

Fructose-1,6-Bisphosphate Protects Astrocytes from Hypoxic Damage

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Summary: To determine the effects of glucose and fructose-1,6-bisphosphate (FDP) on hypoxic cell damage, primary cultures of astrocytes were incubated for 18 h in an air-tight chamber that had been flushed with 95% N₂/5% CO₂ for 15 min before it was sealed. Cultures containing 7.5 mM glucose without FDP or FDP without glucose showed evidence of significant cell injury after 18 h of hypoxia (increased lactate dehydrogenase content in the culture medium; cell edema and disruption by phase-contrast microscopy). Cultures exposed to glucose + FDP had normal lactate dehydrogenase concentrations and appeared normal microscopically. Maximal protection of hypoxic cells occurred at 6.0 mM FDP. Lactate concentrations of the culture medium of hypoxic cells increased 2.5 times above normoxic control values when glucose was present, but neither FDP alone nor glucose

+ FDP caused the lactate concentrations to increase further. This implies that anaerobic glycolysis was not increased by adding FDP to the medium. Cell volumes (water space) measured with [¹⁴C]-3-O-methyl-D-glucose were normal with glucose + FDP in the culture medium of hypoxic cells but were significantly larger than normal when glucose alone was present. Increases in cell volume paralleled changes in lactate dehydrogenase in the culture medium. Uptake of [¹⁴C]FDP occurred rapidly in normoxic cells and was maximal after 5 min of incubation. The data indicate that the presence of glucose + FDP in the culture medium protects primary cultures of hypoxic astrocytes from cell damage. **Key Words:** Hypoxia—Astrocytes—Fructose-1,6-bisphosphate—Glucose—Oxygen—Cell injury—Culture.

Fructose-1,6-bisphosphate (FDP) reduces the tissue damage associated with cardiac arrest (Jones et al., 1980), myocardial infarction (Markov et al., 1980; 1986), myocardial ischemia (Marchionni et al., 1985), and renal ischemia (Didlake et al., 1985). It also reduces ischemic damage to skin flaps (Heckler et al., 1984), and it increases the concentrations of ATP, phosphocreatine, and lactate in ischemic muscle (Heckler et al., 1983). We showed that hypoxic FDP-treated rabbits breathed nearly 15 times longer than glucose (glc)-treated rabbits (20.9 ± 4.9 versus 1.4 ± 0.2 min) before respiratory arrest occurred and that we could resuscitate 100% of the FDP-treated, 18% of glc-treated, and 14% of

saline-treated animals, despite the fact that the FDP-treated rabbits were much more acidotic at cardiac arrest (Farias et al., 1986). Thirty minutes after resuscitation, FDP-treated rabbits had normal pupil and eyelid reflexes and responded normally to pain. Rabbits treated with either glc or saline had no response to pain.

To eliminate the multiple factors that may affect the brain in vivo, we determined whether FDP protected intact, primary cultures of cortical astrocytes from hypoxic injury. This model is described in detail in the article by Yu et al. (1989), which demonstrates the time course of hypoxic injury and its multifactorial nature.

MATERIALS AND METHODS

Cell culture

Primary cultures of cerebral astrocytes were prepared by the method of Yu et al. (1986) using newborn Sprague-Dawley rats (Simonsen, Gilroy, CA, U.S.A.). The cerebral hemispheres were removed from the skull aseptically

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Abbreviations used: FCS, fetal calf serum; FDP, fructose-1,6-bisphosphate; fru, fructose; glc, glucose; LDH, lactate dehydrogenase; MEM, modified Eagle's minimum essential tissue culture medium; PFK, phosphofructokinase.

and the meninges, olfactory bulbs, basal ganglia, and hippocampus were discarded, leaving the neopallium, i.e., the cortex dorso-lateral to the lateral ventricle. The cleaned neopallium was placed in modified Eagle's minimum essential tissue culture medium (MEM) (Hertz et al., 1985) that contained 20% fetal calf serum (FCS) (Sterile Systems, Logan, UT, U.S.A.) and cut into ~1 mm cubes. Tissues were disrupted by vortex-mixing for 1 min (Bullaro and Brookman, 1976) and passed through two sterile nylon Nitex sieves (L. and S. H. Thompson, Montreal, P.Q., Canada) with pore sizes of 80 μ m (first sieving) and 10 μ m (second sieving). One-thirtieth of each brain-cell suspension was placed in a 60-mm Falcon tissue culture dish (Becton Dickinson, Oxnard, CA, U.S.A.) and sufficient fresh culture medium (with 10% FCS) added to bring the final volume to 3 ml. Next we incubated the culture at 37°C with 95% air/5% CO₂ (vol/vol) and a humidity of 95%. The medium was first changed 3 days after the cultures were plated and then twice weekly. After 2 weeks of incubation, when the cultures had reached confluency, they were exposed to 0.25 mM dibutyryl cyclic AMP (Sigma, St. Louis, MO, U.S.A.) to induce morphologic differentiation into cells that resemble mature astrocytes (Hertz et al., 1985). All studies were done after the cultures were at least 4 weeks old.

Hypoxia studies

To determine whether several phosphorus-containing intermediates of glycolysis prevented hypoxic damage to astrocytes, we replaced the growth medium with MEM* or MEM + 7.5 mM FDP, fructose (fru), fructose-6-phosphate (fru-6-P), glucose-6-phosphate (glc-6-P), glucose-1,6-bisphosphate (glc-1,6-P), or glyceraldehyde-3-phosphate (gly-3-P), or with glc-free MEM and each of these compounds. All culture medium contained 10% FCS. The cultures were placed in a humidified Modular Incubator Chamber (Billups-Rothenberg, Inc., Del Mar, CA, U.S.A.), and the chamber was purged of oxygen with 95% N₂/5% CO₂ (10 L/min) for 15 min. It then was sealed and placed in an incubator at 37°C for 18 h. The oxygen concentration inside the chamber was 0 mm Hg after it was sealed (for details, see Yu et al., 1989). At the end of incubation, the cultures were removed from the chamber, and the medium was decanted. The cells were rapidly washed three times with ice-cold MEM to remove FCS and debris and were then digested with 1 N NaOH. A dose-dependent study of MEM + FDP (0.0, 0.5, 1.5, 3.0, 4.5, 6.0, and 7.5 mM FDP) was done under similar conditions.

Lactate and lactate dehydrogenase measurement

Lactate dehydrogenase (LDH) was determined by the method of Amador et al. (1963) (Sigma Diagnostics, St. Louis, MO, U.S.A.) (for details, see Yu et al., 1989).

Lactate concentrations were determined by the method of Henry (1968) (Sigma Diagnostics) after adding ice-cold 8% perchloric acid to the culture medium to precipitate the protein, centrifuging the medium at 2,500 r/min for 15 min, and incubating the sample with the reaction medium at 37°C for 15 min. Changes of absorbance were determined with a Hewlett-Packard 8451A Diode Array Spectrophotometer (Hewlett-Packard, Palo Alto, CA, U.S.A.)

* Normal MEM contains 7.5 mM glucose.

at room temperature. LDH and lactate were quantified by standard curves.

Water space measurement with [¹⁴C]-3-O-methyl-D-glucose

Water space was determined by replacing the growth medium with MEM or MEM + 3.5 mM FDP that contained 1 mM [¹⁴C]-3-O-methyl-D-glucose (radioactivity = 1 μ Ci/ml) in the medium (Kletzien et al., 1975; Yu et al., 1989). In addition, the culture medium contained 5% FCS. The cultures were made hypoxic for 18, 24, 30, or 36 h. At the end of incubation, they were removed from the chamber, the medium was decanted, and the cultures were washed three times with cold MEM containing 1 mM phloretin dissolved in 1% ethanol to inhibit the loss of [¹⁴C]-3-O-methyl-D-glucose from cells. Then the cells were digested in 1 N NaOH and samples were used for protein determination (Lowry et al., 1951) and scintillation counting (Beckman LS7000, Beckman Instruments, Palo Alto, CA, U.S.A.).

Uptake of radioactive FDP

Uptake of FDP was determined by incubating normoxic cells at 37°C or at 0°C with MEM + 3.5 mM FDP and 0.5 μ Ci of uniformly labeled [¹⁴C]-fructose-1,6-bisphosphate ([¹⁴C]FDP) (0.5 μ Ci/3 ml; 3.5 mM FDP) (Amersham, Arlington Heights, IL, U.S.A.). After 10, 20, 30, 40, or 60 s or 5, 10, 30, 60, or 120 min of incubation, the medium was rapidly decanted, and the cells were quickly washed three times with ice-cold culture medium. Then they were dissolved in 1 ml of 1 N NaOH, and their protein content and radioactivity were determined by the method described above. [¹⁴C]FDP uptake was calcu-

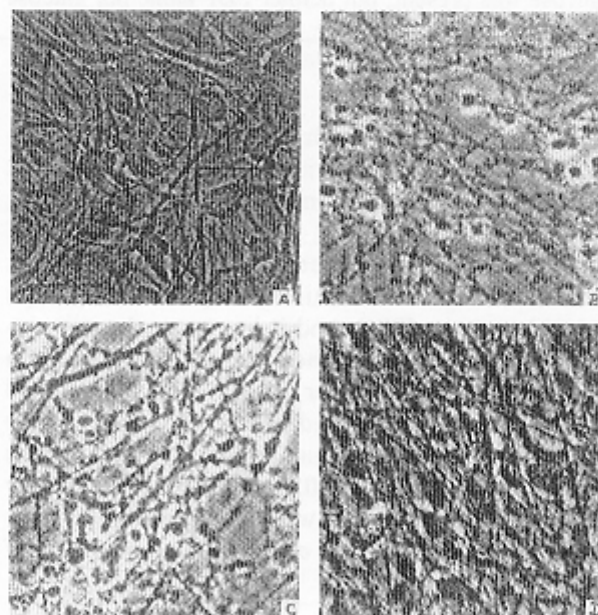


FIG. 1. The effects of glucose + fructose-1,6-bisphosphate (FDP) (A), FDP alone (B), and glucose alone (C) on the microscopic appearance of astrocytes after 18 h of hypoxia (95% N₂ 5% CO₂). D: Photomicrograph of normoxic cells. Hypoxic cells treated with glucose + FDP were not discernibly different from normoxic controls. Photomicrographs of cells treated with glucose alone (C) or FDP alone (B) show edema, vacuolization, and cell dropout.

lated from the amount of radioactivity per milligram of protein and the specific activity of the incubation medium.

Statistics

Data were analyzed by analysis of variance and the Neuman-Keuls test within groups and by unpaired *t* test between groups. Data are presented as means \pm 1 SEM.

RESULTS

After 18 h of hypoxia, the PO_2 , PCO_2 , and pH of the culture medium were 18 ± 0.9 mm Hg, 43.6 ± 1.2 mm Hg, and 6.92 ± 0.04 , respectively. There was no difference among these variables between glc- and glc + FDP-treated cultures. The PCO_2 and pH of similarly treated MEM without cells were 42.5 ± 1.8 mm Hg and 7.30 ± 0.06 respectively. Absence of glc from or the presence of glc or FDP alone in MEM was associated with gross cell damage by phase-contrast microscopy (Fig. 1). Glc + FDP-treated cells appeared normal. After 18 h of hypoxia, cultures without glc or FDP had LDH concentrations of 460.75 ± 15.94 U/L in their medium. Medium containing glc alone had a similar LDH

content (360.93 ± 23.44) and medium with FDP alone had a slightly lower LDH content (280.64 ± 22.83 U/L) (Fig. 2). Addition of Glc + FDP to the medium resulted in similar LDH contents after 18 h of hypoxia to those of normoxic cells [32.26 ± 3.37 U/L (FDP) and 36.84 ± 2.48 U/L (normoxic control)].

Fructose, fru-6-P, glc-6-P, glc-1,6-P, or glyc-3-P (with or without glc) did not protect the cells from hypoxic damage (Fig. 2). Only glc + FDP prevented the rise in LDH and microscopic changes seen with hypoxia. The protein content of the cells was inversely related to the LDH content of the culture medium (Fig. 3). The lactate content of hypoxic glc-containing cultures was 2.5 times that of normoxic controls (Fig. 4), whereas that of glc + FDP-treated cultures was similar to the lactate content of cultures incubated with glc alone.

Adding 0.5–7.5 mM FDP to MEM significantly reduced the LDH content of the medium (Fig. 5) (0.5 mM FDP significantly decreased it; 6.0 mM FDP reduced it to normoxic control levels).

Normoxic astrocytes rapidly took up [^{14}C]FDP at both 37 and 0°C (Fig. 6). After 5 min of incubation, the concentration of [^{14}C]FDP in the cell fraction was 11.67 ± 0.49 nmol/mg protein at 37°C and 4.48 ± 0.49 nmol/mg protein at 0°C ($p < 0.05$).

After 18 and 24 h of incubation, hypoxic glc-

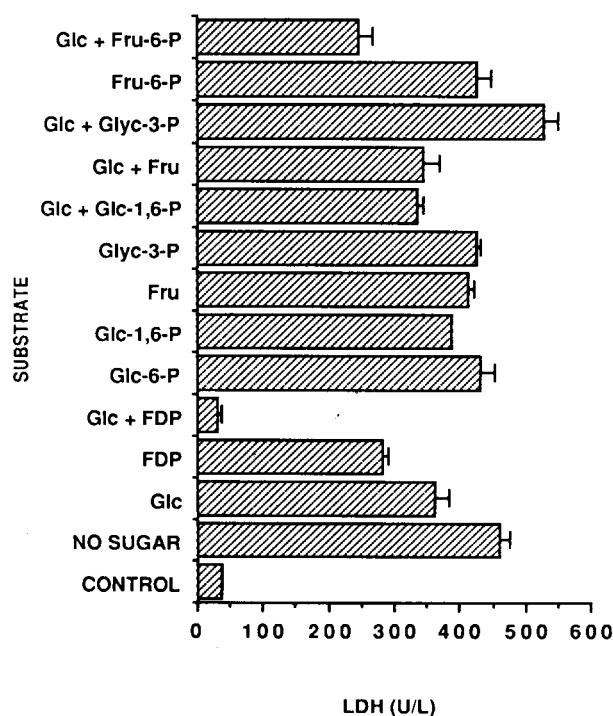


FIG. 2. The effects of fructose (Fru), fructose-1,6-bisphosphate (FDP), glucose-6-phosphate (Glc-6-P), glucose-1,6-bisphosphate (Glc-1,6-P), glyceraldehyde-3-phosphate (Glyc-3-P), fructose-6-phosphate (Fru-6-P), with and without glucose (Glc) on the lactate dehydrogenase (LDH) content of the culture medium after 18 h of hypoxia. Only Glc + FDP prevented increases in LDH. $N =$ six cultures per point. Values are means \pm SEM. All values are significantly different from control (normoxia) and Glc + FDP at $p < 0.001$. Control and Glc + FDP are not different from each other.

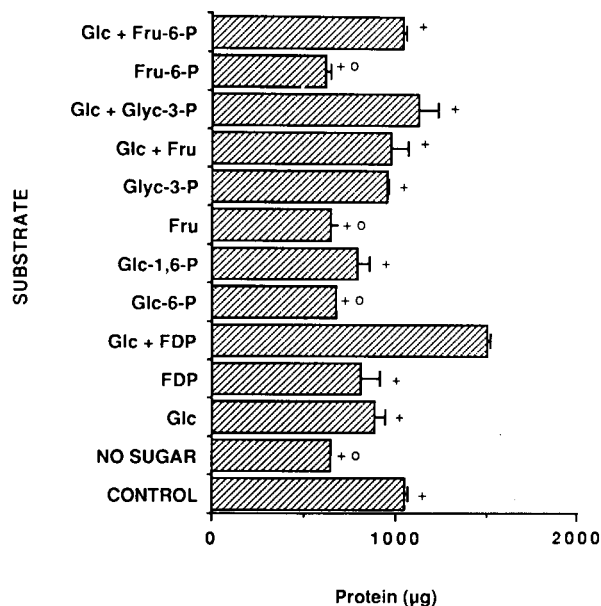


FIG. 3. The effects of FDP on the protein content of hypoxic astrocytes treated with several phosphorus-containing glycolytic intermediates, with and without glucose, after 18 h of hypoxia (see Fig. 2 legend for abbreviations). The protein content is from cells left attached to the culture dish at the end of the 18-h hypoxic period. $N =$ six cultures per point. Values are means \pm SEM. + = $p < 0.001$ versus Glc + FDP; $\circ = p < 0.01$ versus control (normoxia).

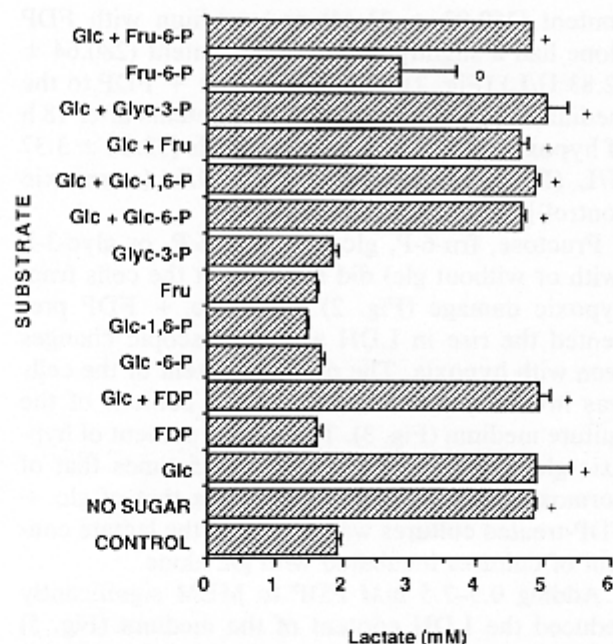


FIG. 4. The effects of FDP on the lactate content of the culture medium of hypoxic astrocytes treated with one of several phosphorus-containing intermediates of glycolysis (see Fig. 2 legend for abbreviations). Hypoxia lasted 18 h. The lactate content of the medium only increased when glucose was present. Glc + FDP did not increase the lactate content of the medium above that with glucose alone. The lactate content of cells with "no sugar" probably increased because they had normal glycogen contents at the start of hypoxia. N = six cultures per point. Values are means \pm SEM. + = $p < 0.001$ versus control (normoxia); ○ = $p < 0.01$ versus control.

treated astrocytes had similar, normal water spaces (cell volumes) to glc + FDP-treated cells (Table 1). After 36 h of hypoxia, the volume of glc-treated cells was 331% the volume after 24 h of hypoxia. Glc + FDP-treated cells, on the other hand, had volumes of $7.62 \pm 1.00 \mu\text{L}$ after 36 h of hypoxia, which is similar to the volume of glc-treated cells after 30 h of hypoxia. The volume of glc + FDP-treated cells was only 45% of that of glc-treated cells after 36 h of hypoxia. The LDH content of the culture medium of glc + FDP-treated cells increased from a normal value of $17.4 \pm 1.7 \text{ U/L}$ to $142.1 \pm 30.7 \text{ U/L}$ after 36 h of hypoxia, whereas the LDH content of glc-treated cultures increased to $429.51 \pm 30.01 \text{ U/L}$ (Table 1).

DISCUSSION

These data indicate that glc + FDP prevents hypoxic damage in cultured astrocytes. Neither glc or FDP alone, nor the phosphate-containing intermediates of glycolysis tested, prevented the loss of LDH from the cells or the microscopic changes induced by hypoxia.

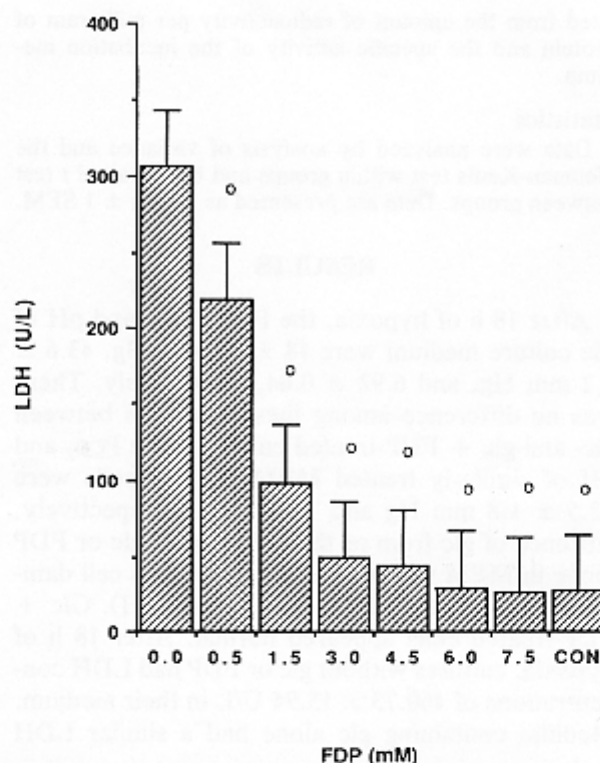


FIG. 5. The effect of increasing concentrations of FDP on the LDH content of the culture medium of hypoxic astrocytes. The medium of all cultures also contained 7.5 mM glucose. More than 6.0 mM FDP resulted in LDH concentrations that were similar to those of normoxic controls. CON = normoxic control. All values are significantly different from no FDP ($p < 0.001$). Values are means \pm SEM. N = six cultures per point.

How glc + FDP protects these cells is unknown. Markov et al. (Markov, 1983, 1986) suggested that FDP enters cells, bypasses the reaction involving phosphofructokinase (PFK) (which is inhibited by hypoxia and acidosis) (Mansour, 1963), and increases glycolysis and ATP production. They point out that this would effectively provide twice as much ATP as glc metabolism because the phosphorylation steps of both glc and fru-6-P would be bypassed.

It is unclear, however, whether exogenous FDP enters cells. Reports suggesting that it does are based on indirect evidence (Markov, 1986; Didlake et al., 1982; Markov et al., 1983); reports suggesting that it does not are based on [^{14}C]FDP uptake studies in erythrocytes (Rigobello et al., 1982; Bernasconi et al., 1983). There are no published data on whether FDP is taken up by brain or brain cells.

We found that FDP was rapidly incorporated into the cell fraction of normoxic astrocytes at 37°C (Fig. 6) and that the steady-state concentrations of FDP were similar to those of glc (Yu and Hertz, 1983). However, we do not know whether the FDP

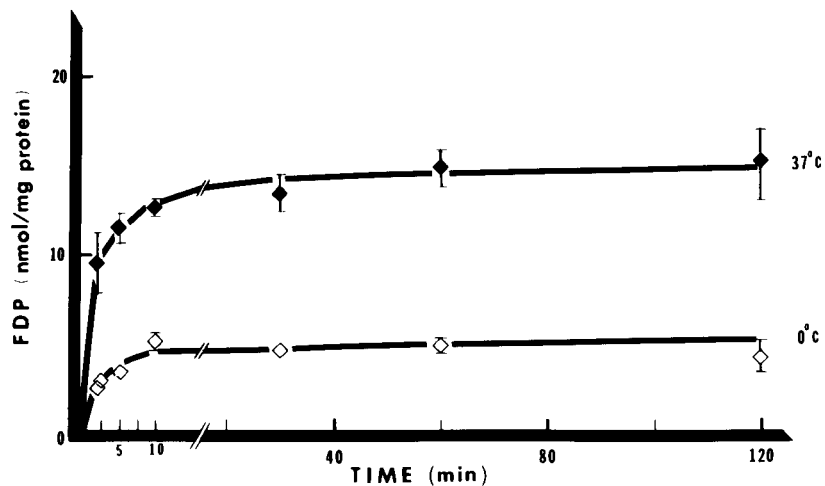


FIG. 6. Uptake of [^{14}C]FDP by normoxic astrocytes. $N = 6-10$ cultures per point. Values are means \pm SEM.

entered the cells or whether it was bound to cell membranes because the concentration of [^{14}C]FDP at 0°C was higher than expected. Some of the "uptake" was, however, energy dependent because "uptake" decreased by nearly 70% between 37 and 0°C .

Adding [^{14}C]FDP to the suspension medium of normoxic red blood cells increases the intracellular FDP concentration (Bernasconi et al., 1983), possibly by increasing glycolysis. Bernasconi et al. suggested that glycolysis increased because the exchange of H^+ for K^+ increased, which raised the intracellular pH. They also demonstrated an increase in intracellular ATP, which they postulated occurred because membrane phosphatases removed phosphorus from extracellular FDP, which then was incorporated into ATP. However, when they added phosphorus, fru, or fru + phosphorus to the medium, intracellular FDP and ATP concentrations did not increase.

Hood and Holloway (1976) found that $5\ \mu\text{M}$ FDP maximized the activity of PFK and that $400\ \mu\text{M}$ fru-6-P, the natural substrate of PFK, had little effect on its activity, whereas Taylor and Bailey (1967) found that FDP also increased the activity of

pyruvate kinase. However, it is unlikely that FDP increased anaerobic glycolysis in our hypoxic cultures because the lactate content of the culture medium was similar with glc + FDP and glc alone. FDP might have increased aerobic metabolism, because the Po_2 of the MEM was 18 ± 0.9 mm Hg after 18 h of hypoxia. Increased aerobic glc metabolism would explain the high ATP levels reported by Heckler et al. (1983) and the normal cell volumes of our glc + FDP-treated cultures.

Myers (1979) and Rehnacrona et al. (1981) suggested that lactic acidosis damages the CNS. However, despite a mean pH of 6.92, we found no evidence of damage in glc + FDP-treated cells, whereas glc-treated cultures (without FDP but with the same low pH) had abnormal appearances, lower protein concentrations, and high LDH contents in their medium. This suggests that pH per se does not always damage cells, which was also shown by the in vivo studies of Litt et al. (1985). They showed that hypercarbia-induced brain intracellular pH of 6.45 did not damage the CNS of adult rats if the concentration of ATP in their brains remained normal. Our preliminary findings (Yu et al., unpublished data) indicate that glc + FDP-treated cul-

TABLE 1. Cell volumes and lactate dehydrogenase (LDH) concentrations (of the culture medium) of cultures treated with glucose (glc) or glucose + fructose-1,6-bisphosphate (glc + FDP) after 18–36 h of hypoxia

Duration of hypoxia (h)	Cell volume ($\mu\text{L}/\text{mg}$ protein)		LDH (U/L)	
	glc	glc + FDP	glc	glc + FDP
18	4.62 ± 0.25	3.91 ± 0.30	30.68 ± 5.29	17.43 ± 1.71
24	5.06 ± 0.32	3.84 ± 0.36	194.63 ± 34.80^a	27.75 ± 4.69
30	7.06 ± 1.37^a	4.71 ± 0.37^b	392.92 ± 31.49^c	92.29 ± 15.61^a
36	$16.76 \pm 2.05^{b,c}$	$7.62 \pm 1.00^{b,c}$	429.51 ± 30.01^c	142.11 ± 30.68^c

Values are means \pm SEM.

^a $p < 0.05$ versus 18 h.

^b $p < 0.05$ versus 30 h.

^c $p < 0.01$ versus 18 h.

tures have normal concentrations of ATP despite 24 h of hypoxia. Cell volumes were also normal.

In summary, glc + FDP protected cultured astrocytes from hypoxic damage, whereas FDP or glc alone did not. The mechanism of this protein is unknown but probably includes increased metabolism and ATP production.

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