Diadenosine Tetraphosphate Protects against Injuries Induced by Ischemia and 6-Hydroxydopamine in Rat Brain

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Diadenosine tetraphosphate (AP₄A), an endogenous diadenosine polyphosphate, reduces ischemic injury in the heart. In this study, we report the potent and protective effects of AP₄A in rodent models of stroke and Parkinson's disease. AP₄A, given intracerebroventricularly before middle cerebral artery (MCA) ligation, reduced cerebral infarction size and enhanced locomotor activity in adult rats. The intravenous administration of AP₄A also induced protection when given early after MCA ligation. AP₄A suppressed terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) induced by hypoxia/reperfusion in primary cortical cultures, and reduced both ischemia-induced translocation of mitochondrial cytochrome *c* and the increase in cytoplasmic caspase-3 activity *in vivo*. The purinergic P₂/P₄ antagonist di-inosine pentaphosphate or P₁-receptor antagonist sulfonylphenyl theophylline, but not the P₂-receptor antagonist suramin, antagonized the effect of AP₄A, suggesting that the observed protection is mediated through an antiapoptotic mechanism and the activation of P₁- and P₄-purinergic receptors.

AP₄A also afforded protection from toxicity induced by unilateral medial forebrain bundle injection of 6-hydroxydopamine (6-OHDA). One month after lesioning, vehicle-treated rats exhibited amphetamine-induced rotation. Minimal tyrosine hydroxylase immunoreactivity was detected in the lesioned nigra or striatum. No KCl-induced dopamine release was found in the lesioned striatum. All of these indices of dopaminergic degeneration were attenuated by pretreatment with AP₄A. In addition, AP₄A reduced TUNEL in the lesioned nigra 2 d after 6-OHDA administration. Collectively, our data suggest that AP₄A is protective against neuronal injuries induced by ischemia or 6-OHDA through the inhibition of apoptosis. We propose that AP₄A may be a potentially useful target molecule in the therapy of stroke and Parkinson's disease.

Key words: ischemia; stroke; Parkinson's disease; repair; protection; diadenosine tetraphosphate; apoptosis

Introduction

Diadenosine polyphosphates (AP_{*n*}As) are a group of compounds that contain two adenosine moieties bridged by three to six phosphates. Diadenosine tetraphosphate (AP₄A), a major representative of AP_{*n*}As, has been found to be highly concentrated in tears (Pintor et al., 2002), and it may be involved in extracellular and intracellular signaling in the brain (Oaknin et al., 2001; Emanuelli et al., 1998; Kisselev et al., 1998). AP₄A is stored in secretory granules with ATP and can be released after stimulation in a Ca²⁺-dependent manner (Pivorum and Nordone, 1996) and during insults (Pintor et al., 1995) or oxidative stress (Bochner et al., 1984). AP₄A interacts with purinergic P₁- (Klishin et al., 1994) and P₂- (Lazarowski et al., 1995) receptors. Recent studies have indicated that high-affinity binding sites for AP₄A (Pintor et al., 1993), as differentiated from P₁- or P₂-receptors, are found in the olfactory bulb, cerebral cortex, and striatum, as well as several other brain areas (Rodriguez-Pascual et al., 1997). These recep-

tors have been identified as purinergic P₄-receptors (Rodriguez-Pascual et al., 1997). In the synaptic terminals of rat midbrain and cerebellum, the interaction of AP_{*n*}As and P₄ receptors cannot be cross-desensitized by ATP (Pintor and Miras-Portugal, 1995). Taken together, these data suggest that AP₄A may use unique signal transduction pathways and may activate physiological responses different from those caused by ATP or adenosine.

Previous studies have indicated that AP₄A reduces ischemic injury in the heart (Ahmet et al., 2000; Khattab et al., 1998). The role of AP₄A in the CNS is not clear. AP₄A can be released from the striatum after the systemic administration of amphetamine (Pintor et al., 1995). ϵ -AP₄A, an AP₄A analog, has been found to decrease extracellular glutamate levels in the striatum (Oaknin et al., 2001). We now report that AP₄A has protective effects in the cortex and midbrain in defined rat models of stroke and Parkinson's disease, which have predictive value for therapeutic development.

Materials and Methods

In vitro primary neuronal cultures, hypoxia, and cell death. Cells were isolated at embryonic day 15 from timed-pregnant rats. Cells were plated in Neurobasal media containing B₂₇ supplement, 200 mM L-glutamine, and 25 μ M L-glutamate for 4 d. Cells were treated with AP₄A (Sigma-Aldrich Chemical, St. Louis, MO) or vehicle and incubated for 18 hr in a

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Figure 1. Neuroprotective effects of AP₄A against hypoxia/reoxygenation *in vitro*. Exposure to AP₄A (100 nM) significantly reduced the hypoxia/reoxygenation-induced release of LDH (A), increase in caspase-3 enzymatic activity (B), and increase in TUNEL labeling in primary cortical cultures (C). * $p < 0.05$, one-way ANOVA. Photomicrographs demonstrate that hypoxia/reoxygenation increased TUNEL in primary cortical cells (D) and pretreatment with AP₄A reduced the density of TUNEL (E). Scale bars, 20 μm.

hypoxic incubator (3% O₂, 5% CO₂; Thermo Forma). After hypoxia, cells were returned to normoxia conditions (21% O₂, 5% CO₂) for 24 hr. Media were collected and assayed for lactate dehydrogenase (LDH) activity (Cytotoxicity Detection Kit; Roche Products, Penzberg, Germany). Cultures were assayed for DNA fragmentation using a terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL)-based method (In Situ Cell Death Detection Kit; Roche) or activated caspase-3 activity (see below) using the ApoAlert kit (Clontech, Palo Alto, CA).

Animals and drug administration for *in vivo* studies. Adult male Sprague Dawley rats were used for this study. For intraventricular administration of AP₄A, animals were anesthetized with chloral hydrate (0.4 gm/kg, i.p.). AP₄A (3 or 30 nmol/20 μl) was administered through a Hamilton syringe over 10 min intracerebroventricularly. The coordinates were 0.8 mm posterior to the bregma, 1.5 mm lateral to the midline, and 3.5 mm below the dura surface. The speed of injection was controlled by a syringe pump at a rate of 2.5 μl/min. A systemic injection of AP₄A (10 mg/kg) or vehicle (saline, 1 ml) was given through the tail vein at 5–10 min after removal of the ligature on the right MCA (see below).

Brain ischemia/reperfusion. Anesthetized rats were subjected to cerebral ischemia. The ligation of the right middle cerebral artery (MCA) and bilateral common carotids (CCAs) was performed using methods described previously (Chen et al., 1986; Wang et al., 2001a). The right MCA was ligated with 10–0 suture. The ligature was removed after 60 min of ischemia to allow reperfusion. Body temperature was monitored with a thermistor probe and maintained at 37°C throughout the period of surgery and recovery.

Cerebral blood flow in stroke animals. Cortical blood flow was continuously measured using a laser Doppler flowmeter (PF-5010, Periflux system, SE) in anesthetized animals (Wang et al., 2001b). A Doppler probe (0.45 mm in diameter) was stereotactically placed in the right frontoparietal cortex (1.3 mm posterior, 2.8 mm lateral to the bregma and 1.0 mm below the dura).

Behavioral measurements in stroke animals.

Animals were placed in an Accuscan activity monitor (Columbus, OH) 2 d after ischemia for behavioral recording (Wang et al., 2001a). The monitor contained 16 horizontal and 8 vertical infrared sensors spaced 2.5 cm apart. The vertical sensors were situated 10.5 cm from the floor of the chamber. Motor activities, such as horizontal activity (total number of beam interruptions that occurred in the horizontal sensors), total distance traveled, number of horizontal or vertical movements, movement and rest time, were calculated by the number of beams broken by the animals from 0 to 30 min after being placed in the chamber.

Triphenyltetrazolium chloride staining in stroke animals. Two days after stroke, some animals were killed for triphenyltetrazolium chloride (TTC) staining (Wang et al., 2001a). The brain tissue was then removed and sliced into 2.0 mm thick sections. The brain slices were incubated in a 2% TTC solution. The area of infarction on each brain slice was measured double blind using a digital scanner. The total infarction volume in each animal was obtained from the product of average slice thickness (2 mm) and the sum of the area of infarction in all brain slices.

Cytochrome *c* in stroke animals. Brain tissues from stroke rats were harvested at 8 hr after the onset of reperfusion for Western blot analysis of cytochrome *c* (Tsao and Su, 2001). The fourth 2 mm coronal section (6–8 mm from rostral end) from each brain was dissected and homogenized in lysis buffer. We have demonstrated previously that this section contains the largest

area of infarction (Chang et al., 2002). The brain-tissue homogenates were centrifuged at 800 × *g* for 20 min at 4°C. The resulting supernatant was additionally centrifuged at 10,000 × *g* for 20 min at 4°C to obtain the heavy membrane pellet enriched for mitochondria. The supernatants were centrifuged again at 100,000 × *g* for 60 min at 4°C. The resulting supernatant was used as the soluble cytosolic fraction. The mitochondrial pellet was dissolved in lysis buffer and centrifuged at 10,000 × *g* for 20 min at 4°C to make soluble protein. Fifty micrograms of soluble protein samples from subcellular fractionations were separated by electrophoresis using 10–12% SDS-PAGE gel. Gels were electrotransferred onto nitrocellulose membranes. The membranes were blocked for 1 hr in TBS/0.05% Tween 20 containing 5% nonfat dried milk and then probed with monoclonal mouse anti-cytochrome *c* antibody (BD PharMingen, San Diego, CA) at a 1:500 dilution at 4°C overnight. The amounts of mitochondria were normalized with a mitochondrial marker (voltage-dependent anion channel, VDAC). Immunoblot analysis was performed by probing with horseradish peroxidase (HRP)-labeled secondary antibodies, developing with enhanced chemiluminescence (ECL) Western blotting detection reagents, and exposing to Hyperfilm-ECL (Amersham Biosciences, Arlington Heights, IL).

Caspase-3 enzymatic activity in stroke animals. Activated caspase-3 enzyme activity was measured using the ApoAlert kit (Clontech) (Wang et al., 2001a). Eight hours after the onset of reperfusion, animals were killed and the brain tissue was removed. The fourth 2 mm coronal section from each brain was dissected and homogenized in lysis buffer for the same rationale noted previously. Caspase-3 activity was determined fluorometrically by the formation of 7-amino-4-trifluoromethyl coumarin (AFC) from Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (DEVD-AFC). The selective caspase-3 inhibitor DEVD-aldehyde (DEVD-CHO) was included, at a concentration of 10 μM, to ensure that the enzymatic reaction was specific.

6-OHDA lesioning and amphetamine-induced rotation. Rats were anes-

thetized with chloral hydrate (0.4 gm/kg, i.p.) and were injected with 6-OHDA (9 μ g/4 μ l in normal saline containing 0.2 mg/ml ascorbic acid) over 4 min, into the left medial forebrain bundle [−4.4 mm anteroposterior (AP), 1.2 mm mediolateral relative to the bregma, and 7.8 mm below the dura]. One month after lesioning, the unilaterally 6-OHDA-lesioned rats were tested for rotational behavior in response to subcutaneous amphetamine injections (5 mg/kg) in an automated rotometer (Chiang et al., 2001).

TUNEL and tyrosine hydroxylase immunostaining in 6-OHDA-lesioned animals. The 6-OHDA-lesioned animals were killed at 48 hr for TUNEL (Zuch et al., 2000). Some animals treated with 6-OHDA were killed 1 month after 6-OHDA lesioning for tyrosine hydroxylase (TH) immunostaining (Chiang et al., 2001), respectively. The TUNEL assay was conducted according to the manufacturer's recommendations (In Situ Cell Death Detection Kit, Roche). Brains sections (30 μ m thick) were immunolabeled using an anti-tyrosine hydroxylase (TH) monoclonal, primary antibody (Boehringer Mannheim, Indianapolis, IN) and either a fluorescent Alexa 568 secondary antibody (Molecular Probes) or an HRP-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (Molecular Probes).

In vivo electrochemistry in 6-OHDA-lesioned animals. Six months after lesioning, some 6-OHDA-lesioned animals were anesthetized with urethane (1.25 gm/kg, i.p.). *In vivo* chronoamperometric measurements of extracellular dopamine (DA) concentration were performed as described previously (Zhou et al., 1996). The recordings were taken at rates of 10 Hz continuously using Nafion-coated carbon-fiber working electrodes (SF1A: Quanteon, Nicholasville, KY). The release of DA was measured by changes of extracellular DA concentration after the microinjection of KCl into the striatal parenchyma. KCl (70 mM) was applied locally through micropipettes. The working electrode and the micropipette were mounted together with sticky wax; tips were separated by 150 μ m. The electrode/pipette assembly was lowered into striatum (AP 1.0 mm, 2.5 mm lateral relative to the bregma and 4.0–7.0 mm below the dura). Local application of KCl from the micropipettes was performed by pressure ejection using a pneumatic pump. The ejected volume was monitored by recording the change in the fluid meniscus in the pipette before and after ejection using a dissection microscope.

Blood pressure, heart rate, electrolytes, and blood CO₂ measurements. Physiological parameters were measured in 15 rats as described previously (Wang et al., 2001b). Animals were anesthetized with chloral hydrate, and a polyethylene catheter was inserted into the right femoral artery. Mean arterial pressure was recorded through a strain gauge transducer and recorded on a strip chart recorder. Arterial blood (<1 ml) was withdrawn from the artery 30 min after the intracerebroventricular administration of AP₄A (30 nmol) or vehicle. Blood gas, serum electrolytes and glucose were analyzed using standard methods.

Statistical analysis. Statistical analyses were performed with the *t* test, one- or two-way ANOVA, and the *post hoc* Newman–Keuls test. Significance was inferred at *p* < 0.05. Data are presented as means $\pm$ SEM.

Figure 2. Pretreatment with AP₄A reduced ischemia-induced infarction and bradykinesia. *A*, TTC staining indicates marked infarction (white areas) in the right cerebral cortex in a rat pretreated intracerebroventricularly with vehicle. Pretreatment with AP₄A (30 nmol, i.c.v.) reduced the infarction area. *B*, AP₄A dose-dependently reduced the volume of infarction (*B*) and the area of the largest infarction in a slice in 22 animals (*C*) (**p* < 0.05). *D*, AP₄A significantly increased horizontal activity (hact), total distance traveled (totdist), number of horizontal (movno) or vertical (vmovno) movements, movement time (movtime), while reducing immobilized time (restime), in stroke animals 2 d after stroke (*p* < 0.05, *t* test). In this and the following figures, the volume of infarction and each locomotor activity from the AP₄A group were normalized by comparison with the mean of those indices in stroke animals receiving vehicle.

RESULTS

Ischemia

AP₄A-induced protection against hypoxia/re-oxygenation *in vitro* Primary neuronal cultures were treated with vehicle or AP₄A (100 nM) followed by either hypoxia (3% O₂ for 18 hr)/re-oxygenation (21% O₂ for 24 hr) or normoxia (21% O₂ for 42 hr). AP₄A itself did not alter LDH production or specific caspase-3 activity in a normoxic environment (*p* > 0.05, *t* test). Nonspecific caspase-3 activity, defined as the activity found in the presence of the caspase-3 inhibitor DEVD-CHO (inhibitor) in the assay medium, was not detectable. Hypoxia/re-oxygenation enhanced LDH production (Fig. 1*A*) and caspase-3 activity (Fig. 1*B*) and elicited an increase in TUNEL (Fig. 1*C,D*). All of these responses

Figure 3. Systemic post-stroke treatment with AP₄A reduced ischemia-induced infarction and bradykinesia. *A*, TTC staining indicated that the intravenous administration of AP₄A (10 mg/kg), 5–10 min after the onset of reperfusion, reduced cerebral infarction. The volume of infarction (*B*) and the area of the largest infarction (*C*) in a slice were significantly reduced by intravenous AP₄A treatment ($n = 17$, $p < 0.05$). *D*, Intravenous administration of AP₄A reduced bradykinesia in stroke animals ($*p < 0.05$).

to hypoxia/re-oxygenation were significantly reduced by pretreatment with AP₄A (Fig. 1*A–C*) ($p < 0.05$, one-way ANOVA) (Fig. 1*D,E*).

Pretreatment with AP₄A increases locomotor activity and reduces infarction in stroke rats

Rats were pretreated with either vehicle ($n = 9$) or AP₄A (30 nmol, i.c.v.; $n = 10$) 20–30 min before middle cerebral artery occlusion. Animals were individually placed in activity chambers to measure their locomotor activity on the second day after stroke. Pretreatment with AP₄A, compared with vehicle, significantly enhanced the horizontal activity, total distance traveled, number of horizontal or vertical movements, and movement time in stroke animals ($p < 0.05$, t test) (Fig. 2*D*).

One hour MCA ligation and 48 hr reperfusion resulted in clear-cut infarction of the cortex in animals pretreated with vehicle or AP₄A, as examined by TTC staining (Fig. 2*A*). Infarction was additionally analyzed by two methods: (1) The volume of

infarction, which equals 2 mm [thickness of the slice] $\times$ [sum of the infarction area in all brain slices (mm²)]. (2) The area of the largest infarction in a slice from each rat. In 31 animals studied, pretreatment with AP₄A (3 or 30 nmol, i.c.v.) dose-dependently reduced the volume of infarction (Fig. 2*B*) ($p < 0.05$, $F_{(2,28)} = 10.303$, one-way ANOVA and Newman–Keuls test). Similarly, pretreatment with AP₄A dose-dependently reduced the area of largest infarction in a given slice (Fig. 2*C*) ($p < 0.002$, $F_{(2,28)} = 8.410$, one-way ANOVA; $p < 0.05$, *post hoc* Newman–Keuls test).

Parenteral treatment with AP₄A after ischemia

The blood–brain barrier (BBB) can be transiently disrupted during ischemia, which provides a temporal window for the parenteral administration of AP₄A to enter the brain. To test the hypothesis that the systemic administration of AP₄A may reduce infarct volume, 17 adult rats were subjected to a 60 min right MCA ligation and treated at 5–10 min after removal of the ligature with an intravenous injection of either AP₄A (10 mg/kg, $n = 8$) or vehicle ($n = 9$). Rats that had been treated with a systemic injection of AP₄A had significantly higher locomotor activity than their respective controls (Fig. 3*D*; $p < 0.05$, t test). Most notably, the volume of infarct, determined at 48 hr after MCA occlusion, was significantly smaller in rats that had been treated with a systemic injection of AP₄A early in the reperfusion period (Fig. 3*A–C*) ($p < 0.05$, t test). These data demonstrate that AP₄A, given intravenously in the early reperfusion period, exhibits a protective effect against ischemic injury.

Blood gas, electrolytes, and systemic blood pressure

We found that the intracerebroventricular administration of AP₄A (30 nmol/20 μ l)

did not acutely (25–30 min) alter systemic blood pressure, heart rate, or serum concentrations of glucose, electrolytes, and blood gases ($p > 0.05$, t test) in 15 non-stroke animals (Fig. 4*A,B*).

Cerebral blood flow during stroke

Cerebral cortical blood flow was measured continuously using a fine (0.45 mm diameter) laser Doppler probe. Previous studies have indicated that this modified laser Doppler flowmetry can provide a high degree of spatial and temporal resolution (Wang et al., 2001a,b). We found that cortical blood flow was significantly reduced to the same degree after MCA ligation in both vehicle- and AP₄A-treated animals (Fig. 4*C1,C2*) ($p = 0.894$, t test), suggesting that pretreatment of rats with AP₄A did not alter cortical blood flow during ischemia.

Translocation of cytochrome c

In vehicle-treated animals ($n = 4$), 60 min MCA occlusion and 8 hr reperfusion resulted in a significant decrease in the levels of

cytochrome *c* protein in the mitochondria (Fig. 5A). The intravenous injection of AP₄A ($n = 3$) in the early reperfusion period attenuated the reduction in mitochondrial levels of cytochrome *c* caused by cerebral ischemia and reperfusion ($p < 0.05$, $F_{(3,10)} = 357.168$, one-way ANOVA and Newman–Keuls test). The ratio between cytochrome *c* levels in cytosol and that in mitochondria was significantly increased in the ischemic hemisphere in animals subjected to MCA occlusion and reperfusion, and treated with vehicle. This increase was attenuated by the administration of AP₄A in the early reperfusion period. These data suggest that AP₄A reduces the ischemia/reperfusion-induced translocation of cytochrome *c* from the mitochondria into the cytosol, which is thought to be a critical mechanism in apoptosis.

Caspase-3 activity at 8 hr after ischemia

Previous studies have indicated that the activity of caspase-3, a marker of apoptosis, is greatly increased as early as 8 hr after ischemia (Sasaki et al., 2000). To investigate the effect of AP₄A on caspase-3 activity, animals were subjected to 60 min MCA occlusion and 8 hr of reperfusion, and treated in the early reperfusion period with intravenous AP₄A or vehicle. In vehicle-treated control animals, MCA-occlusion and reperfusion resulted in a significant increase in the enzymatic activity of caspase-3 (Fig. 5B). Compared with non-stroke animals, MCA occlusion increased caspase-3 activity $274.1 \pm 34.3\%$ and $167.5 \pm 25.8\%$ in the ipsilateral (ischemic side) and contralateral hemisphere, respectively ($p < 0.05$, $F_{(2,11)} = 43.514$, one-way ANOVA and Newman–Keuls test). Caspase-3 enzymatic activity was significantly decreased in the brain sections obtained from animals treated with AP₄A in the early reperfusion period ($p < 0.05$, $F_{(5,24)} = 90.736$, one-way ANOVA and Newman–Keuls test) (Fig. 5B).

P₁-, P₂-, and P₄-receptor mediation

Numerous studies have indicated that adenosine has neuroprotective effects against ischemia. We also found that pretreatment with adenosine (30 nmol, i.c.v.) significantly reduced cerebral infarction in eight stroke rats (Fig. 6A) ($p < 0.05$, $F_{(2,33)} = 17.095$, one-way ANOVA and Newman–Keuls test). However, such protective effects of adenosine are much less than those of AP₄A ($p < 0.05$, one-way ANOVA) (Fig. 6A), suggesting that mechanisms other than adenosine signaling may be involved. To further characterize the interactions of AP₄A with various purinergic P₁-, P₂-, or P₄-receptors, AP₄A was co-administered with the selective P₁-receptor antagonist sulfonylphenyl theophylline, the nonselective P₂-receptor antagonist suramin, or the P₂/P₄-receptor antagonist di-inosine pentaphosphate (IP₅I). We found that suramin ($n = 15$) did not antagonize the reduction in total infarct volume caused by AP₄A (Fig. 6B) ($p > 0.05$, $F_{(2,22)} = 2.940$, one-way ANOVA). Suramin (100 nmol) induced seizures in six of seven rats and was lethal in three. In contrast to suramin, AP₄A-induced protection was significantly attenuated by IP₅I (Fig. 6C) ($p < 0.05$, $F_{(2,26)} = 6.329$, one-way ANOVA) or sulfo-

Figure 4. Intracerebroventricular pretreatment with AP₄A did not alter systemic physiological parameters and cerebral blood flow (CBF). *A*, Pretreatment with AP₄A (30 nmol, i.c.v.) did not acutely alter systemic blood pressure. Δ BP or Δ HR, Difference in systemic blood pressure or heart rate before and 25–30 min after intracerebroventricular administration. *B*, AP₄A pretreatment did not alter blood gases or blood constituents and electrolytes. The concentrations of blood gases, constituents, and electrolytes from the AP₄A group were normalized by comparison with the mean of each index in animals receiving vehicle. *C*, CBF was measured using laser Doppler flow (LDF) perfusion in stroke rats. LDF was normalized (% control) by comparison with the mean blood flow before MCA ligation in each animal. *C1*, Real-time tracing from animals receiving vehicle or AP₄A (30 nmol) before treatment demonstrates that AP₄A did not increase CBF during ischemic periods (arrowheads). *C2*, Histograms showing that the average CBF was significantly reduced to a similar degree after MCA ligation in vehicle- and AP₄A-treated animals.

nylphenyl theophylline (Fig. 6D) ($p < 0.05$, $F_{(2,23)} = 5.103$, one-way ANOVA). These data suggest that activation of P₁- and P₄-, but not P₂-, receptors, is involved in the observed neuroprotective effects of AP₄A in the stroke rats.

6-OHDA lesioning

TUNEL in 6-OHDA-lesioned rats

Six rats were pretreated intracerebroventricularly with AP₄A or vehicle and lesioned unilaterally with 6-OHDA in the medial forebrain bundle. Animals were killed 2 d after 6-OHDA lesioning. An increase in TUNEL was found in the lesioned nigra in rats receiving vehicle pretreatment (Fig. 7A1,A2). TUNEL was mainly colocalized with TH immunoreactivity in the nigral cells (Fig. 7A3). AP₄A pretreatment reduced the density of TUNEL (Fig. 7B1,B2).

Amphetamine-induced ipsilateral rotation in 6-OHDA-lesioned rats

Amphetamine-induced rotation was evaluated 1 month after 6-OHDA lesioning in 31 rats. We found that control animals developed a marked ipsilateral rotation after injection (472 ± 41 turns/hr). This response was significantly attenuated in animals pretreated with AP₄A (93 ± 29 turns/hr, $p < 0.05$, *t* test).

TH immunostaining in 6-OHDA-lesioned rats

Immunoreactivity to TH (Fig. 7C,D) was used to identify dopaminergic neurons and processes in 12 rats 1 month after lesioning. Very low TH immunoreactivity was noted in the striatum of

Figure 5. AP₄A reduced both translocation of cytochrome *c* and caspase-3 activity in stroke brain. *A*, Mitochondrial cytochrome *c* protein levels were reduced in the ischemic (R) hemisphere of the vehicle-control animals. The intravenous administration of AP₄A significantly increased the mitochondrial cytochrome *c* levels in the ischemic hemisphere compared with vehicle-treated animals. A representative Western blot showing the decrease in mitochondrial cytochrome *c* (top), but not mitochondrial marker VDAC (bottom), in the lesioned cortex is shown above the histogram. OD, Optical density. *B*, Increased caspase-3 enzymatic activity was reduced in the ischemic brains of AP₄A-treated rats at 8 hr after stroke. Caspase-3 enzymatic activity was significantly increased in the ischemic (R) compared with the non-ischemic (L) hemisphere in vehicle-pretreated rats. The administration of AP₄A in the early reperfusion period significantly reduced the ischemia-induced increase in caspase-3 activity. Specific caspase-3 activity, as examined by the addition of the caspase-3 inhibitor DEVD-CHO (inhibitor) in the assay medium, is shown above the dotted line. **p* < 0.05.

6-OHDA-lesioned rats, which had received vehicle (Fig. 7C). There were minimal TH-positive neurons in the lesion-side nigra. In contrast, AP₄A partially prevented the loss of TH immunoreactivity in the 6-OHDA-lesioned striatum and nigra (Fig. 7D).

KCl-induced DA release in striatum

To determine whether the release of DA was preserved in animals treated with AP₄A, 17 rats were used to detect dopamine release by *in vivo* chronoamperometry at 6 months after the injection of 6-OHDA. Of these, 10 rats (four pretreated with AP₄A and six with saline) were lesioned with 6-OHDA, whereas seven rats,

Figure 6. AP₄A-induced protection against ischemia involves purinergic receptors. AP₄A (30 nmol, i.c.v.) is more potent than adenosine (30 nmol, i.c.v.) to reduce cerebral infarction examined at 48 hr after stroke (*p* < 0.05, one-way ANOVA). Co-administration of IP₅I (C) or sulfonphenyl theophylline (D), but not suramin (B), antagonized AP₄A (30 nmol)-induced protection.

housed in the same environment, served as sham-operated controls. In the sham-operated rats, the local application of KCl resulted in a significant release of DA in the anterior striatum (Fig. 8A,B). In rats that had been lesioned with 6-OHDA, KCl caused only a very small release of DA (Fig. 8A,B). In contrast, the release of DA elicited by KCl in the striatum of rats treated with AP₄A before the administration of 6-OHDA, was significantly greater (*p* < 0.05, two-way ANOVA and Newman–Keuls test) (Fig. 8B). It should be noted that the peak release of DA in 6-OHDA-lesioned rats treated with AP₄A was significantly reduced compared with the sham-operated rats (*p* < 0.05, two-way ANOVA and Newman–Keuls test) (Fig. 8B), suggesting that AP₄A attenuates, but does not abolish, the reduction in the release of dopamine caused by 6-OHDA in the striatum.

Discussion

In this study, we have documented the efficacy of a novel and potent neuroprotective agent AP₄A in rodent models of Parkinson's disease and stroke. Pretreatment with AP₄A at 100 nM, a physiological concentration found in tears (Pintor et al., 2002), reduced hypoxia/reoxygenation-induced increases in LDH in cortical cultures. Similarly, AP₄A, given before MCA ligation attenuated cerebral infarction as well as the associated bradykinesia in a dose-related manner in stroke animals. These data suggest that AP₄A has protective effects against hypoxic and ischemic injuries in the CNS. Because AP₄A did not alter BP, blood gases, and blood electrolytes and did not alter cerebral blood flow during ischemia, the neuroprotective effect of AP₄A *in vivo* is probably not indirectly mediated through the changes in systemic physiological parameters. We found that the application of AP₄A even after an ischemic event reduced cerebral infarction and increased motor function. Such a protection may have clinical significance because treatment can be given after the occlusion in stroke patients. Interestingly, animals that received systemic AP₄A, compared with intracerebroventricular AP₄A, tended to recover better in one (number of horizontal movements in 30

min, $p = 0.060$, t test), but not all, of the locomotor behavioral test parameters. It is possible that the increased number of surgical procedures (or trauma) associated with intracerebroventricular injection may have caused less horizontal movement after stroke.

Pro-apoptotic mechanisms are activated during ischemia–reperfusion. For example, in ischemia or brain trauma, the translocation of cytochrome *c* from the mitochondria to the cytosol activates caspase-3 and facilitates apoptosis (Sasaki et al., 2000). Prevention of apoptosis reduces brain damage (Kitagawa et al., 1998; Wang et al., 2001a,b). In the current study, AP₄A prevented both translocation of cytochrome *c* and activation of caspase-3 induced by ischemia/reperfusion. AP₄A also reduced hypoxia/reoxygenation-induced TUNEL in cerebrocortical cells. Thus, our data support the view that the neuroprotective effects of AP₄A involve the inhibition of several molecular steps that lead to the development of apoptotic cell death during ischemia–reperfusion of the brain.

Previous studies have indicated that AP₄A increases intracellular calcium levels through P₂ receptors in cortical synaptic terminals and through suramin-insensitive dinucleotide P₄ receptors in the midbrain or cerebellum (Pintor et al., 1997). Activation of P₄ receptors in rat midbrain synaptic terminals produces an initial voltage-independent calcium entry followed by a voltage-dependent increase in N-type calcium currents (Pintor and Miras-Portugal, 1995). These data suggest that AP₄A exhibits differential functions in the cortex and midbrain through the purinergic receptors. In the present study, the neuroprotective effect of AP₄A against ischemic injury was reduced by P₁ and P₂/P₄, but not P₂, purinergic antagonists. Thus, it is likely that both P₁- and P₄-receptors are involved in AP₄A-induced protection.

We have demonstrated that the protective effect of AP₄A can be reproduced in other models of neuronal injury, in which neurons not located within the cortex are affected. The neurodegeneration induced by 6-OHDA in nigrostriatal dopaminergic neurons was reduced by pretreatment with AP₄A. All of the effects of 6-OHDA, including motor bias, decrease in TH immunoreactivity in the substantia nigra and striatum, and attenuation of DA release in the striatum, were significantly reduced by pretreatment with AP₄A. Because 6-OHDA can induce toxicity through the production of reactive oxygen species and apoptosis in nigral neurons (Zuch et al., 2000) and AP₄A suppressed the 6-OHDA-induced increase in DNA fragmentation in nigra, we propose that AP₄A partially attenuates the toxic effects of 6-OHDA, also through anti-apoptotic mechanism.

In conclusion, our results demonstrate that AP₄A has neuroprotective effects against the injuries induced by ischemia in cortex and by 6-OHDA in nigrostriatal dopaminergic neurons. We propose that prevention of the activation of apoptosis by AP₄A contributes importantly to the neuroprotective effects of this endogenous compound. These data may well be of clinical importance, insofar as systemic administration of AP₄A after an isch-

Figure 7. AP₄A protects against 6-OHDA-induced lesioning in hemiparkinsonian rats. *A*, Administration of 6-OHDA into the medial forebrain bundle induced TUNEL in the ipsilateral nigral region, 2 d after the lesioning, in a vehicle-treated animal (*A1*, *A2*, low and high magnification). *A3*, TUNEL (+) labeling (green) was predominantly colocalized to the nuclei (blue) of TH (+) cells (red; e.g., large arrow). Some TUNEL (–) and TH (+) cells (small arrow) were also present in the lesioned nigral area. *B*, Pretreatment with AP₄A (*B1*, *B2*, low and high magnification) reduced 6-OHDA-induced TUNEL in nigra. Scale bars: (in *A1*) *A1*, *B1*, 200 μ m; (in *A2*) *A2*, *B2*, 50 μ m. *C*, TH immunoreactivity was virtually abolished in the ipsilateral striatum and nigra in a 6-OHDA-lesioned animal receiving vehicle pretreatment 1 month after lesioning. *D*, In another 6-OHDA-lesioned animal pretreated with AP₄A, TH immunoreactivity was relatively preserved in the striatum and nigra.

emic episode may reduce reperfusion injury and improve outcome in patients with stroke.

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Figure 8. AP₄A elevated KCl-induced DA release in the 6-OHDA-lesioned striatum. *A*, *In vivo* chronoamperometric recordings demonstrate that the local application of 70 mM KCl (150 nl) to the anterior–dorsal striatum induced DA release in a non-lesioned rat (sham control). Lesioning the medial forebrain bundle abolished KCl-evoked DA release in an animal pretreated with vehicle. DA release was partially preserved by pretreatment with AP₄A. Note that DA release in the 6-OHDA-lesioned rat receiving AP₄A pretreatment has a lower amplitude but a longer half-life compared with that in a non-lesioned rat. *B*, DA release in the anterior striatum (4.0–7.0 mm below the cortical surface, 2.5 mm lateral and 1.0 mm anterior to the bregma) was virtually abolished by 6-OHDA lesioning in four animals receiving vehicle pretreatment. Pretreatment with AP₄A significantly enhanced DA release in 6-OHDA-lesioned rats ($p < 0.05$, two-way ANOVA).

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