Fructose-1,6-Bisphosphate Stabilizes Brain Intracellular Calcium During Hypoxia in Rats

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Background and Purpose: Exogenously administered fructose-1,6-bisphosphate reduces neuronal injury from hypoxic or ischemic brain insults. To test the hypothesis that fructose-1,6-bisphosphate prevents changes in intracellular calcium ([Ca$^{2+}$]), intracellular pH (pH$_i$), and high-energy phosphate levels, we measured [Ca$^{2+}$], intracellular pH (pH$_i$), and adenosine triphosphate in cultured rat cortical astrocytes and cortical brain slices during hypoxia.

Methods: The fluorescent indicators fura-2 and bis-carboxyethyl-carboxyfluorescein were used to simultaneously measure [Ca$^{2+}$]$_i$ and pH$_i$ with a fluorometer.

Results: Exposure to hypoxia (95% N$_2$, 5% CO$_2$) or 100 &micro;m sodium cyanide produced transient increases in [Ca$^{2+}$]$_i$ in astrocytes and sustained increases in [Ca$^{2+}$]$_i$ in brain slices. Adenosine triphosphate levels fell in slices exposed to hypoxia or cyanide. Fructose-1,6-bisphosphate (3.5 mM) blocked increases in [Ca$^{2+}$]$_i$ and prevented depletion of adenosine triphosphate. Fructose-1,6-bisphosphate also partially prevented adenosine triphosphate depletion in brain slices incubated in glucose-free medium. Iodoacetate (a specific inhibitor of glycolysis) elevated [Ca$^{2+}$]$_i$ and partially prevented these actions of fructose-1,6-bisphosphate. Changes in pH$_i$ during hypoxia were not affected by fructose-1,6-bisphosphate.

Conclusions: Fructose-1,6-bisphosphate supports adenosine triphosphate production via stimulation of glycolysis and results in the maintenance of normal [Ca$^{2+}$]$_i$ during hypoxia or hypoglycemia. (Stroke 1992;23:1617-1622)

KEY WORDS • astrocytes • brain injuries • calcium • fructose-1,6-bisphosphate

Treatment of animals with fructose-1,6-bisphosphate (FBP) reduces brain injury during global cerebral hypoxia and ischemia,1 insulin-induced hypoglycemia,2 and hemorrhagic or cardiogenic shock.3,4 FBP also reduces damage to ischemic kidneys5 and prevents adverse biochemical and histological changes in hypoxic myocardium.6 The mechanism responsible for these protective effects of FBP is unclear.

Although it is a phosphorylated sugar, FBP crosses the blood–brain barrier and enters neurons.7 Possible intracellular actions of FBP during hypoxia or ischemia include 1) stimulation of carbohydrate metabolism through activation of phosphofructokinase8,9; 2) increased carbon flux through the pentose phosphate pathway (G. Gregory, J. Kelleher, T.Y. Chan, and P. Chan, unpublished observations); 3) direct glycolytic metabolism of FBP, with resulting ATP production7; and 4) prevention of oxygen-derived free radical injury.10 Another possible mechanism of protection, suggested to occur in the hypoxic myocardium11 and in ethanol-exposed baby hamster kidney cells,12 is prevention of the cytotoxic effects of increased intracellular Ca$^{2+}$ (Ca$^{2+}$).

We hypothesize that prevention of elevated [Ca$^{2+}$]$_i$ may be a plausible mechanism for the protective effect of FBP because elevations in [Ca$^{2+}$]$_i$ may play a central role in brain injuries caused by hypoxic or ischemic insults.13 Causes of elevated [Ca$^{2+}$]$_i$ from these insults include membrane depolarization from failure of cellular energy production, excitatory neurotransmitter activation of Ca$^{2+}$ channels,14 and release of Ca$^{2+}$ from mitochondria or endoplasmic reticulum.13 Based on previous studies suggesting direct metabolism of FBP or stimulation of glycolysis, prevention of energy failure seems one of the more plausible of these mechanisms.

In addition, recent work has shown that 3.5 mM FBP prevents ATP depletion during hypoxia in astrocytes and increases their glutamate utilization.15 We therefore wished to test the hypothesis that 3.5 mM FBP prevents elevation in [Ca$^{2+}$]$_i$ in hypoxic astrocytes and brain slices and prevents ATP depletion in brain slices.

Stimulation of carbohydrate metabolism by FBP may lower intracellular pH (pH$_i$). Although low pH$_i$ may exacerbate injury from hypoxia,10 recent evidence suggests that it may actually attenuate glutamate neurotoxicity in cortical neuronal cultures.17 Therefore, we also measured pH$_i$ during hypoxia in FBP-treated brain slices and astrocytes.

Living cortical brain slices and cultured astrocytes were chosen as models to study cerebral hypoxia. Astrocytes were examined because they are known to be relatively tolerant of hypoxia compared with neurons and because FBP has recently been shown to prevent...
ATP depletion during hypoxia. In both tissues we have measured [Ca\textsuperscript{2+}], and pH, during hypoxia, simulated anoxia with cyanide, and blockade of glycolysis with iodoacetate (an inhibitor of the glycolytic enzyme glyceraldehyde phosphate dehydrogenase) and after glucose deprivation. These responses were compared with those seen when 3.5 mM FBP was present in the medium.

**Materials and Methods**

**Brain Slice Preparation**

Cortical brain slices were prepared from adult Sprague-Dawley rats. After rapid decapitation and dissection, 5×8-mm portions of cortex were placed in chilled (3–5°C) buffered salt solution (Earle's balanced salt solution with 5 mM glucose and 25 mM NaHCO\textsubscript{3} bubbled with 95% O\textsubscript{2}/5% CO\textsubscript{2}, pH 7.4). The brain pieces were then attached to a mounting block with cyanoacrylate cement, and 175–250-μm slices were prepared with a vibroslicer (Campden Instruments, Cambridge, England). The slices were transferred to room-temperature buffered salt solution and continuously bubbled with 95% O\textsubscript{2}/5% CO\textsubscript{2}. Fura-2 acetoxy-methyl ester and 2',7'-bis-(2-carboxyethyl)-5-(and -6) carboxyfluorescein acetoxyethyl ester (BCECF) (Molecular Probes, Eugene, Ore.) were both added (from a 1-mM stock solution in dimethyl sulfoxide) to achieve final concentrations of 3–5 μM. For both dyes, cell loading was found to be facilitated by allowing at least 1 hour of incubation. The slices were then transferred to petri dishes filled with buffered salt solution and washed for 5 minutes. The slices were mounted on a mesh baffle and fitted into a cuvette filled with O\textsubscript{2}/CO\textsubscript{2} equilibrated buffer. A cap, fitted with stainless steel inlet and outlet tubing to perfuse the slice with gas equilibrated buffer, was used to seal the cuvette. Fluorescence measurements were then made in a Hitachi Instruments F-2000 fluorometer (Tokyo) with samples positioned so that excitation light fell within the confines of the tissue. The cuvette holder and perfusing solution were both temperature controlled to 37±0.1°C.

**Astrocyte Cultures**

Primary cultures of cerebral astrocytes were prepared by the method of Boorher and Sensenbrenner, as modified by Yu et al., using newborn Sprague-Dawley rats. Astrocytes were loaded with fluorescent dyes by incubation in buffered Earl's salt solution containing 3 μM fura-2 and 3 μM BCECF for 45–180 minutes. The coverslips were then rinsed in a petri dish of buffered salt solution and washed for 5 minutes. The coverslips were then attached to a mounting block with cyanoacrylate cement, and 175–250-μm slices were prepared with a vibroslicer (Campden Instruments, Cambridge, England). The slices were transferred to room-temperature buffered salt solution and continuously bubbled with 95% O\textsubscript{2}/5% CO\textsubscript{2}. Fura-2 acetoxy-methyl ester and 2',7'-bis-(2-carboxyethyl)-5-(and -6) carboxyfluorescein acetoxyethyl ester (BCECF) (Molecular Probes, Eugene, Ore.) were both added (from a 1-mM stock solution in dimethyl sulfoxide) to achieve final concentrations of 3–5 μM. For both dyes, cell loading was found to be facilitated by allowing at least 1 hour of incubation. The slices were then transferred to petri dishes filled with buffered salt solution and washed for 5 minutes. The slices were mounted on a mesh baffle and fitted into a cuvette filled with O\textsubscript{2}/CO\textsubscript{2} equilibrated buffer. A cap, fitted with stainless steel inlet and outlet tubing to perfuse the slice with gas equilibrated buffer, was used to seal the cuvette. Fluorescence measurements were then made in a Hitachi Instruments F-2000 fluorometer (Tokyo) with samples positioned so that excitation light fell within the confines of the tissue. The cuvette holder and perfusing solution were both temperature controlled to 37±0.1°C.

**Calibration of Fura-2 and BCECF**

An in vivo calibration protocol was used for each dye. Calibration for fura-2 was done essentially as described by Jensen and Chiu. Slices were alternatively excited with 340- and 380-nm wavelengths, and fluorescence intensity at 510 nm was recorded every 1.0 second with a Hitachi F-2000 fluorometer. To reduce scattered excitation light, a 505-nm cut-off filter was placed over the emission port.

Measurement of pH, was done simultaneously with that of Ca\textsuperscript{2+}, by interleaving excitation at wavelengths of 440 and 495 nm with those for fura-2. Emission intensity for BCECF was recorded every 1.0 second at 530 nm. Excitation at 495 nm (instead of the usual 500 nm) was used with BCECF, as this greatly reduced the intensity of scattered light detected at 530 nm and improved sensitivity to pH changes. Calibration of BCECF was done by two methods. First, equilibration of intracellular and extracellular hydrogen ion concentrations was achieved by application of the K\textsuperscript{+}/H\textsuperscript{+} exchanger nigericin. At the end of some of the studies, the cells were perfused with a high-K\textsuperscript{+} Earle's solution containing 100 mM KCl and 10 mM nigericin. The pH was calculated from the equation presented by Eisner et al. The second method relied on exposing cells to Earle's solution equilibrated with different levels of Pco\textsubscript{2}. Because the effect of acute changes in Pco\textsubscript{2} on pH in brain is known, changing Pco\textsubscript{2} can be used as a method of pH calibration. Pco\textsubscript{2} values of 0, 40, and 80 mm Hg were used. The latter method does not give an absolute value for pH, but rather a calibration for evaluating changes in pH. The nigericin and Pco\textsubscript{2} methods were used in combination.

**ATP Assays**

ATP was extracted from brain slices by immersing individual slices in 0.5 ml 20-mM Tris buffer (pH 7.75) at 100°C for 10 minutes. After centrifugation, ATP was measured in 0.1 ml of the supernatant with a luciferin/luciferase bioluminescent assay in a Model 2001 luminescence meter. The sensitivity of the assay was approximately 1 pmol ATP, and ATP standards were used for calibration. Total protein was measured with a Coomassie blue assay and results expressed as micro-moles of ATP per milligram of protein.

**Study Protocol**

Before study, brain slices or astrocyte cultures were mounted in a fluorometer cuvette and incubated in oxygenated medium with or without 3.5 mM FBP for 10 minutes. The temperature was held at 37°C. Changes in medium composition, addition of cyanide or iodoacetate, were then made, and continuous measurements of [Ca\textsuperscript{2+}], and pH, were made for 30–40 minutes. ATP levels were determined in brain slices treated identically except that they were not loaded with fluorescent dyes.

**Statistical Methods**

ANOVA was used to compare [Ca\textsuperscript{2+}], ATP, and pH values between different treatment groups. Student's t test, with a correction for multiple comparisons, was applied to examine the difference between a given treatment with and without FBP in the medium. Values are reported as mean±SD, and p<0.05 was required for statistical significance.

**Results**

After 45 minutes of exposure to 3–5 μM fura-2 and BCECF, both brain slices and astrocytes were loaded with sufficient dye to permit calcium and pH measurements. Dye leakage was sufficiently slow to allow measurements to be made for at least 1 hour after removal of tissue from the dye-loading bath.
Intracellular Ca$^{2+}$, pH, and ATP Levels During Hypoxia and Inhibition of Glycolysis With Iodoacetate

[$\text{Ca}^{2+}$]$_i$ was $140\pm50$ nM ($n=29$) in cortical brain slices and $160\pm35$ nM in astrocytes ($n=27$) maintained in oxygenated buffered salt solution containing 5.5 mM glucose. Exposure to hypoxic buffer medium (equilibrated with 95% N$_2$/5% CO$_2$, P$_{O_2}<5$ mm Hg) resulted in a mean increase in [$\text{Ca}^{2+}$]$_i$ of $950\pm210$ nM. A typical trace is shown in Figure 1. In astrocytes, this elevation in [$\text{Ca}^{2+}$]$_i$ persisted for only 10–20 minutes, but in brain slices, [$\text{Ca}^{2+}$]$_i$ did not return to control levels during 45 minutes of observation. Cyanide (100 μM), a specific blocker of mitochondrial oxidative metabolism, also elevated [$\text{Ca}^{2+}$]$_i$ in both astrocytes and brain slices (Figure 2). When added to hypoxic slices, cyanide often caused a further increase in Ca$^{2+}$. This was presumably due to incomplete removal of O$_2$ from the incubation medium. Blockade of glycolysis with iodoacetate caused large increases in [$\text{Ca}^{2+}$], similar to those elicited by hypoxia or cyanide in both brain slices and astrocytes (Figure 3). No recovery of [$\text{Ca}^{2+}$]$_i$ was seen in either tissue. Removal of glucose from the perfusion medium also resulted in increases in [$\text{Ca}^{2+}$]$_i$ in brain slices (Figure 3).

ATP in control brain slices was approximately 114 nmol/mg protein or approximately 2.5 mmol/kg wet wt (Table 1). After 10 minutes' exposure to cyanide, ATP declined to about 4 nmol/mg protein (4% of control). Hypoxia produced a similar fall in ATP levels (5±1.0 nmol ATP/mg protein). Iodoacetate treatment, which blocks glycolysis but leaves mitochondrial oxidative capacity intact, resulted in a 68±10% decrease in ATP levels in brain slices. Perfusion of tissue in glucose-free medium resulted in a 95% drop in ATP levels.

Intracellular pH declined by approximately 0.35 units in brain slices exposed to hypoxia or cyanide (Table 2). In astrocytes, pH fell by about the same amount. These changes were statistically significant ($p<0.05$). In contrast, iodoacetate produced alkalinization of pH, by about 0.15 units.

Effects of FBP Treatment

Addition of 3.5 mM FBP greatly reduced the increase in Ca$^{2+}$, that resulted from exposure to cyanide or hypoxia in both brain slices and astrocytes (Figure 4). In
TABLE 1. ATP Levels (10⁻³ mol ATP·mg protein⁻¹) in 175–200-μm-Thick Rat Cortical Brain Slices

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Without FBP</th>
<th>With 3.5 mM FBP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>114±10</td>
<td>129±15</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>5.1±1.0</td>
<td>45±8</td>
</tr>
<tr>
<td>Cyanide</td>
<td>4.3±2.2</td>
<td>32±10</td>
</tr>
<tr>
<td>Iodoacetate</td>
<td>36±8</td>
<td>18±5</td>
</tr>
<tr>
<td>Cyanide+iodoacetate</td>
<td>5.4±3.4</td>
<td>3.2±2.7</td>
</tr>
<tr>
<td>Glucose-free medium</td>
<td>5.9±1.0</td>
<td>29±5</td>
</tr>
</tbody>
</table>

Slices were incubated in artificial cerebrospinal fluid (CSF) with and without 3.5 mM fructose-1,6-biphosphate (FBP), with 100 μM NaCN, 3.5 mM iodoacetate, or only artificial CSF medium with 5.5 mM glucose (Control).

*Statistically different from same treatment without FBP by two-tailed t test (p<0.05).

FBP-treated brain slices, the increase in [Ca²⁺], caused by blockade of mitochondrial oxidative ATP production with cyanide was only 36±12% of that seen without FBP. However, as shown in Figure 4, when cyanide and iodoacetate were combined, [Ca²⁺], increased as in untreated slices.

FBP partially prevented depletion of ATP in brain slices exposed to hypoxia or cyanide (Table 1). However, FBP did not prevent a fall in ATP in slices treated with iodoacetate. Similarly, FBP was ineffective in preventing ATP depletion when both iodoacetate and cyanide were present in the medium. In glucose-free medium, FBP treatment resulted in ATP levels that were approximately twice as high as without FBP (p<0.005).

The pH declined by about 0.35 units (from 7.05±0.10 to 6.70±0.13, n=10; p<0.05) upon exposure of brain slices to hypoxia. Similar results were seen when FBP was present in the medium (pH changed from 7.05±0.13 to 6.74±0.10, n=10; difference from control not significant) and when cyanide was used instead of hypoxia. In astrocytes with no FBP present, hypoxia produced a drop in pH of 0.34 units; when 3.5 mM FBP was added, the drop in pH was 0.25 units (not significant). FBP treatment therefore did not change the degree of intracellular acidification caused by hypoxia or cyanide.

TABLE 2. Intracellular pH in 300-μm-Thick Cortical Brain Slices and Monolayer Cultures of Astrocytes During Exposure to Anoxia, Cyanide, or Metabolic Inhibition With Iodoacetate

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Brain slices</th>
<th>Astrocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without FBP</td>
<td>With FBP</td>
</tr>
<tr>
<td></td>
<td>Without FBP</td>
<td>With FBP</td>
</tr>
<tr>
<td>Control</td>
<td>7.05±0.10</td>
<td>7.05±0.07</td>
</tr>
<tr>
<td></td>
<td>7.11±0.13</td>
<td>7.08±0.10</td>
</tr>
<tr>
<td>Anoxia/cyanide</td>
<td>6.70±0.13</td>
<td>6.72±0.10</td>
</tr>
<tr>
<td></td>
<td>6.74±0.17</td>
<td>6.83±0.08</td>
</tr>
<tr>
<td>Iodoacetate</td>
<td>7.20±0.05</td>
<td>7.22±0.08</td>
</tr>
</tbody>
</table>

Values are mean±SD. n, Number of brain slices or astrocytes (values in parentheses).

As pretreatment, 3.5 mM fructose-1,6-biphosphate (FBP) was administered 5–10 minutes before exposure to anoxia, cyanide, or iodoacetate.
vented FBP from sustaining ATP production in the pentose phosphate pathway may be responsible for ATP conditions. This conclusion is based on our findings that preserves ATP in astrocytes exposed to anoxic conditions.15 In astrocytes, increased carbon flux through the presence of cyanide. It has been shown recently that FBP resulted in significantly higher ATP levels in the absence of glucose and the fact that iodoacetate pre-depletes in neuronal tissue.7 Our results suggest, but do not prove, that stimulation of glycolysis during hypoxia may be the mechanism by which FBP promotes sustained ATP generation in the brain under anaerobic conditions. This conclusion is based on our findings that FBP resulted in significantly higher ATP levels in the absence of glucose and the fact that iodoacetate prevented FBP from sustaining ATP production in the presence of cyanide. It has been shown recently that FBP preserves ATP in astrocytes exposed to anoxic conditions.15 In astrocytes, increased carbon flux through the pentose phosphate pathway may be responsible for ATP generation (G.A. Gregory, J.A. Kelleher, and P. Chan, unpublished data). This would also account for the inability of FBP to completely reverse the ATP reduction due to hypoxia.

Reduction in cellular ATP levels appears to correlate directly to elevations in [Ca^{2+}], in both brain slices and astrocytes, but a causal relation has not been demonstrated. ATP depletion leading to cell membrane depolarization with subsequent Ca^{2+} entry through voltage-gated calcium channels has been suggested as a cause of elevation in [Ca^{2+}]. In anoxic or ischemic brain insults (reviewed in reference 13). Maintenance of low [Ca^{2+}] and improved ATP levels during hypoxia or hypoglycemia in the presence of FBP is thus circumstantial evidence that cellular energy failure has not occurred. Elevations in [Ca^{2+}], during hypoxic or ischemic insults may result from several other mechanisms: release of calcium from intracellular stores (e.g., mitochondria, endoplasmic reticula) and activation of ligand-gated calcium channels by excitatory neurotransmitters.13 Cellular energy failure plays a direct role in the first two mechanisms. Because cyanide and iodoacetate together (i.e., when both glycolytic and oxidative ATP production is halted) caused elevations in Ca^{2+}, even with FBP treatment, this is evidence that energy failure is most likely the cause for elevated [Ca^{2+}], in our preparations.

We observed that while hypoxia produces a transient increase in [Ca^{2+}], in astrocytes, it produces a sustained increase in brain slices. Although the mechanisms responsible for the restoration of normal [Ca^{2+}], in anoxic astrocytes were not studied, it could be due to induction of increased anaerobic ATP production and subsequent recovery of the energy-requiring mechanisms that regulate cytosolic calcium concentrations. Astrocytes are tolerant of hypoxia,15 but the contribution of [Ca^{2+}] regulation to anoxic survival has not been studied.

In addition to preservation of ATP necessary for ionic regulation, it is possible that FBP may prevent increases in [Ca^{2+}], by other mechanisms as well. These include chelation of Ca^{2+}, (although ionized calcium in the perfusion medium was unchanged by FBP) or blockade of calcium entry through effects on cell membrane calcium channels or at sites of intracellular sequestration. We have not explored either of these possibilities.

Changes in pH, during hypoxic insults were not affected by FBP treatment; thus, buffering of hypoxia-induced changes in pH by FBP seems an unlikely mechanism for the protective effects of FBP. It should be noted that the changes in pH seen in this study were relatively small compared with changes seen in hypoxic or ischemic brain in vivo, probably because of diffusion and dilution of metabolic acids into the medium rather than accumulation in solid tissue. As expected, cyanide and hypoxia caused acidification of the intracellular compartment in both astrocytes and brain slices. Iodoacetate caused intracellular alkalosis, presumably because of reduced glycolytic flux. The combination of both cyanide and iodoacetate produced an even more profound alkalization, which may be due to the nearly complete conversion of phosphocreatine to ATP (which consumes protons) or from equilibration of intracellular and extracellular pH caused by massive cell injury.

Our results could mean that FBP may be most useful as a brain protectant when present at the time of insult.

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**Figure 4.** Traces showing effects of 100 μM cyanide and 3.5 mM iodoacetate on intracellular [Ca^{2+}] in a brain slice and a culture of astrocytes pretreated with 3.5 mM FBP for 10 minutes.

**Discussion**

Our results demonstrate that FBP prevents increased [Ca^{2+}], in brain slices and astrocytes exposed to hypoxia, cyanide, or glucose-free medium. We have also shown that FBP prevents ATP depletion in brain slices during these insults. These findings may explain the protective effects of FBP in preventing brain injury during hypoglycemia, hypoxia, or circulatory failure previously noted by other investigators.1-3 Our conclusions are consistent with the hypothesis that FBP is protective by supporting energy metabolism and preventing ATP depletion in neuronal tissue.7 Our results suggest, but do not prove, that stimulation of glycolysis during hypoxia may be the mechanism by which FBP promotes sustained ATP generation in the brain under anaerobic conditions. This conclusion is based on our findings that FBP resulted in significantly higher ATP levels in the absence of glucose and the fact that iodoacetate prevented FBP from sustaining ATP production in the presence of cyanide. It has been shown recently that FBP preserves ATP in astrocytes exposed to anoxic conditions. In astrocytes, increased carbon flux through the pentose phosphate pathway may be responsible for ATP generation (G.A. Gregory, J.A. Kelleher, and P. Chan, unpublished data). This would also account for the inability of FBP to completely reverse the ATP reduction due to hypoxia.
This is because FBP's mechanism of protection may involve metabolic support of hypoxic or ischemic tissue. However, FBP may be of use in salvaging moderately injured tissue (e.g., in the penumbra of an infarct) that would otherwise suffer energy failure and elevations in [Ca\(^{2+}\)].

We conclude that 3.5 mM FBP prevents elevations in [Ca\(^{2+}\)], during hypoxia in cortical brain slices and cultured astrocytes and that maintenance of ATP may be the proximate cause of this effect.

Acknowledgments

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References


Editorial Comment

A large number of agents have been shown to protect neurons from ischemic or hypoxic injury. There is evidence that a number of different mechanisms may account for such protective effects. Perhaps the best studied mechanisms involve inhibition of NMDA receptors or scavenging of radicals. Bikler and Kelleher, in the study reported above, showed that the protective effect of fructose-1,6-bisphosphate on neurons and astrocytes is mediated by acceleration of glycolysis, improvement in ATP stores, and inhibition of accumulation of calcium ions.

It is very important to identify the mechanisms by which different agents exert their protective effects. One might reasonably expect that agents that have different mechanisms of action might have additive effects. By combining several such agents with independent mechanisms of action, it would be expected that the protective effects would be maximized.

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