

Cerebral Ischemia Enhances Polyamine Oxidation: Identification of Enzymatically Formed 3-Aminopropanal as an Endogenous Mediator of Neuronal and Glial Cell Death

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Summary

To elucidate endogenous mechanisms underlying cerebral damage during ischemia, brain polyamine oxidase activity was measured in rats subjected to permanent occlusion of the middle cerebral artery. Brain polyamine oxidase activity was increased significantly within 2 h after the onset of ischemia in brain homogenates (15.8 ± 0.9 nmol/h/mg protein) as compared with homogenates prepared from the normally perfused contralateral side (7.4 ± 0.5 nmol/h/mg protein) ($P < 0.05$). The major catabolic products of polyamine oxidase are putrescine and 3-aminopropanal. Although 3-aminopropanal is a potent cytotoxin, essential information was previously lacking on whether 3-aminopropanal is produced during cerebral ischemia. We now report that 3-aminopropanal accumulates in the ischemic brain within 2 h after permanent forebrain ischemia in rats. Cytotoxic levels of 3-aminopropanal are achieved before the onset of significant cerebral cell damage, and increase in a time-dependent manner with spreading neuronal and glial cell death. Glial cell cultures exposed to 3-aminopropanal undergo apoptosis ($LD_{50} = 160 \mu\text{M}$), whereas neurons are killed by necrotic mechanisms ($LD_{50} = 90 \mu\text{M}$). The tetrapeptide caspase 1 inhibitor (Ac-YVAD-CMK) prevents 3-aminopropanal-mediated apoptosis in glial cells. Finally, treatment of rats with two structurally distinct inhibitors of polyamine oxidase (aminoguanidine and chloroquine) attenuates brain polyamine oxidase activity, prevents the production of 3-aminopropanal, and significantly protects against the development of ischemic brain damage in vivo. Considered together, these results indicate that polyamine oxidase-derived 3-aminopropanal is a mediator of the brain damaging sequelae of cerebral ischemia, which can be therapeutically modulated.

Key words: stroke • infarction • spermine • apoptosis • caspase

Cerebral ischemia, a leading cause of disability and mortality world-wide, is mediated by a cascade of molecular cytotoxins that kill potentially viable cells in the brain. The polyamines spermine, spermidine, and putrescine, which are among the most abundant molecules in mammalian brain, have been implicated in the pathogenesis of ischemic brain

damage (1–8). Polyamine biosynthesis is increased after the onset of cerebral ischemia, due to an ischemia-mediated induction of ornithine decarboxylase, a key synthetic enzyme in the polyamine biosynthetic pathway (9–13). Spermine was recently linked to the development of glutamate-mediated cytotoxicity, because it can bind to the NR1 subunit

of the NMDA receptor and potentiate glutamate-mediated cell damage (14–17). Administration of experimental therapeutics that inhibit ornithine decarboxylase prevents the development of ischemic brain damage, suggesting that the accumulation of polyamines in the ischemic brain occupies an important role in the pathogenesis of stroke (9).

Somewhat paradoxically, however, brain spermine and spermidine levels decrease during cerebral ischemia (5, 18, 19). This decline of tissue spermine and spermidine levels is accompanied by an increase in brain putrescine levels (13, 19–21). Furthermore, intracerebral putrescine levels correlate significantly with the volume of dead brain, suggesting that putrescine may be an endogenous molecular marker for the extent of ischemia-induced damage. Notably, putrescine does not interact with the *N*-methyl-*D*-aspartate (NMDA) receptor, and does not potentiate its function. Therefore, we reasoned that a possible explanation for these results could be found in the catabolism of polyamines via the “interconversion pathway”, which is dependent upon the activity of tissue polyamine oxidase (20, 22–25). This ubiquitous enzyme, which is present in high levels in brain and other mammalian tissues, cleaves spermine and spermidine via oxidative deamination to generate the end products putrescine and 3-aminopropanal (22, 26–28). 3-Aminopropanal is widely known for its cytotoxicity to primary endothelial cells, fibroblasts, and a variety of transformed mammalian cell lines (29–33). 3-Aminopropanal has also been implicated as a mediator of programmed cell death in murine embryonic limb buds, and may contribute to the development of necrosis in some tumors (34, 35). Inhibition of polyamine oxidase with aminoguanidine, a well-characterized inhibitor, blocks the generation of 3-aminopropanal in cell cultures after the addition of spermine, and prevents subsequent cytotoxicity (30, 36, 37). The LD₅₀ concentration of 3-aminopropanal to cells is similar to that of glutamate excitotoxicity to neurons (38). In contrast, putrescine is not cytotoxic to cells (even in the millimolar range) but its rate of production through polyamine oxidation correlates with the rate of formation of the cytotoxin (3-aminopropanal). Reasoning that increased production of 3-aminopropanal might therefore mediate cytotoxic brain damage during cerebral ischemia, we investigated the activity of polyamine oxidase, and measured the levels of 3-aminopropanal in an animal model of cerebral infarction.

We now report that cerebral ischemia mediates the induction of brain polyamine oxidase activity, and that the cytotoxic end product 3-aminopropanal accumulates in the brain at levels that are lethal to neurons and glial cells. In glial cells, 3-aminopropanal mediates apoptosis by activation of a caspase 1-dependent signaling pathway, whereas in neurons it causes necrotic cell death. Inhibition of polyamine oxidase activity with structurally distinct compounds prevents the formation of 3-aminopropanal and provides significant protection against the development of cerebral damage after permanent cerebral artery occlusion in rats. This is the first direct evidence that polyamine oxidase-derived 3-aminopropanal is a potential therapeutic target in cerebral ischemia.

Materials and Methods

Animal Model of Permanent Middle Cerebral Artery Occlusion. All procedures involving animals were conducted in conformity with institutional guidelines and under the approval of the Animal Care and Use Committee of North Shore University Hospital–New York University Medical School. Middle cerebral infarction was performed as previously described in detail (39, 40). In brief, the ipsilateral common carotid artery was ligated and divided, the middle cerebral artery was coagulated and divided distal to the lenticulostriate branch, and the contralateral common carotid was occluded for 1 h. The onset of ischemia in these experiments was defined as the time the middle cerebral artery was cut. For measurement of infarct volume, the animals were killed at the times indicated and fresh brain sections were prepared (1 mm) and immersed in 2,3,5-triphenyltetrazolium chloride (TTC)¹ in 154 mM NaCl for 30 min at 37°C, and total cerebral infarct volume was measured by computerized quantitative planimetry as previously described (39–41). Similar measurements of stroke volume were obtained in separate experiments using planimetric analysis of brain sections stained with hematoxylin and eosin (data not shown).

Polyamine Oxidase Assay. Polyamine oxidase in brain homogenate was assayed as previously described (25, 26, 42). In brief, 2 h after occlusion of the middle cerebral artery, a 4-mm-thick coronal section of ipsilateral hemisphere encompassing the zone of ischemia (beginning 3 mm caudal from the frontal pole) was manually homogenized on ice in 1.5 ml of Hanks media containing 1 mM PMSF, and was centrifuged at 43,000 *g* for 30 min. Brain polyamine oxidase activity in the homogenates was determined by addition of spermine to the homogenate at time zero (50 μl of a 1 mM stock solution added per 1 ml of supernatant). Where indicated in some experiments the enzyme inhibitors aminoguanidine or chloroquine (50 μM–5 mM) were added 5 min before spermine. Homogenates were maintained at 37°C, and duplicate 200-μl samples were removed at time points up to 60 min after the addition of spermine; enzyme activity in the samples was stopped by addition of 10 μl of 60% perchloric acid (PCA). Samples for HPLC analysis to detect spermine were prepared as described below. Enzyme activity was corrected for the protein content of the supernatants using a commercially available protein assay (Bio-Rad Protein Assay; Bio-Rad, Hercules, CA) with BSA (GIBCO BRL, Gaithersburg, MD) as a standard.

3-Aminopropanal. 3-Aminopropanal was prepared by hydrolysis of 3-aminopropanal diethyl acetal (145 mM; TCI America, Portland, OR) in 1.5 M of HCl for 5 h at room temperature. The reaction mixture was applied to a column (3 × 6 cm) of Dowex-50 (H⁺ form; Sigma Chemical Co., St. Louis, MO) ion exchange resin and eluted with a step gradient of HCl (0–3 M; 160 ml; flow rate 0.7 ml/min). Fractions containing aldehyde, as determined by the method of Bacrach and Reches (43), were concentrated in a centrifugal evaporator at room temperature. The concentration of 3-aminopropanal was determined spectrophotometrically at λ = 531 nm, based on a reaction of aldehydes with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald; Aldrich Chemical Co., Milwaukee, WI; reference 44) with reference to a standard curve using propionaldehyde (Sigma Chemical

¹Abbreviations used in this paper: EIMS, Electrospray ionization mass spectroscopy; iNOS, inducible nitric oxide synthase; NMDA, *N*-methyl-*D*-aspartate; NMR, nuclear magnetic resonance; PAO, polyamine oxidase; PCA, perchloric acid; TTC, 2,3,5-triphenyltetrazolium chloride; TUNEL, Tdt-mediated dUTP-biotin nick-end labeling.

Co.). Acidic solutions of the aldehyde were neutralized with NaOH to physiological pH immediately before use. Vehicle control solutions consisted of the same stoichiometric amounts of HCl and NaOH.

Derivatization of 3-Aminopropanal with 2,4-Dinitrophenylhydrazine. 2,4-dinitrophenylhydrazine (0.5 g) in concentrated HCl/ethanol (1:10, vol/vol; 11 ml) was refluxed for 10 s with the aqueous 3-aminopropanal. The resulting 3-aminopropionaldehyde-2,4-dinitrophenylhydrazone derivative was precipitated at room temperature and collected by filtration. ¹H-nuclear magnetic resonance (NMR) spectroscopy (DMSO-*d*₆ and CDCl₃, 270 MHz) of purified 2,4-dinitrophenylhydrazone derivative was employed to confirm its structure. The NMR spectrum revealed the presence of *syn* and *anti* isomers (1:1) with resonance at δ8.83 and δ11.35. A standard curve was generated by an HPLC assay of the dansylated derivative of the compound (see below). An additional standard curve was constructed to quantify recovery of the compound from brain homogenates. In brief, a 4-mm-thick brain slice obtained from the region of the middle cerebral artery perfusion zone was homogenized manually, followed by addition of 3-aminopropanal (final concentration = 10, 100, 150, 200, 300, or 1,000 nmol/ml) in 1.5 ml of 2,4-dinitrophenylhydrazine reagent. The samples were refluxed in the 2,4-dinitrophenylhydrazine reagent for 10 s, then 20 μl of 60% PCA added to stop the reaction, followed by addition of water (200 μl). The samples were vigorously vortexed and centrifuged at 14,000 rpm for 30 min, and the supernatant was concentrated to near dryness in a centrifugal evaporator. Samples were redissolved in 100 μl of water, centrifuged for 10 min at 14,000 rpm to clear precipitates, and then subjected to HPLC.

HPLC Detection of the Derivatization Products of 3-Aminopropanal and 2,4-Dinitrophenylhydrazine. A liquid chromatograph (model 1090; Hewlett-Packard, Wilmington, DE), equipped with an autosampler, photo diode-array, fluorescence detectors, and Chemstation operating software, was used for all analyses. We used detection by fluorescence, based on the reaction of 5-dimethylaminonaphthalene sulfonyl-chloride (dansyl chloride; Molecular Probes, Eugene, OR; relative fluorescence intensity 280–340 out of 430 nm) with primary and secondary amines. Dansylation was performed by reacting 50 μl of the sample with 200 μl of 10 mg/ml dansyl chloride solution in acetone, 200 μl of saturated Na₂CO₃ solution, 3 μl of 60% PCA, and 3 μl of 1-mM 1,7-diaminoheptane (Sigma Chemical Co.), followed by incubation at 65°C for 10 min. 20 μl of the resulting supernatant was injected onto a C-4 250 × 4.6 mm column (Vydac, Hesperia, CA) with 5-μm particle size. Using a flow rate of 1.0 ml/min, runs were initiated at 100% A (dH₂O) and a linear gradient to 100% B (methanol) performed over 45 min, followed by 5 min of 100% B and a return to 100% A over 5 min.

For detection of the presence of 3-aminopropanal in ischemic brain, animals were subjected to permanent middle cerebral artery occlusion and killed at the times indicated. Brain sections corresponding to the area of focal infarction caused by middle cerebral artery occlusion (4-mm-thick located 3 mm caudal to the frontal lobes) were quickly excised. Control brain slices were taken from sham-operated animals. Manual homogenization was performed in 1.5 ml of 2,4-dinitrophenylhydrazine reagent followed by concentration and HPLC analysis as described above. The limit of detection of 3-aminopropanal with this assay is 180–200 nmol/ml. Results are normalized for protein content using a commercially available assay (Bio-Rad Protein Assay; Bio-Rad) and corrected for HPLC injection volume using an internal standard of 1,7-diaminoheptane.

Stereotactically Guided Microinjections of Polyamines into the Cerebral Cortex. Male Lewis rats (270–300 g) were anesthetized and placed in a stereotactic head frame (Stoelting Co., Wood Dale, IL). The incisor bar was adjusted until the plane defined by the lambda and bregma was parallel to the base plate. A microsurgical craniotomy was performed 1.7 mm anterior to bregma, and 5 mm right of the midline, and the tip of a 29-gauge needle was advanced 2 mm deep to the dural opening. Polyamine- or 3-aminopropanal-containing solutions (25 μg) prepared in 2 μl of sterile saline (NaCl; 154 mM) were injected over 3 min, and the needle was left undisturbed for 5 min and then removed. Animals were killed 48 h later, and the brains were excised and sectioned in 1-mm-thick slices in the coronal plane, and then immersed for 30 min at 37°C in a solution containing 2,3,5-triphenyl-2H-tetrazolium chloride (2% in NaCl; 154 mM). Brain infarction was visualized as areas of unstained (white) tissue which were easily contrasted with viable tissue (stained red). Slices were placed in buffered 10% formalin and infarct size was quantitatively assessed by planimetric analysis. In separate studies, histopathological analysis of brain sections verified the location of the injectate and the correlation of necrosis revealed by TTC staining. Groups of three or four animals were used for each of the experimental conditions as noted.

Tissue Culture. The glial (HTB14; reference 45) and neuronal (HTB11; reference 46) cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and cultured in DMEM (GIBCO BRL) containing fetal bovine serum (10%; Hyclone, Logan, UT), sodium pyruvate (1 mM; Sigma Chemical Co.), penicillin and streptomycin (0.5%; Sigma Chemical Co.) in a humidified atmosphere (5% CO₂; 37°C). For all experiments involving exposure to 3-aminopropanal, cells were grown in 96-well microtiter plates to 90–95% confluence and medium was replaced with fresh serum-free medium (Opti-MEM I; GIBCO BRL) to prevent nonspecific interaction of 3-aminopropanal with serum proteins. For all experiments using a short duration of 3-aminopropanal exposure (5 min to 2 h in 96-well plates), the cells were washed at the times indicated, and then incubated in Opti-MEM I for up to 20 h. Where indicated, cells were pretreated with the caspase 1 inhibitor II Ac-YVAD-CMK (BACHEM, Torrance, CA) or the caspase-3 inhibitor Ac-DEVD-CHO (Peptides International, Louisville, KY) in DMSO for 3 h followed by addition of 3-aminopropanal for an additional 5 h. DMSO vehicle controls were performed concurrently. Cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma Chemical Co.) as previously described (47). Data are expressed as mean ± SE; *n* = 3–6 wells per condition; experiments were performed in triplicate.

TUNEL Staining by FACS®. Cells were treated with 3-aminopropanal as indicated and then harvested by centrifugation (1,500 rpm for 5 min). The pellets were fixed with 1× ORTHO Permeafix (Orthodiagnostics, Raritan, NJ) at room temperature for 40 min. After washing with Dulbecco's PBS containing 1% BSA (PBS-BSA), cells were stained by the TUNEL (Tdt-mediated dUTP-biotin nick-end labeling) method using the ApoptTag Direct Fluorescein kit (Oncor, Gaithersburg, MD). Negative controls were performed using a reaction mixture devoid of TdT. A FACScan® (Becton Dickinson, Sunnyvale, CA) was used for all analyses; 5,000–10,000 events (ungated) were collected using single color histogram for FITC.

Annexin V/Propidium Iodide Staining. Annexin V/propidium iodide (PI) staining was performed using a commercially available kit (The Apoptosis Detection Kit; R&D Systems, Minneapolis,

MN). Cells were analyzed by flow cytometry within 1 h of completion of staining.

DNA Electrophoresis. HTB11 or HTB14 cells were harvested ($2-3 \times 10^7$ cells) by centrifugation (1,000 rpm for 5 min), resuspended in a reaction buffer containing proteinase K, and incubated overnight at 55°C. RNAase was added to a final concentration of 50 $\mu\text{g}/\text{ml}$, and samples were incubated at 37°C for 1 h. DNA was extracted three times with phenol/chloroform and two times with chloroform and precipitated in 2 vol of 100% cold ethanol and 0.3 M of sodium acetate (pH 5.2). DNA was resuspended in 50 μl of dH_2O , fractionated by 1.5% agarose gel electrophoresis, and stained with SYBR Green I nucleic acid stain (Molecular Probes).

Results

Cerebral Ischemia Enhances Brain Polyamine Oxidase Activity. To determine the role of brain polyamine oxidase activity in cerebral ischemia, Lewis rats were subjected to focal cerebral infarction by microsurgical occlusion of the middle cerebral artery in a standardized model as described previously (39, 40, 48). Brain homogenates were prepared from the anatomic region perfused by the middle cerebral artery, and total polyamine oxidase (PAO) activity was determined using a method described previously (25, 26, 42). Polyamine oxidase activity was significantly higher in homogenates prepared from ischemic hemispheres as compared with normally perfused contralateral hemispheres (PAO activity after ischemia = 15.8 ± 0.9 nmol/h/mg protein; versus PAO activity in sham-operated controls = 7.4 ± 0.5 nmol/h/mg protein; $P < 0.05$; Fig. 1). The increase of

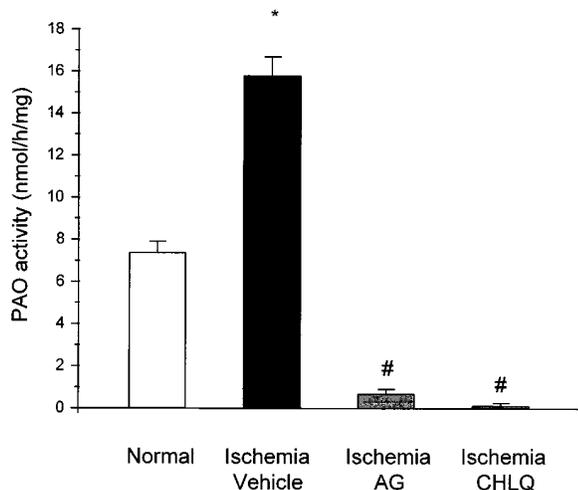


Figure 1. Polyamine oxidase activity increases during cerebral ischemia, and is inhibited by aminoguanidine and chloroquine. Polyamine oxidase activity was measured in brain homogenates prepared as described in Methods. Data shown are mean \pm SE; $n = 3$. *Normal*, sham-operated control brain homogenate; *Ischemia Vehicle*, homogenate prepared 2 h after the onset of middle cerebral artery occlusion; *Ischemia AG*, addition of aminoguanidine (1 mM) at time = -5 min before spermine; *Ischemia CHLQ*, addition of chloroquine (1 mM) at time = -5 min before spermine. *, $P < 0.05$ versus normal; #, $P < 0.05$ versus ischemia vehicle.

brain polyamine oxidase activity was detected within 2 h after the onset of cerebral ischemia. Two structurally distinct inhibitors of polyamine oxidase activity (aminoguanidine and chloroquine) were used to assess specificity (26, 49–51). Addition of either agent to the ischemic brain homogenates dose-dependently inhibited polyamine oxidase activity; chloroquine, $\text{IC}_{50} = 40 \mu\text{M}$; aminoguanidine, $\text{IC}_{50} = 400 \mu\text{M}$. This indicates that within 2 h after the onset of cerebral ischemia there is a specific induction of brain polyamine oxidase activity, and that this activity can be pharmacologically inhibited.

Cerebral Ischemia Enhances 3-Aminopropanal Production. To obtain direct evidence that the cytotoxin 3-aminopropanal is produced during cerebral ischemia, we developed a method to detect brain 3-aminopropanal using HPLC and mass spectroscopy. 3-Aminopropanal was prepared by hydrolysis of the diethyl acetal and then derivatized using 2,4-dinitrophenylhydrazine (Fig. 2 a). HPLC analysis of the derivatized products revealed two peaks (at 24 and 27 min, respectively) in equal ratio (Fig. 2 b, inset). $^1\text{H-NMR}$ spectroscopy revealed the presence of *anti* and *syn* isomers, as predicted by the structures of the principle condensation products (Fig. 2 a). Electrospray ionization mass spectroscopy (EIMS) of the HPLC-purified products detected the expected mass ion m/z 251 (Fig. 2 b). We then subjected rats to permanent focal cerebral ischemia, and derivatized brain homogenates with 2,4-dinitrophenylhydrazine. HPLC analysis of the derivatized brain homogenate revealed the appearance of the two expected peaks, and EIMS confirmed identity as the isomeric 3-aminopropanal-2,4-dinitrophenylhydrazone reaction products (Fig. 2 c). The 3-aminopropanal derivatization products could not be detected in brain homogenates prepared from sham-operated, normally perfused control animals (Fig. 3). 3-Aminopropanal became significantly elevated within 2 h after the onset of ischemia, and increased in a time-dependent manner for at least 25 h after the onset of ischemia (Fig. 3). The HPLC assay we used may well have underestimated the amount of 3-aminopropanal produced in the ischemic brain, because 3-aminopropanal is a reactive molecule that can bind to the amino and sulfhydryl groups of proteins (33, 52), thereby decreasing its availability for derivatization and detection. Nonetheless, after correcting the measured levels for total brain protein (213 g/liter), brain 3-aminopropanal concentrations after ischemia reach a highly cytotoxic range (0.5–2.7 mM). When considered with our previous observation that cerebral ischemia mediates an early induction in the activity of brain polyamine oxidase, these findings indicate that this enzyme pathway continually generates 3-aminopropanal during the first 25 h after the onset of cerebral ischemia.

Production of 3-Aminopropanal Precedes the Development of Significant Brain Cell Death. We next examined whether the enzymatic formation of 3-aminopropanal preceded the onset of ischemic cell death. Accordingly, the volume of dead brain was measured by staining brain sections with the vital dye TTC. For the first 3 h of ischemia, cells in the re-

gion of the occluded middle cerebral artery were observed to be largely viable (total volume of cell death = 2 ± 2 mm³). Histological examination of hematoxylin and eosin-stained brain sections (data not shown) confirmed that cells were morphologically intact and had not yet developed degenerative changes at a time when 3-aminopropanal levels were already significantly increased (Fig. 3). Over the next 25 h, spreading cell death developed in association with increasing 3-aminopropanal levels (infarct volume at 25 h = 71 ± 24 mm³; versus infarct volume at 3 h = 2 ± 2 mm³; $P < 0.05$). These findings give evidence that 3-aminopropanal accumulates during the early response to cerebral ischemia, and precedes the development of progressive, spreading brain cell death.

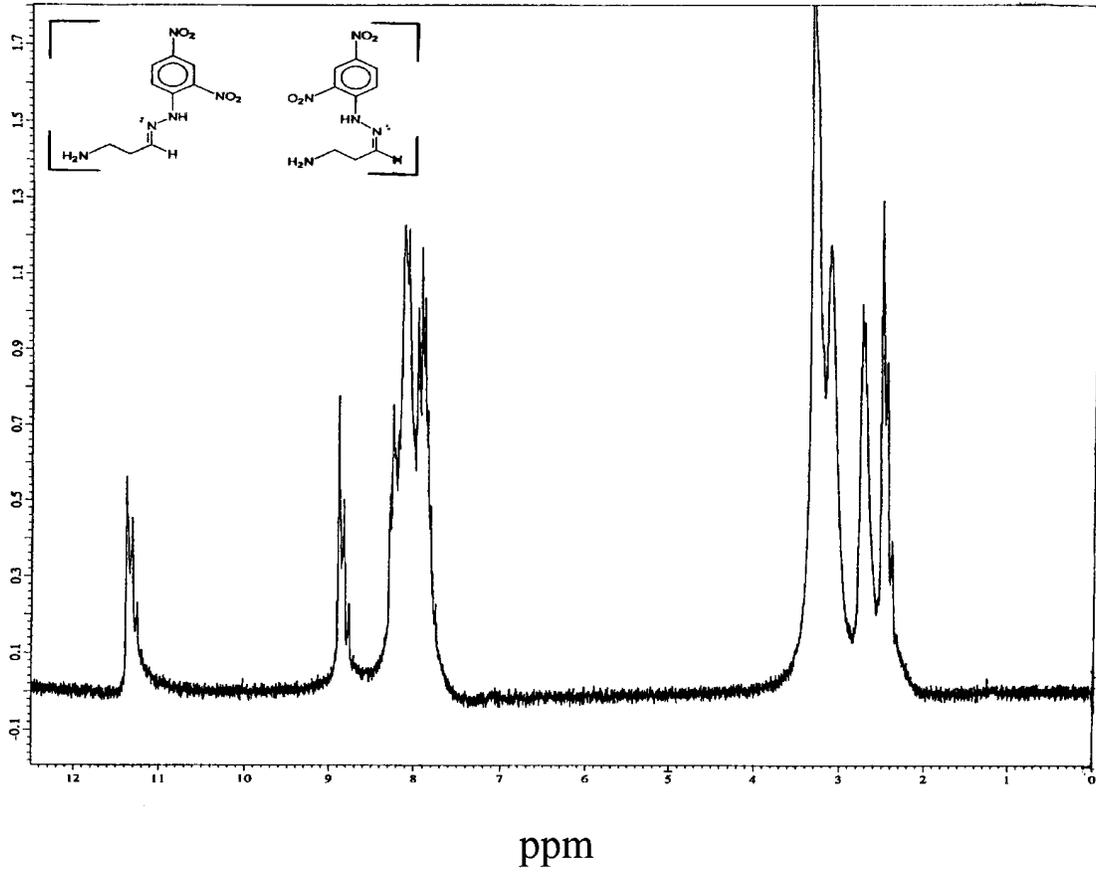
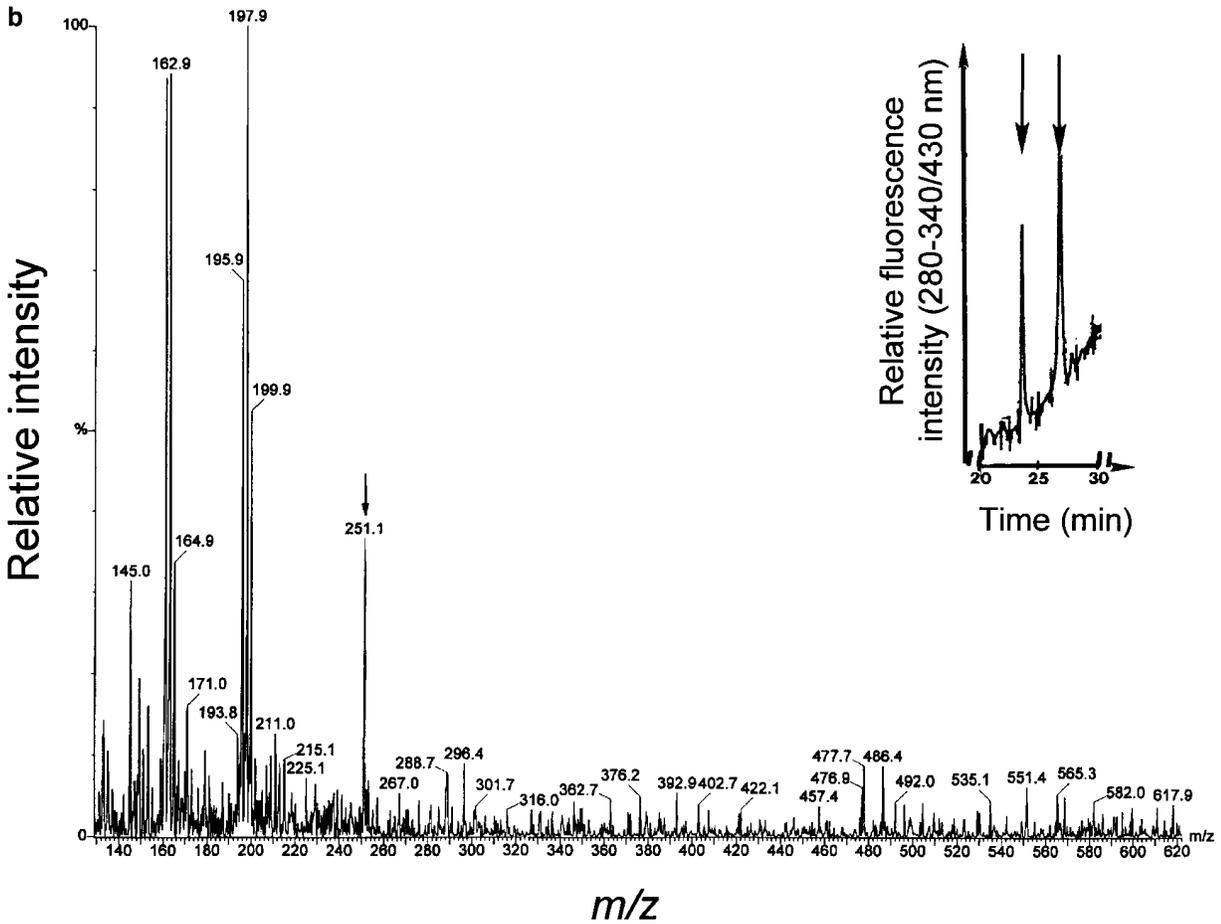
Brain Damage Is Mediated by Intracortical Microinjection of Spermine, Spermidine, or 3-Aminopropanal, but Not by Putrescine. Since polyamine oxidase activity is present in normal mammalian brain (Fig. 1 and references 22, 25), we wished to investigate whether increased extracellular levels of substrate (e.g., spermine or spermidine) would induce local cell death. Accordingly, spermine and spermidine were administered into rat cerebral cortex by direct stereotactic microinjection, and the volume of cell death was measured by TTC staining brain sections. We observed significant cortical cell death after spermine or spermidine administration, but not after that of putrescine, a polyamine that cannot be degraded by polyamine oxidase (Fig. 4 a). Microinjection of 3-aminopropanal mediated significant cell death in the cerebral cortex (Fig. 4 a). The quantity of 3-aminopropanal administered (25 μ g/injection) is similar to the amounts endogenously produced during ischemia (~ 350 μ M assuming a volume of distribution of a typical middle cerebral artery infarction in this model). Systemic administration of the polyamine oxidase inhibitors (chloroquine or aminoguanidine) conferred significant protection against the development of spermine-mediated intracortical damage (Fig. 4 b), suggesting that polyamine oxidase activity is necessary to mediate the cytotoxicity of extracellular spermine. Intracortical administration of aminoguanidine also conferred significant protection against intracortical spermine-mediated cell death (Fig. 4 b), indicating that the cerebroprotective effects of enzyme inhibition occur locally in brain, and not via some unanticipated peripheral drug action. Of note, aminoguanidine failed to significantly attenuate the direct cytotoxicity of intracortical 3-aminopropanal (Fig. 4 b), suggesting that the protective mechanism of aminoguanidine against spermine cytotoxicity is through inhibition of polyamine oxidase activity, and not through direct inhibition of 3-aminopropanal. Thus, increased extracellular levels of spermine, spermidine, or 3-aminopropanal (but not putrescine) are cytotoxic to cerebral cortical cells in vivo.

Intracortical Administration of 3-Aminopropanal Induces Apoptosis and Necrosis. We performed histopathologic examination of brain sections 24 h after intracortical 3-aminopropanal microinjection and observed localized degenerative changes surrounding the injection zone (Fig. 5 A). Cells

within the 3-aminopropanal injection site were necrotic, as evidenced by eosin-positive staining. Moreover, in the same region we also observed cells undergoing programmed cell death, as evidenced by TUNEL-positive staining (Fig. 5, B and C). These changes were not observed in the injection zone when either vehicle or putrescine was administered (data not shown). Direct intracortical administration of spermine also caused cell necrosis and apoptosis, and these findings were significantly inhibited by administration of aminoguanidine (data not shown). Thus, the accumulation of extracellular 3-aminopropanal induces brain cell damage in vivo, which occurs through both necrosis and programmed cell death.

3-Aminopropanal Induces Apoptosis in Glial Cells, but Necrosis in Neurons. To investigate directly the cytotoxic signaling mechanisms of 3-aminopropanal, we exposed cultured human glial (HTB14) and neuronal (HTB11) cell lines to 3-aminopropanal. After 20 h of incubation, the LD₅₀'s for 3-aminopropanal were 160 ± 10 μ M for the glial cell line and 90 ± 20 μ M for the neuronal cell line (HTB11). 3-Aminopropanal was somewhat more cytotoxic in primary rat astroglial cell cultures (LD₅₀ = 80 ± 9 μ M). A time-course study revealed that 3-aminopropanal exposure for as little as 5 min was significantly cytotoxic to neuronal cells, but a longer exposure was required to mediate significant cytotoxicity in glial cells (Table 1). This delayed onset suggested that glial cell death might be dependent upon apoptosis-mediated pathways, and in agreement with this possibility, we observed apoptosis-specific DNA fragmentation after exposure of glial cells to 3-aminopropanal (Fig. 6 a). We obtained additional evidence of apoptosis by flow cytometric detection of DNA strand breaks using the TUNEL method (53). In these experiments, 76% of the glial cells stained TUNEL-positive after 13 h of exposure to 160 μ M of 3-aminopropanal (Fig. 6 b), whereas vehicle-treated control cells were uniformly negative (Fig. 6 c). Multiparameter flow cytometry revealed that glial cells exposed to 3-aminopropanal exhibited a decrease in cellular forward light scatter and an increase in side scatter, in agreement with typical cell shrinkage, chromatin condensation, and nuclear fragmentation. Apoptosis of glial cells was also confirmed by subdiploid staining with propidium iodide and annexin V/PI (data not shown).

In contrast to the results in the glial cell line, 3-aminopropanal did not induce apoptosis in neuronal cell cultures (HTB11) using similar experimental methods. DNA electrophoresis of 3-aminopropanal-treated neurons revealed no evidence of chromosomal DNA degradation (Fig. 6 a). In addition, we observed no increase in TUNEL positivity under these conditions, although a forward/side scatter analysis revealed significant cell death after 3-aminopropanal treatment (55.7%), but not in vehicle-treated controls (9.1%). There was also no evidence of apoptosis as measured with annexin V, a method used to detect loss of cell membrane phospholipid asymmetry that can be associated with apoptosis (Fig. 6, f and g). Apoptosis could be induced in neuronal cells by exposure to camptothecin (15 μ g/ml

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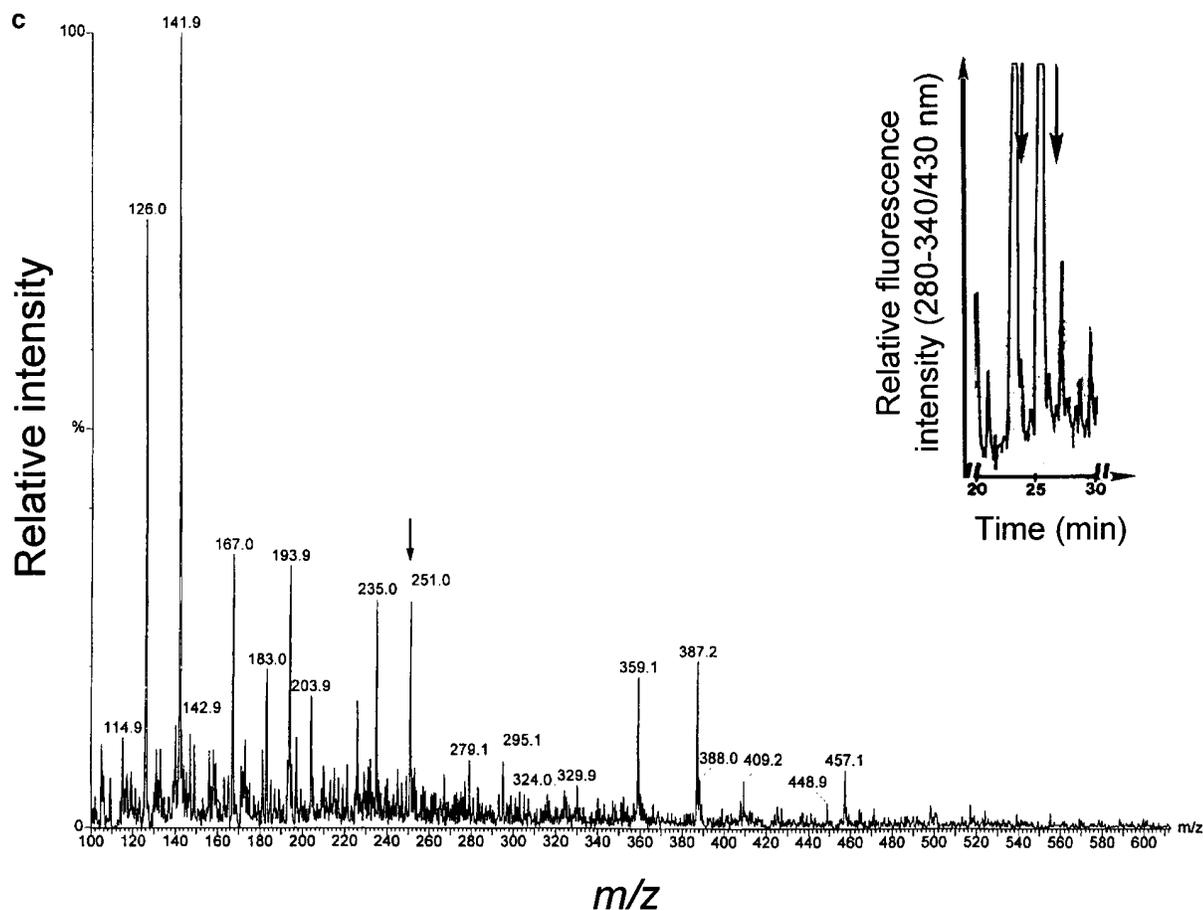


Figure 2. (a) $^1\text{H-NMR}$ spectroscopy ($\text{DMSO-}d_6$ and CDCl_3 , 270 MHz) of the products of reacting 3-aminopropanal with 2,4-dinitrophenylhydrazine. NMR revealed the presence of *anti* and *syn* isomers with resonance at $\delta 8.83$ and $\delta 11.35$. (Inset) Structure of the principle condensation products. (b) Electrospray ionization mass spectrum of synthetic dansylated 3-aminopropanal-2,4-dinitrophenylhydrazone. 3-aminopropanal was derivatized with 2,4-dinitrophenylhydrazine and dansyl chloride, and the reaction products were subjected to HPLC as outlined in Materials and Methods. The inset shows the HPLC profile of the separable geometric isomers. Note that the EIMS of the HPLC-purified fractions revealed the expected molecular ion at m/z 251. (c) EIMS of derivatized ischemic brain homogenate. Animals were subjected to permanent focal cerebral ischemia and after 25 h, brain tissue was obtained for homogenization and derivatization as described in Materials and Methods. The inset shows the HPLC profile, and the mass spectrum confirms the expected molecular ion of the HPLC-purified fractions at m/z 251.

for 20 h; reference 54) as assessed by TUNEL and Annexin V methods (data not shown), indicating that the absence of apoptosis after 3-aminopropanal exposure was not due to some unanticipated generalized cellular defect in this neuronal cell line. Thus, in contrast to glial cells, exposure of neurons to 3-aminopropanal causes primarily necrotic cell death.

Inhibition of Caspase 1 Prevents 3-Aminopropanal-induced Apoptosis in Glial Cells. The cysteine proteases caspase 1 and caspase 3 have been implicated in the cellular signaling pathways mediating apoptosis during cerebral ischemia (55–59). To investigate whether these proteases were required for the induction of apoptosis by 3-aminopropanal in glial cells, HTB14 cells were treated for 3 h with a tetrapeptide caspase 1 inhibitor (Ac-YVAD-CMK), or with a caspase 3 inhibitor (Ac-DEVD-CHO), followed by a 5-h treatment with 3-aminopropanal. Treatment with the caspase 1 inhibitor, but not the caspase 3 inhibitor, conferred dose-depen-

dent inhibition of 3-aminopropanal-induced cell death (Fig. 7, a and b). These data give evidence for a specific role of the caspase 1 proteases in the 3-aminopropanal-induced signaling that mediates apoptosis in glial cells.

Administration of Polyamine Oxidase Inhibitors In Vivo Attenuates 3-Aminopropanal Production and Protects against Brain Damage, even after the Onset of Cerebral Ischemia. The mechanism of ischemic brain cell damage proposed here predicts that administration of polyamine oxidase inhibitors during cerebral ischemia in vivo will reduce both the accumulation of 3-aminopropanal and the volume of cerebral infarction. Accordingly, we measured these end points after administering two structurally distinct polyamine oxidase inhibitors to rats in the standardized model of permanent middle cerebral artery occlusion. We reported previously that aminoguanidine administered after the onset of cerebral ischemia (320 mg/kg intraperitoneally 15 min after ischemia) significantly reduces the volume of cerebral dam-

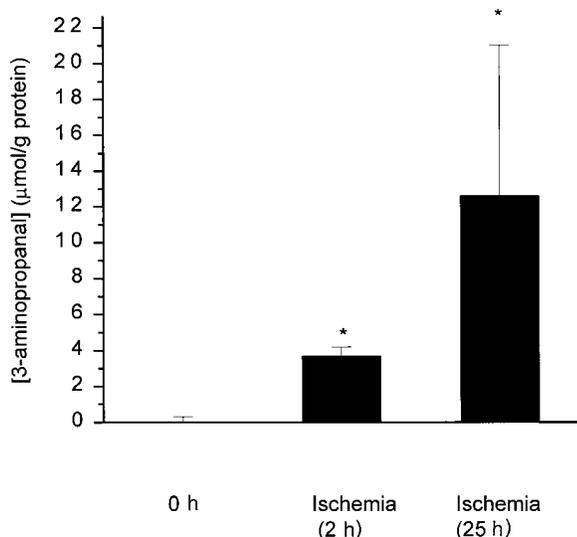


Figure 3. Brain 3-aminopropanal levels increase during cerebral ischemia. Brain 3-aminopropanal levels were measured by HPLC in rats subjected to permanent focal cerebral ischemia. 3-Aminopropanal was not detected in sham-operated controls. Note that 3-aminopropanal tissue levels increased markedly within 2 h after middle cerebral artery occlusion, and continued to increase for at least 25 h. Data shown are mean \pm SE, $n = 3$ animals/group. *, $P < 0.05$ versus $t = 0$ h by analysis of variance.

age (40). In this study we administered aminoguanidine by this established treatment protocol and observed that it effectively prevented the increase of brain 3-aminopropanal levels (Table 2). In agreement with the proposed mechanism of inhibiting polyamine oxidase, the administration of the structurally distinct enzyme inhibitor chloroquine also conferred effective cerebroprotection against ischemic cell damage, even when the administration was delayed 15 min after occlusion of the middle cerebral artery (Table 2). We previously reported that the protective effects of aminoguanidine are not attributable to altering peripheral cardiovascular parameters that influence the volume of brain damage (40). In this study, physiological parameters determined before and during ischemia (blood pressure, heart rate, body temperature, and arterial blood gases) did not differ among groups treated with vehicle or chloroquine (data not shown). Thus, the cerebroprotective effects of chloroquine cannot be attributed to altering the peripheral cardiovascular response to cerebral ischemia.

Previously, Zhang et al. reported that iNOS is upregulated 24–48 h after cerebral ischemia, and that delayed administration of aminoguanidine can prevent secondary NO-mediated brain damage in a delayed therapeutic window (60). Our results here indicate that polyamine oxidase activity is upregulated much earlier after cerebral ischemia (within 2 h), and that early administration of aminoguanidine inhibits the generation of 3-aminopropanal. Although the most direct interpretation of our results is that two structurally distinct inhibitors of polyamine oxidase prevent ischemic damage by preventing the formation of 3-amino-

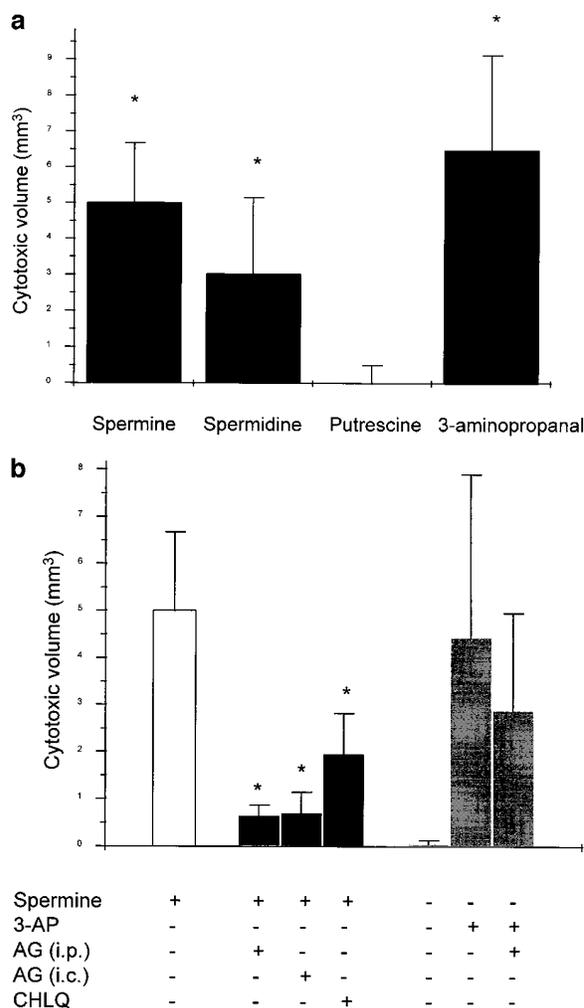


Figure 4. Brain damaging effects of intracortically administered polyamines, and protection with polyamine oxidase inhibitors. (a) Brain damage mediated by intracortical microinjection of spermine, spermidine, and 3-aminopropanal, but not putrescine. Data shown are volume of brain damage (mm^3) as measured by integrating the area of negative TTC staining over the entire brain hemisphere in animals injected with the polyamines shown; mean \pm SE, $n = 6$ –8/group. *, $P < 0.05$ versus vehicle. (b) Aminoguanidine and chloroquine protection against intracortical spermine toxicity. All animals received intracortical spermine (25 μg in 2 μl) by stereotactically guided microinjection. Experimental animals were treated with aminoguanidine or chloroquine simultaneously with the intracortical spermine injectate in the following doses: systemic aminoguanidine was 320 mg/kg intraperitoneal 30-min pretreatment followed by subsequent doses of 110 mg/kg intraperitoneally each 8 h after intracortical spermine; intracortical aminoguanidine was administered by a single dose (320 mg/kg) given simultaneously with intracortical spermine; chloroquine was administered by a single intraperitoneal dose (25 mg/kg) 30 min before the spermine injection. Data shown are infarct volume (mm^3) assessed quantitatively 48 h after the intracortical spermine injection (mean \pm SE, $n = 6$ –8/group). *, $P < 0.05$ versus spermine/vehicle.

propanal, we nonetheless performed a series of additional experiments to exclude other possibilities.

First, we wished to exclude the unlikely possibility that chloroquine protection occurred through an unanticipated inhibition of iNOS. Addition of even suprapharmacological amounts of chloroquine (1 mM) failed to inhibit iNOS

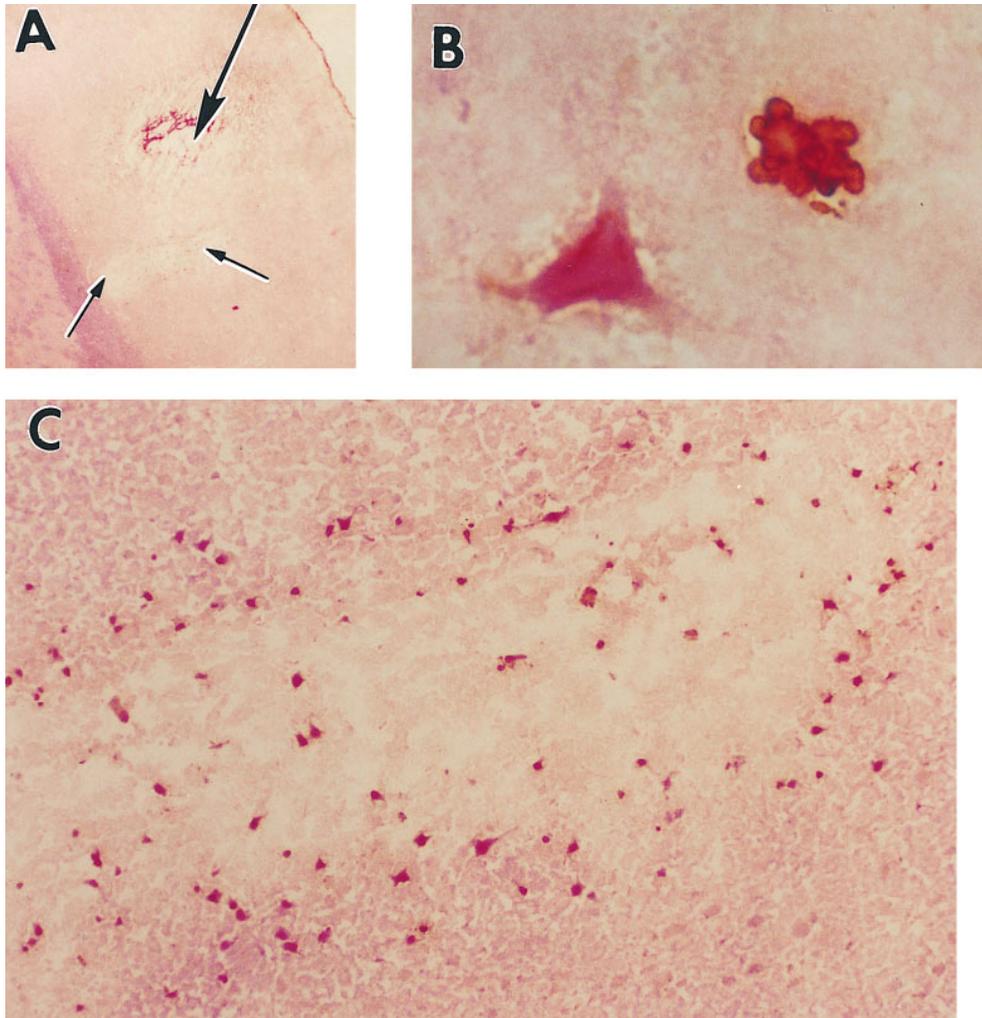


Figure 5. Histology of 3-aminopropanal-induced cell death. Animals were anesthetized and perfused intracardially with 100 ml saline followed by 200–300 ml of 4% formaldehyde in PBS. Brains were removed, postfixed for 2 hr in the same fixative, and then incubated overnight in 30% sucrose at 4°C. Ten micrometer sections were prepared and air-dried. TUNEL staining was performed as outlined in Methods. (A) Low magnification microphotograph demonstrating the site of microinjecting needle (*large arrow*) and areas of 3-aminopropanal-induced prominent cell death (*small arrows*). (B) High power microphotograph taken within the marked area showing coexistence of apoptosis (TUNEL-positive cell on the right) and necrosis (eosin-stained cell on the left). (C) High magnification of area indicated in A showing the extent of cellular degeneration.

activity measured in murine macrophage-like RAW264.7 cell lysates (control iNOS activity = $13,254 \pm 250$ DPM/ μ g protein; versus chloroquine iNOS activity = $11,755 \pm 883$ DPM/ μ g protein; $P > 0.05$). We also wished to exclude the unlikely possibility that aminoguanidine or chloroquine might protect cells by directly inactivating the cytotoxicity of 3-aminopropanal. Cell cytotoxicity was measured in the presence of inhibitors, and we observed that the LD₅₀ for 3-aminopropanal after overnight incubation in HTB11 cells was similar whether or not aminoguanidine or chloroquine were added (data not shown). We also wished to exclude the unlikely possibility that the mechanism of aminoguanidine protection is not mediated via altering the sensitivity of cells to the cytotoxicity of glutamate. When aminoguanidine was added to primary neuronal cultures treated with NMDA we observed no significant attenuation of cytotoxicity (Table 3). We also addressed whether 3-aminopropanal mediates cell death through induction of iNOS activity. Addition of iNOS inhibitors (L-NMMA or aminoguanidine) to 3-aminopropanal-treated glial cells failed to attenuate the development of

TUNEL positivity as measured by FACS® (data not shown).

Although we have excluded a number of plausible alternative mechanisms through which aminoguanidine might protect against cerebral ischemia, it remains theoretically possible that other nonspecific activities of chloroquine might additionally contribute to the observed protection against infarction (i.e., inhibition of free radical formation, phospholipase activity, or protein synthesis). However, these mechanisms are not supported by our direct observations that (a) inhibiting polyamine oxidase activity reduces the formation of cytotoxic concentrations of 3-aminopropanal; (b) 3-aminopropanal cytotoxicity cannot be blocked with aminoguanidine or chloroquine; and (c) either chloroquine or aminoguanidine prevent the brain damaging effects of either intracortical spermine or ischemia.

Discussion

Four closely related lines of evidence therefore support the role of 3-aminopropanal as a cytotoxic mediator of

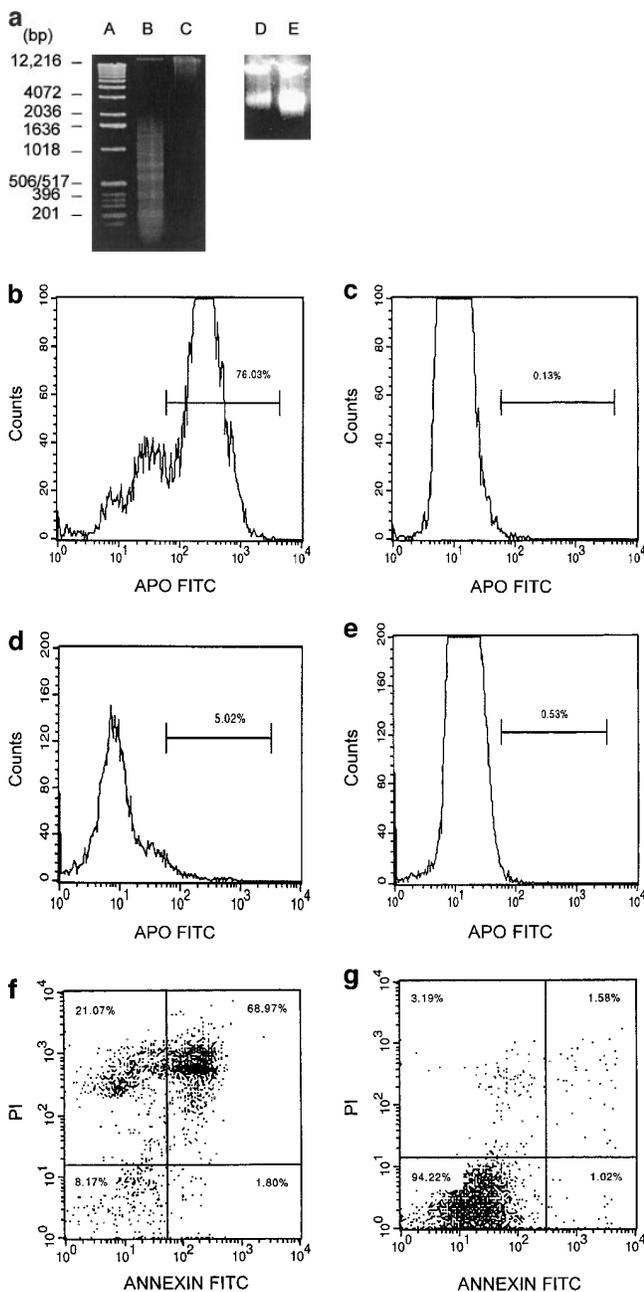


Figure 6. Apoptosis in glial cells exposed to 3-aminopropanol. (a) DNA gel electrophoresis of glial-like and neuronal-like cells exposed to 3-aminopropanol for 13 h as described in Materials and Methods; 7.5 μg of DNA was loaded per lane for the glial cells (lanes B and C), and 15 μg was loaded per lane for the neuronal cells (lanes D and E). Lane A, 1 kb DNA size marker; lane B, 3-aminopropanol-treated (160 μM); lane C, vehicle control; lane D, vehicle-treated; lane E, 3-aminopropanol-treated (90 μM). (b) FACS[®] histogram showing TUNEL staining of 3-aminopropanol-treated glial cells (160 μM , 13 h) as outlined in Materials and Methods. As indicated by the marker, 76% of the cell population stained TUNEL positive. (c) FACS[®] histogram of vehicle-treated glial cells revealed no evidence of apoptosis (0.13% TUNEL-positive cells). (d) FACS[®] histogram showing TUNEL staining of 3-aminopropanol-treated neuronal cells (90 μM , 13 h) as outlined in Materials and Methods. As indicated by the marker, only 5.02% of the cell population of stained TUNEL positive, though a forward/side scatter of the same cell population revealed 55.7% cell death upon electronic gating (data not shown).

Table 1. Time Course Study of Cell Viability after Exposure to 3-Aminopropanol

Time (min)	Cell viability (% alive)*	
	Glial cells (HTB14)	Neurons (HTB11)
5	96 \pm 3	29 \pm 6
60	92 \pm 1	13 \pm 4
120	78 \pm 7	6 \pm 2
1,200	5 \pm 5	3 \pm 1

Glial or neuronal cells were exposed to an LD₁₀₀ concentration of 3-aminopropanol (750 and 350 μM , respectively) for the times indicated, and then the media was replaced by fresh OPTI-MEM for a total incubation time of 20 h. Cell viability was then determined by MTT assay (data shown are mean \pm SE).

*Cell viability was 100 \pm 4% for all times in vehicle-treated controls.

brain damage in cerebral ischemia. First, cerebral ischemia mediates an early induction of polyamine oxidase activity. Second, the cytotoxic enzyme product 3-aminopropanol accumulates during the early response to cerebral ischemia, but is not produced in normally perfused controls. Third, 3-aminopropanol production in the ischemic brain increases before the onset of significant cellular degeneration, with tissue 3-aminopropanol levels rising further during the period of progressive cell death. Fourth, 3-aminopropanol is a potent cytotoxin that activates apoptosis via a caspase 1-dependent mechanism in glial cells and necrosis in neurons. Considered together, these data offer an explanation for the correlation between brain levels of putrescine, a stable end-product of terminal polyamine oxidation, and infarct volume (5, 18, 61), since catabolism of spermine and spermidine by polyamine oxidase produces both a stable, nontoxic end product (putrescine) and a potent cytotoxin (3-aminopropanol). The latter product mediates cell death, and the former accumulates in correlation to the extent of damage.

Previous observations suggest that polyamines can prevent apoptosis in neuronal cultures (62, 63), or can amplify glutamate-mediated cell cytotoxicity (14). Cell survival in the ischemic zone is likely to be critically dependent upon the balance between the direct effects of polyamines and the cytotoxic effects of 3-aminopropanol. We have yet to address the contribution to brain damage of an alternative

(e) FACS[®] histogram of vehicle-treated neuronal cells showed no evidence of apoptosis (0.53% TUNEL-positive cells). Electronic gating for forward/side scatter showed 9.1% cell death under these conditions. (f) Annexin V FITC/propidium iodide (PI) staining of 3-aminopropanol-treated neuronal cells, as above, revealed no evidence of apoptosis (1.8% apoptotic cells). Necrotic cells comprised 68.97% of the cell population. (g) Annexin V FITC/propidium iodide staining of vehicle-treated neuronal cells revealed no evidence of apoptosis (1.02% of the cell population) or necrosis (1.58% of the cell population).

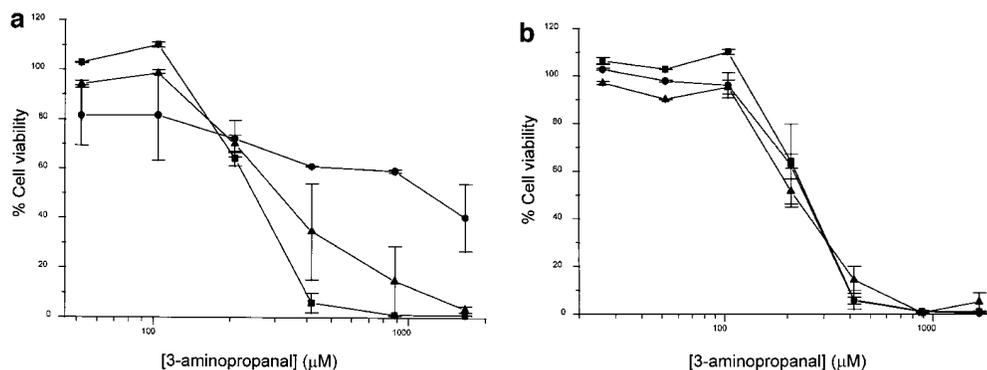


Figure 7. Inhibition of caspase 1 but not of caspase 3 blocks 3-aminopropanal-induced glial apoptosis. Cells were pretreated with (a) the caspase 1 inhibitor (Ac-YVAD-CMK) or (b) the caspase 3 inhibitor (Ac-DEVD-CHO) at concentrations 0.4 (*triangles*) or 40 μM (*circles*) for 3 h, followed by treatment with 3-aminopropanal for an additional 5 h, and then were analyzed for cell viability by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Controls consisted of DMSO-treated cells (*squares*) to assess for nonspecific solvent effects. Data are mean \pm SE, $n = 3$ wells/experiment.

pathway of polyamine catabolism, the acetylation pathway, but it is reasonable to speculate that this pathway could provide an additional source of potentially toxic aldehyde products, e.g., 3-acetamidopropanal (64, 65). There has been some controversy as to whether both 3-aminopropanal and 3-acetamidopropanal can produce acrolein *in vivo*, a known mediator of cytotoxicity and apoptosis (66, 67). It is likely that several products of polyamine oxidation further augment the cytotoxicity of 3-aminopropanal. When considered together, these observations add credence to the previously uninvestigated hypothesis that enhanced polyamine oxidation during cerebral ischemia is deleterious.

Our results now suggest the following mechanism of brain cell death during cerebral ischemia: dead and dying cells in the densely hypoxic core release stores of intracellular spermine and

spermidine, which are catabolized by polyamine oxidase. The resultant production of 3-aminopropanal causes apoptosis in surrounding glial cells, and necrosis of neurons, which in turn release more spermine and spermidine as substrate for polyamine oxidase. This cytotoxic mechanism spreads to involve a larger volume of potentially viable cells surrounding the ischemic core. It is likely that 3-aminopropanal is positioned proximally in the mediator cascade elicited by cerebral ischemia, which includes the excitatory amino acids, activated oxygen species, nitric oxide, TNF, IL-1, IL-6, and platelet-activating factor (48, 60, 68–75). It is interesting to reconsider previous observations that expression of dominant negative mutants of IL-1 β converting enzyme (caspase 1) protects against the development of apoptosis during cerebral ischemia (58, 76). Based on our results, it is now plausible that inhibition of caspase 1 activity prevents the damaging effects of 3-aminopropanal. Further,

Table 2. Structurally Distinct Inhibitors of Polyamine Oxidase Attenuate Cerebral Infarction

	Infarct volume	Brain 3-aminopropanal level
	mm^3	$\mu\text{mol/g protein}$
Vehicle	71 \pm 24	13 \pm 8
Aminoguanidine	12 \pm 2* (reference 40)	ND
Chloroquine	27 \pm 8*	—

Rats were subjected to permanent middle cerebral artery occlusion ($n = 4/\text{group}$), and infarct volume was measured 25 h after the onset of ischemia as described in the legend to Fig. 2a. Treated animals received aminoguanidine (320 mg/kg intraperitoneally 3 h before ischemia, followed by 110 mg/kg intraperitoneally every 8 h for three doses), chloroquine (25 mg/kg intraperitoneally 15 min after ischemia), or vehicle (saline, intraperitoneally, given 15 min after ischemia). The limit of detection for brain 3-aminopropanal is 1.0 $\mu\text{mol/g protein}$. Data shown are mean \pm SE.

* $P < 0.05$ versus vehicle.

Table 3. Aminoguanidine Is Not Protective against NMDA Neurotoxicity

[Aminoguanidine] (μM)	NMDA (500 μM)	MK-801 (20 μM)	Neurotoxicity (% dead)
0	—	—	3 \pm 1
0	+	—	88 \pm 4
0	+	+	8 \pm 3
1,000	+	—	79 \pm 4
1,000	—	—	7 \pm 2

Cultured hippocampal neurons (12–15 d *in vitro*) were exposed to NMDA (500 μM) for 5 min in MEM without serum supplemented with glutamine (100 μM) and glycine (10 μM), rinsed in Earle's Basic Salt Solution, and incubated for 24 h (37°C). Neuronal survival was assessed by counting somas containing or excluding trypan blue. Aminoguanidine or MK-801 were added just before NMDA and in the final rinse in the concentrations shown. Data are mean \pm SE of nine wells from three replicate experiments.

we previously reported that TNF synthesis is upregulated during the first 12 h of brain ischemia, and that TNF participates in the mediation of brain damage (48). It will now be interesting to explore further the influence of decreasing brain spermine levels after ischemia, because spermine is a direct inhibitor of TNF synthesis in human peripheral blood mononuclear cells (77). We have recently found that centrally administered 3-aminopropanol directly stimulates

intracerebral TNF synthesis (data not shown), indicating that 3-aminopropanol may stimulate this component of the ischemic cytotoxic cascade.

Polyamines and polyamine oxidase are ubiquitous in mammalian tissues (78). This poses the intriguing possibility that the mechanism of polyamine oxidative tissue damage proposed here might be invoked in other ischemic conditions, like myocardial infarction and tumor necrosis.

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