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 review 1). Cyclooxygenase (COX) is a rate-limiting enzyme for the synthesis of prostanoids (see review 2). 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 review 3). 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.
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 0111:B4) and BSA were from Sigma Chemical. 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 to 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 Hank's balanced salt solution containing 4% BSA and centrifuged at 1000 rpm for 5 minutes. The pellet was resuspended with Neurobasal-A/B-27 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 Hank's 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 achieved 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 determined 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 reduction 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
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. 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 al).

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...
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. Cerebral ischemia is associated with a marked inflammatory reaction that contributes to the progression of the damage. In models of cerebral ischemia, as well as in human stroke, COX-2 is upregulated in neurons, inflammatory cells, glial cells, and the vasculature. Inhibition of COX-2 with NS-398 or genetic deletion of COX-2 in null mice attenuates cerebral ischemic damage, 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. 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. Although NS-398 can activate peroxisome proliferator-activated receptors, which are involved in inflammation, such an effect is observed at concentrations of 100 to 300 μmol/L. 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, 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 neuronal-glial cultures.
Effect of TNF-α

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. This possibility is consistent with our own finding that COX-2 inhibition attenuates the production of TNF-α and with reports that PGE₂ enhances cytokine production in other culture systems. In addition, PGE₂ could exacerbate neurotoxicity by facilitating astrocytic glutamate release. However, it remains to be established whether glutamate plays a role in the neuronal death induced by LPS.

Furthermore, other studies have reported that prostanoids protect against glutamate neurotoxicity. Therefore, it remains unclear whether COX-2-derived prostanoids are involved in LPS toxicity through glutamate-dependent mechanisms. Experiments in which LPS-stimulated cultures are treated with glutamate receptor inhibitors would be required to address this issue. Furthermore, the role of arachidonic acid metabolites produced by the 5-lipoxygenase pathway in the mechanisms of LPS-induced cell death remains to be defined.

It is unlikely that the protective effect of COX-2 inhibition is entirely mediated through TNF-α signaling, because the protection exerted by anti–TNF-α antibodies was less than that observed with NS-398. Thus, the beneficial effects of NS-398 are also mediated by mechanisms independent of TNF-α. Furthermore, the fact that TNF-α–blocking antibodies did not completely abolish LPS-induced neurotoxicity suggests that in our model, as in other cell culture systems, TNF-α is not the sole mediator of neurotoxicity. As for the cellular origin of TNF-α, glial cells are a likely source of this cytokine, although a neuronal source cannot be ruled out.

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. It is of interest that in glutamate excitotoxicity, at variance with LPS-induced cytotoxicity, the cellular source of COX-2 is neuronal. COX-2 inhibition is an attractive therapeutic strategy for ischemic stroke because it can counteract pathogenic mechanisms taking place in both the early and late 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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