Aprotinin confers neuroprotection by reducing excitotoxic cell death

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487, 495, and 685.

Objective: Aprotinin is used in cardiac surgery for its anti-inflammatory and hemostatic benefits. Recent reports describe the neuroprotective effects of other serine protease inhibitors via reduced excitotoxic cell death, a common pathway causing cytotoxic edema induced in various neuropathologic conditions. The purpose of this study was to investigate whether aprotinin directly protects against glutamatergic excitotoxicity in cell cultures.

Methods: Mixed cortical cultures containing neuronal and glial cells were prepared from fetal mice at 13 to 15 days' gestation and plated on a layer of confluent astrocytes from 1- to 3-day-old postnatal pups. Near-pure neuronal culture containing less than 5% astrocytes was obtained from the same gestational stage and plated in multiwell vessels previously coated with poly-D-lysine and laminin. Both cultures were used at 12 to 14 days in vitro. Slowly triggered excitotoxicity was induced at 37°C by 24-hour exposure to 12.5 μ M N-methyl-D-aspartate or 50 μ M kainate. Neuronal death was quantified by measuring the release of lactate dehydrogenase from damaged cells into the bathing medium. Data were analyzed by analysis of variance with post hoc Bonferroni comparisons.

Results: Aprotinin at a clinically relevant concentration of 100 KIU/mL significantly reduced N-methyl-D-aspartate-induced neuronal death in both pure and mixed cultures ($P < .001$). Aprotinin also reduced neuronal death induced by kainate from 36% to 23% in mixed cortical culture ($P = .008$) and from 40% to 27% in near-pure culture ($P = .015$), indicating that the neuroprotective effects of aprotinin are mediated directly through neurons.

Conclusion: Aprotinin provides direct neuroprotection against glutamatergic excitotoxicity as demonstrated by reduced neuronal death in near-pure neuronal cell culture. Additional studies are needed to evaluate the potential of aprotinin to reduce neurologic injury in patients at high risk of cerebral injury, including those undergoing circulatory arrest.

Aproinin is a broad-spectrum serine protease inhibitor with a molecular weight of 6512 d. It was first introduced into clinical practice for the treatment of pancreatitis. It is widely used in congenital cardiac surgery for its hemostatic and anti-inflammatory benefits,¹⁻³ although its safety when used in adults undergoing coronary artery surgery has recently been questioned.⁴ The hemostatic effects of aprotinin are mediated by the preservation of platelet function and reduced fibrinolysis through inhibition of multiple proteases, including plasmin, plasminogen, and plasminogen activator.^{5,6} Aprotinin reduces the inflammatory response to cardiopulmonary bypass through reduced complement and neutrophil activation.^{7,8}

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Abbreviations and Acronyms

LDH	= lactate dehydrogenase
NMDA	= N-methyl-D-aspartate
tPA	= tissue plasminogen activator

We previously studied the neuroprotective potential of aprotinin using a piglet model of deep hypothermic circulatory arrest. Aoki and colleagues⁹ described accelerated recovery of cerebral adenosine triphosphate and intracellular pH determined by magnetic resonance spectroscopy, and showed that aprotinin preserved endothelial-mediated vasodilation. Anttila and colleagues¹⁰ demonstrated that aprotinin reduced rolling and adherent leukocytes in the cerebral circulation measured by intravital microscopy and improved neurobehavioral outcomes after a prolonged period of deep hypothermic circulatory arrest or low-flow cardiopulmonary bypass. We concluded from our 2 previous studies that the primary neuroprotective effect of aprotinin was accelerated recovery of the microcirculation.

In addition to microvascular injury, ischemic brain injury can be mediated through “excitotoxicity,” which causes excessive calcium influx through N-methyl-D-aspartate (NMDA), AMPA, and kainate pathways. Nicole and colleagues^{11,12} investigated the mechanism by which ischemic stroke could sometimes be exacerbated by the thrombolytic agent tissue plasminogen activator (tPA). They found that tPA exaggerates NMDA-mediated excitotoxicity and confirmed that inhibition of tPA by the serine protease inhibitor plasminogen activator inhibitor-1 reduces NMDA, but not kainate-induced excitotoxic neuronal death in a cell culture model. This work expanded on a previous report from the same center in which Buisson and colleagues¹³ reported that up-regulation of plasminogen activator inhibitor-1 in astrocytes was neuroprotective against ischemic neuronal death. tPA is present as an endogenous protease even when it is not used as an exogenous thrombolytic agent. We hypothesized that the inhibition of intrinsic tPA by the broad-spectrum serine protease inhibitor aprotinin would be neuroprotective. Lebeurrier and colleagues¹⁴ demonstrated that NMDA excitotoxic neuronal injury could also be reduced by a newly discovered serine protease inhibitor named “neuroserpin,” suggesting that other mechanisms involving serine protease inhibition might be neuroprotective.¹⁵

The purpose of this study was to investigate whether aprotinin has a direct neuroprotective effect against glutamatergic excitotoxicity using both mixed and near-pure neuronal cell culture models.

Materials and Methods**Glial Cultures**

Glial cell cultures were prepared from 1 to 3-day-old postnatal mice (Taconic, Rockville, Md), as described by Rose and colleagues.¹⁶

Dissociated cortical cells were plated in multiwell vessels previously coated with poly-D-lysine (Sigma, St Louis, Mo) using a plating medium of medium stock (Dulbecco's Modified Eagle's Medium with 25 mmol/L glucose; Sigma) supplemented with 10% horse serum (Gibco, Carlsbad, Calif), 10% fetal bovine serum (Gibco), and 2 mmol/L glutamine. Cultures were kept at 37°C in a humidified 5% CO₂-containing atmosphere until they reached confluence 7 to 14 days in vitro. Confluent cultures were then used as a support for mixed cultures.

Mixed Cortical Cultures

Mixed cortical cultures containing both neurons and astrocytes were prepared from fetal mice at 14 to 15 days gestation.¹⁶ Dissociated cortical cells were plated in 24 wells on a layer of confluent astrocytes, using medium stock supplemented with 5% horse serum, 5% fetal bovine serum, and 2 mmol/L glutamine. After 7 days in vitro, non-neuronal cell division was halted by 2 to 3 days of exposure to 10 μ M cytosine arabinoside (Ara-C; Sigma). Subsequent partial medium replacement was performed twice per week, and after 12 days in vitro, cultures were shifted to a maintenance medium identical to the plating medium but lacking serum, because neurons survive without it. Experiments were performed on cortical cultures after 13 to 14 days in vitro.

Near-pure Neuronal Cell Cultures

The cultures containing less than 5% astrocytes were prepared as detailed previously by Rose and colleagues.¹⁶ Dissociated cortical cells in medium stock supplemented with 5% fetal bovine, 5% horse serum, and 2 mmol/L glutamine were plated in multiwell vessels that had been coated with poly-D-lysine and laminin (Invitrogen, Carlsbad, Calif). After 3 days in vitro, non-neuronal cell division was halted by exposure to 10 μ M Ara-C. There was no further exchange of the media except the addition of Dulbecco's Modified Eagle's Medium for evaporation. After 12 days in vitro, the cultures did not need the presence of serum to survive. They were shifted to a maintenance medium identical to the plating medium but lacking serum. Cultures were used after 13 to 14 days in vitro for excitotoxic injury.

Excitotoxicity

Slowly triggered excitotoxicity was induced at 37°C by 24-hour exposure to 12.5 μ M NMDA (Tocris, Ellisville, Mich) or 50 μ M kainate (Tocris) as an excitotoxin in medium stock supplemented with 10 μ M glycine;^{17,18} 10 μ M MK-801 (Tocris) was always added concurrently with kainate to block secondary NMDA receptor activation. Aprotinin (Bayer HealthCare Pharmaceuticals, Wayne, NJ) was co-applied at 3 different concentrations (100, 150, or 200 KIU/mL) with the excitotoxin and left for 24 hours in the bathing medium. Aprotinin concentrations were determined according to the current clinical target level of 200 KIU/mL, because aprotinin inhibits plasmin at the concentration of 50 KIU/mL, kallikrein at the concentration of 200 KIU/mL, and platelet aggregation at the concentration of approximately 160 KIU/mL.

Assessment of Neuronal Cell Death

Neuronal death was confirmed by examining cultures under phase-contrast microscopy and quantified by measurement of lactate dehydrogenase (LDH) release from damaged cells into the bathing medium 1 day after the onset of excitotoxin exposure.¹⁹ LDH was

measured by an enzyme-linked immunosorbent assay kit (Promega, Madison, Wis). The LDH level corresponding to complete neuronal death (without glial death) was determined in sister cultures exposed to 100 μ M 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.

Statistical Analysis

The primary efficacy outcome measure was the percentage of neuronal cell death. The results are expressed as mean $\pm$ standard error. When $n = 12$ is indicated, this value corresponds to 12 different wells from 3 different dissections. Analysis of the data included factorial analysis of variance with the Bonferroni method to protect against false-positive results. The Statistical Package for the Social Sciences was used for statistical analysis (version 15.1, SPSS Inc, Chicago, Ill). Two-tailed values of P less than .05 were used, with Bonferroni correction as appropriate.

Results

Sham wash and the addition of aprotinin alone at the concentration of 200 KIU/mL did not influence neuronal cells in either culture (Figure 1, A and B). Excitotoxicity induced by exposure of the cultures to 12.5 μ M NMDA or 50 μ M kainate for 24 hours resulted in acute swelling of neuronal cell bodies, followed by widespread necrotic neuronal degeneration resulting in disrupted neurons and segmentalized neuritis (Figure 1, C). When aprotinin was co-applied in the medium with the excitotoxin, aprotinin preserved neuronal cells, including neuritis (Figure 1, D). Exposure to 12.5 μ M NMDA caused approximately 70% neuronal cell death by LDH assay measuring LDH levels in bathing

medium in both cultures. In mixed cultures, the percentage of neuronal cell death was significantly reduced from $69.3\% \pm 4.6\%$ to $46.1\% \pm 4.9\%$ with aprotinin concentration of 100 KIU/mL, $40.0\% \pm 4.5\%$ with aprotinin concentration of 150 KIU/mL, and $29.3\% \pm 2.4\%$ with aprotinin concentration of 200 KIU/mL (Figure 2, A). In near-pure cultures, neuronal cell death was reduced from $67.6\% \pm 4.9\%$ to $44.0\% \pm 4.9\%$ with aprotinin concentration of 100 KIU/mL, $21.6\% \pm 3.6\%$ with aprotinin concentration of 150 KIU/mL, and $17.0\% \pm 3.8\%$ with aprotinin concentration of 200 KIU/mL. The degree of reduction depended on the concentration of aprotinin. Aprotinin also reduced the percentage of neuronal cell death induced by exposure to 50 μ M kainate from $36.0\% \pm 5.0\%$ to $22.9\% \pm 2.6\%$ in mixed cultures and from $40.4\% \pm 4.0\%$ to $27.5\% \pm 4.6\%$ in near-pure cultures (Figure 3, A and B). These results suggest that the effect of aprotinin against excitotoxicity induced by NMDA or kainate was not mediated through glial cells but directly through neurons.

Discussion

This study is important because it demonstrates that aprotinin confers direct neuroprotection against excitotoxic cell death. The protective effect is mediated through neurons and is effective against both NMDA and kainate-mediated excitotoxicity.

What Is Excitotoxicity?

Excitotoxicity is one of several mechanisms whereby neurons can be injured or killed after an ischemic insult. High levels of the excitatory neurotransmitter glutamate result in

Figure 1. A, Sham wash (mixed cortical cultures). Scale bars = 100 μ m. B, Aprotinin alone. C, Mixed cortical cultures were exposed to 12.5 μ M NMDA for 24 hours and showed an acute swelling of neuronal cell bodies followed by a widespread necrotic neuronal degeneration resulting in disrupted neurons and segmentalized neuritis. D, Aprotinin at a concentration of 200 KIU/mL was co-applied in the medium with excitotoxin. Aprotinin preserved neuronal cells, including neuritis.

Figure 2. Percentage of neuronal cell death induced by an exposure to 12.5 μ M NMDA revealed approximately 70% neuronal cell death by LDH assay measuring LDH levels in bathing medium in both cultures. Aprotinin at the concentration of 100, 150, and 200 KIU/mL reduced the percentage of neuronal cell death significantly from 69.3% $\pm$ 4.6% to 46.1% $\pm$ 4.9%, 40.0% $\pm$ 4.5%, and 29.3% $\pm$ 2.4% in mixed cultures and from 67.6% $\pm$ 4.9% to 44.0% $\pm$ 4.9%, 21.6% $\pm$ 3.6%, and 17.0% $\pm$ 3.8% in near-pure cultures, respectively (A: mixed cultures; B: near-pure cultures). *Significantly different from NMDA alone by analysis of variance with Bonferroni correction (mean $\pm$ standard error). NMDA, N-methyl-D-aspartate.

overactivation of glutamate receptors, resulting in excessive influx of calcium into cerebral neurons.^{17,18} Calcium influx activates a number of enzymes, including phospholipases, endonucleases, and proteases (eg, calpain). These enzymes damage cell structures, such as components of the cytoskeleton, cell membrane, and DNA.

Glutamate Receptor Subtypes

The glutamate receptors involved in excitotoxic injury caused by ischemia are normally responsible for excitatory synaptic transmissions. There are 3 groups of ionotropic glutamate receptors that principally control ion flux: NMDA, AMPA, and kainate. NMDA receptors are structurally distinct from kainate receptors and are named for their specific response to the nonphysiologic agent NMDA. Kainate receptors respond specifically to kainic acid, another nonphysiologic agent known to trigger seizures. Both groups of receptors in the physiologic setting participate in glutamate-mediated synaptic transmission. Differences in the response to the excitotoxins NMDA and kainate in the experimental setting allow a greater understanding of the mechanism of excitotoxic injury and specifically the role of subgroups of glutamate receptors.

Previous studies of the neuroprotective mechanism of the serine protease inhibitor plasminogen activator inhibitor-1 against excitotoxic injury suggested that tPA specifically enhanced NMDA-mediated excitotoxicity but not kainate-mediated excitotoxic injury.¹³ On the other hand Tsirka and colleagues²⁰ reported that tPA is the mediator by which kainate induces neuronal death caused by intrahippocampal injection of kainic acid. Intrahippocampal injection of kainic acid induced a loss of laminin immunoreactivity that pre-

ceded neuronal death. Laminin cleavage and the neuronal death subsequent to kainate injection were abolished in tPA-deficient mice or when kainate was co-injected with alpha 2-antiplasmin in the hippocampus of wild-type mice. These results suggest that the cleavage of plasminogen into plasmin by tPA is a key step in the excitotoxicity cascade mediated through kainate/glutamate receptors.²¹

The Role of Glial Cells in Excitotoxic Injury

Glial cells play an important role in plasmin-mediated excitotoxic injury. It has been suggested that ischemic injury leads to increased tPA expression and subsequent extracellular release from injured neurons.²² tPA cleaves plasminogen, and the resulting plasmin degrades extracellular matrix molecules. Signaling from the injured neurons causes hypersensitivity to direct attacks by tPA, recruitment and activation of microglia, subsequent release of microglial neurotoxic factors, and further degradation of extracellular matrix molecules by other proteases activated by plasmin.²³ Glial cells play an important role in the rescue of neurons from ischemic injury, for example, by release of neuroserpin.¹⁴ The present study demonstrates that aprotinin reduces excitotoxic brain injury through a direct neuronal effect and without the support of glial cells.

Our study has documented that NMDA-mediated excitotoxic injury and kainate-mediated excitotoxic injury are reduced by aprotinin. Furthermore, the effect of aprotinin is not affected by the presence of glial cells. Aprotinin was equally effective in reducing both NMDA excitotoxic injury and kainate-induced injury in both near-pure neuronal cultures and mixed glial and neuronal cultures. This is not surprising in view of aprotinin's broad spectrum of action.

Figure 3. A, Percentage of neuronal cell death induced by an exposure to 50 μ M kainate revealed approximately 40% neuronal cell death. Aprotinin reduced percentage of neuronal cell death induced by an exposure to 50 μ M kainate from $36.0 \pm 5.0\%$ to $22.9 \pm 2.6\%$ in mixed cultures and from $40.4 \pm 4.0\%$ to $27.5 \pm 4.6\%$ in near-pure cultures (A: mixed cultures; B: near-pure cultures). *Significantly different from NMDA alone by analysis of variance with Bonferroni correction (mean $\pm$ standard error).

Aprotinin Is a Broad-spectrum Protease Inhibitor

Aprotinin belongs to the group of Kunitz protease inhibitors. It is a naturally occurring broad-spectrum serpin obtained from bovine lung. Aprotinin inhibits a wide range of serine proteases with varying efficacy. It inhibits proteases such as kallikrein, trypsin, and plasmin, and reduces complement activation. Aprotinin has been reported to inhibit neutrophil elastase release and superoxide anion formation. Further studies with other specific protease inhibitors are needed to elucidate the mechanisms by which aprotinin reduces NMDA and kainate-mediated excitotoxic injury.

Aprotinin and the Blood-Brain Barrier

A question central to this study is the ease with which aprotinin crosses the blood-brain barrier. Although an early report from Bayer Pharmaceuticals suggested that aprotinin does not pass through the blood-brain barrier, it has been detected in cerebrospinal fluid after venous administration.²⁴ Further-

more, previous studies have suggested that the blood-brain barrier is at least partially broken down during cardiopulmonary bypass. For example, Cavaglia and colleagues²⁵ detected fluorescent-labeled albumin leaking from microarteries into the brain during cardiopulmonary bypass. They demonstrated that normothermic cardiopulmonary bypass and low temperature increased this leakage of fluorescein isothiocyanate-labeled albumin after a brief exposure of 15 minutes. Given that the blood-brain barrier is immature in fetuses and newborns, studies are needed to determine the blood-brain barrier's permeability to aprotinin in young individuals undergoing cardiopulmonary bypass.

Other Ways Aprotinin May Reduce Cerebral Injury

Our own previous studies have focused on the vascular protective effects of aprotinin in whole animal models of cardiopulmonary bypass. Direct observation of the cerebral microcirculation confirmed that aprotinin reduced white cell activation.¹⁰ Kamiya²⁶ reported that venous administration of aprotinin improves the preservation of cerebral adenosine triphosphate and reduces cerebral water content after ischemia. He concluded that cerebral edema is closely related to plasma and brain tissue bradykinin levels. Murtomaki and colleagues²⁷ reported that aprotinin rescues dying neurons from cell death in the weaver mutated mouse as a result of the restoration of resting membrane potentials of granule neurons to near normal and neurite outgrowth to normal. These results suggest that aprotinin may be neuroprotective against ischemia by reducing inflammation.

Conclusions

This study provides insight into a possible mechanism whereby aprotinin may be neuroprotective in the setting of ischemia.

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Discussion

Dr J. Conte (Baltimore, Md). The purpose of your study was to investigate whether aprotinin directly protects against glutamatergic

excitotoxic injury. I think as far as your data are concerned, you've shown that very nicely. I think we can believe that.

I have 3 methodological questions, and I'd be remiss if I didn't go for the low-hanging fruit on this one as well.

The first has to do with the use of fetal tissue. Now, is there anything particular about fetal tissue in cell culture studies like this that would make it particularly amenable to the prevention of that type of injury and does it accurately reflect the type of glutamatergic-stimulated injury that we would see in adolescents or adults in the whole animal?

The second has to do with the use of a mechanism of slowly stimulated or slowly triggered excitotoxicity as your slide nicely demonstrated. Does that accurately reflect the type of injury we would see with a short period of negative stimulation, such as hypothermic circulatory arrest, or in situations where we have embolic types of ischemic injury? Does that slowly triggered type of mechanism reflect what we would see in that type of real-life situation?

The third question has to do with aprotinin. Aprotinin was left in culture for 24 hours. Does that really reflect the mechanism of action of aprotinin that we would see clinically? Can we translate aprotinin's benefit in a situation where we've left it in culture for 24 hours to a situation where we would use it in near juxtaposition to when an injury would occur, either hypothermic circulatory arrest or an ischemic event?

The low-hanging fruit is obvious. In the United States, the Food and Drug Administration has come down on aprotinin, and many surgeons are looking twice at whether they want to risk using aprotinin because of the reported adverse events and the flurry of lawsuits. Many offices, including our own, are inundated with calls from patients asking whether they received aprotinin, a week ago, a year ago, 5 years ago. We've even been contacted by malpractice lawyers who asked if we used aprotinin in a certain patient. Is it worth it to use it and would you recommend that on the basis of these studies or on your previous work? If the answer is no, are there other serine protease inhibitors that might be able to confer the same advantages?

Dr Iwata. We used fetal cells in our study because adult cells cannot be grown in an incubator. Therefore we had to use cortical cells from fetal mice, because they still have potential for growth.

Your second question was why we used slowly triggered excitotoxicity. It's a very good question. However, there would be a technical problem if we used a higher concentration with brief exposure to NMDA or kainate. We would then need to remove NMDA or kainate to parallel the clinical setting. This is technically difficult, because if we expose the cells to the air, those cells are going to die. Neurons cannot be exposed to air. Complete replacement or partial replacement of the bathing medium to allow a briefer exposure to aprotinin is also difficult because neurons in culture are very sensitive.

In regard to the advisability of using aprotinin clinically, we realize that this is controversial for adults. However, the majority of pediatric centers continue to use aprotinin either routinely or frequently without observing adverse effects.