Ibuprofen for neuroprotection after cerebral ischemia

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Objective: Ibuprofen has been shown to reduce cerebral ischemic injury, such as may occur after deep hypothermic circulatory arrest. We investigated whether ibuprofen has direct protective effects against excitotoxic neuronal injury, as may be seen after cerebral ischemia, by using a cell culture model.

Methods: Mixed cortical cultures containing neuronal and glial cells were prepared from fetal mice at 13 to 15 days gestation, plated on a layer of confluent astrocytes from 1- to 3-day-old postnatal pups. Near-pure neuronal cultures containing less than 5% astrocytes were obtained from mice of the same gestational stage. Slowly triggered excitotoxic injury was induced at 37°C by 24-hour exposure to 12.5 μmol/L N-methyl-D-aspartate or 50 μmol/L kainate. Neuronal death was quantified by release of lactate dehydrogenase from damaged cells. Data were analyzed using 1-way analysis of variance with Tukey post hoc multiple comparisons.

Results: In *mixed* cultures, ibuprofen concentrations of 25 μg/mL, 50 μg/mL, and 100 μg/mL all significantly reduced N-methyl-D-aspartate–induced neuronal cell death from 74.5% to 56.1%, 38.7%, and 12.3%, respectively, revealing a strong dose response ($P < .001$). In *near-pure* cultures, ibuprofen at a concentration of 25 μg/mL failed to protect neurons, indicating that the neuroprotective effects of ibuprofen require interaction with glial cells. Furthermore, ibuprofen at 100 μg/mL was not protective against neuronal cell death induced by kainate exitotoxicity in near-pure culture but was effective in mixed cultures.

Conclusion: Ibuprofen provides neuroprotection through glial cells against excitotoxic neuronal injury caused by glutamatergic excitotoxicity after cerebral ischemia as demonstrated by reduced neuronal cell death in mixed cell cultures. Further studies are needed to evaluate the potential of ibuprofen to reduce neurologic injury in patients experiencing an hypoxic/ischemic insult. (J Thorac Cardiovasc Surg 2010;139:489-93)

Low-flow cardiopulmonary bypass and deep hypothermic circulatory arrest are used to facilitate pediatric cardiac surgery, although they carry a risk of ischemic brain damage. There are also deleterious effects of cardiopulmonary bypass, such as inflammation, which may exacerbate ischemic brain injury. Ibuprofen is a nonsteroidal anti-inflammatory drug that is widely used to reduce pain, fever, and inflammation. The drug inhibits cyclooxygenase and activates peroxisome proliferator-activated receptors; both of these actions result in reduced inflammation.[1-3] Ibuprofen has been shown to reduce cerebral ischemic injury and infarct size, such as may occur after deep hypothermic circulatory arrest.[4,5] Ibuprofen has been reported to improve cerebral blood flow after global cerebral ischemia and to restore cerebral metabolism after cerebral traumatic injury.[6,7]

It is well known that ischemia can cause neuronal cell death through the mechanism of glutamatergic excitotoxicity. An ischemic insult initially impairs glutamate transportation at the postsynaptic level and increases extracellular glutamate levels, resulting in glutamatergic excitotoxicity, which is excessive neuronal excitation mediated by excessive calcium influx. Thus, neurologic problems after cardiac surgery, such as may occur with circulatory arrest, embolic injury, or low-flow states, may be a result of excitotoxicity after cerebral ischemia.

Glutamate is the principal neurotransmitter at the majority of synapses throughout the brain and spinal cord. N-methyl-D-aspartate (NMDA), AMPA, and kainate are glutamate receptor subtypes defined by their response to these agents; the NMDA pathway causes neuronal injury more rapidly than the AMPA or kainate pathway, reflecting a greater ability to induce calcium influx. Rapidly triggered excitotoxicity can be separated into 2 components: (1) immediate neuronal cell swelling induced by the influx of extracellular Na and (2) delayed neuronal cell degeneration induced by excessive Ca influx. AMPA, kainate, and NMDA receptors all contribute to the neuronal cell swelling seen with excitotoxic injury. In contrast, NMDA receptor activation is necessary and sufficient to induce delayed neuronal cell degeneration. Although either the acute cell swelling or delayed neuronal cell degeneration can alone produce irreversible neuronal injury, under certain conditions the majority of cells can

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Abbreviations and Acronyms
DIV = days in vitro
LDH = lactate dehydrogenase
NMDA = N-methyl-D-aspartate

recover and survive from cell swelling. Generally most cortical neurons die through delayed neuronal degeneration.[8]

The purpose of the current study was to investigate whether ibuprofen has direct neuroprotective effects against excitotoxic neuronal cell death induced by NMDA and kainate using a neuronal cell culture model.

MATERIALS AND METHODS

Near-Pure Neuronal Cultures

Near-pure neuronal cultures containing less than 5% astrocytes were obtained from fetal mice at 13 to 15 days gestation (Taconic, Rockville, Md). Dissociated cortical cells in plating medium of media stock (Dulbecco's modified Eagle's medium with 25 mmol/L glucose; Sigma, St Louis, MO) supplemented with 5% fetal bovine serum (Gibco, Carlsbad, Calif), 5% horse serum (Gibco), and 2 mmol/L glutamine (Sigma) were plated in 24-well plates coated with poly-D-lysine (0.1 mg/dL; Invitrogen, Carlsbad, Calif) and laminin (0.02 mg/mL; Invitrogen). After 3 days in vitro (DIV), non-neuronal cell division was halted by exposure to 10 μmol/L cytosine arabinoside (Ara-C; Sigma). There was no further exchange of the media except adding Dulbecco's modified Eagle's medium for evaporation. After 12 DIV, cultures did not need the presence of serum to survive. They were shifted to a maintenance medium identical to plating medium but lacking serum. All cultures were kept at 37°C in a humidified 5% CO_2 incubator. Cultures were used after 13 to 14 DIV for excitotoxic injury.[9]

Glial Cultures

Glial cell cultures were prepared from 1- to 3-day-old postnatal mice (1–3 days after birth from Taconic, Rockville, Md). Dissociated cortical cells were plated in 24-well plates previously coated with poly-D-lysine (0.1 mg/dL) using a plating medium of media stock supplemented with 10% horse serum, 10% fetal bovine serum, and 2 mmol/L glutamine. Cultures were kept at 37°C in a humidified 5% CO_2-containing atmosphere until they reached confluency, 7 to 14 DIV. Confluent cultures were then used as a support for mixed cultures.[9]

Mixed Cortical Cultures

Mixed cortical cultures containing both neurons and astrocytes were prepared from fetal mice at 13 to 15 days gestation. Dissociated cortical cells were plated in 24 wells on a layer of confluent astrocytes, using plating medium supplemented with 5% horse serum, 5% fetal bovine serum, and 2 mmol/L glutamine. After 7 DIV, non-neuronal cell division was halted by a 2 to 3 days of exposure to 10 μmol/L Ara-C. Subsequent partial medium replacement was performed twice per week, and after 12 DIV, cultures were shifted to a maintenance medium identical to plating medium although lacking serum because neurons survive without it. Experiments were performed on cortical cultures after 13 to 14 DIV.[9]

Slowly Triggered Excitotoxicity

Because lower levels of glutamate exposure induce delayed neuronal cell death in most cortical neurons, slowly triggered excitotoxicity was induced at 37°C by 24-hour exposure to 12.5 μmol/L NMDA or 50 μmol/L kainate as an excitotoxin in media stock supplemented with 10 μmol/L glycine. These concentrations do not influence glial cells; 10 μmol/L MK-801 was always added concurrently with kainate to block secondary NMDA receptor

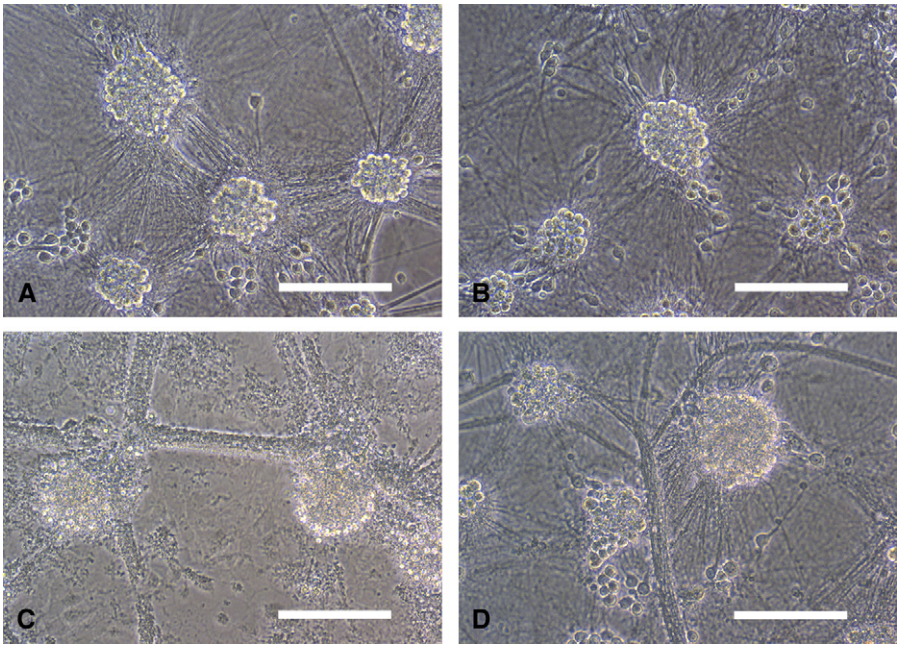

FIGURE 1. A, Sham wash (mixed cortical cultures). B, Ibuprofen (100 μg/mL) alone. C, Mixed cortical cultures were exposed to 12.5 μmol/L NMDA for 24 hours and demonstrated an acute swelling of neuronal cell bodies followed by a widespread necrotic neuronal degeneration resulting in disrupted neurons and segmentalized neuritis. D, Ibuprofen at a concentration of 100 μg/mL was co-applied in the medium with excitotoxin (NMDA). Ibuprofen preserved neuronal cells and protected neurites. Original magnification is 200×. Scale bars = 100 μm. *NMDA,* N-methyl-D-aspartate; *Ib,* ibuprofen.

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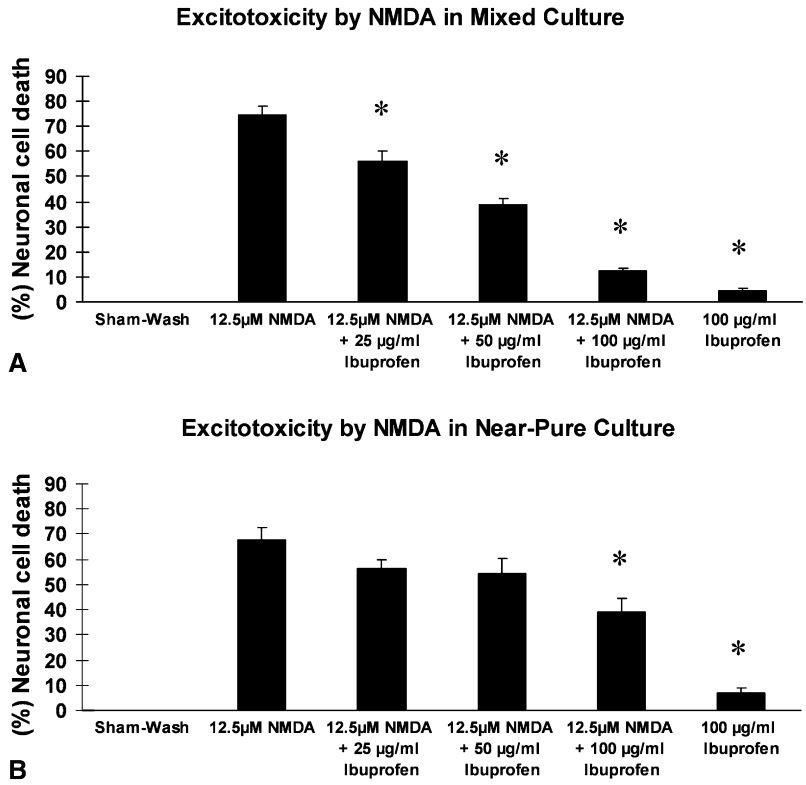

FIGURE 2. Exposure to 12.5 μmol/L NMDA revealed approximately 70% neuronal cell death by LDH assay measuring LDH levels in bathing medium in both cultures. Ibuprofen at a concentration of 25 μg/mL, 50 μg/mL, and 100 μg/mL reduced neuronal cell death significantly from 74.5% $\pm$ 3.8% to 56.1% $\pm$ 4.0%, 38.7% $\pm$ 2.8%, and 12.3% $\pm$ 1.4% in mixed cultures, respectively, revealing a strong dose response ($P < .001$). However low-concentration ibuprofen failed to protect neurons in near-pure cultures. A, Mixed cultures. B, Near-pure cultures. *Significantly different from NMDA alone by analysis of variance with Tukey correction (mean $\pm$ standard error).

activation.[9] Ibuprofen was co-applied at 3 different concentrations, 25 μg/mL, 50 μg/mL, or 100 μg/mL, with the excitotoxin and left for 24 hours in the bathing medium. A clinically effective concentration of ibuprofen is between 25 and 100 μg/mL because the inhibitory effect of ibuprofen on neutrophil activation and migration generally occurs at concentrations greater than 50 μg/mL.[10]

Assessment of Neuronal Cell Death

The percentage of neuronal cell death was calculated by lactate dehydrogenase (LDH) assay, which is a standard method that is widely applied for quantitation of cell death in neuronal cell culture.[8,9,11] Briefly, neuronal death was confirmed qualitatively by examining cultures under phase-contrast microscopy and was quantified by measurement of LDH release from damaged cells into the bathing medium 24 hours after the onset of excitotoxin exposure. Only damaged cells (dead neurons) release LDH. LDH was measured by ELISA kit (Promega, Madison, Wis). The percentage of neuronal cell death was calculated by LDH levels in the bathing medium. The LDH level corresponding to complete neuronal death (without glial death) was determined in sister cultures exposed to 100 μmol/L NMDA. Background LDH levels were determined in sister cultures with sham wash as a control and subtracted from experimental values to yield the signal specific for experimentally induced injury.[11]

Statistical Analysis

The primary outcome measure for evaluating efficacy was percentage of neuronal cell death calculated by LDH levels. Results are expressed as mean $\pm$ standard error. When n = 12 is indicated, this value corresponds to 12 different well pools derived from 3 different dissections. Analysis of these data included factorial 1-way analysis of variance with the Tukey post hoc method for multiple group comparisons to protect against type I (false-positive) results.[12] The Statistical Package for the Social Sciences was used for statistical analysis (version 15.1; SPSS Inc, Chicago, Ill). Power analysis indicated that the sample sizes provided 80% power to detect a significant difference in neuronal cell death of 15% or more between treatment groups assuming a standard deviation of 5% to 10% (version 7.0; nQuery Advisor, Statistical Solutions, Saugus, Mass).

RESULTS

Sham wash and addition of ibuprofen alone at the concentration of 100 μg/mL did not influence neuronal cells in either mixed or pure neuronal culture (Figure 1, *A, B*). Excitotoxicity was induced by exposure to 12.5 μmol/L NMDA or 50 μmol/L kainate for 24 hours and demonstrated an acute swelling of neuronal cell bodies followed by widespread necrotic neuronal degeneration resulting in disrupted neurons and segmentalized neurites in both near-pure and mixed cultures (Figure 1, *C*). Ibuprofen at a concentration of 100 μg/mL was co-applied in the medium with excitotoxin (NMDA). Ibuprofen was able to preserve neuronal cells, including neurites in both cultures (Figure 1, *D*). Exposure to 12.5 μmol/L NMDA revealed approximately 70%

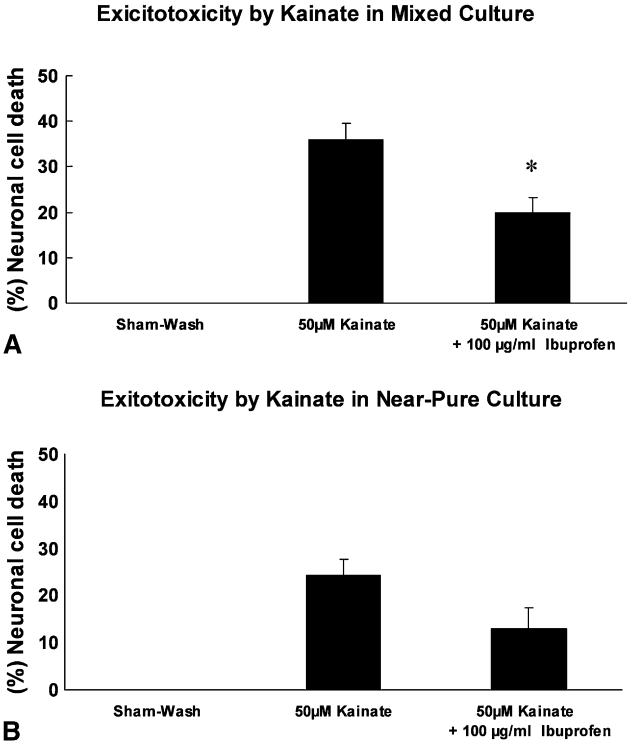

FIGURE 3. Exposure to 50 μmol/L kainate revealed approximately 30% neuronal cell death. Ibuprofen reduced neuronal cell death induced by exposure to 50 μmol/L kainate from 36.1% $\pm$ 3.5% to 20.0% $\pm$ 3.1% in mixed cultures but failed in near-pure cultures. A, Mixed cultures. B, Near-pure cultures. *Significantly different from kainate alone by analysis of variance with Tukey correction (mean $\pm$ standard error).

neuronal cell death by LDH assay, measuring LDH levels in bathing medium in both cultures. Ibuprofen at a concentration of 25 μg/mL, 50 μg/mL, and 100 μg/mL reduced neuronal cell death significantly from 74.5% $\pm$ 3.8% to 56.1% $\pm$ 4.0%, 38.7% $\pm$ 2.8%, and 12.3% $\pm$ 1.4% in *mixed* cultures, respectively, revealing a strong dose-response ($P < .001$). However, low-concentration ibuprofen failed to protect neurons in *near-pure* cultures (Figure 2, *A, B*). Exposure to 50 μmol/L kainate revealed approximately 30% neuronal cell death. Ibuprofen also reduced neuronal cell death induced by exposure to 50 μmol/L kainate from 36.1% $\pm$ 3.5% to 20.0% $\pm$ 3.1% in mixed cultures but failed in near-pure cultures (Figure 3, *A, B*). These results suggest that ibuprofen may reduce glutamatergic excitotoxic neuronal cell death induced by NMDA or kainate, resulting from ischemia, although it may require interaction with glial cells.

DISCUSSION

This study demonstrated that ibuprofen protects neurons against glutamatergic excitotoxic neuronal cell death induced by NMDA or kainate in mixed cortical cultures. However, ibuprofen fails to reduce neuronal cell death in near-pure cultures. This result suggests that protection of neurons by ibuprofen requires interaction with glial cells and is not a result of a direct effect on neurons. Further evidence for the role of glial cells is the observation that the percentage of neuronal cell death was the same whether induced by NMDA or kainate in both cultures. It is possible that ibuprofen stimulates glial cells to release a neuroprotective mediator, such as a cytokine, or inhibits the deleterious effects of mediators released by glial cells.

It is thought that inflammation contributes to post-ischemic brain injury. Although cytokines are produced in the central nervous system by other cells, including astrocytes, neurons, and endothelial cells, microglia rapidly respond to injury by secreting inflammatory cytokines on injury. Proinflammatory cytokines, such as interleukin-1β and tumor necrosis factor-α, are involved in ischemia/hypoxia and trauma-induced brain injury.[13,14] Ibuprofen is a nonsteroidal anti-inflammatory drug and is widely used to reduce pain, fever, and inflammation. The drug inhibits cyclooxygenase and activates peroxisome proliferator-activated receptors; both of these actions result in reduced inflammation.[1-3] Park and coworkers[5] described ibuprofen protecting neurons against ischemia via up-regulating interleukin-1 receptor antagonist expression. However, inhibition of cyclooxygenase has been controversial in adult cardiac surgery in regard to cardiac protection. McGuinness and coworkers[15] reported that glutamine pretreatment conferred infarct protection through up-regulation of cyclooxygenase-2. Wong and colleagues[16] reported that a selective cyclooxygenase-2 inhibitor did not affect the myocardium in patients undergoing coronary artery bypass grafting.

Local cerebral glucose use decreases after traumatic brain injury as live cells decrease in number. Pappius and Wolfe[6] demonstrated that pretreatment with ibuprofen prevented the depression of local cerebral glucose use after traumatic brain injury. They suggested that ibuprofen protected neurons and improved cerebral metabolism after brain injury and that these effects might be associated with suppression of prostaglandin synthesis.

Cerebral blood flow usually decreases after cerebral ischemia because of vasoconstriction resulting from hypoperfusion during ischemia and subsequent inflammation. Grice and colleagues[7] reported that ibuprofen improved post-ischemic hypoperfusion after normothermic global cerebral ischemia in dogs, although cerebral blood flow did not return to normal. They demonstrated that ibuprofen suppressed the production of thromboxane B2 and prostaglandin I2 after global cerebral ischemia.[7] Ibuprofen has been reported to reduce infarct size after temporary cerebral ischemia, for example, after deep hypothermic circulatory arrest.[4] They suggested that the beneficial effect of cyclooxygenase inhibition occurs during reperfusion.

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CONCLUSIONS

Ibuprofen provides neuroprotection through glial cells against glutamatergic excitotoxicity as demonstrated by reduced neuronal cell death in mixed cell cultures. Further studies regarding mechanisms at the molecular level and with whole animal models are needed to assess the potential of ibuprofen to reduce neurologic injury in patients undergoing circulatory arrest and cardiopulmonary bypass.

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