Inosine Reduces Ischemic Brain Injury in Rats

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Background and Purpose—Purinergic nucleoside inosine elicits protection and regeneration during various injuries. The purpose of this study was to examine the protective effects of inosine against cerebral ischemia.

Methods—Adult Sprague–Dawley rats were anesthetized. Inosine, hypoxanthine, or vehicle was administered intracerebroventricularly before transient right middle cerebral artery occlusion (MCAo). Animals were placed in behavioral chambers 2 days to 2 weeks after MCAo and then euthanized for triphenyltetrazolium chloride staining. Glutamate release was measured by microdialysis/high-performance liquid chromatography, and single-unit action potentials were recorded from neurons in the parietal cortex.

Results—Stroke animals receiving inosine pretreatment demonstrated a higher level of locomotor activity and less cerebral infarction. Intracerebroventricular administration of the same dose of hypoxanthine did not confer protection. Coadministration of selective A3 receptor antagonist 3-ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (MRS1191) significantly reduced inosine-mediated protection. Inosine did not alter basal glutamate release, nor did it reduce ischemia-evoked glutamate overflow from cerebral cortex. However, inosine antagonized glutamate-induced electrophysiological excitation in cerebral cortical neurons.

Conclusions—Inosine inhibits glutamate postsynaptic responses and reduces cerebral infarction. Its protective effect against ischemia/reperfusion-related insults may involve activation of adenosine A3 receptors. (Stroke. 2005;36:000-000.)

Key Words: inosine, adenosine ■ neuroprotection ■ stroke

The purinergic nucleoside inosine elicits protection and regeneration during various injuries. Inosine reduced zinc-induced injury in PC12 cells,1 reduced toxicity after oxygen-glucose deprivation (OGD) in rat astrocyte cultures,2 and preserved cell viability during chemical hypoxia induced by rotenone in spinal cord cell culture.3 The protective effect of inosine has also been demonstrated in vivo. Infusion of inosine reduced ischemic/hypoxic injury in myocardium.4 The roles of inosine in central nervous system (CNS) during ischemia have not been studied extensively. Middle cerebral artery occlusion (MCAo) led to release of inosine and its metabolite hypoxanthine from the ischemic cortex in stroke animals.5 Inosine, given after stroke, stimulated axonal outgrowth and improved behavioral outcome in stroke animals.6 These data suggest that inosine promotes neuroregeneration after stroke. It is still not clear whether inosine, given before cerebral ischemia, is neuroprotective.

Several protective mechanisms of inosine have been proposed from in vitro studies. Inosine binds selectively to A3, but not A1 or A2A, receptors in human embryonic kidney (HEK)-293 cells. Exogenous application of inosine increased ATP level in PC12 cells. Such an effect was not found with selective A1, A2A, or A3 agonists, indicating that the inosine-induced elevation of ATP levels did not relate to these purinergic receptors in PC12 cells.7 Inosine or its analog inosine 5'-triphosphate reduced glutamate receptor–mediated responses in hippocampal CA1 synapse8 or N-methyl-D-aspartate–mediated neurotoxicity in cultured hippocampal neurons.9 Activation of purinergic receptors reduced glutamate toxicity in retinal neuronal culture10 and K⁺-induced glutamate release in hippocampus.11 These data suggest that inosine interacts with purinergic receptors, increases ATP production, and alters glutamate responses in vitro. It is not known whether any of these mechanisms are involved in inosine-related neuroprotection in CNS injury.

In the present study, we examined the protective effect of inosine against ischemia-induced cerebral injury. Our data suggest that pretreatment with inosine reduced cerebral infarction and improved locomotor behavior in stroke rats.

Materials and Methods

Animals and Surgery

Adult male Sprague–Dawley rats (n=159; Charles River Laboratories, Wilmington, Mass) were used. Animals were anesthetized with chloral hydrate (0.4 g/kg IP). Inosine, hypoxanthine (25 nmol/L in 25 μL), or vehicle was administered intracerebroventricularly (0.8-mm...
posterior, 1.5-mm lateral to the bregma, and 3.7-mm below dura) through a Hamilton syringe.

Thirty minutes after intracerebroventricular administration, animals were subjected to cerebral ischemia. The ligation of the right MCA and bilateral common carotids was performed using methods described previously. The right MCA was ligated with 10-O suture for 60 minutes. Core body temperature was monitored with a thermistor probe and maintained at 37°C with a heating pad during surgery. After surgery, animals were kept in a temperature-controlled incubator to maintain body temperature at 37°C. After recovery from the anesthesia, animals were returned to their home cages.

Cerebral Blood Flow
Cortical blood flow was continuously measured using a laser Doppler flowmeter (PF-5010; Perimed). A burr hole was made in the right frontoparietal region. A blood flow probe (Probe 411; Perimed) was stereotaxically placed in the cortex (3.5 to 4.0 mm posterior, 3.5 to 4.0 mm lateral to bregma, and 1.0 mm below dura).

Blood Pressure and Blood Gas Measurements
Physiological parameters were measured as described previously. Brain temperature was continuously monitored through a fine thermo-probe (PF-5020; Perimed). A polyethylene catheter was inserted into the right femoral artery. Mean arterial pressure was monitored by a blood pressure recorder (Windo-Graf 930; Gould). Arterial blood was analyzed by a blood gas analyzer (GEM Premier-3000).

Quantification of Glutamate and Adenosine Release
Glutamate and adenosine were measured using microdialysis techniques. The probe was stereotaxically placed in the parietal cerebral cortex (−4.0 mm posterior, 4.5 mm lateral to the bregma, and −3.0 mm below brain surface). Artificial cerebrospinal fluid (aCSF) was advanced through the probe at a rate of 2 μL/min. Samples were collected at 20-minute intervals. In some animals, inosine (1 mM) was included in the aCSF.

The concentration of glutamate in the dialysis samples was determined using high-performance liquid chromatography (HPLC). The mobile phase consisted of 25% methanol and 100 mM Na,HPO₄, pH 6.75. A reversed-phase column was used to separate the amino acids, and precolumn derivatization of amino acids with ophthalaldehyde was performed using an ESA model 542 autosampler. Glutamate was detected using a fluorescence spectrophotometer with an excitation wavelength of 356 nm and an emission wavelength of 420 nm.

Adenosine content in the samples was analyzed by HPLC coupled to a spectrofluorometric detector with an excitation wavelength of 270 nm and an emission wavelength of 394 nm. Adenosine was detected as a fluorescent derivative ethenoadenosine after mixing with chloroacetaldehyde. A Nucleosil C18 column (Waters) with a mobile phase of 50 mM/L acetate buffer, pH 5, with 5% acetonitrile and 1 mM/L 1-octanesulfonic acid sodium salt.

Locomotor Measurements
Animals were placed in an Accuscan activity monitor 2, 7, and 14 days after MCAo. Each animal was placed in a 420×420×310 mm Plexiglas open box for 30 minutes. Motor activity was calculated using the number of beams broken by the animals. The following variables were measured: (1) horizontal activity (the total number of beam interruptions that occurred in the horizontal sensor in 30 minutes), and (2) total distance traveled (the distance, in centimeters, traveled by animals in 30 minutes).

Triphenyltetrazolium Chloride Staining
Animals were euthanized 2 or 21 days after MCAo. Brains were removed and sliced into 2.0-mm-thick sections. Brain slices were incubated in 20 g/L triphenyltetrazolium chloride (TTC) and then transferred into a 40 g/L paraformaldehyde solution for fixation. The area of infarction was measured by blinded investigators using a digital scanner.

Electrophysiology
Naive rats were anesthetized and placed in a stereotaxic frame. Extracellular recordings were made from neurons in the parietal cortex. Coordinates for recordings from bregma with flat skull were anteroposterior −2.0 to −2.5 mm; mediolateral 1.5 to 3 mm; and dorsoventral 0.5 to 1.2 mm, a region corresponding to the part of the ischemic core in stroke rats. Neuronal signals were amplified, filtered, and displayed on a storage oscilloscope. Action potentials were isolated using a window discriminator, and the firing rate was displayed using Datawave. Single units had a signal-to-noise ratio of ≥2.1. Multibarrel glass micropipettes were used for single cell recording and local drug application via pressure microjection. Glutamate (10 μmol/L, pH 7.2) and inosine (100 μmol/L, pH 7.2) were loaded into the barrels of the pipette.

Statistics
Student t test, 1- or 2-way ANOVA, and post hoc Newman–Keuls test were used for statistical comparison. Data are presented as mean±SEM.

Results
Neuroprotective Effect of Inosine
Forty-eight rats were treated with vehicle (n=28), inosine (n=11), or hypoxanthine (n=9). Animals were subjected to 1 hour of MCAo. Of these, 35 rats were used for behavioral measurements 2 days after MCAo. Animals pretreated with inosine had significantly higher locomotor activity than controls. Horizontal motor activity and total distance traveled were enhanced by pretreatment with inosine (P<0.05; 1-way ANOVA; Figure 1A1 and 1A2). Administration of hypoxanthine (25 nmol/L) did not enhance these locomotor behaviors (Figure 1A1 and 1A2; P>0.05; Newman–Keuls test). Another set of animals, injected with inosine (n=10) or vehicle (n=10), were examined for the recovery of locomotor function 7 and 14 days after stroke. The animals receiving inosine pretreatment had significant increase in horizontal and total distance traveled up to 2 weeks after MCAo (Figure 1B1 and 1B2; P<0.05; 1-way ANOVA).

Animals were euthanized 2 days (n=48) or 21 days (n=20) after stroke for TTC staining. The volume of infarction was significantly reduced by inosine but not hypoxanthine 2 days after stroke (vehicle 187.1±9.0 mm³ versus inosine 118.5±18.4 mm³; P<0.05; Figure 1A3). Because of the gliosis and dissolution of the infarcted tissue in rats euthanized at 21 days after stroke, the ischemia-induced loss of brain was indirectly measured by subtracting the noninfarcted area in the ischemic side hemisphere from the total area of the nonischemic hemisphere in animals euthanized 3 weeks after stroke. Pretreatment with inosine significantly reduced tissue loss in these animals (Figure 1B3; P<0.05; t test).

To determine whether A3 receptors are involved in inosine-mediated protection, we administered 3ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1, 4-(±)-dihydropyridine-3,5-dicarboxylate (MRS1191; 25 nmol/L ICV), a selective A3 antagonist for rodents, to 11 rats. MRS1191 alone (n=6) did not alter volume of infarction in stroke rats. In another 5 rats, MRS1191 was coadministered with inosine before MCAo. MRS1191 significantly antagonized the
Inosine-mediated reduction of infarction volume (Figure 2; \( P < 0.05 \); 1-way ANOVA plus Newman–Keuls test).

Cerebral blood flow (CBF) was measured in 6 nonstroke and 11 stroke animals. CBF was normalized (percent control) by comparison with the mean blood flow before administration of chemicals or MCAo in each animal. Inosine or vehicle did not alter CBF in nonstroke rats. MCAo significantly reduced CBF. There was no difference in CBF during MCAo between animals treated with vehicle or inosine (\( P = 0.214 \); t test).

Glutamate levels were measured from the dialysates in 26 rats. In the nonstroke animals, perfusion with inosine (n=6) did not alter the basal glutamate overflow compared with animals treated with vehicle (n=6; \( P = 0.981 \); 2-way ANOVA; Figure 3A). In the stroke rats, ischemia significantly increased overflow of glutamate (Figure 3B; \( P < 0.05 \); 1-way ANOVA). There was no difference in glutamate release in stroke animals perfused with inosine (n=7) or vehicle (n=7) during ischemia (\( P = 0.994 \); 2-way ANOVA).

Adenosine levels were measured from the dialysates in 15 stroke animals. Of these, 8 were perfused with inosine and 7 were perfused with vehicle. Samples were compared 0 to 20 minutes before and 40 to 60 minutes after MCAo. Ischemia significantly increased adenosine level in vehicle controls (Figure 3C; \( P < 0.05 \); 1-way ANOVA plus Newman–Keuls test). Inosine did not alter the basal adenosine level. There is no significant difference in adenosine overflow before and after MCAo in animals perfused with inosine (Figure 3C).

Electrophysiological recordings were made from 12 cortical neurons from 5 rats. Baseline responses to glutamate were established, and inosine was subsequently applied out of an adjacent barrel of the microelectrode assembly. In 10 of 12 neurons, inosine was able to

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**Figure 1.** Inosine decreased ischemia-induced bradykinesia and cerebral infarction. Pretreatment with inosine but not hypoxanthine increased (A1) horizontal activity and (A2) total distance traveled 2 days after stroke (n=35). A3. The volume of infarction was significantly less in the animals treated with inosine compared with the vehicle control 2 days after stroke. B1 and B2. In another set of rats, inosine pretreatment enhanced locomotor activity 1 and 2 weeks after stroke. B3. Animals receiving inosine pretreatment had significantly less tissue loss 3 weeks after MCAo. n=20; *\( P < 0.05 \).

**Figure 2.** A3 antagonist MRS1191 attenuated inosine-induced protection. MRS1191 alone did not alter the volume of infarction. Coadministration of MRS1191 antagonized inosine-mediated protection in cerebral infarction. *\( P < 0.05 \); n=56.
diminish or completely block the excitatory actions of locally applied glutamate on the cortical neurons. An example of this reaction is shown in Figure 4A. Inosine increased the firing rate in 8 of 12 neurons recorded; this effect was observed within 10 to 20 seconds of inosine application. Although there was an increase in the background firing rate, the effect of glutamate was diminished. The degree of excitation (percent excitation) was calculated by comparing the increase in average firing rate during glutamate application with the average firing rate before each drug application. Local application of inosine significantly antagonized the excitatory effects of glutamate (*P<0.001; n=12).

Figure 4. Inosine reduces electrophysiological excitation by glutamate. A, A typical ratemeter record obtained from a cerebral cortical neuron. Inosine or glutamate was applied directly to the neuron through a multibarrel micropipette. Local application of glutamate produced an increase in neuronal activity. Coadministration of inosine reversibly reduced glutamate-mediated excitation. The vertical calibration is in spikes per second, and the duration of application of drug is indicated by solid lines above the trace. B, Bar graphs represent the average neuronal excitation induced by glutamate application before and during inosine application. The degree of excitation was normalized by comparing the increase in average firing rate during glutamate application with the average firing rate before each application. Local application of inosine significantly antagonized the excitatory effects of glutamate (*P<0.001; n=12).

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Figure 3. Glutamate and adenosine overflow from cerebral cortex. A, In the nonstroke rats, perfusion with inosine or aCSF did not alter the glutamate overflow (n=12). B, In the stroke rats (n=14), overflow of glutamate was increased during ischemia and reperfusion. Coadministration of inosine did not attenuate ischemia-induced glutamate release. C, Ischemia increased adenosine overflow. Samples were compared before and 40 to 60 minutes after MCAo (n=15). Ischemia significantly increased adenosine level. Such a difference was not found in the animals perfused with inosine. *P<0.05.

A total of 17 rats were used to measure physiological parameters. Inosine did not alter the mean blood pressure, arterial Pao2, Paco2, pH, or brain temperature (Table; P>0.05).
In this study, pretreatment with inosine (25 nmol/L) reduced MCAo-induced bradykinesia and cerebral infarction. The improvement of motor function lasted up to 2 weeks after stroke. The reduction of cerebral infarction was dose dependent (2.5 to 50 nmol/L), and using nonlinear regression analysis, ED50 was ≈10 nmol per rat (data not shown). Inosine, given 1 hour after MCAo, did not reduce cerebral infarction (data not shown). These data suggest that inosine is neuroprotective in vivo.

We found that the inosine did not alter basal CBF, blood pressure, blood gas, or brain temperature in naïve rats. Moreover, inosine did not alter CBF during ischemia. The protective effect of inosine reported here is thus not related to a change in CBF or systemic physiological parameters.

Inosine can be converted to hypoxanthine by purine nucleoside phosphorylase (PNP), mainly in glia, and used as an energy donor to form ATP through hypoxanthine-guanine phosphoribosyl transferase (HGPRT) in the salvage pathway. In vitro studies have indicated that the protective effect of inosine depends on the conversion inosine to hypoxanthine by PNP.3 On the other hand, hypoxathine did not induce protection against OGD in astrocyte cultures.2 We found that hypoxanthine, at the same dose of inosine that produced protection, did not reduce cerebral infarction and bradykinesia in stroke rats. Because hypoxanthine is the main substrate for HGPRT to form inosine 5’-monophosphate (IMP) and then ATP, the lack of protection by hypoxanthine indicates that inosine-mediated protection is not through the production of active metabolite hypoxanthine or ATP in vivo.

There are no known inosine-specific receptors; however, inosine can induce physiological responses through the activation of A3 receptors. Inosine has a high affinity for A3 but not A1 or A2 receptors; it lowered cAMP production in HEK-293 cells expressing rat A3A receptors (A3AR).19 The protective effect of inosine against concanavalin A-induced fulminant hepatitis can only be found in A3R-expressing and not wild-type mice.17 Chronic pretreatment or post-treatment with the A3 agonist N[6]-(3-iodobenzyl)-5’-[(N-methylcarbamoyl)adenosine (IB-MECA) reduced transient focal ischemia–induced infarction volume in mice. Acute pretreatment with A3 agonist IB-MECA, given systemically, potentiated the damage, possibly secondary to the activation of histamine release and hypotension.18 Repeated systemic administration of A3 agonist chlorobenzyl–IB-MECA did not produce cardiovascular action and histamine release19 but reduced cerebral infarction in stroke rats (data not shown). Mice with a deficiency in A3 receptors (A3AR−/−) were more vulnerable than wild-type controls (A3AR+/+) to hypoxic injury of hippocampal pyramidal neurons. Similarly, A3AR+/+ mice pretreated with the A3 antagonist MRS1523 had high sensitivity to hypoxia.20 These data suggest that A3 receptors modulate protective responses during ischemic/hypoxic brain injury. In this study, we found that A3 antagonist MRS1191 attenuated inosine-mediated protection, suggesting the activation of A3 receptors contributes to these protective effects of inosine in the CNS.

Purinergic receptors have been reported to regulate glutamate release21 and the response to excitatory amino acids.22 We found that ischemia caused glutamate release in the ischemic cortex. The overflow of glutamate during ischemia was unaffected by inosine. However, microinjection of inosine reversibly antagonized electrophysiological excitation by glutamate in cerebral cortical neurons, suggesting a postsynaptic interaction between inosine and glutamate. This assumption is further supported by the observation that activation of A3 receptors inhibits metabotropic glutamate receptor–mediated function at hippocampal Schaffer collateral–CA1 synapses.3 It is thus possible that blocking glutamate is a potential, but not the sole, mechanism in inosine-induced neural protection. More detailed experiments are required to elucidate the mechanisms of such an interaction.

Adenosine is neuroprotective against ischemia.12 Similar to previous reports,14 we found the adenosine release was enhanced during ischemia. Perfusion of inosine did not increase the concentration of adenosine in the perfusate, suggesting that the inosine-mediated protection is not secondary to the overflow of adenosine.

In this study, we administered inosine intracerebroventricularly before stroke and observed neuroprotective effects. The mechanism of action is independent of changes in blood flow or glutamate release but may involve suppression of glutamate responses and A3 receptor activation. These results suggest that inosine and A3 agonists may have therapeutic potential as protective agents against stroke-induced damage.

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