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Pharmacokinetics of fructose-1,6-diphosphate after intraperitoneal and oral administration to adult rats

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Abstract

Exogenously administered fructose-1,6-diphosphate (FDP) has been studied for its ability to protect tissue during hypoxia or ischemia. Recently, a clear effect of FDP on the central nervous system has raised the question whether FDP can get into the brain. FDP levels were measured in blood, brain, liver, kidney, muscle and fat after intraperitoneal administration of a single 0.5g kg⁻¹ dose of FDP to adult male Sprague-Dawley rats. A complete time course of the levels in blood and brain was determined. The levels of FDP in the blood and brain increase simultaneously, i.e. there is no lag in the increase in the brain. The levels of FDP fall to baseline in liver, kidney, muscle and fat by 12 hours, but remain elevated in blood and brain. However, levels in the blood at 12 hours are significantly decreased from the peak levels, while those in brain are not different from the peak levels, suggesting that the kinetics of FDP in blood and brain are quite different. Stripping the endothelial cells from the brain tissue sample did not change the levels of FDP indicating that FDP is not trapped in the capillary cells. Incubation of brain slices in a solution of FDP, followed by washing, raised tissue levels of FDP indicating that FDP is taken up into cells within the brain. Finally, the experiments demonstrate a significant increase in brain levels of FDP after oral administration. These data suggest that an oral formulation of FDP might be developed for treatment of neurological disease.

Keywords

blood brain barrier; fructose-1,6-bisphosphate; fructose-1,6-diphosphate; pharmacokinetics; rat

Introduction

Exogenously administered fructose-1,6-diphosphate (FDP; also called fructose-1,6-bisphosphate) has been studied for its ability to protect ischemic or hypoxic tissue and to facilitate the recovery of that tissue (1). The mechanism of its action has been debated, in part, because of the general belief that charged, phosphorylated sugars cannot cross cell membranes, particularly the blood brain barrier. However, it has been shown that FDP is capable of entering cells and serving as a glycolytic intermediate. This was done with ¹³C-labeled FDP in smooth muscle cells from pig artery *in vitro* (2). FDP has also been shown to diffuse across a membrane bilayer in a dose-dependent fashion (3). The same study also showed dose-dependent uptake of ¹⁴C-FDP into endothelial cells. The data indicate that FDP crosses the membrane intact.

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Recently, it has been shown that exogenous administration of FDP can reduce the duration and severity of seizures in laboratory animals (4). In these experiments, the FDP was given into the peritoneal cavity. Despite the clear effect of FDP on seizure activity, the question remains whether FDP can get into the brain. In earlier experiments it was shown that administration of FDP to rabbits during hypoglycemic coma (5) or ischemia-hypoxia and reperfusion (6) improved outcomes. Experiments have also shown alterations in pyruvate levels in the brain of pigs after intravenous administration of FDP (7). Finally, exogenous administration has been shown to have neuroprotective activity in pigs (8) and mice (9). These results also suggest that FDP is taken up and utilized by the brain, but the evidence is indirect. In these experiments, peripheral administration of FDP could be altering metabolism within the brain without actually crossing the blood brain barrier. Therefore, direct measurements of FDP levels in the brain after peripheral administration are needed to begin to understand the effect of exogenously administered FDP on cerebral function.

Methods

Adult male Sprague-Dawley rats (170–220g, Harlan Sprague-Dawley, Indianapolis IN, USA) were used to determine the kinetics of fructose-1,6-diphosphate (FDP). For the duration of the experiments, animals were housed individually with free access to food and water under standard conditions (controlled temperature and humidity with 12 hour light:dark cycles). Experiments were performed following the “Principles of Laboratory Animals Care” (NIH Publication no 85–23, revised 1996) and with approval of the local Animal Care and Use Committee. Levels of FDP were determined in blood and tissue samples as previously described (10). All chemicals and enzymes for the assay were obtained from Sigma Chemical Co (St. Louis MO). Animals were administered a single intraperitoneal dose of 0.5 g kg⁻¹ FDP (250 mg ml⁻¹ dissolved in 0.1M phosphate buffered saline, pH 7) or vehicle (control). In addition, naïve animals, not treated with vehicle or drug, were also tested. *In vivo* experiments utilized the dicalcium salt of FDP, which has a purity of ~70%. Preliminary experiments demonstrated no difference in efficacy between the trisodium salt, with a purity of >98% and the dicalcium salt. At the designated time, animals were anesthetized with 1 g kg⁻¹ urethane. After deep anesthesia, whole blood was obtained from a cardiac puncture. One ml of whole blood was added to 5 ml of perchloric acid (0.6 ml l⁻¹) and mixed. After centrifugation at 3,500 rev min⁻¹ for 10 min at 4°C, the supernatant was removed and set aside. The sediment was re-suspended in 1 ml of the perchloric acid solution and 1 ml of distilled water and centrifuged. The resulting supernatant was combined with the first one and the pH was adjusted to 3.5 with potassium carbonate (5 mol l⁻¹). The final volume was brought to 7 ml and the solution was allowed to sit in ice for 15 min. The supernatant was used for determination of FDP levels.

Immediately after removal of the whole blood sample, the rat was perfused through the heart with ice-cold 0.01M phosphate buffered saline. Tissue samples from liver, kidney, skeletal muscle (from thigh) and intra-abdominal fat were obtained and then the brain was removed and dissected into hippocampus, cerebral cortex, cerebellum and rest of brain. In initial experiments, the different brain regions did not give statistically different results, so the values for these 4 samples were averaged to give a mean FDP level in the brain for each animal. All tissue samples were weighed and then homogenized in 5 ml of ice-cold perchloric acid (0.6 mol l⁻¹) as quickly as possible. The homogenates were then treated as described above for whole blood.

Actual levels of FDP were determined by monitoring absorbance of reduced nicotinamide adenine dinucleotide (NADH) and treating the sample with aldolase (EC 4.1.2.13, 45 units mg⁻¹ diluted 1:27 with distilled water), the enzyme which cleaves FDP into dihydroxyacetone phosphate (DAP) and D-glyceraldehyde 3-phosphate (GAP). DAP and GAP are interconverted by the enzyme triosephosphate isomerase (TIM, EC 5.3.3.1, 5290 units mg⁻¹ diluted 1:120

with distilled water). Glycerol-3-phosphate dehydrogenase (GDH, EC 1.1.1.8, 252 units mg⁻¹ diluted 1:100 with distilled water) catalyzes the reduction of DAP by NADH. For the FDP measurements, 1.5 ml of tetraethyl ammonium buffer (TEA, 0.4 mol l⁻¹, pH 7.6 with EDTA 40 mmol l⁻¹) was added to 1 ml of the sample in a 4 ml cuvette. Then 0.1 ml of 5 mmol l⁻¹ β-NADH, 0.4 ml of distilled water and 0.01 ml of the enzymes TIM and GDH were added and the cuvette inverted to mix the solutions. After 5 min the absorbance was read 3 times at 340 nm, each reading 3 min apart. The average of these readings is the initial absorbance (A_i). This step removes any DAP or GAP in the sample and determines the baseline absorbance. Aldolase (0.01 ml) was then added to cleave FDP into DAP and GAP and the solution was mixed. Nine minutes after addition of the aldolase, the final absorbance (A_f) was determined by 3 readings at 340 nm, each 6 minutes apart. The concentration of FDP in the sample was proportional to the difference in the initial and final absorbance. Two moles of NADH are oxidized for each mole of FDP. Blanks and a FDP standard sample were run in parallel with every assay. Each blank had 1.6 ml of the TEA buffer, 1.4 ml water and the mixture of TIM and GDH. For the positive control, the sample was replaced with 1 ml of a solution containing 200 μg FDP per ml of phosphate buffered saline. The trisodium salt of FDP, with a purity of >98%, was utilized for all of the positive control samples and for the standard curves. Levels of FDP in blood were determined per ml, while levels in tissue samples were determined as a function of tissue weight.

In additional samples, a capillary depletion protocol was carried out to remove endothelial cells from the brain samples (11). The cortex was harvested as described above and homogenized in 3 ml perchloric acid. Four milliliters of a 26% dextran (low fraction) solution was then added and the sample was homogenized again. The homogenates were then centrifuged at 5400 rev min⁻¹ for 15 min at 4°C. The supernatant and pellet were carefully separated and processed separately for FDP levels as described above.

Additional experiments determined the uptake of FDP in brain slices *in vitro*. Male Sprague-Dawley rats (150–160 g, n=5) were anesthetized with a ketamine cocktail (mixture of ketamine (42.8 mg ml⁻¹), xylazine (8.6 mg ml⁻¹), acepromazine (1.4 mg ml⁻¹); dose 0.5–0.7 ml kg⁻¹) and then perfused through the heart with an ice-cold solution containing 110 mM choline Cl, 2.5 mM KCl, 1.25 mM NaH₂PO₄, 25 mM NaHCO₃, 10 mM glucose, 0.5 mM CaCl₂, and 7.5 mM MgCl₂ and oxygenated with 95% O₂/5% CO₂. The brain was rapidly removed and cut transversely along the septo-temporal axis. Both halves of the brain were cut into 6–8 sagittal sections, 400 μm thick on a Vibratome (Technical Products, St. Louis, MO). The slices were incubated at 32 °C for at least 30 min in an artificial cerebrospinal fluid (CSF) solution containing 125 mM NaCl, 2.5 mM KCl, 1.25 mM NaH₂PO₄, 25 mM NaHCO₃, 10 mM glucose, 2 mM CaCl₂, 2 mM MgCl₂, 1.3 mM ascorbate and 3mM pyruvate, equilibrated with 95% O₂/5% CO₂. Half of the slices were then transferred to a container with artificial CSF plus 500 μg ml⁻¹ FDP (~ 1mM). The other half of the slices remained in artificial CSF. After 1 hour of incubation, all slices were washed 3 times for 15 min each in ice-cold artificial CSF. Immediately after washing, both control and FDP-treated slices were weighed and then homogenized in 5 ml ice-cold perchloric acid. Determination of FDP levels was carried out as described above.

All results were expressed as means ± S.E.M. The time course for FDP in blood and brain was analyzed with a 2-way ANOVA comparing to control levels as a function of time. Other comparisons were done with a 1-way ANOVA or grouped t-test as appropriate. Significance was set at p<0.05.

Results

Initial results with the FDP assay demonstrated that the changes in absorbance were linear up to at least 45 $\mu\text{g ml}^{-1}$, which is in the range of values obtained in both blood and tissue. In addition, when 1 ml of 200 $\mu\text{g ml}^{-1}$ of fructose was added in place of the sample in the assay (for a final concentration of 66.7 $\mu\text{g ml}^{-1}$), there was no change in the absorbance. This indicates that the assay is specific for the phosphorylated form of fructose. A sample blank and FDP-positive control (final concentration of 33.5 $\mu\text{g ml}^{-1}$) were included in every assay run. The results for naïve animals in blood are within the range previously reported for humans at baseline (1.13 mg dl⁻¹) and after administration of FDP (3.39 mg dl⁻¹, 12). The control group includes both vehicle control (n=3) and naïve (n=9) animals. There was no difference in these groups in any tissue, so the results have been pooled.

Adult male Sprague-Dawley rats were administered a single intraperitoneal dose of 0.5 g kg⁻¹ FDP (250 mg ml⁻¹ dissolved in 0.01M phosphate buffered saline, pH 7) and sacrificed at various times (Fig. 1). This dose and route of administration has previously been shown to be an effective anticonvulsant (Lian et al, 2007). There was a relatively rapid rise in FDP levels in blood. An increase was seen as early as 30 minutes after administration and there was a significant increase at 1 hour. The levels peaked at 2 hours after administration and by 6 hours had fallen more than halfway back to baseline levels. The levels then remained elevated out to 72 hours after a single dose. The level of FDP in the blood at 12 hours after administration was significantly elevated compared to control values, but also significantly decreased compared to the peak levels at 2 hours. The levels in the brain also rose very promptly, peaking 1–2 hours after administration of FDP. The levels then fell slightly, but remained significantly elevated at 36 hours. The level of FDP in the brain at 12 hours was not significantly different than the levels at 1 and 2 hours after administration of the FDP indicating a sustained elevation of FDP in the brain. Thus the levels in the blood and brain do not follow the same kinetic profile.

To test the handling of FDP in other tissues, animals were perfused with ice cold phosphate buffered saline to remove blood from the tissues of interest. The levels of FDP in liver, kidney, skeletal muscle and fat were determined in naïve animals (n=6) and 1 (n=6) and 12 (n=6) hours after intraperitoneal administration of a single 0.5 g kg⁻¹ dose of FDP (Fig. 2). Based on the data from blood and brain, the 1 hour time point was expected to have the peak levels of FDP. The 12 hour time point was chosen because at this time the levels in the blood have returned closer to baseline, while the levels in the brain are still elevated. Baseline levels in the tissues were quite different, but the increase in FDP at 1 hour appeared to be roughly equivalent in all tissues measured. There was an increase of around 0.2 mg g⁻¹ (increase of 0.17 mg g⁻¹ (muscle and liver) to 0.26 (kidney)). The increase at 1 hour only reached statistical significance in muscle and fat, most likely due to the low baseline levels in these tissues. In liver, kidney, muscle and fat, the levels of FDP had returned to near baseline at 12 hours indicating that the levels in these tissues mirror more closely the levels in blood than the levels of FDP in brain tissue.

To determine whether the FDP was getting into the brain tissue, two additional experiments were carried out. First, the endothelial cells were stripped from the brain samples. The ratio of FDP in the stripped cortex to the FDP in whole cortex was 0.86 ± 0.04 in naïve animals (n=3). In animals treated with a single intraperitoneal dose of 0.5 g kg⁻¹ of FDP, the ratio was 0.91 ± 0.01 (n=7), which is not statistically different from the naïve animals (grouped t-test). Within the group of animals treated with FDP, 4 were sacrificed ≤ 2 hours after treatment and the remaining 3 were sacrificed more than 12 hours after treatment with FDP. There was no difference in the levels of FDP in the endothelial cells in any of these groups compared to control. These results show that FDP is not trapped in the endothelial cells, but does cross into the brain parenchyma. In the second experiment brain slices were prepared and incubated in

artificial CSF containing 500 $\mu\text{g ml}^{-1}$ FDP to determine whether FDP could be transported into cells within the brain tissue. After incubation, slices were washed to remove FDP from the extracellular space. Incubation for 1 hour in 500 $\mu\text{g ml}^{-1}$ FDP resulted in a significant increase in tissue levels of FDP ($0.22 \pm 0.01 \text{ mg g}^{-1}$ compared to $0.14 \pm 0.004 \text{ mg g}^{-1}$ in control slices, $p=0.0006$ with a grouped t-test).

Because FDP does appear to cross membranes into a variety of tissues in the body after intraperitoneal administration, we chose to determine whether FDP had oral bioavailability. FDP was added to water at a concentration of 0.5% ($\text{pH}=7$). Five animals received only this water to drink for 7 days. By measuring the amount of solution in the water bottle at the beginning and again at the end of the 7 day treatment period, it was estimated that an average of $226 \pm 13 \text{ ml}$ (mean $\pm$ SEM) was consumed by each animal. This translates into approximately 160 mg of FDP consumed per day per animal. Using the weight of the animals on the last day, this corresponds to an average daily dose of $658 \pm 51 \text{ mg kg}^{-1}$ (mean $\pm$ SEM). If there was significant water spillage or leakage, then the total FDP consumption would be less. Levels of FDP in the blood and brain of these animals was significantly increased compared to naïve animals. After oral administration, the levels in the blood were $22.2 \pm 3.3 \mu\text{g ml}^{-1}$ (mean $\pm$ SEM, $n=5$, compared to $15.0 \pm 1.0 \mu\text{g ml}^{-1}$ in naïve animals, $n=11$) and in the brain were $0.50 \pm 0.02 \text{ mg g}^{-1}$ (mean $\pm$ SEM, $n=5$, compared to $0.42 \pm 0.01 \text{ mg g}^{-1}$ in naïve animals, $n=11$), which are not significantly different from the levels in blood and brain 12 hours after a single intraperitoneal dose.

Discussion

The experiments presented here demonstrate that peripheral administration of FDP raises levels in tissues throughout the body. The levels of FDP in the blood and brain increase simultaneously, i.e. there is no lag in the increase in the brain. The levels of FDP fall to baseline in liver, kidney, muscle and fat by 12 hours, but remain elevated in blood and brain. However, levels in the blood at 12 hours are significantly decreased from the peak levels, while those in brain are not different from the peak levels, suggesting that the kinetics of FDP in blood and brain are quite different. Further experiments indicate that FDP is taken up into the cells in the brain and not trapped in the endothelial cells of the brain. Finally, the experiments demonstrate a significant increase in brain levels of FDP after oral administration. These data demonstrate that exogenous administration of FDP results in a significant increase in levels of FDP in the brain and also suggest that an oral formulation of FDP might be developed for treatment of neurological disease.

Experiments have shown that FDP can cross lipid bilayers in a dose-dependent manner (3). It has also been hypothesized that FDP can cross cell membranes via either a band 3 or a dicarboxylate transporter. This was tested in isolated rat heart myocytes (13) where it was concluded that since fumarate and malate could cross the plasma membrane that a dicarboxylate transport system is present on these cells. A band 3 inhibitor had no effect on production of [^{13}C]lactate from [^{13}C]FDP in these cells, but fumarate, which will compete for transport on the dicarboxylate transporter, did inhibit metabolism of [^{13}C]FDP. The data in this study is consistent with transport of FDP into cardiac myocytes by a dicarboxylate transport system. This study also found no conversion of FDP to fructose. The sodium-dicarboxylate cotransporter family, includes 2 proteins found in humans (SLC13A2 and SLC13A3, 14) which are reported to transport succinate, citrate and α -ketoglutarate. SLC13A3 is reported to be present in brain tissue. Searching the Allen Institute Brain Atlas (<http://www.brain-map.org/welcome.do>), the mRNA for SLC13A3 appears to be in very low levels in the brain. In some sections, positive staining appears in choroids plexus and possibly ependymal cells. The expression levels for the mitochondrial dicarboxylate transporter (SLC25A10) show staining in a more uniform distribution throughout the brain in cell bodies.

In the hippocampus, it appears that the mRNA for this transporter is found at moderate levels in principal neuronal cells only. Lower levels are seen in the cortex in layers II and IV/V. It does not appear to be expressed in interneurons or glial cells. Therefore, if FDP crosses the blood brain barrier and is transported into cells via SLC25A10, then metabolism in neurons would be predicted to be changed more than metabolism in glial cells. If FDP is moving through by body by diffusion through membranes, then both neuronal and glial metabolism should be altered in a similar fashion. More than one process may also be involved in the movement of FDP into and through the brain. Evidence in cardiac myocytes suggests that at least 2 processes are involved in the entry of FDP (15).

The most intriguing question is why do the levels of FDP remain elevated in the brain long after they have fallen in the peripheral tissues? The answer to this question is not readily apparent. FDP is a normal cellular constituent and, as such, has a normal route of metabolism and cellular regulation. When exogenous FDP is administered, one might predict that a cell would respond by increasing the metabolism of FDP. This is not consistent with the data in the present study. Alternatively, the exogenously administered FDP could alter metabolism to the extent that more FDP is generated within the cells, maintaining the overall level. Again, while possible, this does not seem likely since there are no other examples of this type of regulation. The ability of various tissues to hydrolyze FDP has been measured in organ extracts and brain had the lowest level (16). Therefore, it is possible that levels remain elevated because of decreased metabolism. However, if FDP can diffuse across membranes, then one would expect FDP to diffuse back out of the brain as the levels fall in the blood. The data suggest that the FDP is trapped in the brain. This supports a one-way transport system with limited tissue metabolism of FDP. Irrespective of the mechanism, the kinetics of FDP in the brain suggest that less frequent dosing would be needed to maintain therapeutic levels of FDP in the brain compared to other tissues. The data also suggest that oral dosing can result in a significant elevation of levels of FDP in the brain.

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Figure 1.

Kinetics of fructose 1,6-diphosphate (FDP) after intraperitoneal administration. Levels of FDP in whole blood (top) and brain (bottom) are presented as a function of time after administration of a single dose of 0.5 g kg⁻¹. Each point represents the mean $\pm$ SEM and the number of animals in each group is indicated beside each point. The asterisk indicates a significant difference compared to the control values, which are combined vehicle control animals (n=3) and naïve animals (n=9). The dashed line on both graphs extends the mean value for the control and naïve animals for visual comparison.

Figure 2.

Levels of fructose 1,6-diphosphate (FDP) after intraperitoneal administration in peripheral tissues. Levels of FDP were determined in 4 additional tissues in naïve animals and animals treated with a single dose of 0.5 g kg⁻¹ FDP then sacrificed at either 1 or 12 hours. There were 6 samples in each tissue group. A 1-way ANOVA was used to compare the levels within each tissue and only the 1 hour samples were significantly different from control in muscle and fat.