Protein Kinase C η Mediates Lipopolysaccharide-induced Nitric-oxyde Synthase Expression in Primary Astrocytes*

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The signaling pathway involved in protein kinase C (PKC) activation and role of PKC isoforms in lipopolysaccharide (LPS)-induced nitric oxide (NO) release were studied in primary cerebellar astrocytes. LPS caused a dose- and time-dependent increase in NO release and inducible NO synthase (iNOS) expression. The tyrosine kinase inhibitor, genistein, the phosphatidycholine-phospholipase C inhibitor, D609, and the phosphatidate phosphohydrolase inhibitor, propanolol, attenuated the LPS effects, whereas the PI-PLC inhibitor, U73122, had no effect. The PKC inhibitors (staurosporine, Ro 31–8220, Go 6976, and calphostin C) also inhibited LPS-induced NO release and iNOS expression. However, long term (24 h) pretreatment of cells with 12-O-tetradecanoyl phorbol-13-acetate (TPA) did not affect the LPS response. Previous results have shown that TPA-induced translocation, but not down-regulation, of PKCη occurs in astrocytes (Chen, C. C., and Chen, W. C. (1996) G3a1 17, 63–71), suggesting possible involvement of PKCη in LPS-mediated effects. Treatment with antisense oligonucleotides for PKCη or δ, another isoform abundantly expressed in astrocytes, demonstrated the involvement of PKCη, but not δ, in LPS-mediated effects. Stimulation of cells for 1 h with LPS caused activation of nuclear factor (NF)-kB in the nuclei as detected by the formation of a NF-kB-specific DNA-protein complex; this effect was inhibited by genistein, D609, propanolol, or Ro 31–8220 or by PKCη antisense oligonucleotides, but not by long term TPA treatment. These data suggest that in astrocytes, LPS might activate phosphatidylcholine-phospholipase C and phosphatidylycholine-phospholipase D through an upstream protein tyrosine kinase to induce PKC activation. Of the PKC isoforms present in these cells, only activation of PKCη by LPS resulted in the stimulation of NF-kB-specific DNA-protein binding and then initiated the iNOS expression and NO release. This is further evidence demonstrating that different members of the PKC family within a single cell are involved in specific physiological responses.

Nitric oxide (NO), 1 a bioactive free radical, is involved in various physiological and pathological processes in many systems (1). Low concentrations of NO play a role in neurotransmission and vasodilation. However, when secreted at higher concentrations, NO is implicated in the pathogenesis of stroke and other degenerative diseases, such as demyelinating conditions and ischemic and traumatic injury (2). NO is formed enzymatically from l-arginine by nitric-oxide synthase (NOS). NOS enzymes are classified into two groups. Type 1 (cNOS) is constitutively present in several cell types (e.g., neurons and endothelial cells) and is regulated predominantly at the post-transcriptional level by calmodulin in a Ca2+-dependent manner (2). In contrast, the inducible form (iNOS), expressed in various cell types, including vascular smooth muscle cells, macrophages, hepatocytes, and astrocytes, is induced in response to proinflammatory cytokines and bacterial lipopolysaccharide (LPS) (3–6). Cellular NO release following iNOS induction in astrocytes and microglia has been implicated in oligodendrocyte degeneration in demyelinating diseases and in neuronal death during trauma (7–9).

The mechanism of the signal transduction cascade involved in the induction of iNOS in response to LPS and cytokines is an active area of investigation. Although LPS-produced iNOS induction in primary astrocytes has been reported (6, 10), the molecular events involved are not understood. Previous reports have shown a potential role for tyrosine kinase in LPS-produced iNOS induction (11, 12). The murine iNOS promotor contains 24 transcriptional factor binding sites, including those for NF-kB and activator protein-1 (13, 14). Proteins of the NF-kB family appear to be essential for the enhanced iNOS gene expression seen in macrophages exposed to LPS (15), and the p65 NF-kB also seems to be responsible for iNOS induction in astrocytes (16). In the present study, the intracellular signaling pathway by which LPS induces iNOS expression in primary astrocytes was studied. The results show that LPS might activate phosphatidylycholine-phospholipases C and D (PC-PLC and PC-PLD) via tyrosine phosphorylation to produce PKC and NF-kB activation, iNOS expression, and, finally, NO release. Of the PKC isoforms α, δ, θ, η, and ζ expressed in astrocytes (17, 18), only PKCη is involved in the regulation of LPS-induced NF-kB activation, iNOS expression, and NO release.

EXPERIMENTAL PROCEDURES

Materials—Affinity-purified rabbit polyclonal antibody to iNOS was obtained from Transduction Laboratories (Lexington, KY). Basal modified Eagle’s medium, fetal calf serum, glutamine, gentamycin, penicillin, and streptomycin were purchased from Life Technologies, Inc. Rabbit polyclonal antibodies to PKCδ and η and the NF-kB probe were purchased from Santa Cruz Biotechnology. TPA was from L. C. Services Corp. (Woburn, MA). LPS (from Escherichia coli serotype 0127:B8), staurosporine, pyridoline dithiocarbamate, sulfanilamide, and N-(1-naphthyl)-ethylenediamine were from Sigma. Genestein, calphostin C, and other reagents used are: NO, nitric oxide, iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; PC-PLC, phosphatidylcholine-phospholipase C; PC-PLD, phosphatidylcholine-phospholipase D; PI-PLC, phosphoinositide-phospholipase C; NF-kB, nuclear factor kB.

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Go 6976, and Ro 31–8220 were from Calbiochem (San Diego, CA). D609, U73122, and U73343 were from RBI (Natick, MA). T4 polynucleotide kinase was from New England Biolabs (Beverly, MA). Poly(dI-dC) was from Amersham Pharmacia Biotech. Reagents for SDS-polyacrylamide gel electrophoresis were from Bio-Rad. [γ-32P]ATP (3000 Ci/mmol) was from New England Science Products. The horseradish peroxidase-labeled donkey anti-rabbit second antibody and ECL detecting reagent were purchased from Amersham Pharmacia Biotech.

Primary Cultures of Astrocytes—Glia l cells were prepared from the cerebellum of 8-day Wistar rats as described previously (17). Briefly, the cerebella were dissected and dissociated by mechanical chopping and trypsinization to obtain a cell suspension. Cells were plated at a density of 10^5 cells/well in poly-L-lysine precoated 12-well plates for the nitrate assay and at a density of 10^6 cells/10-cm dish for iNOS and PKCα and δ isoform expression tests and the NK-β gel shift assay. Cultures were maintained in basal modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM glutamine, and 50 μg/ml gentamycin, which was changed twice each week. Cells grown in an atmosphere of 5% CO2/95% humidified air at 37 °C were used after 10–12 days in culture, at which time they consisted of confluent glial cell plates, which stained positively for glial fibrillary acidic protein (17).

Determination of NO Concentration—NO production in culture supernatant was evaluated by measuring nitrite, its stable degradation product, using the Griess reagent. The basal modified Eagle’s medium from nonstimulated cells was immediately assayed. NO generation in response to LPS (10 μg/ml LPS for 24 h) and then the isolated supernatant was centrifuged and mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylene diamine dihydrochloride, 2% phosphoric acid) and incubated at room temperature for 10 min before the absorbance was measured at 550 nm in a microplate reader. Sodium nitrite (NaNO2) was used as a standard. In pretreatment experiments, cells were incubated with genistein (a tyrosine kinase inhibitor), U73122 (a PI-PLC inhibitor), D609 (a PC-PLC inhibitor), propranolol (a phosphatase hydrolysase inhibitor), staurosporine, calphostin C, Go 6976, or Ro 31–8220 (PKC inhibitors) for 30 min or with TPA for 24 h before the addition of LPS.

Preparation of Cell Extracts and Western Blot Analysis of iNOS and PKCα and δ—Following treatment with LPS, or pretreatment with inhibitors, TPA or antisense oligonucleotides (see below) followed by LPS, the cells were harvested and collected. For studies of iNOS expression or PKCα and δ expression (antisense oligonucleotides treatment), cells homogenates were prepared and subjected to SDS-polyacrylamide gel electrophoresis using 7.5% (iNOS) or 10% (PKC isoform) gels. The proteins were transferred to nitrocellulose paper, and immunoblot analyses were performed as described previously (17). Briefly, the membrane was incubated successively at room temperature with 0.1% milk in Tris-buffered saline/Tween 20 (TBBS) for 1 h, with rabbit antibodies specific for iNOS or PKCα or δ for 1 h and with horseradish peroxidase-labeled anti-rabbit antibody for 30 min. After each incubation, the membrane was washed extensively with TBBS. The immunoreactive bands were detected with ECL detecting reagent purchased from Amersham Pharmacia Biotech.

Preparation of Nuclear Extracts and the Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts were isolated from the cells using concentrated ammonia water by a standard procedure. After the DNA was precipitated with ethanol, the DNA was resuspended in binding buffer and incubated with the NF-κB probe at room temperature. After addition of the labeled oligonucleotide probe, the samples were incubated for 15 min at 37 °C. The DNA-protein complexes were separated by electrophoresis on a 4% polyacrylamide gel, and then the gels were vacuum-dried and subjected to autoradiography with an intensifying screen at −80 °C.

RESULTS

Signaling Pathways for LPS-induced NO Production and 130-kDa iNOS Expression—Expression of cells to LPS resulted in concentration- and time-dependent nitrite production and expression of the 130-kDa iNOS (Fig. 1). Using a 24-h exposure period, maximum nitrite release (65 ± 9 nmol/10^5 cells/24 h; n = 3) was obtained at 10 μg/ml LPS, and the basal nitrite release was 2 ± 1 nmol/10^5 cells/24 h (n = 3) (Fig. 1A). When cells were treated with 1 μg/ml LPS for various times, nitrite release was significant at 12 h (8 ± 5 nmol/10^5 cells/24 h; n = 3) and maximal at 48 h (Fig. 1B). In the following NO release experiments, the cells were treated with 1 μg/ml LPS for 24 h.

In order to study the intracellular signaling pathway involved in the LPS-induced NO production and iNOS expression, the tyrosine kinase inhibitor genistein was used. When cells were pretreated for 30 min with 30 μM genistein, LPS-induced nitrite production was inhibited by 35% and iNOS expression decreased (Fig. 2A). When cells were pretreated for 30 min with 10 μM U73122 or U73343 (an inactive analog of U73122), 50 μM D609, or 50 μM propranolol, the LPS-induced nitrite production was inhibited 50 and 38% by D609 and propranolol, respectively, whereas U73122 and U73343 had no effect (Fig. 2B). iNOS protein expression was also inhibited by D609 or propranolol but not by U73122 (Fig. 2C).

Inhibitory Effect of PKC Inhibitors and Lack of Effect of Long Term TPA Treatment on LPS-induced NO Production and iNOS Expression—LPS-induced nitrite production and iNOS expression were both inhibited by D609 and propranolol, indicating the involvement of the PC-PLC and PC-PLD pathways. Both pathways can increase diacylglycerol levels and then activate PKC. To determine whether activation of PKC by LPS was involved in the regulation of LPS-induced NO production,
PKC inhibitors were used. Pretreatment of cells for 30 min with 100 nM staurosporine, 0.5 μM Ro 31–8220, 3 μM Go 6976, or 100 nM calphostin C inhibited LPS-induced nitrite production by 48, 73, 47, or 26%, respectively; iNOS protein expression was also inhibited by Ro 31–8220 (Fig. 3A). When cells were treated with 1 μM TPA for 24 h, NO release (106 ±1 nmol/105 cells/24 h; n = 4) was seen; under these conditions, the LPS-induced nitrite production was 66 ± 5 nmol/105 cells/24 h (n = 7), which was greater than with LPS alone (48 ± 5 nmol/105 cells/24 h; n = 3), although not statistically significant by the t test (Fig. 3B). Similar time-dependent results were obtained for TPA-induced iNOS expression (Fig. 3B). Previous studies have shown that in macrophages, MDCK cells and astrocytes, PKCγ is translocated but not down-regulated by TPA treatment (21). The inhibition of the LPS-induced effects by PKC inhibitors, but not by long term TPA treatment, suggested the possible involvement of PKCγ in LPS-induced NO production.

Because PKCγ is involved in the LPS-mediated NO production and iNOS expression, the effect of direct TPA-mediated activation of PKC on NO release and iNOS expression was examined. As shown in Fig. 4, 1 μM TPA also induced a time-dependent increase in nitrite release. However, the increase was much smaller than that induced by LPS (compare with Fig. 1B). Similar time-dependent results were seen for TPA-induced iNOS expression (Fig. 4).

Inhibitory Effect of PKCγ Antisense Oligonucleotides but Not PKCδ Antisense Oligonucleotides on LPS-Induced NO Production and iNOS Expression—To further study the involvement of PKCγ and the lack of involvement of other isoforms in the LPS-induced NO release and iNOS expression, PKCγ antisense and scrambled control oligonucleotides and antisense oligonucleotides for PKCδ, another isoform abundantly expressed in astrocytes (17), were used. Following treatment of primary astrocyte cultures with PKCγ or PKCδ antisense oligonucleotides for 9 days, the expression levels of PKCγ or δ were determined by Western blotting. As shown in Fig. 5A, 10 μM of PKCγ or δ antisense oligonucleotides caused a specific reduction in the level of the corresponding immunoreactive

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**Fig. 1.** Concentration- and time-dependent LPS-induced stimulation of nitrite release and iNOS expression in astrocytes. Cells were incubated at 37 °C with various concentrations of LPS for 24 h (A) or with 1 μg/ml LPS for various time intervals (B), and then the medium was removed and analyzed for nitrite release. The results are expressed as the mean ± S.E. of three independent experiments performed in triplicate. In C, cells were incubated with the indicated concentrations of LPS for 24 h or with 1 μg/ml of LPS for the indicated time intervals, and then cell lysates were subjected to Western blotting using iNOS-specific antibody as described under “Experimental Procedures.”

**Fig. 2.** Effect of genestein, U73122, U73343, D609, or propranolol on LPS-induced nitrite release and iNOS expression in astrocytes. Cells were pretreated with 10 or 30 μM genestein (A), 10 μM U73122 or U73343, 50 μM D609, or 50 μM propranolol (B) for 30 min before incubation with 1 μg/ml of LPS for 24 h; the medium was then removed and analyzed for nitrite release. The results are expressed as the mean ± S.E. for a minimum of three independent experiments performed in triplicate. *, p < 0.05 as compared with LPS alone. For iNOS expression studies, cells were pretreated with 30 μM genestein (A) or 10 μM U73122, 50 μM D609, or 50 μM propranolol (C) for 30 min before incubation with 1 μg/ml LPS for 24 h, and then cell lysates were subject to Western blotting using iNOS-specific antibody as described under “Experimental Procedures.”
isoform protein, e.g. PKCγ antisense oligonucleotides specifically inhibited the expression of PKCγ protein but had no effect on the expression of PKCδ. Because cerebellar astrocytes grow confluent after 10–12 days in culture, PKCγ or δ antisense oligonucleotides were added for entire culture periods, and the reduction in the level of PKCγ and PKCδ was similar to those shown in Fig. 5A.

When cells were treated for 9 days with PKCγ or δ antisense oligonucleotides, LPS-induced nitrite production was inhibited 39% by the PKCγ antisense oligonucleotides, whereas the PKCδ antisense oligonucleotides, although they inhibited the expression of PKCδ, had no effect (Fig. 5B). The control sequences for PKCγ, which did not affect the expression of PKCγ, also had no effect on the LPS response. Similar results were seen for LPS-induced iNOS expression (Fig. 5B).

Induction of NF-kB in the Nuclei of LPS-stimulated Astrocytes and the Inhibitory Effect of PKCγ Antisense Oligonucleotides—In resting cells, the NF-kB heterodimer is held in the cytosol by binding to IκB (22); after stimulation of the cells with various agents, the cytosolic NF-kB/IκB complex dissociates and free NF-kB translocates to the nuclei. Pyridine dithiocarbamate, an antioxidant that acts as a specific inhibitor of NF-kB activation (23), blocks the ability of astrocytes to produce nitrite production and the nuclear binding activity for NF-kB normally seen in response to LPS.\(^5\) Thus, activation of NF-kB is critical in the induction of iNOS by LPS in astrocytes. We performed an EMSA using oligonucleotides containing NF-kB recognition site-like sequences present in the iNOS gene (13) and nuclear extracts prepared from LPS-stimulated cells. In nuclear extracts of unstimulated astrocytes, one faint NF-kB-specific DNA-protein complex was identified, the intensity of which markedly increased following exposure of the cells to 1 \(\mu\)g/ml LPS for 10 min and was even greater after 1 h of treatment (Fig. 6A). For the EMSA, cells were treated with LPS for 1 h.

After pretreatment of cells for 30 min with 30 \(\mu\)M genistein, 50 \(\mu\)M D609, 50 \(\mu\)M propranolol, or 0.5 \(\mu\)M Ro 31–8220, the LPS-induced activation of NF-kB-specific DNA-protein complex formation was inhibited (Fig. 6B and C). However, overnight pretreatment with TPA, which, in astrocytes, down-regulates conventional and new PKC isoforms, but not PKCγ (17, 18, 21), did not affect the LPS-induced NF-kB activation (Fig. 6C). Following exposure of cells to 1 \(\mu\)M TPA for 1 h, activation of NF-kB-specific DNA-protein complex formation was also seen (Fig. 7A). When cells were treated with 10 \(\mu\)M of PKCγ antisense oligonucleotide for 9 days, the LPS-induced NF-kB activation was inhibited, whereas PKCγ control sequences had no effect (Fig. 7B).

**DISCUSSION**

In both cultured astrocytes and C6 glioma cells, LPS induces NO production (6, 24). In macrophages, activation by LPS requires the presence of a 53-kDa glycoprotein, mCD14 (25), which is attached to the cell surface by a glycosylphosphatidylinositol moiety (26). A second form of CD14, soluble CD14, is found at high concentrations in the serum (27), and can confer LPS responsiveness upon cells that lack mCD14, including astrocytoma cells (28). Complexes formed between LPS and either mCD14 or soluble CD14 are thought to lead to transfer of bound LPS to a distinct signaling molecule, which may be either transmembrane or intracellular (29). The formation of

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\(^5\) C.-C. Chen, J.-K. Wang, D.-Y. Lin, and W.-C. Chen, unpublished data.
LPS/CD14 complexes is accelerated by LPS-binding protein (30), which is also present at high concentrations in normal serum and thus can contribute to the serum effects (31). Recent studies have demonstrated the presence of CD14 mRNA and protein in rat astrocytes and that LPS-produced iNOS induction requires membrane and soluble forms of CD14 (32). Thus, in astrocytes, formation of a LPS/LPS-binding protein complex might allow binding to, and activation of, CD14, and then trigger signal transduction to initiate the expression of iNOS and NO release.

Although PKC has been shown to be involved in LPS-induced iNOS expression and NO production in macrophages (33, 34), it has been reported to be unnecessary for iNOS induction by LPS in astrocytes (11). However, in the present study, four PKC inhibitors, calphostin C, Go 6976, Ro 31–8220, and staurosporine, inhibited LPS-stimulated NO production and iNOS expression, indicating that PKC activation is an obligatory event in the LPS-mediated regulation of NO release and iNOS expression in astrocytes. PKC is activated by the physiological activator diacylglycerol, which can be generated either directly by the action of PLC or indirectly by a pathway involving the production of phosphatidic acid by PLD, followed by a dephosphorylation reaction catalyzed by phosphatidate phosphohydrolase. Normally, the PLC involved in the production of diacylglycerol is PI-PLC, but PC-PLC may also be involved (36, 37). The PC-PLC inhibitor, D609, and the phosphatidate phos-
activate PC-PLC and PC-PLD via an upstream protein tyrosine phosphorylation to elicit PKC activation and, finally, iNOS expression and NO production.

Although PKC inhibitors attenuated LPS-induced iNOS expression and NO production, long term TPA pretreatment, which down-regulates PKCα, δ, and θ, but not PKCγ, in astrocytes (17, 18), had no effect, indicating the possible involvement of PKCγ in LPS-mediated effects. To confirm the involvement of PKCγ, PKCγ antisense oligonucleotides and the scrambled controls or antisense oligonucleotides for PKCδ, which is abundantly expressed in astrocytes and down-regulated by TPA, were used. The specificity of the PKCγ and PKCδ antisense oligonucleotides was demonstrated (Fig. 5A), and the results showed inhibition of LPS-stimulated iNOS expression and NO production by PKCγ antisense oligonucleotides but not by PKCδ antisense or control oligonucleotides. Thus, a crucial role for PKCγ in the LPS-induced stimulation of NO production and iNOS expression has been demonstrated. The PKC family, which consists of phospholipid-dependent serine/threonine kinases, is believed to play a major role in cellular functions. Molecular cloning has shown that it consists of at least 12 isoforms with different tissue expressions (36), which have been shown to be related to specialized cell functions (36). Primary cerebellar astrocytes express the new types of PKCs, θ, and η, which are not expressed in neuronal granule cells (17, 18).3 PKCδ and θ, but not PKCγ, have been shown to be involved in the regulation of receptor-mediated PI hydrolysis (17, 18). However, in this study, PKCγ, but not other isoforms, was shown to be involved in the LPS-induced iNOS expression and NO production. This is further evidence that different members of the PKC family within a single cell elicit specific physiological responses. However, PKCγ, which is abundantly expressed in RAW 264.7 macrophages (21), was not involved in the LPS-induced iNOS expression and NO production (38).

The transcriptional factor, NF-κB, is critical in the induction of iNOS by LPS in macrophages (13, 14). In astrocytes, the NF-κB blocker, pyrrolidine dithiocarbamate, inhibits LPS-induced NO production and iNOS expression, indicating that NF-κB is also critical in the induction of iNOS by LPS in these cells. LPS increased the levels of the NF-κB-specific DNA-protein complex in nuclear extracts (Fig. 6A); this activation was inhibited by genistein, D609, propranolol, or Ro 31–8220 but not by long term TPA treatment. Furthermore, PKCγ antisense oligonucleotides, but not the scrambled controls, attenuated NF-κB activation, indicating the involvement of PKCγ in the LPS-stimulated up-regulation of iNOS in astrocytes. Direct activation of PKC by TPA does induce NF-κB activation, NO production, and iNOS expression in astrocytes (Figs. 4 and 7A). Similar findings have been reported in peritoneal macrophages, hepatocytes, and human umbilical vein endothelial cells (42–44) but not in RAW 264.7 macrophages, in which TPA alone did not induce NF-κB activation and NO production (38, 45). Although the role of PKCγ has been clearly demonstrated, other LPS-activated components are also involved in the co-stimulation of NF-κB and iNOS expression, because direct activation of PKC by TPA induced less NF-κB activation and NO production than LPS stimulation (Figs. 1B, 4, and 7A).

Glia cells, including astrocytes, microglia, and oligodendrocytes, are involved in lesion and plaque formation in multiple sclerosis and experimental allergic encephalomyelitis, a model for multiple sclerosis. Multiple sclerosis is a central nervous system disorder with immune-mediated destruction of myelin and the myelin-producing cells, oligodendrocytes. The presence of iNOS in tissues of patients with multiple sclerosis and in animals with experimental allergic encephalomyelitis suggests that NO may play a role in the central nervous system autoimmune diseases (35, 46, 47). Rodent astrocytes and microglia express a high level of iNOS and release significant amounts of NO within hours of LPS stimulation (Refs. 6, 7, and 10 and the present study). Astrocytes are the major cell population in the central nervous system; therefore, induction of iNOS in astrocytes may be an important source of NO in central nervous system inflammatory disorders associated with neuronal and oligodendrocytes death (9).

In summary, the signaling pathway involved in the LPS-induced activation of PKC in primary astrocytes was explored, and the PKC isoform η, but not other isoforms, was found to be involved in the regulation of LPS-induced NF-κB activation, iNOS expression, and NO release. This is the first study showing the involvement of these two mechanisms in LPS-stimulated NO release in such cells.

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