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Protective role of fructose in the metabolism of astroglial C6 cells exposed to hydrogen peroxide

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ABSTRACT

Astroglial cells represent the main line of defence against oxidative damage related to neurodegeneration. Therefore, protection of astroglia from an excess of reactive oxygen species could represent an important target of the treatment of such conditions. The aim of our study was to compare the abilities of glucose and fructose, the two monosaccharides used in diet and infusion, to protect C6 cells from hydrogen peroxide (H_2O_2)-mediated oxidative stress. It was observed using confocal microscopy with fluorescent labels and the MTT test that fructose prevents changes of oxidative status of the cells exposed to H_2O_2 and preserves their viability. Even more pronounced protective effects were observed for fructose 1,6-bis(phosphate). We propose that fructose and its intracellular forms prevent H_2O_2 from participating in the Fenton reaction via iron sequestration. As fructose and fructose 1,6-bis(phosphate) are able to pass the blood–brain barrier, they could provide antioxidative protection of nervous tissue in vivo. So, in contrast to the well-known negative effects of frequent consumption of fructose under physiological conditions, acute infusion or ingestion of fructose or fructose 1,6-bis(phosphate) could be of benefit in the cytoprotective therapy of neurodegenerative disorders related to oxidative stress.

1. Introduction

Neurodegeneration is related to promoted production of hydrogen peroxide (H_2O_2) via several different pathways.[1–3] In addition, breakdown of metal homeostasis and formation of depositions of redox-active metals in Alzheimer's disease (AD),[4] Parkinson's disease (PD)[5] and amyotrophic lateral sclerosis (ALS),[6,7] set up the conditions for promotion of oxidative stress via hydroxyl radical (·OH)-generating Fenton chemistry.[8–10] Relatively low antioxidant levels, low regenerative capacity and specific cellular geometry in combination with high oxygen consumption result in neurons being susceptible to oxidative damage provoked by reactive oxygen species (ROS).[4] Pertinent to this, it has been proposed that astroglia represents the main line of defence of the brain tissue against oxidative stress.[11,12] Astroglial cells supply neurons with substrates for energy metabolism[13,14] and glutathione (GSH) synthesis.[15,16] Moreover, they protect neurons from extracellular oxidants such as H_2O_2.[17,18] Since H_2O_2 permeates through the cellular membrane, it overflows to the extracellular space where it is detoxified by astroglia. Hence, antioxidative support of astroglial cells exposed to excess of H_2O_2 and other ROS could be highly important element of cytoprotective therapy in neurodegeneration. However, the administration of exogenous antioxidants in an attempt to fight oxidative damage is generally ineffective. The difficulty of antioxidative therapy is that the body acts to maintain flexible and responsive intracellular redox poise, enabling a swift genetic response to stress.[19,20] The double-agent theory makes a clear prediction that even if long-term supplementation raises blood levels of antioxidants, this will have a limited effect on intracellular levels or redox status because homeostatic mechanisms will correct for the rise above the physiological level.[21] This arises a question as to how to overcome refractory mechanisms and help the organism to fight oxidative stress against its own 'will'.

We have proposed recently that, in contrast to supplemented antioxidants, the organism will not reject the 'energy supply' in the form of fructose if presented in times of crisis.[22] Fructose has been shown to protect tissues against oxidative stress related with anoxia and hypoxia.[23] Fructose 1,6-bis(phosphate) (F16BP) has anticonvulsant activity,[24] prevents reperfusion injury,[25] protects against septic shock[26] and provides protection against the cell damage associated with mitochondrial poisons and prooxidants. A protective effect of adding exogenous F16BP to preservation solution (University of Wisconsin storage solution) used during an experimental procedure of small bowel transplantation in rats has also been reported.[27] An exogenous supply of F16BP can inhibit neutrophil free-radical production,[28] maintain the correct xanthine dehydrogenase/xanthine oxidase ratio,[29] put off changes in

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intracellular calcium[30] and prevent excitotoxicity.[31] Finally, deletion of fructose 1,6-bisphosphatase leads to decreased sensitivity of cells to oxidative stress.[32]

Some protective effects of fructose are independent of the tissue or experimental model used. We have shown using in vitro chemical systems that fructose and its phosphorylated forms (F16BP, fructose 1-phosphate (F1P) and fructose 6-phosphate (F6P)) show surprisingly high antioxidative capacities against ROS, when compared to two other sugars used in infusion solutions—glucose and mannitol.[22] Fructose and its intracellular forms scavenge ·OH and sequestrate iron, thus preventing the destructive Fenton reaction.[22] In addition, fructose and F16BP could shift glucose towards pentose phosphate pathway (PPP),[24] thus increasing the production of NADPH and GSH,[25] an important free-radical scavenger in the mammalian nervous system.[33] As fructose and F16BP are able to pass the blood–brain barrier,[34] they could provide antioxidative protection of nervous tissue in vivo.

In the present study we tested the antioxidative capacity of fructose and its phosphorylated forms in a biological setup. Confocal microscopy with fluorescent markers, as well as the MTT assay of viability, was applied to evaluate ability of fructose to protect C6 astroglial cells exposed to H_2O_2-mediated oxidative stress. The aim was to explore potential benefits of acute application of fructose in cytoprotective therapy of neurodegenerative conditions and to establish intracellular mechanisms of antioxidative actions of fructose.

2. Results and discussion

Figure 1 shows confocal micrographs of C6 astroglial cells cultured in the media with glucose, fructose and glucose + fructose and stained with MitoTracker® Orange (MTO). MitoTracker dyes are fluorescent mitochondrial markers that covalently bind free sulfhydryls. The impact of alterations in mitochondrial membrane potential and oxidative stress on MitoTracker staining of mitochondria in cultured neurons and astrocytes have been reported.[35] MTO exhibited H_2O_2 concentration-dependant rise in fluorescence intensity,[36] and it has been applied previously in determining the 'relative intracellular ROS level' ('relative oxidative status' as indicated here).[37] We used the fluorescence intensity of treated cells relative to fluorescence intensity obtained in control samples to index oxidative status of the cells.

Cells exposed to H_2O_2 (3 mM) are compared to controls. Hydrogen peroxide is spontaneously emitted by the nervous tissue into the extracellular space where even under physiological conditions its concentration can reach up to 1 mM. In pathophysiological states its level may be substantially higher. In many studies dealing with neurodegeneration-related oxidative stress and the role of antioxidants, H_2O_2 has been used as a model compound to initiate prooxidative conditions. Figure 1 shows that in contrast to glucose, fructose prevented prooxidative changes in C6 cells exposed to exogenous H_2O_2, as no increase of MTO fluorescence could be observed. In addition, exposure to H_2O_2 led to significant changes in cellular morphology, which seems to be more pronounced in glucose medium, when compared to other media. After the treatment cells have rounded and shrunken, and the cellular processes are withdrawn (see Fig. 2). This is most likely a consequence of H_2O_2-provoked aggregation of F actin microfilaments.[38]

Hydrogen peroxide has been reported to provoke oxidative damage, lipid peroxidation and at higher concentrations/times of exposure even cell death.[39] In order to test whether applied treatment provokes death of C6 cells, we used FDA/PI (alive/dead) staining. Figure 2 shows confocal micrographs of C6 cells simultaneously stained with FDA and PI. It can be observed that the H_2O_2 treatment in our experimental setup did not provoke significant level of cellular death. As micrographs presented in Figure 2 are imaged with higher optical thickness (8 μm) when compared to those presented in Figure 1 (optical thickness, 3.5 μm), they also provide more complete information on the morphological changes. The withdrawal of cell processes, as well as cell rounding can be clearly observed in cells exposed to H_2O_2, particularly in glucose-based medium.

Although the treatment with H_2O_2 did not lead to cell death, it may provoke decrease in the cell viability. Therefore, the MTT assay was applied to test the viability and to compare effects of fructose and glucose. Figure 3 represents quantifications of the results that were obtained using confocal microscopy (illustrated in Fig. 1) and the MTT assay. Relative intensity of MTO fluorescence represents a measure of oxidative status of cells. Hydrogen peroxide provoked a statistically significant increase of oxidative status of C6 cells in glucose medium, as reported previously for neurons and astrocytes.[35] Some changes of the oxidative status were quantified for cells cultured in fructose medium and in a medium with both glucose and fructose, but these are non-significant. Hydrogen peroxide provoked significant decrease of C6 viability in all investigated media, but the viability was the lowest in glucose. Significantly higher viability was obtained in the medium containing equal amounts of fructose and glucose (5 mM each) when compared to the glucose-only (10 mM) medium. At this point it should be stressed out that there was no statistically significant difference in the oxidative status and viability between untreated controls cultured in different media during 48 h.

Although relatively stabile, when present in excess, H_2O_2 provokes an increase in cellular oxidative status. In addition it oxidises catalytically active transition metals (e.g., iron) to produce dangerous ·OH radicals via the Fenton mechanism ($Fe^{II} + H_2O_2 \rightarrow Fe^{III} + {\cdot}OH + OH^-$).[40] Glucose and fructose have shown similar scavenging capacities towards ·OH.[22] However, fructose and its phosphorylated forms interfere with the Fenton chemistry also by iron sequestration. We proposed that in the presence of the fructose/iron complex and H_2O_2, the Fenton-like mechanism occurs, but instead of resulting in ·OH generation, it leads to degradation of fructose to glycolate,[22] which has been observed to develop in the Fenton system in the presence of fructose.[41] In addition, it has been proposed that fructose can directly react with peroxides.[42] This could partially account for the protective effects of fructose as compared to those of glucose that were observed here.

Fructose is introduced into the cells of the nervous tissue via the GLUT-5 transporter.[43] This monosaccharide is involved in metabolic pathways in brain cells generating phosphorylated forms of fructose—F16BP, F1P and F6P,[43] which can further modulate the intracellular oxidative status based on their high antioxidative capacity.[22] These phosphorylated forms were shown to be more efficient in ·OH scavenging and iron sequestration than fructose itself.[22] In order to establish their potential role in the antioxidative performance of fructose, as well as to explore their applicability in pathophysiological conditions related with uncontrolled oxidation, the effects of exogenous phosphorylated fructose forms were tested on C6 cells cultured in glucose and treated with H_2O_2. Figure 4 shows confocal micrographs of C6 cells exposed to H_2O_2 in the glucose-based medium with or without supplementation of phosphorylated forms of fructose. Quantification of fluorescence intensity is presented in Figure 5. It can be observed that F16BP prevented changes of oxidative status of cells exposed to H_2O_2. Application of F1P protected the cells from oxidative stress, but the protective effects were less distinct when compared to the effect of F16BP. In contrast to its antioxidative capacity observed in chemical systems,[22] F6P did not provide any protection in C6 cells. F16BP crosses the cellular membrane by diffusion in a dose-dependant manner,[44] and to a lesser extent via active transport by dicarboxylate transporters present in brain cells.[34] Similar to F16BP, F1P contains the phosphorylated carboxyl moiety, enabling it, unlike

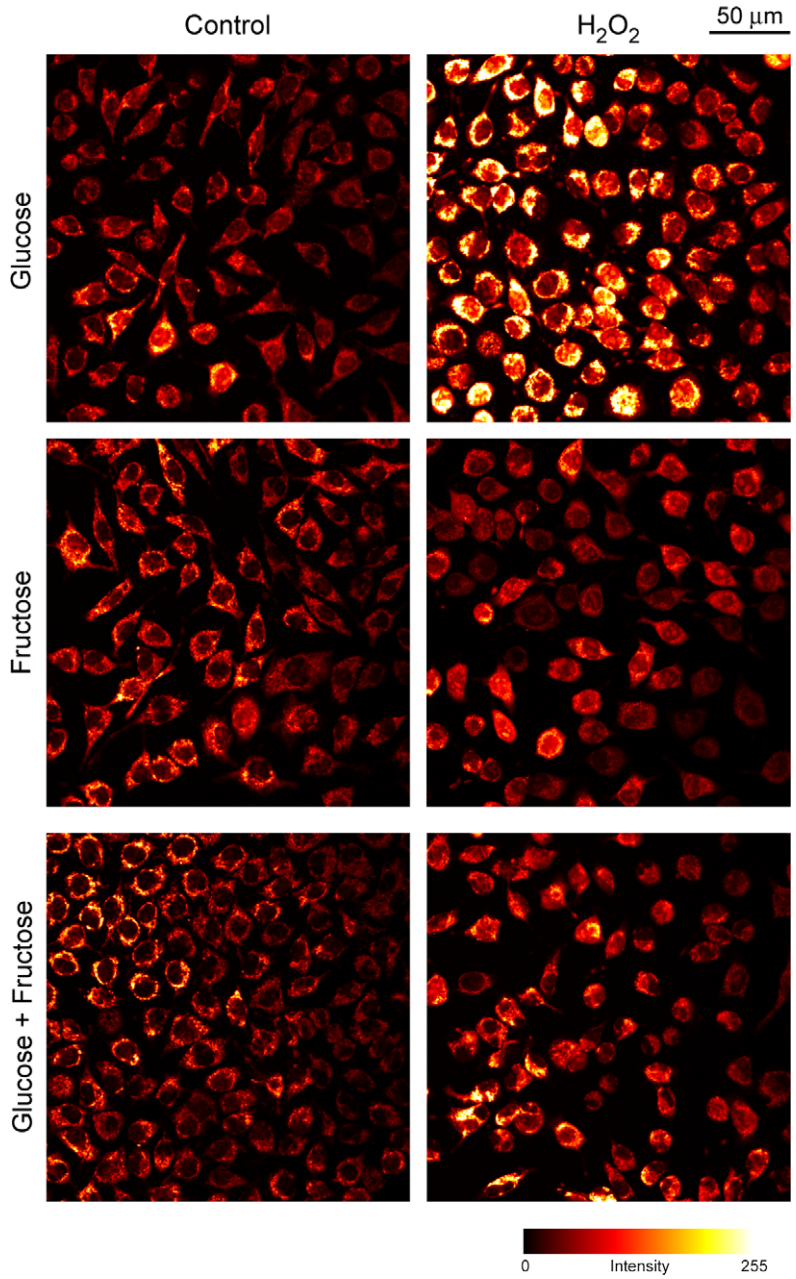

Figure 1. Confocal micrographs (optical thickness, 3.5 μm) of C6 astroglial cells cultured in media with glucose (10 mM), fructose (10 mM) and glucose + fructose (5 + 5 mM) for 48 h. Left column: untreated cells (controls); right column: cells treated with H_2O_2 (3 mM) for 10 min, all subsequently labelled with MTO.

F6P, to be picked up by dicarboxylate transporters. In addition, F6P has been even observed to be channelled out of some cells.[45] The ability of F16BP and F1P to enter the cell could account for their higher antioxidative activities relative to F6P. Intracellular conversion of fructose into phosphorylated forms, which have high antioxidative capacities, could, in addition to its direct antioxidative effects, explain why fructose is more protective than glucose.

The ROS-system and the antioxidative defence system are just two intertwined components present within global physiological mechanisms of homeostasis.[46] Our results clearly demonstrate that fructose and its phosphorylated form, F16BP, which represents a key intracellular metabolite, exhibit high antioxidative capacities, the latter even higher than fructose. It is rather interesting that, after intraperitoneal or oral administration of F16BP, its level in the brain, which is in the range of concentration used here, remains high long after it has fallen in peripheral tissues.[34] In addition, H_2O_2 inhibits the glycolytic enzyme glyceraldehydephosphate dehydrogenase (*GAPDH*), which could lead to H_2O_2-dependent F16BP accumulation.[47] Thus F16BP could show prolonged antioxidative actions in the nervous tissue in vivo. In addition to the effects on intracellular oxidative status, fructose–iron complexes could be formed in the extracellular or cerebrospinal fluid, where they could remove H_2O_2 emitted by damaged cells before it reaches other cells. In this way supplemented fructose or F16BP could prevent the propagation of oxidative stress in the tissue. It should be stressed that 'chronic' consumption of high fructose could provoke an imbalance in the redox status of cells under physiological conditions, thus affecting their normal functioning,[48] which is in accordance with the double-agent theory.[21,22] A significant role of the chronic consumption of fructose has been proposed in hypertension, obesity, metabolic syndrome, diabetes, kidney and heart disease,[49] so any excess of fructose should be

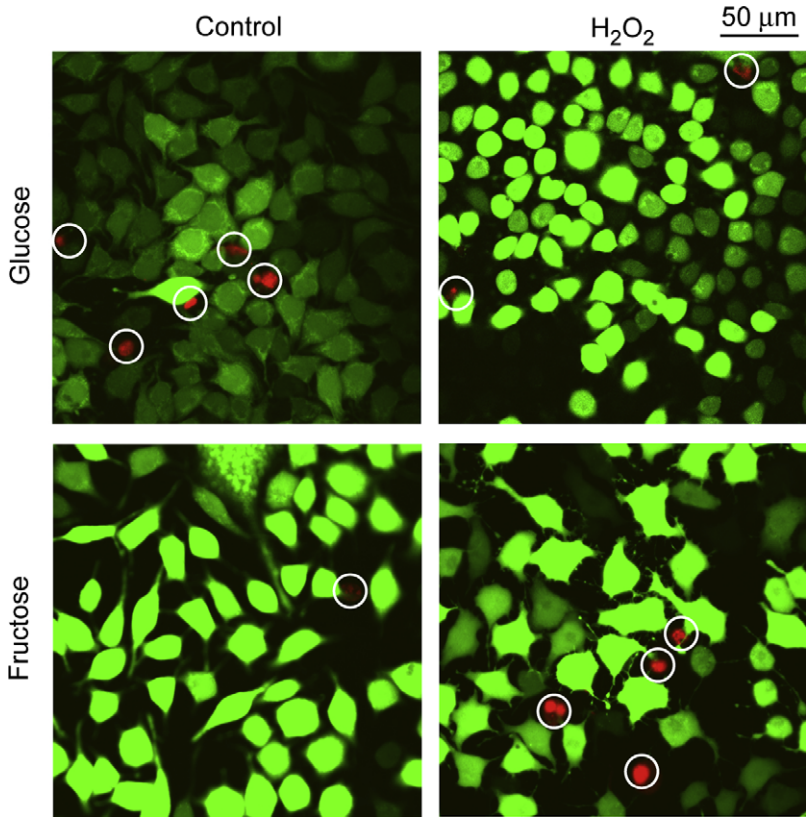

Figure 2. Confocal micrographs (optical thickness, 8 μm) of C6 cells cultured in media with glucose (10 mM) or fructose (10 mM). Left column: untreated cells (controls); right column: cells treated with H_2O_2 (3 mM) for 10 min, all subsequently stained with FDA and PI. Alive cells, green (FDA); dead cells, red in white circles (PI).

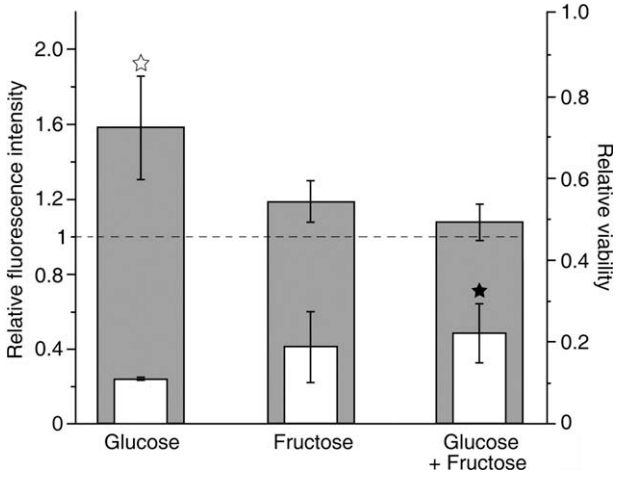

Figure 3. Relative intensity of MTO fluorescence (grey) and relative viability (white) of C6 cells exposed to H_2O_2 in media with different monosaccharides. Values are presented relative to controls cultured in corresponding media with no H_2O_2. The relative MTO fluorescence intensity (dashed horizontal line) and relative viability of untreated cells are equal to 1. Stars indicate a statistical significance ($p < 0.05$) relative to corresponding control (white) or relative to cells treated in glucose (black); concentrations as indicated in Figure 1. Viability of C6 cells in control samples did not vary significantly in different media.

eliminated from normal diets. On the other hand, short-term application of fructose or F16BP has protective effects under pathophysiological conditions related to oxidative stress, as shown here on astroglial cells exposed to excessive exogenous H_2O_2. Therefore, acute supplementation of fructose and F16BP in the diet or infusion could be of benefit in cytoprotective therapy of neurodegenerative conditions related to uncontrolled oxidation.

3. Experimental

3.1. Chemicals

Chemicals of the highest grade available were obtained from commercial providers: 20% w/v commercial monosaccharide infusion solutions of fructose (Mayrhofer Pharmazeutika GmbH, Linz, Austria) and glucose (Leopold Pharma, Graz, Austria); F1P, F6P and F16BP (Sigma–Aldrich, St. Louis, MO, USA); H_2O_2 (Renal, Budapest, Hungary); culture medium RPMI-1640 (Sigma–Aldrich); TrypLE™ Express, MitoTracker® Orange, propidium iodide (PI), Fluorescein diacetate (FDA) (Invitrogen, Carlsbad, CA, USA); MTT ((3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide), Sigma–Aldrich); all other chemicals (Sigma–Aldrich).

3.2. Cell culture and experimental design

Rat C6 astroglioma cells, generously provided by Dr. Vladimir Trajković (Faculty of Medicine, Belgrade), were cultured in 25-cm^2 flasks in a humidified atmosphere with 5% CO_2 at 37 °C and passaged once a week. The culture medium was RPMI-1640 supplemented with $NaHCO_3$ (2 g/L), glucose (10 mM), foetal bovine serum (5%) and gentamicin (50 μg/mL). For the experiments, the cells were detached with TrypLE™ Express and seeded into 35-mm dishes at a density of 2×10^5. Two days of culture preconditioning in mediums with glucose (10 mM), fructose (10 mM) or combination of these sugars (5 mM each) preceded treatment with H_2O_2. The cells exhibited differentiated phenotype and formed a semiconfluent layer prior to the start of experiment.

For the assessment of oxidative status of the cells cultured under normal glucose concentration and partial or complete glucose replacement with fructose, two days after seeding, cultures were

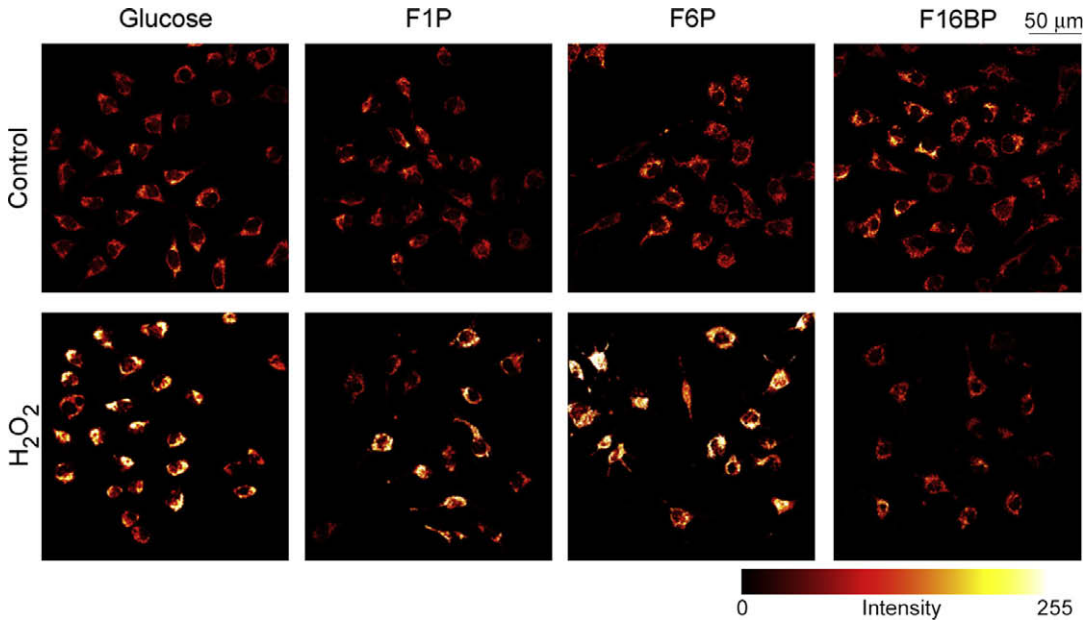

Figure 4. Confocal micrographs (optical thickness, 3.5 μm) of C6 astroglial cells cultured in media with glucose (10 mM) and exposed to H_2O_2 after addition of (5 mM): glucose, F1P, F6P or F16BP. Upper row: untreated cells (controls); bottom row: cells treated with H_2O_2 (3 mM) for 10 min.

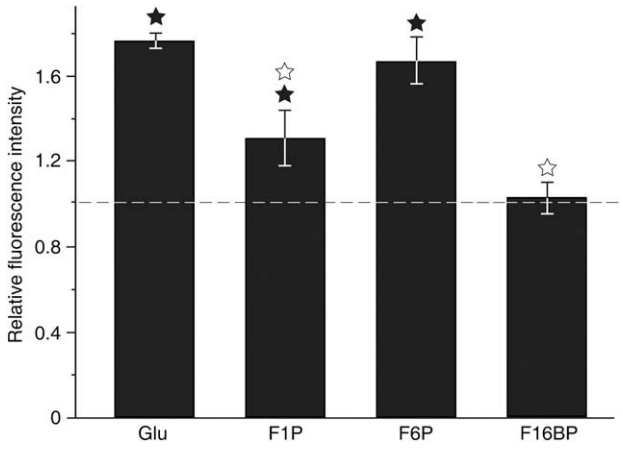

Figure 5. Relative intensity of MTO fluorescence of C6 cells cultured in glucose (10 mM)-based medium and exposed to H_2O_2 (3 mM) after addition of (5 mM): glucose, F1P, F6P or F16BP. Values are presented relative to controls cultured in glucose-based medium, after addition of 5 mM of glucose, F1P, F6P or F16BP with no subsequent H_2O_2 treatment. Relative fluorescence intensity of untreated samples equals 1 (dashed horizontal line). Stars indicate a statistical significance ($p < 0.05$) relative to untreated cells (black) or relative to cells treated with H_2O_2 in glucose-based medium (white).

washed with tempered ECS (extracellular solution: 135 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES) with 10 mM hexose—glucose, fructose or a glucose–fructose combination (pH and osmolarity adjusted to pH 7.4 and 290 ± 5 mOsm to match medium conditions). In the next step cells were exposed to H_2O_2 (3 mM) in ECS for 10 min at 37 °C. This was followed by a brief wash and mitochondrion probe loading (MitoTracker® Orange). The dye was diluted in ECS to final concentration of 100 nM, and cultures were labelled for 10 min at 37 °C. The cells were then rinsed once again and left for 10 min to accommodate at room temperature before imaging. Hydrogen peroxide was omitted from solutions during preparation of control dishes.

For experiments with phosphorylated fructose, two days after plating cells into 35-mm dishes with glucose culture medium (10 mM), fructose 1-phosphate, fructose 6-phosphate and fructose 1,6-bis(phosphate) were added at final concentration of 5 mM. Glucose was added to attain 10 + 5 mM concentration in the control sample. Twenty minutes after addition of sugar, the culture dishes were exposed to H_2O_2 (3 mM) for 10 min at 37 °C, rinsed in ECS and loaded with MitoTracker® Orange. Cell culture with 10 mM glucose and 5 mM of glucose, F1P, F6P or F16BP was used as a control and was not treated with H_2O_2 before labelling.

3.3. Fluorescein diacetate and propidium Iodide staining

To test whether treatment with H_2O_2 provokes the death of C6 astroglioma cells, cultures were loaded with 5 μg/mL of fluorescein diacetate (FDA) and 50 μg/mL of propidium iodide (PI) in ECS, and incubated for 10 min at 37 °C. Cells were preconditioned in mediums with glucose (10 mM) or fructose (10 mM) for two days before the experiment. After a brief rinse with glucose or fructose, the ECS solution controls were loaded with FDA–PI. Treated cells were exposed to hydrogen peroxide for 15 min before FDA–PI staining.

3.4. Confocal microscopy

Fluorescence signals were recorded and analysed using an LSM 510 confocal system (software Version 3.2) built around an Axioscop 2 FS-mot microscope (Carl Zeiss, Göttingen, Germany). All images were acquired with a Zeiss 'Achroplan' 40× objective with numerical aperture of 0.8. The mitochondrion probe was stimulated with a 543-nm laser line, and emission from a 3.5 μm optical section was collected with an LP560 filter. At least five different fields were imaged from each dish. Mean per-pixel fluorescence intensities were evaluated from whole images after setting the threshold level to exclude 'out-of-cell' pixels. Fluorescence intensities of all images obtained from a dish were averaged. Relative fluorescence intensity was calculated by comparing fluorescence obtained in a treated sample with fluorescence of the control, obtained on the same experimental day. During FDA–PI imaging, dyes were excited with a 488-nm argon laser light. Green FDA emission was collected with a BP505-530 filter, while a red PI signal was

collected with an LP650-nm filter. Optical thickness was set to 8 μm. All experiments were performed in quadruplicate.

3.5. MTT assay

The MTT assay measures the metabolic activity of living cells via mitochondrial dehydrogenases. Hence, MTT reduction corresponds to the integrity of mitochondrial function and cell viability.[50] For the experiments, the cells were detached with TrypLE™ Express and seeded into 96 wells at a density of 8×10^3 cells/well. Three days after seeding, the cells were exhibiting a differentiated phenotype and formed an almost confluent layer. At this point treatment with H_2O_2 (3 mM) was performed for 20 min before adding MTT at a final concentration of 0.5 mg/mL. MTT reduction was stopped after 3 h with SDS (10%) in HCl (10 mM). The optical density at 540 nm was measured using an LKB 5060 multiplate reader. Measurements were performed in triplicate on each of four experimental days. The viability after treatment(s) was expressed relative to the value obtained for untreated cells on the same experimental day.

3.6. Statistical analysis

The data are presented as mean ± SD. Statistical differences between the values obtained in different experimental settings were evaluated by means of the non-parametric two-tailed Mann–Whitney test ($p < 0.05$) using STATISTICA 6.0 (StatSoft Inc, Tulsa, OK, USA).

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