twobin et al. After transfer, the nitrocellulose papers were blocked with blocking solution containing 5% skim milk in TBST containing 10 mM Tris (pH 7.5), 100 mM NaCl and 0.1% Tween 20 for 1 h at room temperature. The nitrocellulose membrane was incubated with a solution containing primary antibody in the blocking buffer. Finally, the nitrocellulose membrane was incubated with peroxidase-linked secondary antibody for 1 h and then developed using a commercially available chemiluminescence kit (from Amersham).

*Immunoprecipitation*

Rat cortical neuronal cell cultures at DIV 13–15 were exposed to LTA (3 μg/ml) for indicated time interval. In some experiments, cells were exposed to LTA (3 μg/ml), or pretreatment with specific inhibitors as indicated followed by LTA (3 μg/ml). After incubation, cells were harvested. Protein was extracted with lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM $Na_3VO_4$, 1 μg/ml leupeptin, 1 mM PMSF) and measured. From each sample, 200 μg was removed for immunoprecipitation. The sample was added 10 μl primary antibody and 20 μl Protein A/G Plus-agarose, and then incubated with gentle rocking overnight at 4 °C. The beads were subsequently washed twice with lysis buffer and twice with kinase buffer (25 mM Tris pH 7.5, 5 mM β-glycerolphosphate, 2 mM DTT, 0.1 mM $Na_3VO_4$ and 10 mM $MgCl_2$).

*Kinase activity assay*

After the relevant kinase was immunoprecipitated from cells, the protein-containing pellet was suspended in 50 μl kinase buffer, and the following were added: 200 μM ATP, 2 μg Elk-1 fusion protein. The reaction was continued for 1 h at 30 °C and then stopped by the addition of 25 μl/sample 3× sample buffer. The samples were boiled for 5 min and run on a 10% SDS-PAGE gel. Samples were analyzed by Western blotting and probed with phospho-Elk-1 antibody.

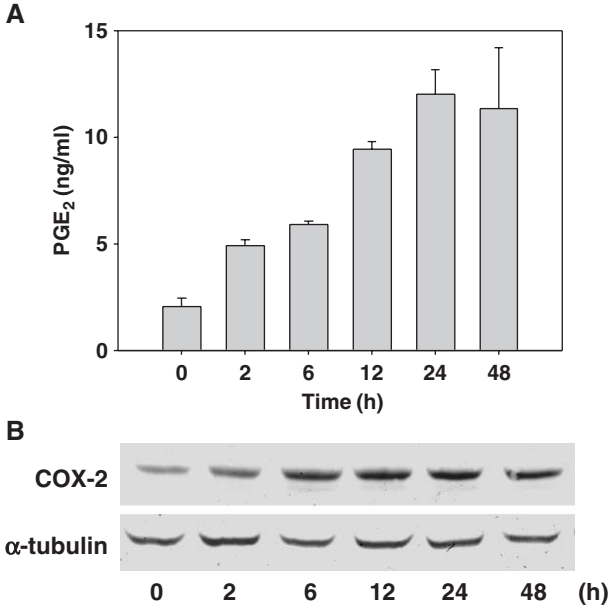


Fig. 3. LTA stimulated time-dependent increase in $PGE_2$ release and COX-2 expression in rat cortical neuronal cells. A. Rat cortical neuronal cells were treated with LTA (3 μg/ml) for indicated time interval. The supernatant was collected and analyzed for $PGE_2$ release. The results are expressed as the mean±SD of three independent experiments. B. Rat cortical neuronal cells were treated with LTA (3 μg/ml) for indicated time interval. Whole cell protein was obtained, and 30 μg/sample were run on a 10% SDS-PAGE gel. Western analysis was performed for COX-2 protein and α-tubulin. Immunoreactive bands were visualized using chemiluminescence and Hyperfilm.

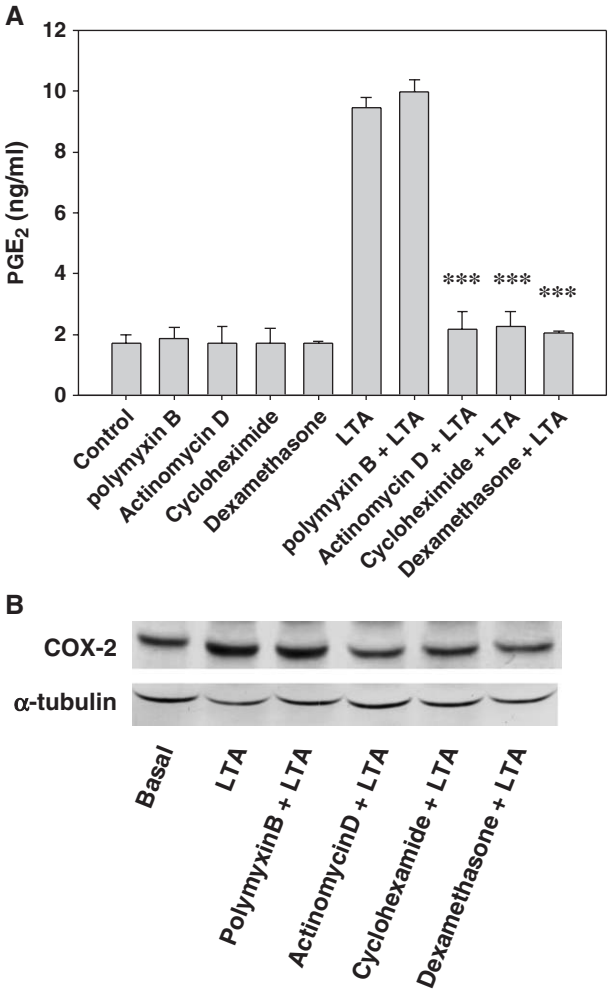


Fig. 4. LTA-stimulated increase of $PGE_2$ release in rat cortical neuronal cells is due to an induction of COX-2 protein synthesis. A. Rat cortical neuronal cells were pretreated with actinomycin D (1 μM; a transcription inhibitor), cyclohexamide (10 μM; a translation inhibitor), dexamethasone (1 μM; a synthetic glucocorticosteroid), or polymyxin B (10 μg/ml; a LPS inhibitor) for 30 min, and then treated with 3 μg/ml LTA for 24 h. The supernatant was collected and analyzed for $PGE_2$ release. The results are expressed as the mean ± SD of three independent experiments. ***: $p<0.001$ as compared with the LTA alone. B. Rat cortical neuronal cells were pretreated with actinomycin D (1 μM; a transcription inhibitor), cyclohexamide (10 μM; a translation inhibitor), dexamethasone (1 μM; a synthetic glucocorticosteroid), or polymyxin B (10 μg/ml; a LPS inhibitor) for 30 min, and then treated with 3 μg/ml LTA for 24 h. Whole cell protein was obtained and 30 μg/sample were run on a 10% SDS-PAGE gel. Western analysis was performed for COX-2 protein and α-tubulin. Immunoreactive bands were visualized using chemiluminescence and Hyperfilm.

### *Analysis of PKC isoforms translocation*

For the detection of protein kinase C (PKC) translocation, cytosolic and membrane fractions were separated as described previously (Li et al., 1998). Briefly, the cells were homogenized in ice-cold homogenization buffer (20 mM Tris–HCl, 2 mM EDTA, 5 mM EGTA, 20% glycerol, 2 mM PMSF, 1% aprotinin, 5 mM DTT, pH 7.5). Cells were centrifuged at 800 *g* for 10 min at 4 °C. The supernatant was recovered, sonicated and centrifuged at 25,000 *g* for 15 min at 4 °C, obtaining supernatant as a soluble (cytosolic) fraction. The pellet, membrane fraction was resolved in homogenization buffer containing 1% NP-40.

### *Quantification of PGE2*

Rat cortical neuronal cell cultures at DIV 13–15 were exposed to LTA (0.1–10 μg/ml) for 24 h or LTA (3 μg/ml) for an indicated time interval. In some experiments, cells were exposed to LTA (3 μg/ml), or pretreatment with specific inhibitors as indicated followed by LTA (3 μg/ml). Supernatant was harvested and centrifuged at 10,000 *g* for 10 min. Level of

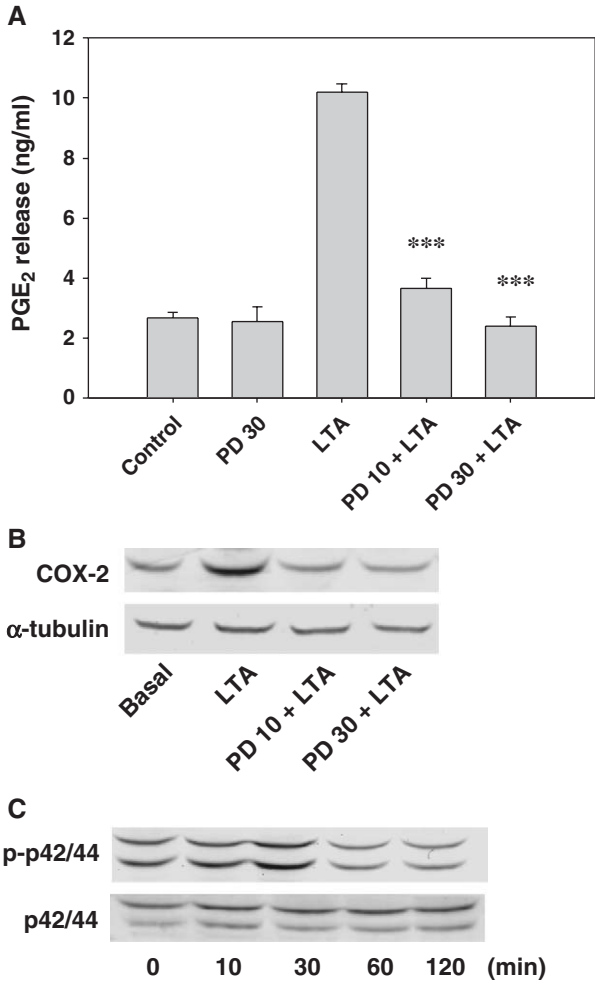


Fig. 5. ERK activation is involved in the LTA induced $PGE_2$ release and COX-2 expression in rat cortical neuronal cells. A. Rat cortical neuronal cells were pretreated with PD98059 (10, 30 μM; a MEK inhibitor) for 30 min and then treated with LTA (3 μg/ml) for 24 h. The supernatant was collected and analyzed for $PGE_2$ release. The results are expressed as the mean ± SD of three independent experiments. ***: $p< 0.001$ as compared with the LTA alone. B. Rat cortical neuronal cells were pretreated with PD98059 (10, 30 μM; a MEK inhibitor) for 30 min and then treated with LTA (3 μg/ml) for 24 h. Whole cell protein was obtained and 30 μg/sample were run on a 10% SDS-PAGE gel. Western analysis was performed for COX-2 protein and α-tubulin. Immunoreactive bands were visualized using chemiluminescence and Hyperfilm. C. Rat cortical neuronal cells were treated with LTA (3 μg/ml) for indicated time interval. Whole cell protein was obtained and 30 μg/sample were run on a 10% SDS-PAGE gel. Western analysis was performed for phospho-ERK protein and ERK. Immunoreactive bands were visualized using chemiluminescence and Hyperfilm.

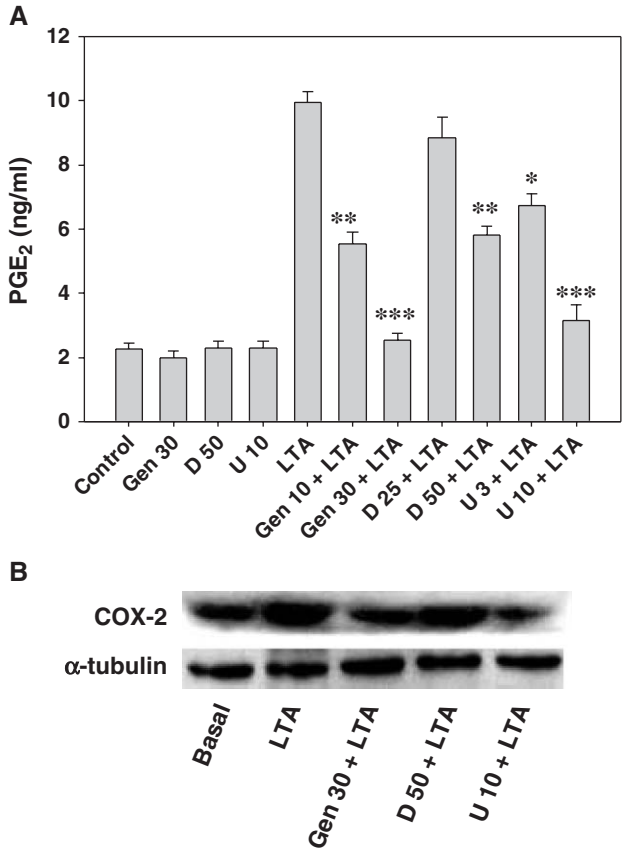


Fig. 6. PTK, PC-PLC, and PI-PLC activation are involved in the LTA induced $PGE_2$ release and COX-2 expression in rat cortical neuronal cells. A. Rat cortical neuronal cells were pretreated with genistein (10, 30 μM; a PTK inhibitor), D-609 (25, 50 μM; a PC-PLC inhibitor), or U-73122 (3, 10 μM; a PI-PLC inhibitor) for 30 min and then treated with LTA (3 μg/ml) for 24 h. The supernatant was collected and analyzed for $PGE_2$ release. The results are expressed as the mean±SD of three independent experiments. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$ as compared with the LTA alone. B. Rat cortical neuronal cells were pretreated with genistein (30 μM; a PTK inhibitor), D-609 (50 μM; a PC-PLC inhibitor), or U-73122 (10 μM; a PI-PLC inhibitor) for 30 min and then treated with LTA (3 μg/ml) for 24 h. Whole cell protein was obtained and 30 μg/sample were run on a 10% SDS-PAGE gel. Western analysis was performed for COX-2 protein and α-tubulin. Immunoreactive bands were visualized using chemiluminescence and Hyperfilm.

$PGE_2$ was measured by an enzyme immunoassay kit (from Cayman Chemical Co., Inc., Ann Arbor, MI) according to the procedure described by the manufacturer.

*Statistical analysis*

The significance of differences between treatment groups was determined by Student's *t* test. Results were considered significant when the calculated *p* value was <0.05.

## Results

*Immunofluorescent microphotographs of rat cortical neuronal cell cultures*

To characterize the purity of primary rat cortical neuronal cell cultures, cell cultures at DIV 13–15 were immunostained with neuron-specific enolase (NSE) for neuronal cells, glial fibrillary acidic protein (GFAP) for astrocytes, ED 8 for microglia/macrophages, and CD 31 for endothelial cells. As shown in Fig. 1(A to D), these cultures were found to contain at least 99% neuronal cells and less than 1% astrocytes. There is no contamination with microglia/macrophages and endothelial cells.

*LTA stimulated dose- and time-dependent increases in PGE2 release and COX-2 expression in rat cortical neuronal cells*

After treatment of rat cortical neuronal cells with LTA (0.1–10 μg/ml) for 24 h, a dose-dependent increase of $PGE_2$ release was induced and reached maximal level at 10 μg/ml LTA (Fig. 2A). LTA treatment (0.3–10 μg/ml for 24 h) also caused COX-2 expression in a dose-dependent manner (Fig. 2B). After treatment of rat cortical neuronal cells with LTA 3

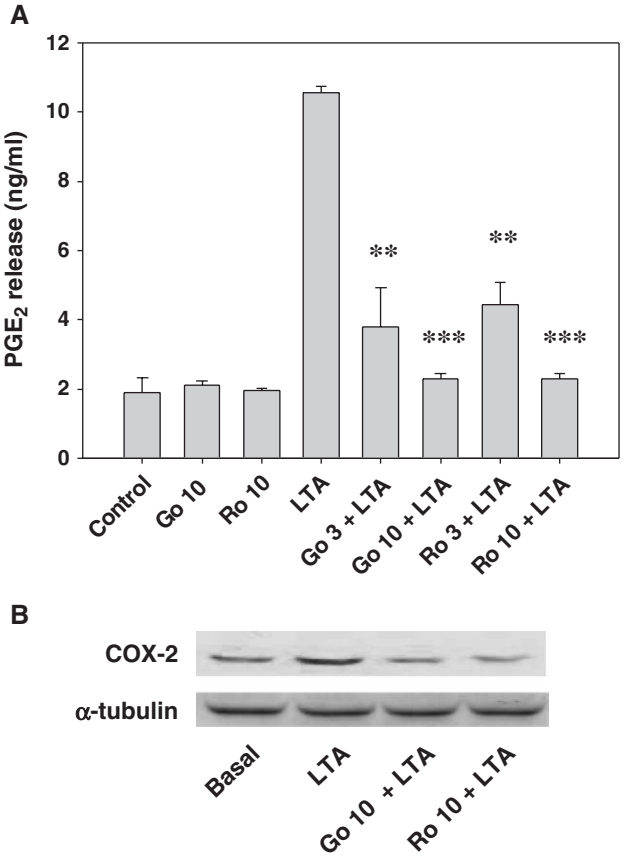


Fig. 7. PKC activation is involved in the LTA induced $PGE_2$ release and COX-2 expression in rat cortical neuronal cells. A. Rat cortical neuronal cells were pretreated with PKC inhibitor Go 6983 (3, 10 μM), or R0 31-8220 (3, 10 μM) for 30 min and then treated with LTA (3 μg/ml) for 24 h. The supernatant was collected and analyzed for $PGE_2$ release. The results are expressed as the mean±SD of three independent experiments. **: $p<0.01$; ***: $p<0.001$ as compared with the LTA alone. B. Rat cortical neuronal cells were pretreated with PKC inhibitor Go 6983 (10 μM), or R0 31-8220 (10 μM) for 30 min and then treated with LTA (3 μg/ml) for 24 h. Whole cell protein was obtained and 30 μg/sample were run on a 10% SDS-PAGE gel. Western analysis was performed for COX-2 protein and α-tubulin. Immunoreactive bands were visualized using chemiluminescence and Hyperfilm.

μg/ml for various time intervals, $PGE_2$ release was induced in a time-dependent manner and peaked at 24 h (Fig. 3A). LTA treatment (3 μg/ml) also induced COX-2 expression in a time-dependent manner (Fig. 3B). To characterize the effects of LTA on $PGE_2$ release and COX-2 expression, rat cortical neuronal cells were pretreated with actinomycin D (1 μM, a transcriptional inhibitor), cyclohexamide (10 μM, a translational inhibitor), or dexamethasone (1 μM, a synthetic glucocorticosteroid) for 30 min, and then treated with 3 μg/ml LTA for 24 h. The LTA-induced $PGE_2$ release was markedly inhibited (Fig. 4A). The LTA-induced COX-2 expression was also inhibited (Fig. 4B). To further confirm this stimulation specifically mediated by LTA without LPS contamination, polymyxin B, a LPS inhibitor, was tested. Polymyxin B (10 μg/ml) did not affect the LTA-induced $PGE_2$ release and COX-2 expression (Fig. 4A, B). Treatment of rat cortical neuronal cells with these inhibitors at the indicated concentrations had no effect on the basal $PGE_2$ release (Fig. 4A).

*The role of ERK on LTA-induced PGE2 release and COX-2 expression in rat cortical neuronal cells*

We further investigated the role of extracellular signal-regulated kinase (ERK) on LTA-induced $PGE_2$ release and COX-2 expression; rat cortical neuronal cells were pretreated with MEK inhibitor PD98059 (10, 30 μM) for 30 min, and then treated with 3 μg/ml LTA for 24 h. The LTA-induced $PGE_2$ release was markedly attenuated (Fig. 5A). The LTA-induced COX-2 expression was also attenuated (Fig. 5B). The inhibitor PD98059, at the concentration used, did not affect the basal $PGE_2$ release (Fig. 5A). To directly determine the activation of ERK in LTA (3 μg/ml)-treated rat cortical neuronal cells, the phosphospecific antibody recognizing the phosphorylated and thus activated forms of ERK was used. LTA-stimulated phospho-ERK reached a maximum at about 30 min, and then decreased by 2 h (Fig. 5C). The results indicate that ERK activation is involved in the signal pathway.

*The role of PTK, PLC on LTA-induced PGE2 release and COX-2 expression in rat cortical neuronal cells*

To examine whether protein tyrosine kinase (PTK) is involved in LTA-induced $PGE_2$ release and COX-2 expression, genistein, a PTK inhibitor, was used. Rat cortical neuronal cells were pretreated with genistein (10, 30 μM) for 30 min, prior to 3 μg/ml LTA treatment for 24 h. The LTA-induced $PGE_2$ release was attenuated (Fig. 6A). The LTA-induced COX-2 expression was also suppressed (Fig. 6B). Next, to further examine whether phosphatidylcholine-specific phospholipase C (PC-PLC) or phosphatidylinositol-specific phospholipase C (PI-PLC) is involved in LTA-induced $PGE_2$ release and COX-2

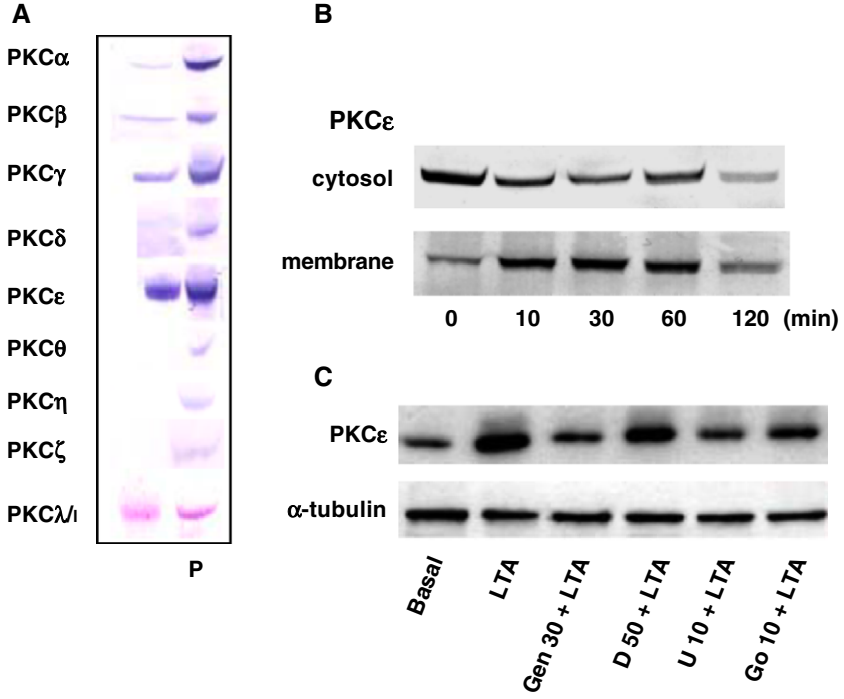


Fig. 8. PKCε activation is involved in the LTA induced $PGE_2$ release and COX-2 expression in rat cortical neuronal cells. A. Whole rat cortical neuronal cell lysate 30 μg/sample were run on a 10% SDS-PAGE gel and immunoblotted with respective PKC isoform antibodies. Immunoreactive bands were visualized using NBT/BCIP. B. Rat cortical neuronal cells were treated with LTA (3 μg/ml) for indicated time interval. The cytosolic and membrane fractions were separated as described in Materials and methods. Samples (30 μg/sample) were run on a 10% SDS-PAGE gel. Western analysis was performed for respective PKC isoform. Immunoreactive bands were visualized using chemiluminescence and Hyperfilm. C. Rat cortical neuronal cells were pretreated with genistein (30 μM; a PTK inhibitor), D-609 (50 μM; a PC-PLC inhibitor), U-73122 (10 μM; a PI-PLC inhibitor), or Go 6983 (10 μM, a PKC inhibitor) for 30 min and then treated with LTA (3 μg/ml) for 30 min. The membrane fractions were separated as described in Materials and methods. Samples (30 μg/sample) were run on a 10% SDS-PAGE gel. Western analysis was performed for PKCε protein and α-tubulin. Immunoreactive bands were visualized using chemiluminescence and Hyperfilm.

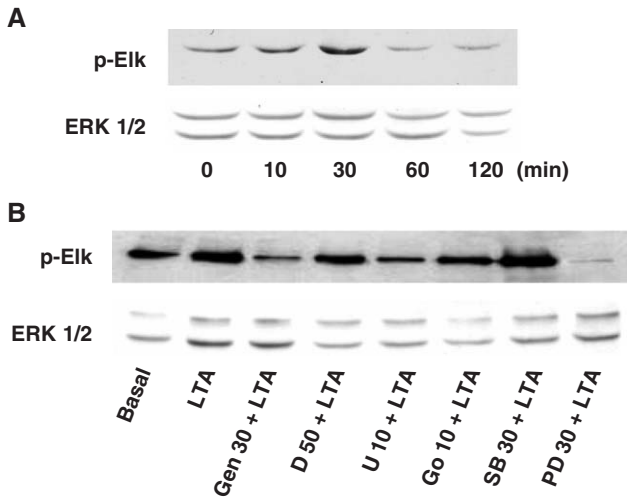


Fig. 9. PTK, PC-PLC, PI-PLC, and PKC are the upstream regulators of ERK. A. Rat cortical neuronal cells were treated with LTA (3 μg/ml) for indicated time interval. Kinase activity assay was performed as described in Materials and methods. Briefly, 200 μg from each sample was used for immunoprecipitation with anti-ERK antibody. After immunoprecipitation, the protein-containing pellet was then suspended in 50 μl kinase buffer, and 200 μM ATP, Elk-1 fusion protein were added. After reaction, the samples were boiled for 5 min and run on a 10% SDS-PAGE gel. Samples were analyzed by Western blotting and probed with phospho-Elk-1 antibody. Immunoreactive bands were visualized using chemiluminescence and Hyperfilm. B. Rat cortical neuronal cells were pretreated with genistein (30 μM; a PTK inhibitor), D-609 (50 μM; a PC-PLC inhibitor), U-73122 (10 μM; a PI-PLC inhibitor), Go 6983 (10 μM; a PKC inhibitor), SB203580 (30 μM; a p38MAPK inhibitor), or PD98059 (30 μM; a MEK inhibitor) for 30 min and then treated with LTA (3 μg/ml) for 30 min. Kinase activity assay was performed as described in Materials and methods. Immunoreactive bands were visualized using chemiluminescence and Hyperfilm.

expression, D-609, a PC-PLC inhibitor, and U-73122, a PI-PLC inhibitor were used. Rat cortical neuronal cells were pretreated with D-609 (25, 50 μM), or U-73122 (3, 10 μM) for 30 min, and then treated with 3 μg/ml LTA for 24 h. The LTA-induced $PGE_2$ release was inhibited (Fig. 6A). The LTA-induced COX-2 expression was also inhibited (Fig. 6B). Treatment of rat cortical neuronal cells with these inhibitors at the indicated concentrations had no effect on the basal $PGE_2$ release (Fig. 6A). Taken together, these results suggest that PTK, PC-PLC, and PI-PLC are involved in the signal pathway.

*The role of PKC on LTA-induced PGE2 release and COX-2 expression in rat cortical neuronal cells*

Activation of PI-PLC could produce two secondary messengers: diacylglycerol (DAG) and inositol 1, 4, 5-triphosphate (IP3). DAG and IP3 activate PKC and release $Ca^{2+}$ from intracellular stores, respectively. Therefore, to further investigate whether PKC activation is involved in the signal pathway, Go 6983 and R0 31-8220, PKC inhibitors, were used. Rat cortical neuronal cells were pretreated with Go 6983 (3, 10 μM), or R0 31-8220 (3, 10 μM) for 30 min, and then treated with 3 μg/ml LTA for 24 h. The LTA-induced $PGE_2$ release was markedly inhibited (Fig. 7A). The LTA-induced COX-2 expression was also suppressed (Fig. 7B). Treatment of rat cortical neuronal cells with these inhibitors at the indicated concentrations had no effect on the basal $PGE_2$ release (Fig. 7A). Immunoblots in whole rat cortical neuronal cell lysate with respective PKC isoform antibodies revealed the presence of α, β, γ, ε, and λ/ι isoforms in rat cortical neuronal cells (Fig. 8A). To examine which PKC isoform is involved in LTA-stimulated responses, the expression of each PKC isoform in cytosol and membrane fractions was examined. Treatment of rat cortical neuronal cells with 3 μg/ml LTA resulted in PKCε translocation. Increases of PKCε in the membrane fraction were seen at 30 min and persisted for 2 h (Fig. 8B). We further examined whether PTK and PLC are involved in PKCε translocation. Rat cortical neuronal cells were pretreated with genistein (30 μM), D-609 (50 μM), U-73122 (10 μM), or Go 6983 (10 μM) for 30 min, and then the cells were treated with 3 μg/ml LTA for 30 min. LTA-stimulated PKCε translocation was inhibited by genistein, D-609, U-73122, and Go 6983 (Fig. 8C). These results suggest that PKCε was involved in the signal pathway and PTK, PC-PLC, and PI-PLC are the upstream regulators of PKCε.

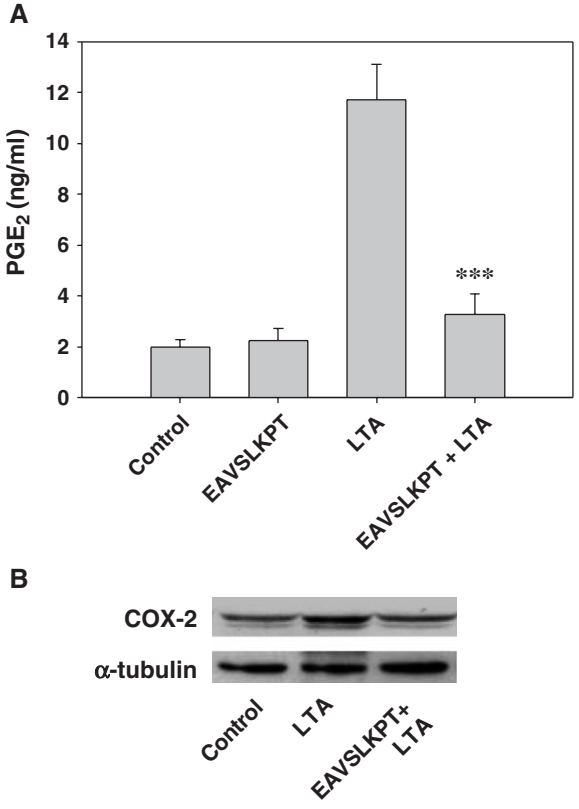


Fig. 10. PKCε translocation inhibitor peptide (EAVSLKPT) blocked the LTA induced $PGE_2$ release and COX-2 expression in rat cortical neuronal cells. A. Rat cortical neuronal cells were pretreated with PKCε translocation inhibitor peptide (EAVSLKPT) 0.1 μM for 30 min and then treated with LTA (3 μg/ml) for 24 h. The supernatant was collected and analyzed for $PGE_2$ release. The results are expressed as the mean±SD of three independent experiments. ***: $p<0.001$ as compared with the LTA alone. B. Rat cortical neuronal cells were pretreated with PKCε translocation inhibitor peptide (EAVSLKPT) 0.1 μM for 30 min and then treated with LTA (3 μg/ml) for 24 h. Whole cell protein was obtained and 30 μg/sample were run on a 10% SDS-PAGE gel. Western analysis was performed for COX-2 protein and α-tubulin. Immunoreactive bands were visualized using chemiluminescence and Hyperfilm.

*The role of PTK, PLC, PKC on LTA-induced ERK activation in rat cortical neuronal cells*

Furthermore, the kinase activity of ERK was determined using Ets-like transcription factor (Elk-1) as a substrate. LTA-stimulated ERK activity reached a maximum at about 30 min, and then decreased by 2 h. The protein level of ERK was not affected by LTA treatment (Fig. 9A). To investigate the role of PTK, PLC and PKC on LTA-induced ERK activation, PTK inhibitor genistein, PC-PLC inhibitor D-609, PI-PLC inhibitor U-73122, PKC inhibitors Go 6983, p38 MAPK inhibitor SB203580, and MEK inhibitor PD98059 were used. Rat cortical neuronal cells were pretreated with genistein (30 μM), D-609 (50 μM), U-73122 (10 μM), Go 6983 (10μM), SB203580 (30 μM), or PD98059 (30 μM) for 30 min, then the cells were treated with 3 μg/ml LTA for 30 min. LTA-stimulated ERK activity was inhibited by genistein, D-609, U-73122, Go 6983, and PD98059, but not SB203580 (Fig. 9B). The results demonstrated that PTK, PC-PLC, PI-PLC, and PKC are the upstream regulators of ERK.

*The role of PKCε on LTA-induced PGE2 release and COX-2 expression in rat cortical neuronal cells*

To directly confirm that PKCε is involved in LTA-induced $PGE_2$ release and COX-2 expression, rat cortical neuronal cells were pretreated with PKCε translocation inhibitor peptide (EAVSLKPT), prior to 3 μg/ml LTA treatment for 24 h. The LTA-induced $PGE_2$ release was attenuated (Fig. 10A). The LTA-induced COX-2 expression was also suppressed (Fig. 10B). The PKCε translocation inhibitor peptide, at the concentration used, did not affect the basal $PGE_2$ release (Fig. 10A).

## Discussion

The mortality associated with bacterial meningitis has remained significant despite advances in antimicrobial therapy. Further improvement in outcome of meningitis will require new therapeutic approaches. Therefore a better understanding of the underlying pathogenic mechanisms is needed. More than half of invasive bacterial infections are Gram-positive in origin. Interspersed with the Gram-positive bacterial cell wall meshwork are membrane-anchored LTA, perhaps the structures most closely related chemically to the lipopolysaccharide (LPS) of Gram-negative bacteria (Weber et al., 2003). The molecular mechanisms of LTA implicated in inflammatory responses in neurons are yet to be fully understood. For this reason, we have investigated the role of COX-2 in the rat cortical neuronal cells challenged by LTA, since COX-2 is expressed in the inflammatory response to various stimuli. The major findings of our study are that in rat cortical neuronal cells, LTA stimulated $PGE_2$ release and COX-2 expression, and thorough activation of the PTK/PC-PLC and PI-PLC/PKCε/ERK signaling pathway.

In rat cortical neuronal cells, pretreatment with polymyxin B (a LPS inhibitor) did not affect the LTA-induced $PGE_2$ release and COX-2 expression, indicating that this stimulation is specifically mediated by LTA, not LPS contamination. The effect of actinomycin D (a transcription inhibitor), cyclohexamide (a translation inhibitor), and dexamethasone (a synthetic glucocorticosteroid) to suppress LTA-induced $PGE_2$ release and COX-2 expression suggested that $PGE_2$ production was due to the activity of newly synthesized COX-2 protein. Dexamethasone has previously been reported to suppress neuronal COX-2 expression in vivo (Adams et al., 1996).

Many signaling pathways lead to the activation of mitogen-activated protein kinases (MAPK), which in turn transmit the activation signal to the nucleus. There are three major families of MAPK: extracellular signal-regulated kinase (ERK), p38 and c-Jun $NH_2$-terminal kinase (JNK) (Cobb and Goldsmith, 1995). ERK is mainly associated with cell proliferation, survival, and differentiation. Although the importance of p38 and JNK for the inflammatory process is well documented, there is growing evidence that also the ERK signaling pathway contributes to inflammatory events. Bates et al. demonstrated that IL-5 stimulation of eosinophils results in the ERK-dependent biosynthesis of leukotrienes (Bates et al., 2000). ERK is also known to participate in the CD-40 mediated induction of IL-1β and TGF-α expression in monocytes (Suttles et al., 1999). Moreover, LTA has been shown to trigger signaling transduction through Toll-like receptor (TLR)-2 and activate ERK and p38 MAPK in various cell types (Schwandner et al., 1999). In the rat cortical neuronal cells, pretreatment with PD98059 (a MEK inhibitor) markedly suppressed LTA-induced $PGE_2$ release and COX-2 expression. LTA-stimulated phospho-ERK reached a maximum at about 30 min, and then decreased by 2 h. Taken together, the results indicated that ERK activation is involved in the signaling pathway. Furthermore, LTA-induced $PGE_2$ release and COX-2 expression can be inhibited by genistein (a PTK inhibitor), D-609 (a PC-PLC inhibitor), and U-73122 (a PI-PLC inhibitor) suggesting that PTK, PC-PLC, and PI-PLC are also involved in the signaling pathway.

Previous reports showed that LTA-stimulated activation of PLC could produce two second messengers: DAG and IP3. DAG and IP3 activate PKC and release $Ca^{2+}$ from intracellular stores, respectively, in several cells (Nishizuka, 1995). PKC is a serine–threonine kinase. It proved to be linked to the signal-induced modulation of a wide variety of cellular processes. To date there are multiple isoforms of PKC described, which due to structural and enzymatic differences, can be subdivided into three main groups: (1) conventional PKCs (α, βI, βII, γ) regulated by phosphatidylserine (PS), $Ca^{2+}$, and diacylglycerol (DAG) or phorbol esters; (2) novel PKCs (δ, ε, η, θ), which require PS, DAG or phorbol esters, but are $Ca^{2+}$-independent; (3) atypical PKCs (λ/ι, ζ), which are only activated by PS (Ron and Kazanietz, 1999). In this study we demonstrated that in rat cortical neuronal cells, Go 6983 and R0 31-8220 could markedly inhibit the LTA-induced $PGE_2$ release and COX-2 expression. Immunoblots of whole rat cortical neuronal cell lysate with respective PKC isoform antibodies revealed the presence of α, β, γ, ε, and λ/ι isoforms in rat cortical neuronal cells. We also present evidence that treatment of the rat cortical neuronal cells with LTA resulted in PKCε translocation, but not the other PKC isoforms (data was not shown). Increases of

PKCε were seen in the membrane fraction at 30 min and persisted for 2 h. Moreover, this effect can be suppressed by genistein (a PTK inhibitor), D-609 (a PC-PLC inhibitor), and U-73122 (a PI-PLC inhibitor) suggesting that PTK, PC-PLC, and PI-PLC are the upstream regulators of PKCε.

PKCε has been shown to be a downstream target of LPS signals that leads to NF-κB and MAPK activation. PKCε −/− macrophages display a severe defect in NF-κB activation kinetics upon LPS challenge (Castrillo et al., 2001). In normal lung fibroblasts, increased PKCε leads to ERK activation, leading to increased collagen expression (Tourkina et al., 2005). Furthermore, by using the kinase activity assay, we demonstrated that LTA-stimulated ERK activity reached a maximum at about 30 min, and then decreased by 2 h. Genistein (a PTK inhibitor), D-609 (a PC-PLC inhibitor), U-73122 (a PI-PLC inhibitor), Go 6983 (a PKC inhibitor), and PD98059 (a MEK inhibitor) suppressed LTA-stimulated ERK activity, but SB203580 (a p38 MAPK inhibitor) did not. The data suggested that PTK, PC-PLC, PI-PLC, and PKC are the upstream regulators of ERK. Finally, the LTA-induced $PGE_2$ release and COX-2 expression were attenuated by the PKCε translocation inhibitor peptide (EAVSLKPT). We provided evidence that PKCε is involved in LTA-induced $PGE_2$ release and COX-2 expression.

In conclusion, the findings of our study for the first time showed that in rat cortical neuronal cells, LTA triggers $PGE_2$ release and COX-2 expression. The stimulatory effects of LTA were modulated by protein tyrosine kinase (PTK), phosphatidylcholine-specific phospholipase C (PC-PLC), phosphatidylinositol-specific phospholipase C (PI-PLC), protein kinase Cε, and extracellular signal-regulated kinase (ERK). This is the first study showing that LTA-induced ERK activation may occur through the activation of PKCε in primary rat cortical neuronal cells.

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