Neuroprotective Activity of Fructose-1,6-Bisphosphate Following Transient Forebrain Ischemia in the Mongolian Gerbil

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ABSTRACT—We examined the protective activity of fructose-1,6-bisphosphate (FBP) on mortality and delayed hippocampal cell death induced by transient cerebral ischemia in the Mongolian gerbil. Forebrain ischemia was produced by bilaterally occluding the common carotid arteries for 15 min using microaneurysm clips; then the blood supply to the brain was restored. The number of survivors was counted for 8 days, and the histopathological damage in the CAI region of the hippocampus was scored according to the semiquantitative scale of Rudolphi and colleagues. When injected 15 min before the ischemic insult, FBP (100 and 333 mg/kg, i.v.) significantly reduced the rate of mortality during the 8-day observation period. Equivalent doses of fructose and fructose monophosphate did not improve survival, and neither did low doses (33 mg/kg) of FBP. FBP also produced a significant degree of protection against the CAI pyramidal cell loss in comparison with its vehicle (distilled water). Conversely, when we administered the compound, at the same dose, 15 min after the release of the arterial occlusion, we observed neither a significant reduction of mortality nor significant protection against hippocampal CAI pyramidal cell loss. These results suggest that FBP possesses salutary properties against the damages induced by transient cerebral ischemia, although they are evident only when the compound is administered before the resolution of the ischemic injury.

Keywords: Cerebral ischemia (transient, gerbil), Neuronal death (delayed), Fructose-1,6-bisphosphate

Fructose-1,6-bisphosphate (FBP), a high energy intermediate of glycolysis, has been shown to exert a protective action against the ischemia-induced damage to a variety of different tissues. It has been reported to reduce the tissue damage associated with cardiac arrest (1), myocardial ischemia (2) and myocardial infarct (3). Didlake and colleagues (4) observed a substantial prevention of the ischemic renal injury in rats treated with FBP. It has also been reported to reduce mortality in dogs subjected to bilateral hind limb tourniquet ischemia (5) and to prevent tissue damage and death in rats with an occlusion of the superior mesenteric artery followed by recirculation (6). In addition, FBP seems to exert a significant protective action in a variety of shock states, reducing mortality and attenuating organ damage (7–10). Recently, Gregory and colleagues (11) described a protective effect possessed by FBP in combination with glucose against hypoxic injury on primary cultures of cortical astrocytes. FBP was also demonstrated to possess salutary properties in an experimental in vivo model of ischemic/hypoxic brain injury in the rabbit (12).

Bearing in mind that following a transient interruption of the blood flow to the forebrain, a selective neuronal death is observed in the hippocampus and, in particular, that this damage presents a particularly slow development, being evident several days after the resolution of the ischemic insult (13), we designed the present series of experiments to investigate whether treatment with FBP can ameliorate the delayed damages induced by forebrain ischemia followed by recirculation. In particular, we evaluated the eventual beneficial effects of the compound on the rate of survival and on the delayed death of neurons in the CAI region of the hippocampus, which is known to be a region of selective ischemic vulnerability. We conducted the experiments in the Mongolian gerbil, a rodent in which, due to an unusual cerebral circulation, the bilateral occlusion of common carotid arteries provides a simple and reproducible model of cerebral ischemia.
MATERIALS AND METHODS

Adult, male Mongolian gerbils, weighing 65–75 g, were obtained from S. Morini s.a.s (S. Polo D’Enza, Reggio Emilia, Italy) and maintained on a natural light/dark cycle. They were kept, throughout the study, in conditions of stable temperature (23 ± 1°C) and humidity (55±5%). Gerbils had unlimited access to tap water and were fed with a standard rat laboratory diet (Mil-Morini, S. Morini s.a.s) supplemented with sunflower and barley seeds and with fresh carrots. Food, but not water, was removed 12 hr before the initiation of the experiment.

The first group of gerbils was anesthetized with chloral hydrate (380 mg/kg, i.p.) and injected with 33, 100, 333 mg/kg or 1 g/kg of trisodium FBP (Esafosfina, Biomedica Foscama, Rome, Italy); 148 mg/kg of fructose; 249 mg/kg of fructose-6-phosphate disodium salt; or the same volume (5 ml/kg) of vehicle (distilled water). The drug injection was performed by puncturing with a 28 gauge needle the dorsal vein of the penis. Fifteen minutes after drug or vehicle administration, an anterior midline cervical incision was made, and both common carotid arteries were exposed and isolated from the vagus nerve. Two microaneurysm clips were placed on them so that the blood flow was completely arrested. The blood flow was recirculated in the brain by removing the clips. The patency of the carotid arteries was checked by direct visualization, the skin incision was then sutured and the animals placed in single cages. The second group of animals received the same surgical treatment but was given 333 mg/kg of FBP after the release of the carotid artery occlusion (post-ischemic treatment). The sham operation group consisted of gerbils that underwent all the surgical procedures described above with the exception of artery occlusion. Care was taken to keep the rectal temperature in all animals not below 37°C throughout the surgical procedures and until the recovery from the anesthesia. Animals were kept in single cages and placed in a quiet room for 24 hr in order to avoid post-ischemic seizures triggered by noise. Thereafter, they were transferred to standard cages, with 4 animals in a cage, and unlimited food and water. Animals were continuously observed for 12 hr after recirculation, and then the number of deaths was taken twice a day (9 AM and 6 PM) for 8 days. On day 9, the animals were anesthetized with chloral hydrate (380 mg/kg) and perfused transcardially with 13.3% formaldehyde in 0.15 M NaCl solution. The brains were removed from the skull and placed in 13.3% formaldehyde and maintained at 4°C until the day of processing. At that time, they were rinsed in tap water for 3 hr and then dehydrated with a series of ethanol solutions (from 30% to 100%). The dehydrated samples were placed in xylene for 1 hr and then for 30 min in 50% xylene in paraffin 1 at 37–39°C. Samples were maintained for 12 hr in paraffin 1 and then for a further 12 hr in paraffin 2. Slices containing the hippocampus (7 μ thick) were cut using a microtome and slide-mounted prior to staining with Cresyl violet. The histological analysis was done independently by two of us unaware of the treatment received by the animal, and the results were scored using the scale of Rudolphi et al. (14): 0 = no cell necrosis, 1 = few single cell necrosis, 2 = areas of necrotic cells, 3 = most cells necrotic, 4 = no intact cells.

We determined the histological damage 8 days after recirculation because in a pilot study, we had previously tested the effects of the transient interruption of the carotid blood supply to the brain at different time-points after recirculation. In this experiment, gerbils that underwent the same experimental procedures described above but did not receive any treatment were sacrificed 2, 4 and 8 days after the release of the carotid occlusion, and the pyramidal cell loss in the CA1 subfield of the hippocampus was evaluated using the same protocol previously described.

Survival rates were compared by the Kruskal-Wallis test, whereas the nonparametric Wilcoxon Rank Sum test (independent samples) was used to analyze histopathological data. A probability of less than 0.05 was considered to indicate a significant difference.

RESULTS

The effects of FBP on mortality during the cerebral blood flow interruption

Sham operation produced no mortality in 16 gerbils within the 8-day observation period. During the 15 min of cerebral blood flow interruption, two gerbils out of 20 in the vehicle-treated group and 6 out of 38 in the group with no treatment (animals which will receive FBP or its vehicle at the release of the occlusion) died, showing clear respiratory problems (Fig. 1 and Table 1). Two gerbils in the group treated with 33 mg/kg (N = 12) died, while all the animals administered with FBP at the dose of 100 and 333 mg/kg survived during the 15 min cerebral blood flow interruption (Fig. 1). All gerbils that received 1 g/kg of FBP (N = 8) died either during the ischemic period (six) or within 45 min after recirculation (two). Moreover, this dose of FBP also produced effects in some way toxic, such as sedation and a marked loss in body weight, in the sham-operated animals, and five gerbils out of eight died within 24 hr after the injection. Thus, we did not further use the dose of 1 g/kg of FBP.

The effects of FBP administered before the ischemia on mortality and hippocampal cell death during recirculation
In the vehicle pretreatment group, five out of the eighteen gerbils that survived during the ischemic period died within 48 hr from the release of the occlusion; and by the eighth day, only 9 animals were still alive (Fig. 1). Pretreatment with FBP resulted in a reduction of the mortality and protection of hippocampal CA1 neurons. In fact, no deaths were detected in gerbils that received 333 mg/kg of FBP 15 min before the ischemic insult up to 5 days from the release of the occlusion. Sixteen animals out of 20, however, showed a monolateral ptosis. On the sixth day of recirculation, two animals out of 20 died, whereas the survivors showed a visible loss of fur; and by the eighth day, only 15 animals were alive. Analyzing these data by means of the Kruskal-Wallis test, we found a highly significant (Z=0.00096) enhancement of the survival rate in the FBP-treated group as compared to that in the vehicle-treated one. At the dose of 100 mg/kg, FBP also showed a beneficial effect in comparison to its vehicle in reducing mortality, although it appeared to be of a much lower extent (Z=0.032) in comparison with the higher dose. Conversely, no protection was observed when the compound was administered at the dose of 33 mg/kg (Fig. 1).

The effects of the treatment with FBP (100 and 333 mg/kg, i.v.) 15 min before the cerebral blood flow interruption on the neuronal death in the CA1 region of the hippocampus evaluated 8 days after recirculation are presented in Figs. 2 and 3. Table 2 presents the changes of the overall histological alterations of the different animal groups by using the total scores (sum of individual scores/number). A transient interruption of the carotid blood flow produces profound damages to the pyramidal cells of the CA1 subfield of the hippocampus (Fig. 3). Following 15 min of forebrain ischemia, 87.5% of the

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**Table 1.** Effect of fructose-1,6-bisphosphate (FBP) given post-ischemically on the number of survivors after 15 min of forebrain ischemia followed by recirculation in the Mongolian gerbil

<table>
<thead>
<tr>
<th>Treat.</th>
<th>Days from recirculation</th>
</tr>
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<tbody>
<tr>
<td>Vehicle</td>
<td>0 1 2 3 4 5 6 7 8</td>
</tr>
<tr>
<td>FBP</td>
<td>16/19 15/19 14/19 14/19 13/19 10/19 8/19 8/19 8/19</td>
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Gerbils were intravenously treated 15 min after the release of the carotid artery occlusion. The dose of FBP was 333 mg/kg.

**Table 2.** Intensity of CA1 pyramidal cell damage following 15 min bilateral forebrain ischemia and 8 days recirculation in the Mongolian gerbil

<table>
<thead>
<tr>
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<th>Total group score (sum of individual scores/number)</th>
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<tr>
<td>Pre-ischemic treatmenta</td>
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<tr>
<td>Post-ischemic treatmentb</td>
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The information is given in the form of average total group scores. See the Materials and Methods section for the scoring scale. *animals treated 15 min before the cerebral blood flow interruption, animals treated 15 min after the release of the carotid occlusion. FBP100 = fructose-1,6-bisphosphate, 100 mg/kg, FBP333 = fructose-1,6-bisphosphate, 333 mg/kg.
animals that intravenously received distilled water showed a total pyramidal cell loss. This effect was evident 8 days after the ischemic insult, whereas it was not observable 2 days after. When we sacrificed the gerbils 4 days after recirculation, however, we observed a total score of 2.5. In fact, in this group, 3 out of 8 gerbils received a score of 4, 3 received a score of 2, and 2 were graded as 1 in the Rudolfi’s histological scale.
Intravenous pretreatment with FBP at the dose of 333 mg/kg showed protecting properties against the delayed death of pyramidal cells in the hippocampus; in fact, the percentage of animals showing grade 4 or 3 of histological damage, according to the semiquantitative score used (14), was significantly (P = 0.0206) reduced in comparison to the vehicle-treated animals. However, at the dose of 100 mg/kg, the compound did not elicit any statistically significant (P=0.234) protection (Fig. 2 and Table 2).

To investigate if fructose or fructose monophosphate also shares with FBP the capability of reducing mortality during recirculation after an ischemic insult of the brain, we administered the two compounds at the dose of 148 and 249 mg/kg, respectively, 15 min before the blood flow interruption by the same route as used for FBP. No significant differences were detected in the survival rate in the animals that received fructose or fructose monophosphate 15 min before the ischemic insult as compared to the animals that received distilled water (Fig. 4).

The effects of FBP administered after the release of the carotid artery occlusion

Up to 2 days from the release of the occlusion, we did not observe any deaths of the animals that received FBP (333 mg/kg) post-ischemically. However, by the eighth day, only 42.1% of the gerbils were still alive either in the FBP-group or in the vehicle-treatment group (Table 1). Moreover, FBP elicited no statistically significant protective effects against the ischemia-induced hippocampal neuronal loss as indicated in Fig. 5.

DISCUSSION

The present study demonstrates that FBP exerts a beneficial effect against mortality and degeneration of hippocampal CA1 pyramidal neurons following transient forebrain ischemia. In fact, gerbils treated with 333 mg/kg of FBP 15 min before the ischemic insult to the brain elicited an enhanced rate of survival and a significant reduction in the severity of delayed neuronal loss with respect to animals treated with its vehicle. In contrast, the administration of fructose or fructose monophosphate, at a dose molarly equivalent to 333 mg/kg of FBP, did not produce any significant improvement in survival in this model of cerebral ischemia. Fructose-1,6-bisphosphate, however, demonstrated salutary properties also when given at a dose of 100 mg/kg, but only in reducing mortality, whereas it was not effective in protecting from delayed neuronal loss in the CA1 subfield of the hippocampus. According to this, we should rule out any relation between mortality and delayed pyramidal cell death, although it should be considered that the treatment with FBP at the dose of 100 mg/kg significantly modified the shape of the survival curve (Fig. 1), reducing mortality during the ischemic period and the earlier phase of recirculation, but exerting only a modest protection against the overall mortality after 8 days of recirculation (i.e., when the histological damage was quantified). All these results appear to agree with some previous observations (11, 12, 15, 16). When comparing, however, the results of the present study with others, a couple of factors should be considered. Farias and colleagues (12) reported that in a rabbit model of cerebral ischemia/hypoxia, a bolus injection of 350 mg/kg of FBP followed by a constant infusion for 90 min was able to protect the animals against mortality, changes in EEG and histological damage. In their model, Farias and col-
be speculated that FBP decreases the generation of free radicals by attenuating the depletion of high-energy adenyl nucleotides and their degradation to hypoxanthine. This could also explain why only a pre-ischemic treatment with FBP is effective. Although the role of xanthine oxidase as a major source of free radicals in focal cerebral ischemia has been recently questioned (22), this hypothesis could represent the basis for further investigations.

Another mechanism that should be considered when trying to explain the pharmacological activity of FBP is that the ATP depletion during the blood flow interruption period produces a failure of ATP-dependent pumps which, in turn, results in an accumulation of sodium, calcium, and chloride into the cell, accompanied by a rapid loss of potassium (23). Calcium, in particular, is recognized to play a pivotal role in the pathophysiology of stroke. One of the postulated consequences of calcium influx is the activation of phospholipase C resulting in the breakdown of phospholipids in the cell membrane and liberation of free fatty acids (24). In particular, arachidonic acid seems to increase in neurons in direct correlation with the duration of the ischemic period (25), and this phenomenon appears to be more marked in brain regions known to be particularly sensitive to ischemia/recirculation-induced injury (26). Arachidonic acid is known to produce edema (27), to cause changes in the release and uptake of neurotransmitters such as glutamate and GABA (28, 29) and, once oxygen is again provided by recirculation, is metabolized to prostaglandins, thromboxanes (30, 31) and superoxide (32).

These and other possible mechanisms, such as the reduction of the activation of polymorphonuclear leukocytes entrapped in the cerebral vessels, which may result in the production of chemotactic factors during recirculation (33–35), represents only a speculative attempt to explain the observed effects of the compound and have to be taken as a working hypothesis to initiate investigations into the mode of action of FBP which still must be clarified through intense studies. Indeed, FBP allows gerbils to better tolerate surgery. In fact, no animals died during the surgical procedures when treated with 100 and 333 mg/kg of the compound. On the other hand, in our laboratory, this effect has also been observed in other species and in surgery of different types such as stereotactical surgery in rats (G.R. Trimarchi and R. De Luca, unpublished observation). Furthermore, FBP, but not fructose or fructose-6-phosphate, when pre-ischemically injected at the dose of 333 mg/kg, was able to reduce the delayed neuronal loss in the CA1 subfield of the hippocampus. All these observations, taken together, warrant further investigations of this fascinating compound, which certainly should be pharmacologically characterized through more detailed studies.
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