Dipyridamole Increases Oxygen-Glucose Deprivation-Induced Injury in Cortical Cell Culture

Doug Lobner, PhD; Dennis W. Choi, MD, PhD

Background and Purpose Adenosine transport inhibitors attenuate ischemic central neuronal damage in vivo, but the locus of this protective action is presently unknown. To help address the question of whether adenosine transport inhibitors have a protective effect directly on brain parenchyma, we tested the effect of the adenosine transport inhibitor dipyridamole on neuronal loss induced by oxygen-glucose deprivation in vitro.

Methods Murine cortical cultures were exposed to combined oxygen and glucose deprivation, N-methyl-D-aspartate, or kainate. The extracellular concentrations of glutamate and adenosine were measured by high-performance liquid chromatography; neuronal cell death was assessed by morphological examination and measurement of lactate dehydrogenase release.

Results Cultures exposed to oxygen-glucose deprivation for 30 to 75 minutes exhibited an insult-dependent increase in extracellular adenosine, followed shortly by an increase in extracellular glutamate and 24 hours later by neuronal death. Addition of the A1 receptor antagonist 8-cyclopentyltheophylline during oxygen-glucose deprivation enhanced both glutamate release and neuronal damage. Addition of 10 μmol/L dipyridamole decreased extracellular adenosine and also enhanced extracellular glutamate and neuronal death. In contrast, dipyridamole increased the levels of extracellular adenosine stimulated by N-methyl-D-aspartate or kainate.

Conclusions These results are consistent with the idea that endogenous adenosine has a neuroprotective effect directly on cortical cells exposed to oxygen-glucose deprivation. However, inhibition of adenosine transport with dipyridamole was surprisingly not an effective strategy for enhancing this protective effect. The beneficial effects of adenosine transport inhibitors observed in vivo may be mediated indirectly—for example, by effects on the vasculature. (Stroke. 1994;25:2085-2090.)

Key Words • dipyridamole • cerebral ischemia • adenosine • glutamates

See Editorial Comment, page 2089

Extracellular adenosine levels have been shown to increase during reduced energy conditions both in vivo2,3 and in vitro.4 However, the source of the extracellular adenosine is not known. In vivo studies have shown that inhibition of the adenosine transporter increases extracellular adenosine5,6 and attenuates ischemic damage.6,8 These results are consistent with the possibility that adenosine is formed extracellularly (by the breakdown of ATP and other adenine nucleotides released during the ischemic period), and the adenosine transporter serves to remove extracellular adenosine.

However, adenosine transport inhibitors are known to have multiple effects. Specifically, dipyridamole has been shown to potentiate hypoxic pial arteriolar vasodilation9 and increase cerebral blood flow during anoxia.10 Thus, the protective effects of adenosine transport inhibitors could be primarily due to effects on blood flow. Furthermore, in vitro studies using neurons11 and heart cells12 have shown that during reduced energy conditions, adenosine transport inhibitors trap adenosine intracellularly. Under low energy conditions, adenosine transport mechanisms may act to extrude adenosine.

To help address the question of whether adenosine transport inhibitors have a protective effect directly on brain parenchyma, we tested the effect of dipyridamole on the neuronal loss induced by oxygen-glucose deprivation in murine cortical cell cultures.

Materials and Methods

Culture medium was obtained from Gibco as 10 × concentrated stock lacking bicarbonate and glutamine; serum was from HyClone Laboratories Inc; 8-cyclopentyltheophylline (8-CPT) was obtained from Research Biochemicals Incorporated. All other chemicals were obtained from Sigma. Swiss Webster mice from Simonsen Laboratories were handled in accordance with a protocol approved by our institutional animal care committee.

Mixed cortical cell cultures that contained both neuronal and glial cells were prepared from fetal (15 to 16 days' gestation) mice as previously described.13 Dissociated cortical cells were plated on preexisting glial cultures (see below) in Eagle's Minimal Essential Medium (MEM, Earle's salts, supplied glutamine-free) supplemented with 10% heat-inactivated horse serum, 10% fetal bovine serum, 2 mmol/L glutamine, and glucose (total, 21 mmol/L). Cultures were maintained in humidified 95% CO₂, incubators at 37°C. Glial cell replication was inhibited after 5 to 9 days in vitro by 1 to 3 days' exposure to 10 μmol/L cytosine arabinoside. Medium was exchanged twice weekly with a growth medium identical to the plating medium except that it lacked fetal serum. Neocortical glial cultures were prepared from 1- to 3-day-old mice.
and plated on Primaria 24-well culture plates in plating media supplemented with epidermal growth factor (10 ng/ml).

Mixed cortical cultures (14 to 16 days in vitro) were exposed to oxygen-glucose deprivation in a balanced salt solution (BSS) that contained (mmol/L) NaCl 116, KCl 5.4, MgSO4 0.8, Na2HPO4 1, CaCl2 0.9, and phenol red 10 mg/L, at 37°C, aerated with 95% N2 and 5% CO2. Oxygen-glucose deprivation was terminated by washout of the exposure medium with oxygenated BSS containing 5.5 mmol/L glucose. N-methyl-D-aspartate (NMDA) and kainate toxicity experiments were performed at 37°C in BSS containing 5.5 mmol/L glucose.

Samples of the bathing medium from the cell cultures were assayed for glutamate and adenosine by phenylisothiocyanate (PITC) derivatization, high-performance liquid chromatography reverse-phase separation, and ultraviolet detection at a wavelength of 254 nm. Two hundred microliters of buffer was derivatized with 100 μL of PITC, methanol, and triethylamine (2:7:4) and dried under vacuum. These samples were then reconstituted in solvent consisting of 0.14 mol/L sodium acetate, 0.05% triethylamine, and 6% acetonitrile and brought to pH 6.4 with glacial acetic acid. Samples were run in the same solvent, and the column was washed with 60% acetonitrile/40% water between each sample run.

Neuronal cell injury was quantitatively assessed by the measurement of lactate dehydrogenase (LDH), released from damaged or destroyed cells, in the extracellular fluid 24 hours after the experiment. Control experiments have shown that the efflux of LDH induced by hypoxia is proportional to the number of neurons damaged or destroyed. Neuronal injury was also estimated at the same time by examination of cultures with phase-contrast microscopy.

Data were analyzed with one-way ANOVA and the Student-Newman-Keuls multiple comparisons test.

Results

Glutamate is known to be a mediator of ischemic damage in many systems, and attenuation of its release is a potential action of adenosine. Therefore, we studied the time course of glutamate and adenosine release during oxygen-glucose deprivation and compared those results with neuronal cell death measured by LDH release 24 hours later (Fig 1). Glutamate did not accumulate in the bathing medium after a sham wash, and adenosine increased only slightly. Extracellular adenosine levels became elevated after 30 minutes of oxygen-glucose deprivation. Increases in extracellular glutamate and neuronal cell death were detected after 45 minutes of oxygen-glucose deprivation.

Adenosine A1 receptor agonists can attenuate hypoxia-ischemia–induced glutamate release40 and neuronal death, whereas A1 receptor antagonists can potentiate damage. Consistent with these prior studies, addition of 10 μmol/L 8-CPT, a selective adenosine A1 receptor antagonist, during oxygen-glucose deprivation produced increases in glutamate release, adenosine release, and subsequent cell death (Fig 2). In this and the following experiment, a 45-minute duration of oxygen-glucose deprivation was chosen because it caused submaximal glutamate release and cell death, allowing for modulation by adenosine.

Surprisingly, addition of the adenosine transport inhibitor dipyridamole at 1 to 100 μmol/L concentrations during oxygen-glucose deprivation produced a concentration-dependent increase in glutamate release and neuronal death (Fig 3). Study of the 100 μmol/L concentration was confounded by mild intrinsic drug neurotoxicity under normoxic conditions (approximately 20% neuronal cell death). However, 10 μmol/L dipyridamole, which lacked intrinsic cytotoxicity, also increased glutamate release and neuronal death. This unexpected result was accompanied by an equally unexpected downward effect on the accumulation of extracellular adenosine (Fig 3).

The ability of 10 μmol/L dipyridamole to attenuate the buildup of extracellular adenosine induced by oxygen-glucose deprivation did not extend to the adenosine increase induced by the direct stimulation of glutamate receptors. Exposure of cultures to 10 μmol/L NMDA or 300 μmol/L kainate (the latter in the presence of 10 μmol/L MK-801 to block NMDA receptors) for 45 minutes produced an increase in extracellular adenosine, albeit less than that produced by oxygen-glucose deprivation, as well as some neuronal death by the next

![Graph](https://via.placeholder.com/150)
FIG 2. Effects of adenosine A1 receptor antagonist 8-cyclopentyltheophylline (8-CPT) (10 μmol/L) during oxygen-glucose deprivation. Experiments were performed using 45 minutes of oxygen-glucose deprivation, as this was a time that induced intermediate levels of damage. Glutamate (A) and adenosine (B) levels were measured at the end of the 45-minute period. C, Lactate dehydrogenase (LDH) levels were measured 24 hours later (mean±SEM; n=19 culture wells per condition). *P<.05 compared with sham wash; †P<.05 compared with control oxygen-glucose deprivation (−02/Glc with no drug).

day (Fig 4). Addition of 10 μmol/L dipyridamole to the exposure medium did not reduce extracellular adenosine or increase neuronal death in either paradigm. In fact, dipyridamole increased the adenosine accumulation and slightly decreased the neuronal death induced by kainate (Fig 4).

Discussion

Present observations confirm and extend previous work that suggested that hypoxia in vitro, or ischemia in vivo, produces an increase in extracellular adenosine that acts on brain parenchyma to improve neuronal survival. In part, this neuroprotective effect is likely due to the ability of adenosine to reduce glutamate release from nerve terminals,2425 which leads to a reduction in excitotoxic neuronal death.17-19 Glutamate efflux during oxygen-glucose deprivation in our system appears to be mediated substantially by Ca²⁺-dependent vesicular release (ie, tetanus toxin–sensitive), and adenosine can attenuate Ca²⁺-dependent glutamate release.2425 While the mechanisms responsible for ischemic glutamate efflux in vivo have not been fully clarified,2728 adenosine agonists reduce this efflux and improve neuronal survival.2029 In addition, adenosine may have other effects on neurons (for example, membrane hyperpolarization30) that could contribute to neuroprotection.

Our finding that dipyridamole decreases extracellular adenosine during oxygen-glucose deprivation argues against transport-mediated uptake as an important factor involved in removing extracellular adenosine. Rather, our data support the alternative idea that dipyridamole-sensitive adenosine transporters serve to export adenosine during reduced energy conditions. The observed effective concentration of dipyridamole, 10 μmol/L, is higher than expected from prior studies with synaptosomes31 but is consistent with studies with intact neuronal systems.3233 Interpretation of the effect of higher (100 μmol/L) concentrations of drug was confounded by the appearance of intrinsic neurotoxicity.

The effect of 10 μmol/L dipyridamole to reduce adenosine accumulation induced by oxygen-glucose deprivation contrasts with its effect to increase adenosine accumulation induced by kainate exposure. The latter observation does fit the transporter-mediated uptake scenario and is consistent with the prior observation of Craig and White,32 who found that dipyridamole increases the levels of extracellular adenosine induced by application of excitatory amino acids to rat cortical slices.

Adenosine transport occurs via both facilitated diffusion and Na⁺-dependent transport.34 At the concentration of interest (10 μmol/L), dipyridamole blocks both facilitated diffusion and Na⁺-dependent transport.35 We chose not to study the selective adenosine transport inhibitor nitrobenzylthioinosine because it blocks only a subset of adenosine transporters.34 Dipyridamole does not cross the blood-brain barrier,36 so the less potent but more permeable adenosine transport inhibitor pro-

Fig 3. Effects of adenosine transport inhibitor dipyridamole (Dipyr) during oxygen-glucose deprivation. Concentrations are in micromoles per liter. Experiments were performed with 45 minutes of oxygen-glucose deprivation. Glutamate (A) and adenosine (B) levels were measured at the end of the 45-minute period. C, Lactate dehydrogenase (LDH) levels were measured 24 hours later (mean±SEM; n=8 to 24 culture wells per condition). *P<.05 compared with sham wash; †P<.05 compared with control oxygen-glucose deprivation (−02/Glc with no drug).
pentofylline is sometimes used in animal studies of brain adenosine compartmentalization. However, propentofylline also has a confounding ability to block adenosine A1 receptors. In preliminary experiments, we found that propentofylline greatly increased oxygen-glucose deprivation–induced neuronal death (control injury, 13±7%; 100 μmol/L propentofylline, 59±8% [mean±SEM; n=8]).

While adenosine transporters normally act to take up adenosine from the extracellular fluid, they may operate in reverse during reduced energy conditions. An increase in ATP breakdown, which leads to an increase in intracellular adenosine levels, may drive the facilitated diffusion transporter to extrude adenosine. Loss of membrane pumps may permit intracellular Na+ to accumulate enough to drive the Na+-dependent transporter to extrude adenosine. Thus, the observed effects of dipyriramole in oxygen-glucose deprivation may be explained by interference with adenosine extrusion, which leads to reduced extracellular adenosine, increased glutamate release, and increased excitotoxic neuronal death. Our observations do not permit any conclusions regarding whether neurons or glia play a larger role in this postulated adenosine export.

The ability of dipyriramole to decrease oxygen-glucose deprivation–induced extracellular adenosine levels and increase neuronal death contrasts with the protective effects observed with the putative adenosine transport inhibitors propentofylline and vinpocetine in animal ischemia models. Furthermore, in such in vivo models propentofylline and dipyriramole increase cerebral interstitial adenosine levels. While further studies will be required to delineate the basis for these differences, possible factors may include the large extracellular space in culture (which makes export of adenosine more likely in vitro than in vivo), effects on sites other than the adenosine transporter, or the contribution of cell types found in vivo but not present in our cell cultures.

In particular, endothelial cells— not present in our cultures— may contribute importantly to the removal of extracellular adenosine in vivo. Inhibition of endothelial cell adenosine uptake may explain the ability of dipyriramole to increase brain extracellular adenosine during ischemia, as dipyriramole crosses the blood-brain barrier poorly. If inhibition of adenosine transport in endothelial cells during ischemia is indeed beneficial, whereas inhibition of adenosine transport in brain parenchyma is deleterious, transport inhibitors that do not cross the blood-brain barrier could be better neuroprotectants than inhibitors that do cross the barrier. Consistent with this idea, a comparison between dipyriramole and a barrier-permeable transport inhibitor, solufrazine, found that dipyriramole increased cerebral interstitial fluid adenosine levels during hypoxia, whereas solufrazine decreased adenosine levels.

Acknowledgments

This study was supported by National Institute of Neurological Diseases and Stroke grant NS-26907 (Dr Choi). The authors thank Dr Jeffrey Gidday for helpful discussion.

References


Editorial Comment

While studies have shown that inhibition of the adenosine transporter increases extracellular levels of adenosine and protects the brain from ischemic damage, the precise source of this protective action is not clear. The goal of the present studies by Lobner and Choi was to determine the effect of an adenosine transport inhibitor (dipyridamole) on neuronal loss induced by oxygen-glucose deprivation in vitro. Using cortical cultures containing both neurons and glial cells, these investigators measured the extracellular concentration of adenosine and glutamate after oxygen-glucose deprivation and measured neuronal cell death via morphological examination and measurement of lactate dehydrogenase. Cultures exposed to oxygen-glucose deprivation exhibited an increase in extracellular levels of adenosine and glutamate that was followed by neuronal cell death. Addition of an A1 receptor antagonist enhanced glutamate release and neuronal damage. Addition of dipyridamole decreased extracellular adenosine but increased extracellular glutamate and neuronal death.

While the results of these studies are consistent with the concept that adenosine is neuroprotective, the findings with dipyridamole were surprising when compared with studies that have shown a protective effect of adenosine transport inhibitors. It is possible that discrepancies between the findings of the present studies and those of studies published previously may be related to differences between in vivo and in vitro methodologies. The present studies examined the effects of adenosine and an adenosine transport inhibitor on specific cell types (neurons and glia), while other studies have used the whole-organ approach to examine the protective effects of adenosine and adenosine transport inhibitors.

William G. Mayhan, PhD, Guest Editor
Department of Physiology and Biophysics
University of Nebraska Medical Center
Omaha, Neb
Dipyridamole increases oxygen-glucose deprivation-induced injury in cortical cell culture.
D Lobner and D W Choi

*Stroke*. 1994;25:2085-2089
doi: 10.1161/01.STR.25.10.2085
*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1994 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://stroke.ahajournals.org/content/25/10/2085