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## Short Communications

# Fructose-1,6-bisphosphate reduces ATP loss from hypoxic astrocytes

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Hypoxia caused injury and metabolic dysfunction of astrocytes, as indicated by a time-dependent loss of lactate dehydrogenase (LDH) activity and ATP content. The combination of 3.5 mM fructose-1,6-bisphosphate (FBP) and 7.5 mM glucose (GLC) reduced the decrease of ATP and prevented the loss of LDH. These data indicate that the combination of GLC + FBP protects astrocytes from hypoxia. The results also suggest that the maintainance of ATP concentration is the mechanism by which FBP prevents hypoxic injury.

We previously showed that severe hypoxia induces swelling and disruption of cortical astrocytes[3,15] in vitro. The mechanism of these changes is unknown but is probably due in part to a failure of energy metabolism, which would lead to cellular dysfunction and to the influx and accumulation of $Na^+$, $Ca^{2+}$, and water[13]. Addition of 3.5 mM fructose-1,6-bisphosphate (FBP) to the culture medium of astrocytes delayed the increase in cell volume caused by hypoxia when these cells were treated with glucose (GLC) alone[3]. In fact, GLC + FBP more effectively maintained normal cell volumes than any of several compounds tested by Yu et al.[15]. FBP is reported to increase the ATP concentration of normoxic red blood cells in vitro[1,12], but this increase does not appear to be the result of uptake and metabolism of FBP. The objective of the present study was to determine whether adding FBP to the culture medium of astrocytes prevents or reduces the decrease of ATP that occurs during hypoxia.

Primary cultures of astrocytes were grown from the cerebral cortex of newborn Sprague–Dawley rats (Simonsen, Gilroy, CA, U.S.A.) using the method of Yu et al.[14]. The cerebral hemispheres were aseptically removed from the skull and the meninges, olfactory bulbs, basal ganglia, and hippocampus discarded, leaving the neopallium, i.e. the cortex dorsal–lateral to the lateral ventricles. The cleaned neopallium was placed in Eagle's minimum essential tissue culture medium (MEM) that contained 20% fetal calf serum (FCS) (Sterile Systems, Logan, UT, U.S.A.) and cut into approximately 1 mm cubes. The tissues were then disrupted by vortex-mixing for 1 min[2] and passed through two sterile nylon Nitex sieves (L. and S.H. Thompson, Montreal, P.Q., Canada) with pore sizes of 80 $\mu$m (first sieving) and 10 $\mu$m (second sieving). One-thirtieth of each brain cell suspension was placed on a 60-mm Falcon tissue culture dish (Becton Dickenson, Oxnard, CA, U.S.A.) and sufficient fresh culture medium (with 10% FCS) added to bring the volume to 3 ml. The cultures were incubated at 37 °C with 95% air/5% $CO_2$ (v/v) and a humidity of 95%. The MEM was first changed 3 days after the cultures were plated and then twice weekly. After two weeks of incubation, when the cultures had reached confluency, the cells were exposed to 0.25 mM dibutyryl cyclic AMP (Sigma, St. Louis, MO, U.S.A.) to induce morphologic differentiation into cells that resemble and function like mature astrocytes[4]. All studies were done at least 4 weeks after the cultures were plated. At the beginning of each study, the culture medium was replaced with MEM containing 7.5 mM GLC or 7.5 mM GLC + 3.5 mM FBP. The MEM for hypoxia experiments was bubbled with 95% N2/5% $CO_2$ for 5 min before it was added to the cultures. All experimental media contained 10% FCS. Hypoxic cultures were placed in a humidified Modular Incubator Chamber (Billups-Rothenberg, Del Mar, CA, U.S.A.) and the chamber was purged of oxygen for 15 min with 10 l/min of 95% $N_2$/5% $CO_2$, sealed, and placed in an incubator at 37 °C for 6–30 h. (The oxygen concentration inside the chamber was 0 mm Hg after the chamber was sealed[15].) At the end of incubation, the cultures were removed from the chamber, the medium decanted, and the cells rapidly washed 3 times with ice-cold saline before metabolism was stopped with 0.1 N NaOH-30% methanol. We tried several extraction meth-

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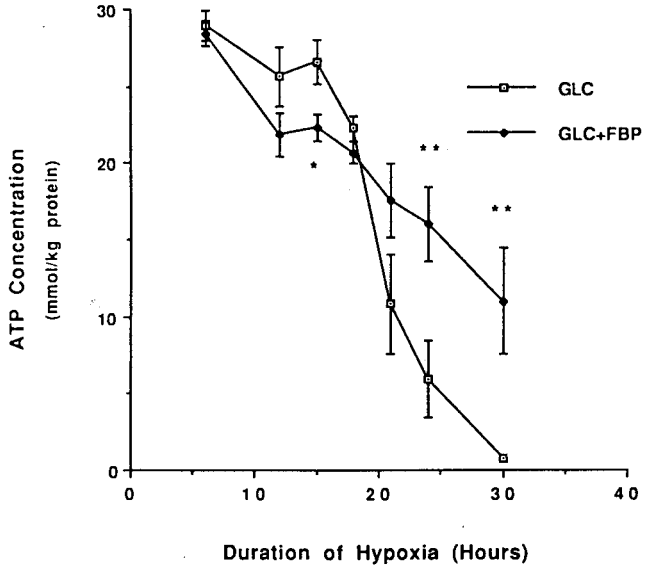


Fig. 1. Effect of FBP on the decrease of ATP in hypoxic astrocytes. Cells were incubated in 95% $N_2$/5% $CO_2$ at 37 °C in the presence of 7.5 mM GLC or 7.5 mM GLC supplemented with 3.5 mM FBP (GLC + FBP). Each point is the mean of 6–9 cultures, with S.E.M. indicated by the vertical brackets. *Significantly different from GLC, $P < 0.05$. **Significantly different from GLC, $P < 0.01$.

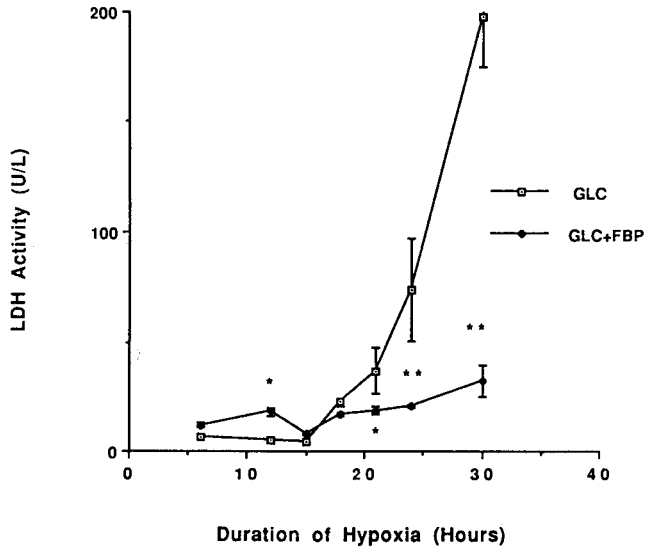


Fig. 2. Effect of FBP on the release of LDH from hypoxic astrocytes. Cells were incubated in the presence of GLC or GLC supplemented with FBP (GLC + FBP). The activity of LDH was determined in the culture media at various intervals of hypoxia. Each point is the mean of 6–9 cultures, with S.E.M. indicated by the vertical brackets. *Significantly different from GLC, $P < 0.05$. **Significantly different from GLC, $P < 0.01$.

ods, including perchloric acid, ethanol, direct lyophilization of the cells, and NaOH-methanol for ATP measurements. The NaOH-methanol method used by Marcy and Welsh[9] was the most reliable and reproducible. ATP was measured using a luciferin-luciferase assay[8], and protein was determined with the Lowry method[7]. The activity of the lactate dehydrogenase (LDH) in the culture media was assayed using a spectrophotometric technique[15]. The GLC concentration of MEM was measured with an enzymatic, fluorometric assay[6]. Data are presented as means ± S.E.M. Differences between GLC and GLC + FBP groups were tested for statistical significance using an unpaired *t*-test.

Under normoxic conditions, the concentration of ATP in astrocytes remained at control levels (30.1 ± 0.6 mmol/kg protein) for 30 h whether or not FBP was added to the culture medium. However, at 21 h of hypoxia ATP levels decreased to 37% of control in cells incubated with GLC alone (Fig. 1). In these cells, ATP fell to 20% and 3% of control at 24 h and 30 h, respectively. By contrast, astrocytes incubated with GLC + FBP maintained significantly higher levels of ATP after 21 h of hypoxia, compared to cells incubated with GLC alone. Thus, at 24 h and 30 h, ATP levels were 53% and 40% of control, respectively, in the presence of FBP.

Twenty-one hours of hypoxia caused an increase in LDH activity in the culture medium of astrocytes incubated with GLC alone (Fig. 2). In these cultures, a marked release of LDH occurred by 24 h and 30 h. In contrast, astrocytes incubated in GLC + FBP showed only a slight increase in LDH in the culture medium. Thus, between 21 h and 30 h, LDH activity was significantly lower in the presence of GLC + FBP, compared to GLC alone.

During hypoxia, the concentration of GLC in the culture medium declined progressively. By 18 h it was 0.5

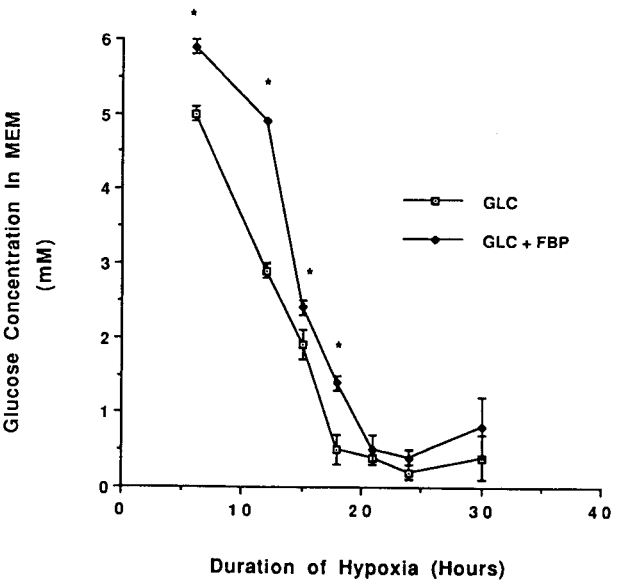


Fig. 3. Effect of FBP on the decline of GLC in the culture medium during hypoxia. Astrocytes were incubated in the presence of GLC or GLC supplemented with FBP (GLC + FBP). The concentration of GLC in the culture medium was determined at various intervals of hypoxia. Each point is the mean of 3 cultures, with S.E.M. indicated by the vertical brackets. *Significantly different from GLC, $P < 0.05$. **Significantly different from GLC, $P < 0.01$.

mM in cultures incubated with GLC alone (Fig. 3). A similar rate of decrease of GLC occurred in the presence of FBP, but the time course was delayed by 2–3 h. Thus, the levels of GLC were significantly higher in FBP-supplemented cultures for the first 18 h of hypoxia.

The major findings of this study were: (1) addition of FBP preserved ATP levels in hypoxic astrocytes; (2) the levels of ATP in astrocytes during hypoxia correlated well with the release of LDH into the culture medium; and (3) the decrease of ATP and release of LDH in cells incubated with GLC alone appeared to occur only after the levels of GLC in the medium fell to 0.5 mM.

The mechanism by which FBP preserves ATP levels is not yet understood. FBP might simply serve as additional substrate for glycolysis[5,10,11], thus delaying the decline of ATP once GLC is depleted from the medium. Since charged molecules like FBP do not readily cross cellular membranes, FBP would first have to undergo hydrolysis to fructose in order to be utilized. However, previous experiments have demonstrated that, unlike addition of FBP, addition of fructose or fructose-6-phosphate did not prevent the release of LDH from hypoxic astrocytes[3]. Thus, it is not likely that FBP protects astrocytes from hypoxia by serving as a glycolytic substrate.

Alternatively, FBP might decrease the rate of energy utilization of astrocytes, thus preserving the limited supply of GLC. Indeed, supplementation with FBP delayed the decline of GLC during hypoxia, however, FBP did not alter the rate of GLC utilization. Further, the delay in the fall of GLC was rather small (2–3 h), in contrast to the delay in the decrease in ATP (9 h). Thus, the preservation of ATP by FBP cannot be explained simply by a decrease in the rate of GLC consumption.

Finally, it is possible that FBP delays the decrease of ATP by augmenting the utilization of other substrates. However, of the amino acids contained in MEM, glutamine is the only one present in a concentration sufficient to serve as substrate[16]. With hypoxic conditions, there is no known pathway to generate ATP from glutamine. Alternatively, FBP might enhance the metabolism of glycogen, which would serve as an intracellular source of substrate. However, compared to the amount of GLC in the culture medium, the amount of glycogen present in astrocytes is too small to significantly alter the total supply of glycosyl units available for glycolysis. Thus, it is not likely that FBP exerts its beneficial effects via utilization of alternative substrates.

In conclusion, FBP protects astrocytes during hypoxia by preserving cellular levels of ATP. In a previous study, astrocytes treated with FBP maintained normal cell volumes despite 30 h hypoxia[3]. The present results also corroborate previous findings that LDH release at 30 h hypoxia was suppressed by supplementation with FBP[3]. Since ATP has fallen to 40% of control levels by this time, it is evident that a decrement in ATP of this magnitude is not sufficient to cause an immediate alteration of cell volume or release of LDH. The mechanism by which FBP preserves ATP levels during hypoxia remains unknown.

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