Cyclooxygenase-2 Inhibitor NS-398 Protects Neuronal Cultures From Lipopolysaccharide-Induced Neurotoxicity

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Background and Purpose—The prostanoid-synthesizing enzyme cyclooxygenase (COX)-2 is markedly upregulated after cerebral ischemia and may participate in the mechanisms by which postischemic inflammation contributes to the late stages of ischemic brain injury. In the present study, we sought to provide additional evidence for a role of COX-2 in the mechanisms of neurotoxicity associated with inflammation.

Methods—Nine-day-old neuronal-glial cultures, prepared from the cerebral cortex of newborn C57BL/6J mice, were exposed to lipopolysaccharide (LPS), a potent proinflammatory agent. The contribution of COX-2 was investigated by using the COX-2 inhibitor NS-398.

Results—LPS produced a dose-dependent (0.001 to 10 μg/mL) and selective neuronal death that was well developed 72 hours after treatment. The effect was associated with a marked increase in the concentration of the COX reaction product prostaglandin E2 (PGE2) and of the cytokine tumor necrosis factor-α (TNF-α). NS-398 (10 μmol/L) blocked the PGE2 increase, attenuated the TNF-α increase, and prevented the neuronal death produced by LPS. TNF-α–blocking antibodies attenuated LPS-induced neuronal death, but the protection was less pronounced than that afforded by NS-398. LPS failed to elevate PGE2 or to produce cell death in neuron-enriched cultures, suggesting that glial cells are required for these effects.

Conclusions—COX-2, in part through TNF-α–related mechanisms, contributes to LPS-induced neuronal death. The data support the hypothesis that COX-2, in addition to its role in glutamate excitotoxicity, participates in the cytotoxicity associated with inflammation. (Stroke. 2001;32:2370-2375.)

Key Words: cerebral ischemia ■ cytokines ■ inflammation ■ lipopolysaccharides ■ neurons ■ mice

Prostanoids are potent biological mediators that play an important role in a wide variety of physiological and pathological processes (see review¹). Cyclooxygenase (COX) is a rate-limiting enzyme for the synthesis of prostanoids (see review²). Two COX isoforms have been described: COX-1 and COX-2.¹ COX-1 is ubiquitously expressed and produces prostanoids that are involved in normal cellular functions. COX-2 expression can be induced in several cell types by cytokines, mitogens, bacterial endotoxins, and growth factors (see review³). In the normal brain, COX-2 is present in selected neurons, and its expression is regulated by synaptic activity.⁴-⁶

There is increasing evidence that COX-2 is involved in the mechanisms of ischemic brain injury. In ischemic stroke, COX-2 is upregulated in the late stages of the injury and is expressed in neurons, glia, inflammatory cells, and blood vessels.⁷-¹¹ Treatment with the COX-2 inhibitor NS-398, starting 6 hours after the induction of ischemia, attenuates cerebral ischemic injury in rodent models of ischemic stroke.¹¹,¹² In addition, COX-2–null mice have reduced brain injury after focal cerebral ischemia.¹³ These data raise the possibility that COX-2 is one of the factors through which the inflammatory reaction that involves the postischemic brain contributes to the delayed progression of ischemic brain injury.

In the present study, we sought to provide further evidence that COX-2 contributes to the mechanisms by which inflammation kills neurons. Using neocortical neuronal-glial cultures, we demonstrate that the endotoxin lipopolysaccharide (LPS) elevates the COX-2 reaction product prostaglandin E2 (PGE2) and produces neuronal death. The COX-2 inhibitor NS-398 blocks PGE2 production and markedly ameliorates LPS-induced neuronal damage. Furthermore, NS-398 attenuates the increase in tumor necrosis factor-α (TNF-α), a cytokine involved in LPS-induced neuronal damage. The data provide new evidence that COX-2, in part through TNF-α, plays a critical role in the brain damage produced by inflammation. Therefore, COX-2 could also be involved in the mechanisms by which inflammation exerts its deleterious effects on the postischemic brain.

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2370
Materials and Methods

Materials
Hibernate-A medium, Neurobasal-A medium, B-27 supplement, N-2 supplement, DMEM, dialyzed horse serum, heat-inactivated horse serum, Glutamax-1, and penicillin-streptomycin were obtained from Life Technologies. Papain and DNase1 were purchased from Worthington Biochemical. LPS (Escherichia coli 011:B4) and BSA were from Sigma Chemical Co. NS-398 was obtained from Cayman Chemical. Anti-mouse TNF-α antibodies, neutralizing antibodies to mouse TNF-α, were purchased from R&D Systems. Anti-microtubule-associated protein (MAP)-2 antibodies were purchased from Upstate Biotechnology. Mac-1 antibodies were obtained from Chemicon. Anti–glial fibrillary acidic protein (GFAP) antibodies were from Boehringer-Mannheim. Prediluted goat anti-mouse IgG antibodies were from Biocare. Rabbit anti-rat IgG antibodies were purchased from Vector Laboratories.

Cell Culture
Primary neuron-glial cultures were prepared from postnatal day 0 from C57BL/6J mice by the method of Brewer, with some modifications. Briefly, the whole cerebral cortex was minced in Hibernate-A medium with B-27 supplement (Hibernate-A/B-27). Individual cells were first treated with papain (25 U/mL) and DNase1 (25 U/mL) in Hibernate-A/B-27 for 30 minutes at 30°C. After they were rinsed, the cells were dissociated mechanically in Hibernate-A/B-27 by using a Pasteur pipette. The cell suspension was laid on Hanks’ balanced salt solution containing 4% BSA and centrifuged at 1000 rpm for 5 minutes. The pellet was resuspended and DNase1 (25 U/mL) in Hibernate-A/B-27 for 30 minutes at 30°C. A/B-27). Individual cells were first treated with papain (25 U/mL) and DNase1 (25 U/mL) in Hibernate-A/B-27 at 30°C. The cell suspension was laid on Hanks’ balanced salt solution containing 4% BSA and centrifuged at 1000 rpm for 5 minutes. The pellet was resuspended in Neurobasal-A medium with B-27 supplement, 0.25 mmol/L glutamate, and the medium was changed. Glial enrichment was studied with the neuronal marker MAP-2, the astroglial marker GFAP, and the microglial marker Mac-1. The cultures were fixed with 4% paraformaldehyde for 30 minutes at room temperature. For Mac-1 staining, cells were incubated with 1% Triton X in Tris-buffered saline for 15 minutes. Cells were incubated overnight at 4°C with a primary antibody in antibody dilution buffer (anti–MAP-2 at 1:2000, anti–GFAP at 1:100, and Mac-1 at 1:100). The cells were then incubated sequentially with a prediluted goat anti-mouse antibody (for anti–MAP-2 and anti–GFAP) and streptavidin-linked horseradish peroxidase (Biocare). Antigen-antibody complexes were visualized by using diaminobenzidine as the chromogen. For Mac-1 staining, cells were incubated with a rabbit anti-rat antibody (1:200) and visualized by using the Vectastain ABC kit (Vector) with diaminobenzidine.

PGE2, TNF-α, and Nitrite Assays
The concentration of PGE2 in the culture medium was measured by using an ELISA kit (Cayman Chemical). Levels of TNF-α in the culture medium were determined with an ELISA kit (Biosource), according to the manufacturer’s instructions. Nitrite was measured in the culture medium by using the Griess reaction system (Promega). Absolute concentrations were derived by comparison with a standard curve.

Statistical Analysis
The data are expressed as mean±SE. Multiple comparisons were evaluated by ANOVA followed by the Tukey or Dunnett test, as indicated. Two-group comparisons were analyzed by the 2-tailed Student t test. For all analyses, a value of P<0.05 was considered significant.

Results
We used 9-day-old neocortical cultures for LPS treatment. At this time, the mixed cultures contained ∼48% neurons, 51% astrocytes, and 1% microglia, as estimated by immunostaining with the neuronal marker MAP-2, the astroglial marker GFAP, and the microglial marker Mac-1. Treatment of the cultures with LPS produced a time-dependent degeneration of neurons and an increase in LDH concentration in the supernatant. The effect was most evident 72 hours after LPS when degenerating neurons were most abundant, and LDH levels were significantly higher than those observed in vehicle-treated cultures (Figure 1A). The effect of LPS on LDH concentration was dose dependent (0.001 to 10 μg/mL, Figure 1B).

Treatment of mixed cultures with NS-398 attenuated the LPS-induced LDH efflux (Figure 2A). NS-398 also attenuated the release of PGE2 produced by LPS treatment (Figure 3A). To confirm that the reduction in LDH reflected a decrease in neuronal death, cultures were immunostained with the neuronal marker MAP-2. In LPS-treated cultures, there was a loss of neuritic network and neuronal perikarya (Figure 4). Although NS-398 itself had no effect in control cultures, it prevented the loss of MAP-2–positive neurons in LPS-treated cultures (Figure 4). Counting of MAP-2–positive neurons showed that NS-398 prevented the loss of neurons in the conversion of lactate and NADH to pyruvate and NADPH. The color intensity is directly proportional to the number of lysed cells and can be quantified by measuring the absorbance (490 nm). Data are expressed as optical density. The ability of this method to detect neuronal death was validated in our system by comparing the LDH data with neuronal counts in cultures immunostained with the neuronal marker MAP-2 (see below). Neuronal counts were made in 4 random fields (0.25 mm²) at ×200 magnification.

Immunocytochemistry
The cultures were fixed with 4% paraformaldehyde for 30 minutes at room temperature. For Mac-1 staining, cells were treated with 1% Triton X in Tris-buffered saline for 15 minutes. Cells were incubated overnight at 4°C with a primary antibody in antibody dilution buffer (anti–MAP-2 at 1:2000, anti–GFAP at 1:100, and Mac-1 at 1:100; Dako). The cells were then incubated sequentially with a prediluted goat anti-mouse antibody (for anti–MAP-2 and anti–GFAP) and streptavidin-linked horseradish peroxidase (Biocare). Antigen-antibody complexes were visualized by using diaminobenzidine as the chromogen. For Mac-1 staining, cells were incubated with a rabbit anti-rat antibody (1:200) and visualized by using the Vectastain ABC kit (Vector) with diaminobenzidine.

Assessment of Neuronal Death
Neuronal death was examined by the lactate dehydrogenase (LDH) assay. Cell culture supernatants were tested for LDH activity by using the CytoTox96 nonradioactive assay (Promega). This assay results in the production of a red formazan product after the
LPS-treated cultures (Figure 2B), a finding consistent with the results obtained with the LDH assay.

To examine the cell type responsible for the toxicity induced by LPS, the effects of LPS were studied in neuron-enriched cultures. In these cultures, LPS did not increase PGE2 (Figure 3A) and failed to produce neurotoxicity, as assessed morphologically or by LDH efflux (Figure 3B). We also studied the effect of LPS in glia-enriched cultures. In such cultures, LPS did not produce glial degeneration, as assessed by morphological criteria, although a small increase in LDH was observed (optical density at 72 hours, 0.05 ± 0.01 for control and 0.08 ± 0.01 for LPS; P < 0.05; n = 5). The lack of nitrite production by LPS stimulation may reflect the fact that LPS alone is not sufficient to induce expression of inducible NO synthase in mouse neuronal-glial cultures (eg, see Jeohn et al17).

TNF-α has been implicated in the mechanisms of the neurotoxicity associated with inflammation. Therefore, we sought to determine whether LPS treatment increases TNF-α and, if so, whether NS-398 attenuates such TNF-α elevation. In control culture, TNF-α concentration was below the detection limit of the assay (19.5 pg/mL). LPS produced a marked increase in TNF-α concentration that was attenuated

LPS-treated cultures (Figure 2B), a finding consistent with the results obtained with the LDH assay.

To examine the cell type responsible for the toxicity induced by LPS, the effects of LPS were studied in neuron-enriched cultures. In these cultures, LPS did not increase PGE2 (Figure 3A) and failed to produce neurotoxicity, as assessed morphologically or by LDH efflux (Figure 3B). We also studied the effect of LPS in glia-enriched cultures. In such cultures, LPS did not produce glial degeneration, as assessed by morphological criteria, although a small increase in LDH was observed (optical density at 72 hours, 0.05 ± 0.01 for control and 0.08 ± 0.01 for LPS; P < 0.05; t test). Therefore, although a component of glial cell death cannot be ruled out, this component is likely to be small and to contribute little to the LDH increase observed in mixed cultures. Therefore, LPS-induced cytotoxicity is observed only in the presence of both neurons and glial cells.

There is evidence that NO enhances COX-2 activity and that such activation contributes to COX-2–induced neurotoxicity.12,16 To determine whether NO participates in the mechanisms of cell death in this preparation, the stable NO metabolite nitrite was measured in mixed cultures 72 hours after LPS treatment. LPS failed to increase nitrite accumulation (2.04 ± 0.16 μmol/L for control and 2.2 ± 0.7 μmol/L for LPS, P > 0.05; n = 6 per group). In contrast, in macrophage cultures, exposure to LPS increased nitrite accumulation markedly (at 24 hours, 1.7 ± 0.6 μmol/L for control and 20.0 ± 1.7 μmol/L for LPS, P < 0.001; n = 5). The lack of nitrite production by LPS stimulation may reflect the fact that LPS alone is not sufficient to induce expression of inducible NO synthase in mouse neuronal-glial cultures (eg, see Jeohn et al17).
by treatment with NS-398 (Figure 5A). Treatment with anti–TNF-α antibodies attenuated the LPS-induced increase in LDH (Figure 5B). However, the attenuation in LDH produced by anti–TNF-α antibodies was less pronounced than that observed with NS-398 (Figure 5B).

Discussion
There is increasing evidence that COX-2 is involved in the pathogenesis of several neurological disorders associated with inflammation, including ischemic brain injury.3 Cerebral ischemia is associated with a marked inflammatory reaction that contributes to the progression of the damage.18 In models of cerebral ischemia, as well as in human stroke, COX-2 is upregulated in neurons, inflammatory cells, glial cells, and the vasculature.7-11 Inhibition of COX-2 with NS-398 or genetic deletion of COX-2 in null mice attenuates cerebral ischemic damage,11,13 suggesting that COX-2 reaction products participate in ischemic injury. In systemic models of inflammation, COX-2 is well known to contribute to the associated tissue damage.19 However, the role of COX-2 in the neuronal damage produced by inflammation in the brain has not been extensively investigated.

In the present study, we sought to examine further the role of COX-2 in the mechanisms of neurotoxicity produced by inflammatory stimuli. To achieve this goal, we treated neocortical neuronal-glial cultures with the endotoxin LPS. LPS produced neuronal death that was well developed 2 or 3 days after LPS exposure and was associated with a marked elevation of the COX reaction product PGE2 but not of the NO metabolite nitrite. Treatment of the cultures with the COX-2 inhibitor NS-398 blocked the increase in PGE2 and prevented neuronal death. These data suggest that COX-2 is involved in the mechanisms of the delayed cell death produced by LPS.

NS-398 was used as a relatively selective COX-2 inhibitor.11,20 Although NS-398 can activate peroxisome proliferator–activated receptors, which are involved in inflammation,21 such an effect is observed at concentrations of 100 to 300 μmol/L.21 Therefore, at the relatively low concentration used in the present study (10 μmol/L), it is unlikely that NS-398 exerts its protective effect through the activation of peroxisome proliferator–activated receptors. Furthermore, NS-398 does not protect against NMDA-induced cytotoxicity in COX-2–null mice,13 demonstrating that COX-2–independent mechanisms do not participate in its neuroprotective effects. Experiments using brain cultures from COX-2–null mice would provide further evidence for a role of COX-2 in LPS-induced cell death.

To gain insight into the cellular sources of COX-2, cultures enriched with neurons were studied. In such cultures, LPS failed to produce PGE2 elevation or elicit neuronal death. This observation is consistent with the hypothesis that glial cells are the source of PGE2 and, presumably, COX-2 in our

Figure 3. Effect of NS-398 on PGE2 and LDH release in neuron-enriched cultures and neuronal-glial cultures. A, Cultures were treated with medium alone (vehicle), LPS (10 μg/mL), or LPS + NS-398. Culture supernatants were analyzed for PGE2 24 hours after treatment. NS-398 (10 μmol/L) inhibited LPS-induced PGE2 release. *P<0.05 (ANOVA and Tukey test). B, LDH release in neuronal-glial cultures and neuron-enriched cultures. LDH was measured in culture supernatants 72 hours after LPS treatment. *P<0.05 compared with respective vehicle-treated cultures (t test).

Figure 4. MAP-2 immunocytochemistry of mixed cultures. Treatment with LPS (10 μg/mL) for 72 hours produced a marked loss of MAP-2–positive neurons (LPS) compared with vehicle-treated cultures (vehicle). Treatment with NS-398 (10 μmol/L) protected MAP-2–positive neurons against the LPS-induced damage (LPS + NS398). Bar =100 μm.
The COX-2 reaction products contributing to LPS-induced neurotoxicity remain to be defined. One possibility is that COX-2 reaction products, including prostanoids and superoxide radicals, are directly responsible for LPS-induced neuronal death. Another possibility is that COX-2 reaction products facilitate neurotoxicity mediated by another mechanism. For example, COX-2 reaction products could enhance the LPS-induced cytokine response, which, in turn, contributes to cytotoxicity. Recent data suggest that COX-2 is also involved in pathogenic events occurring in the early stages of cerebral ischemia. COX-2 inhibition by NS-398 protects neuronal cultures from glutamate neurotoxicity and reduces the brain damage produced by direct injection of N-methyl-D-aspartate into the mouse cerebral cortex. Furthermore, the damage produced by N-methyl-D-aspartate microinjection is attenuated in COX-2–null mice. These observations suggest that COX-2 reaction products participate in the mechanisms of glutamate neurotoxicity, a process that occurs in the early stages of cerebral ischemia.

In conclusion, we have demonstrated that the COX-2 inhibitor NS-398 attenuates the neurotoxicity produced by the proinflammatory agent LPS. The protective effect is associated with a reduction in the COX-2 reaction product PGE2 and the cytokine TNF-α. The data indicate that COX-2 is involved in the mechanisms of neurotoxicity associated with inflammation, and the data are consistent with the hypothesis that COX-2 contributes to the neuronal damage resulting from the inflammatory reaction triggered by cerebral ischemia.

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References

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