




Consequences of a Metabolic Glucose-Depletion on the Survival and the Metabolism of Cultured Rat Astrocytes

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Abstract

Brain astrocytes are considered to be highly glycolytic, but these cells also produce ATP via mitochondrial oxidative phosphorylation. To investigate how a metabolic depletion of glucose will affect the metabolism of astrocytes, we applied glucose at an initial concentration of 2 mM to cultured primary astrocytes and monitored the cell viability and various metabolic parameters during an incubation for up to 2 weeks. Already within 2 days of incubation the cells had completely consumed the applied glucose and lactate had accumulated in the medium to a concentration of around 3 mM. During the subsequent 10 days of incubation, the cell viability was not compromised while the extracellular lactate concentration declined to values of around 0.2 mM, before the cell viability was compromised. Application of known inhibitors of mitochondrial metabolism strongly accelerated glucose consumption and initial lactate production, while the lactate consumption was completely (antimycin A or 8-hydroxy efavirenz) and partially (efavirenz, metformin or tyrphostin 23) inhibited which caused rapid and delayed cell toxicity, respectively. The switch from glycolytic glucose metabolism to mitochondrial metabolism during the incubation was neither accompanied by alterations in the specific cytosolic lactate dehydrogenase activity or in the WST1 reduction capacity nor in the mitochondrial citrate synthase activity, but a cellular redistribution of mitochondria from a perinuclear to a more spread cytoplasmic localization was observed during the lactate consumption phase. These results demonstrate that cultured astrocytes survive a metabolism-induced glucose depletion very well by consuming lactate as fuel for mitochondrial ATP generation.

Keywords Glucose · Glycolysis · Lactate · Metabolism · Mitochondria

Introduction

Astrocytes are the most abundant glial cell type in the brain [1]. These cells have a central role in the metabolism of the brain [2] and are considered to supply neurons with energy substrates in the form of lactate that is derived from glycolysis and/or glycogen mobilization [3–5]. Cultured astrocytes are known for their high glycolytic activity [6, 7], as for example shown by the frequently reported glycolytic

production of almost two lactate molecules from one glucose molecule consumed [8, 9]. The glycolytic phenotype of cultured astrocytes is most likely the consequence of an inactivation of the pyruvate dehydrogenase enzyme complex, which slows down mitochondrial metabolism, thereby favoring aerobic glycolysis in astrocytes [9, 10]. Despite of their high glycolytic potential, cultured astrocytes contain a large number of mitochondria [11, 12], express high levels of mitochondrial tricarboxylic acid (TCA) cycle enzymes [11, 13] and generate quickly TCA intermediates that are derived from glucose [14, 15]. The importance of the mitochondrial metabolism for astroglial ATP production is also supported by the strong acceleration of glycolytic flux that is observed after inactivation of mitochondrial respiration in cultured astrocytes [16, 17].

Astrocytes contain a large number of mitochondria even in their fine processes [18]. However, these organelles do not have a fixed location within astrocytes but are mobile and actively transported within the cells [19]. Mitochondrial motility is

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considered to allow mitochondria to be rapidly recruited to sites of increased metabolic activity [20, 21]. Mitochondrial movement and subsequent retention in astrocytes is strongly coupled with neuronal activity, as glutamate uptake by astrocytes leads to an increase in cellular $[Ca^{2+}]$ [19, 22, 23]. This causes mitochondria to arrest at active regions especially close to synapses [19]. Moreover, individual mitochondria undergo continuous cycles of fusion and fission events which is thought to be a mechanism of quality control [24–26].

Cultured cells are frequently maintained in medium containing unphysiologically high glucose concentrations which creates a hyperglycemic environment. Under such conditions cells appear to generate large amounts of ATP via glycolysis, although sufficient oxygen is present and mitochondria are working properly [27]. The extensive lactate production under such a condition of aerobic glycolysis [28, 29] is known as the Crabtree effect [30]. Interestingly, this is not only restricted to cell cultures exposed to high glucose concentrations but occurs also during incubation with more physiological low millimolar glucose concentrations [31–33].

Primary mouse embryonic fibroblasts, primary mouse astrocytes as well as some cell lines have been shown to metabolize glucose quickly by glycolysis to lactate that is released into the medium and is successively utilized after the glucose has been consumed [34–36]. This switch from a glycolytic glucose consuming phase to a lactate consuming phase is accompanied in peripheral cells by an increase in the expression of mitochondrial enzymes and proteins and in the mitochondrial membrane potential, indicating an adaptation of the cells to the need of an increased mitochondrial activity during the lactate consumption phase [34–36]. To test how brain astrocytes deal with an extended metabolic glucose depletion and whether these cells may also upregulate their mitochondrial metabolism after glucose depletion, we exposed astrocytes to 2 mM glucose and monitored during the next 2 weeks the viability and several metabolic parameters of the cultures. The results obtained demonstrate that cellular consumption of glucose depletes the culture medium already within 2 days completely of glucose and that the cells survive for up to 10 days by consuming lactate as fuel for mitochondrial ATP production. An upregulation of mitochondrial mass or enzyme activity appears not to occur in cultured astrocytes during the lactate consumption phase but a cellular redistribution of mitochondria from a perinuclear to a more spread cytoplasmic localization was observed for the lactate consumption phase.

Materials and Methods

Materials

Glucose-free Dulbecco's modified Eagle's medium (DMEM) powder, antimycin A, menadione, tetramethylrhodamine ethylester perchlorate, Hoechst 33342 (H33342), and alcohol dehydrogenase powder were purchased from Sigma–Aldrich (Steinheim, Germany). All the other enzymes used for photometric assays were from Roche (Mannheim, Germany). Penicillin G/streptomycin solution and fetal calf serum (FCS) were from Biochrom (Berlin, Germany) and MitoTracker Green FM was obtained from ThermoFisher (Bremen, Germany). Glucose-containing (25 mM) DMEM powder was obtained from Gibco (Darmstadt, Germany). Bovine serum albumin, NADH, NAD⁺, NADPH and NADP⁺ were purchased from AppliChem (Darmstadt, Germany). Water-soluble tetrazolium salt 1 (WST1) was obtained from Dojindo (Munich, Germany). Efavirenz and racemic 8-hydroxy efavirenz were purchased from Toronto Research Chemicals (Toronto, Canada) via LGC Standards (Wesel, Germany). Other chemicals of the highest purity available were obtained from Merck (Darmstadt, Germany), VWR International (Hannover, Germany), Riedel-de Haën (Seelze, Germany), Fluka (Buchs, Switzerland), AppliChem (Darmstadt, Germany) or Sigma–Aldrich (Steinheim, Germany). Sterile 24-well cell culture plates and unsterile 96-well microtiter plates were from Sarstedt (Nümbrecht, Germany).

Cell Culture and Experimental Incubation of the Cells

Astrocyte-rich primary cultures are strongly enriched for astrocytes and were prepared as previously described in detail from the brains of newborn Wistar rats [37]. Briefly, approximately 300,000 viable cells were seeded in 1 mL cell culture medium (90% DMEM containing 25 mM glucose, 44.6 mM sodium bicarbonate, 1 mM pyruvate, 20 U/mL penicillin G, 20 µg/mL streptomycin sulfate, supplemented with 10% FCS) into wells of a 24-well plate. The cultures were maintained at 37 °C with 10% CO₂ in a humidified atmosphere in a cell incubator (Sanyo, Osaka, Japan). The culture medium was renewed every seventh day and one day prior to the onset of an experiment. Confluent astrocyte cultures of an age between 14 and 25 days in culture were used to start the incubations.

For cell experiments, the culture medium was completely aspirated and the cells were incubated with 1 mL incubation medium (glucose-free DMEM supplemented

with 2 mM glucose, 44.6 mM sodium bicarbonate, 20 U/mL penicillin G, 20 µg/mL streptomycin sulfate) at 37 °C with 10% CO₂ in the humidified atmosphere of a cell incubator for the time periods indicated in the figures. If not stated otherwise, the incubation medium was harvested after the given incubation time and the cells were washed twice with 1 mL ice-cold (4 °C) phosphate-buffered saline (PBS; 10 mM potassium phosphate buffer pH 7.4 containing 150 mM NaCl) and lysed as described below for the determination of cellular enzyme activities or for the quantification of cellular metabolites. In case of long-term incubations for up to 15 days, media samples of 10 µL were taken for each time point to quantify the concentrations of extracellular glucose and lactate as well as the lactate dehydrogenase (LDH) activity.

Determination of Substrates, Metabolites, Enzyme Activities, Protein and Cell Numbers

The concentration of extracellular glucose or lactate in the incubation medium was determined as previously described for 10 µL media samples [37]. The activities of the enzymes LDH and citrate synthase were measured using the methods described previously in detail [37, 38]. Specific metabolite contents and enzyme activities were calculated by normalizing the values obtained on the protein content per well that had been quantified by the Lowry method [39] using bovine serum albumin as a standard protein. The cell number per well was quantified by counting the number of H33342-stained nuclei in microscopic images (0.068562 mm²) taken for MTG and TMRE stainings and by extrapolating these numbers to the total cell growth area (1.86 cm²) in one well of the culture plates used.

Determination of Cellular NAD(P)(H) Contents

The quantification of cellular contents of NAD⁺ and NADH was performed using a modification of the cycling method previously described [40] for lysates derived from one well of a 24-well plate which contained depending on the incubation conditions between 70 and 160 µg protein/well (Table 1). The cells were washed twice with 1 mL ice-cold (4 °C) PBS and subsequently lysed with 200 µL 50 mM HCl and 20 mM NaOH to extract NAD⁺ and NADH, respectively, for at least 30 min at 4 °C. NaOH lysates were additionally heated for 10 min at 60 °C to decompose NAD⁺, while NADH is labile and rapidly destroyed in the acidic extracts [41]. The lysate from one well was neutralized with 100 µL 0.2 M potassium phosphate buffer pH 7.5 and centrifuged for 3 min at 12,000 g and 4 °C. 100 µL of the supernatant were mixed with 100 µL of reaction mixture containing 300 mM Tris/HCl buffer pH 8.0, 15 mM EDTA, 3 mM phenazinium etho-sulfate (PES), 1.5 mM methylthiazolyldiphenyl-tetrazolium

Table 1 Protein content and cell number during and after metabolic glucose depletion of primary astrocyte cultures

Time of incubation (d)	Protein content (µg per well)	Counted nuclei per image	Cell number per well
0	144 ± 15	193 ± 12	512,784 ± 31,934
1	120 ± 21*	175 ± 30	464,588 ± 80,510
2	109 ± 14**	169 ± 17	448,695 ± 45,410
7	79 ± 11***	117 ± 18**	309,971 ± 48,897**

Primary astrocyte cultures were incubated with 2 mM glucose for up to 7 days. After the given incubation periods the cellular protein content was measured. The cell number per well was quantified by counting the number of H33342-stained nuclei in microscopic images taken for TMRE and MTG stainings and by extrapolating these numbers to the total cell growth area of one well.

The significance of differences (ANOVA) of the data obtained in comparison to the initial values determined for untreated cultures (0 days) was calculated and is indicated by *p<0.05, **p<0.01 and ***p<0.001

bromide (MTT) and 81 U/mL alcohol dehydrogenase. The reaction was started by adding 100 µL 1.8 M ethanol to each well and the increase in absorption at 570 nm was recorded in a Multiskan Sky microtiterplate spectrophotometer (Life Technologies, Darmstadt, Germany). The contents of NAD⁺ and NADH in the extracts were quantified by comparing the slopes of the linear increase in absorbance of the generated MTT formazan with that recorded for standard concentrations of NAD⁺ (0–2 µM final concentration in the assay) or NADH (0–1.5 µM final concentration in the assay).

The contents of cellular NADP⁺ or NADPH were determined with a similar cycling assay [42] for the cell extracts generated as described above, but for the cycling reaction a modified reaction mixture was used which contained 300 mM Tris/HCl buffer pH 8.0, 15 mM EDTA, 3 mM PES, 1.5 mM MTT and 1.5 U/mL glucose-6-phosphate dehydrogenase. The reaction was started by addition of 100 µL 3 mM glucose-6-phosphate to each well. The contents of NADP⁺ and NADPH were quantified by comparing the slopes of the linear increase in absorbance observed for the samples with that determined for standard concentrations of NADP⁺ (0–0.2 µM final concentration in the assay) or NADPH (0–0.4 µM final concentration in the assay).

Cellular WST1 Reduction

The menadione-mediated WST1 reduction of cultured astrocytes was determined by using a slight modification of a recently published method [43]. Briefly, after the given incubation the cells were washed twice with 0.5 mL pre-warmed (37 °C) incubation buffer (IB; 145 mM NaCl, 20 mM HEPES, 5.4 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 0.8 mM Na₂HPO₄, pH adjusted to 7.4 at 37 °C)

and subsequently incubated with 200 μL IB containing 400 μM WST1, 50 μM menadione and 5 mM glucose at 37 °C. After 30 min of incubation, 50 μL of the incubation medium were diluted with 150 μL pure water in wells of a 96-well plate and the absorbance of WST1 formazan was recorded at 405 nm in the microtiterplate spectrophotometer. The concentration of WST1 formazan was calculated by using the Lambert–Beer law and the molecular extinction coefficient of WST1 formazan at 405 nm of $24.14 \text{ mM}^{-1} \times \text{cm}^{-1}$ [43]. The specific WST1 reduction was calculated by normalizing the amount of extracellular WST1 formazan accumulated to the respective protein content per well (depending of the incubation conditions between 70 and 160 μg protein/well; Table 1).

Mitochondrial Staining and Fluorescence Microscopy

After a given incubation period, cultured astrocytes were stained with the mitochondrial dyes MitoTracker Green FM (MTG; staining for mitochondrial mass; [44]) or tetramethylrhodamine ethyl ester (TMRE; staining for mitochondrial membrane potential; [45]). 500 μL of the existing incubation medium were harvested from each well (stored in a closed cup at 37 °C for later use) and the cells were incubated in the remaining incubation medium with 10 μM H33342 plus either 200 nM MTG or 40 nM TMRE for 90 min at 37 °C in the humidified atmosphere of a cell incubator with 10% CO_2 . During this incubation, the cell culture plates were wrapped with aluminum foil to prevent exposure to light. Subsequently, the cells that had been incubated in the presence of MTG were washed twice with 1 mL pre-warmed (37 °C) PBS and incubated with the 500 μL incubation medium that had been harvested from the respective wells earlier and cellular fluorescence was directly analyzed using a fluorescence microscope (Eclipse TE-2000-U with a DS-QiMc camera and imaging software NIS-Elements BR, Nikon, Düsseldorf, Germany). After TMRE treatment, the cells were not washed and the cellular fluorescence was directly documented while the cells were kept in the incubation medium. The following filter settings were used: for the fluorescence of TMRE (excitation at 510–565 nm, emission at 590 nm, dichromatic mirror at 575 nm), for the fluorescence of MTG (excitation at 465–495 nm, emission at 505–515 nm, dichromatic mirror at 505 nm) and for the H33342 staining (excitation at 340–380 nm, emission at 435–485 nm, dichromatic mirror at 400 nm). To allow for direct comparison of the different incubation times within one experiment, all images were taken with the same light intensity and exposure time settings.

Statistical Analysis

All quantitative data are shown as means \pm standard deviations of values obtained in three independently performed experiments on independently prepared astrocyte cultures. Multiple sets of data were compared by ANOVA followed by a Bonferroni post-hoc test using the software GraphPad InStat 3. The level of significance is indicated by: * $p < 0.05$, ** $p < 0.01$ or *** $p < 0.001$. $p > 0.05$ was considered as not significant. Figures showing cell images or mitochondrial stainings are derived from a representative experiment that was reproduced with almost identical outcome on two independently generated cultures.

Results

Metabolic Glucose Depletion and Lactate Consumption in Astrocyte Cultures

To investigate the long time consequences of an exposure of cultured astrocytes to a limited concentration of glucose, the cells were exposed to an initial glucose concentration of 2 mM and various metabolic parameters and the cell viability were monitored for up to 15 days. The cells rapidly consumed the available glucose and the culture medium was already within 2 days completely deprived of glucose (Fig. 1a, control). This cell-dependent metabolic glucose depletion was accompanied by a rapid increase in the extracellular concentration of lactate, which reached after 2 days of incubation a maximal value of around 3 mM (Fig. 1d, control). The viability of the cells, as demonstrated by morphological inspection (Fig. 2a–c) and the absence of any significant increase in extracellular LDH activity (Fig. 1g, control), was not compromised during the glucose consumption phase (initial 2 days of incubation) nor during the following 10 days of incubation (Fig. 1g, control). The lactate consumption phase was characterized by a steady decrease in the extracellular lactate concentration (Fig. 1d, control). Only when the extracellular lactate content had been almost completely consumed by the cells after more than 12 days of incubation (Fig. 1d, control), substantial toxicity was observed as indicated by an increase in the extracellular LDH activity (Fig. 1g, control). Inspection of the cultures during the incubation by phase contrast microscopy revealed no obvious alterations in cell morphology during the initial 2 days period of glucose consumption (Fig. 2a–c) and even after 7 days of incubation the morphology of most of the cells in the incubated cultures remained unchanged (Fig. 2d, e) compared to untreated cultures (Fig. 2a), although a few areas in the cultures showed cells that were characterized by an accumulation of intracellular vesicles (Fig. 2f). These results suggest that the majority of cells in astrocyte cultures appears to survive even after complete depletion of extracellular glucose for many

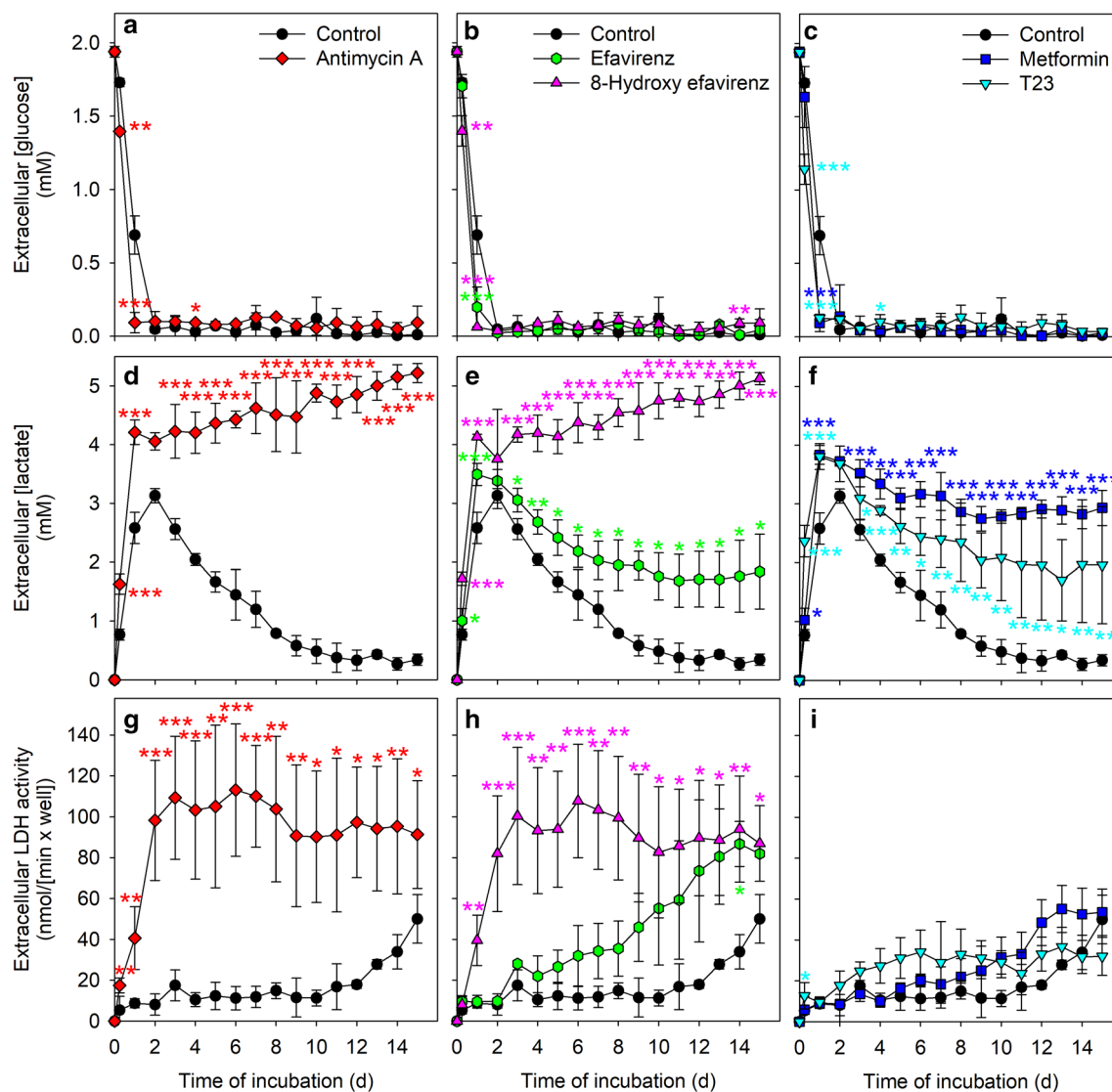


Fig. 1 Alterations in the extracellular concentrations of glucose and lactate and in the cell viability of cultured astrocytes during and after metabolic glucose depletion. Primary astrocyte cultures were exposed to an initial glucose concentration of 2 mM and incubated for up to 15 days in the absence (control) or the presence of 10 μ M antimycin A (**a**, **d**, **g**), 20 μ M efavirenz or 20 μ M 8-hydroxy efavirenz (**b**, **e**, **h**), 1 mM metformin (**c**, **f**, **i**) or 100 μ M T23 (**c**, **f**, **i**). For the indicated time points, the extracellular glucose concentration (**a**–**c**), the extra-

cellular lactate concentration (**d**–**f**) and the extracellular LDH activity (**g**–**i**) as indicator for a loss in cell viability were determined. The initial cellular LDH activity of the cultures was 139 ± 7 nmol/(min \times well) and the initial protein content was 115 ± 16 μ g/well. The significance of differences (ANOVA) between data obtained for the incubation in the presence of modulators of mitochondrial metabolism and data obtained for the control condition is indicated by * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$

days by consuming the extracellular lactate as energy substrate that had been released from the cells during the initial glucose consumption phase.

Effects of Inhibitors of Mitochondrial Metabolism on the Viability and the Metabolism of Astrocytes During and After Glucose Depletion

To confirm that the survival of astrocytes and the utilization of lactate after the metabolic glucose depletion depends on

the mitochondrial oxidative metabolism, cultured astrocytes were incubated in the presence of substances that are known to interfere with astrocytic mitochondrial metabolism, such as the complex III inhibitor antimycin A [46], the antiretroviral drug efavirenz and its primary metabolite 8-hydroxy efavirenz [47, 48] as well as the antidiabetic drug metformin [16, 17, 49] and the protein tyrosine kinase inhibitor tyrphostin 23 (T23; [14, 17]). All these substances significantly accelerated the initial glucose consumption and lactate production compared to the control condition (Fig. 1a–f),

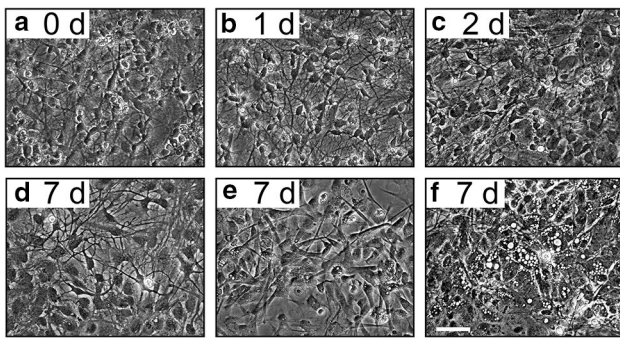


Fig. 2 Morphology of cells in astrocyte primary cultures during and after metabolic glucose depletion. The cells were exposed to 2 mM glucose and then incubated for up to 7 days. Phase contrast microscopic pictures were taken after 0 days (a), 1 days (b), 2 days (c) and 7 days (d–f). The scale bar in panel f represents 50 μ m and applies to all panels

thereby depleting the cultures already within 1 day almost completely of glucose (Fig. 1a–c) and increasing the extracellular lactate concentration to values of around 4 mM (Fig. 1d–f). For cells that had been treated with antimycin A or 8-hydroxy efavirenz severe toxicity was observed already after 1 day of incubation and after 2 days of incubation the

initial cellular LDH was almost completely detectable in the medium (Fig. 1g, h), demonstrating that these conditions were highly toxic for the cells. Accordingly, due to the absence of viable cells the concentration of lactate released from the cells during the initial glucose consumption phase remained at the high level observed after the first day of incubation (Fig. 1d, e). The consequences of a treatment of astrocytes with efavirenz, metformin or T23 were less severe than those observed for astrocytes that had been exposed to antimycin A or 8-hydroxy efavirenz (Fig. 1). The cells remained viable during a treatment with efavirenz, metformin or T23 for several days after the applied glucose had been consumed (Fig. 1h, i). However, compared to control cells (absence of modulators of mitochondrial function), the consumption of lactate was significantly slower (Fig. 1e–f) and some LDH release was found during the lactate consumption phase (Fig. 1h, i). These data suggest that the ability of cultured astrocytes to survive metabolic glucose depletion depends on their ability to metabolize lactate for mitochondrial ATP production.

In order to test whether the toxicity observed for cultured astrocytes that had been incubated with 2 mM glucose in the presence of antimycin A (Fig. 1) was a direct consequence of the rapid depletion of glucose, astrocytes were exposed

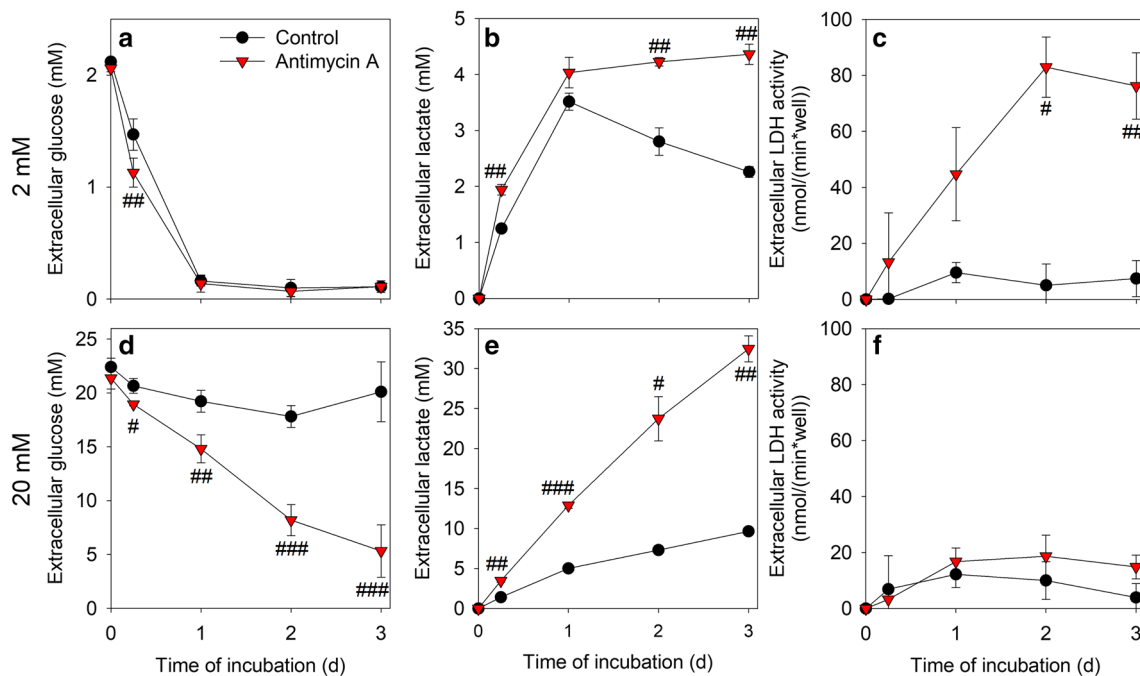


Fig. 3 Alterations in the extracellular concentrations of glucose and lactate and in the cell viability of cultured astrocytes during exposure without or with antimycin A. Primary astrocyte cultures were exposed to an initial glucose concentration of 2 mM (a–c) or 20 mM (d–f) and incubated for up to 3 days in the absence or the presence of 10 μ M antimycin A. For the indicated time points, the extracellular glucose concentration (a, d), the extracellular lactate concentra-

tion (b, e) and the extracellular LDH activity (c, f) as indicator for a potential loss in cell viability were determined. The initial cellular LDH activity of the cultures was 155 ± 23 nmol/(min \times well) with an initial protein content of 129 ± 12 μ g/well. The significance of differences (paired *t*-test) between data obtained for the incubation without and with antimycin A are indicated by #*p*<0.05, ##*p*<0.01 and ###*p*<0.001

to medium containing either 2 mM or 20 mM glucose in the absence or the presence of antimycin A (Fig. 3). As described before (Fig. 1a, d, g), the rapid glucose depletion (Fig. 3a) and lactate production (Fig. 3b) observed for cells that had been exposed to 2 mM glucose was accelerated in the presence of antimycin A and severe toxicity was observed for antimycin A-treated cells (Fig. 3c). For astrocytes that had been exposed to 20 mM glucose, a moderate glucose consumption (Fig. 3d) and extracellular lactate accumulation was observed. In contrast, if astrocytes were incubated with 20 mM glucose in the presence of antimycin A, a strongly accelerated and almost linear glucose consumption (Fig. 3d) and lactate production (Fig. 3e) were found and the severe toxicity observed for astrocytes that had been exposed to 2 mM glucose in the presence of antimycin A (Fig. 3c) was completely prevented (Fig. 3f).

Investigation of Basic Metabolic Parameters for the Glucose Consumption and the Lactate Consumption Phase

To test for a potential adaptation of cellular metabolism due to the switch from glucose consumption to lactate consumption, several metabolic parameters were determined for untreated astrocyte cultures (0 day) as well as for cultures that had been incubated for 1 day (glucose consumption phase), 2 days (glucose completely depleted) or 7 days (lactate consumption phase). As described above (Fig. 1), astrocytes consumed the initially applied 2 mM glucose completely within 2 days and the lactate concentration had increased to around 3 mM during this time (Fig. 4a), while after 7 days of incubation the extracellular lactate concentration had been lowered by cellular consumption from 3 mM to around 0.8 mM (Fig. 4a). During the incubation, the amount of protein per well decreased significantly from an initial value of 144 ± 15 $\mu\text{g}/\text{well}$ to 79 ± 11 $\mu\text{g}/\text{well}$ 7 days after onset of the incubation (Table 1). Also the number of cells calculated for the cultures declined during the incubation and at least after 7 days of incubation the number of cell nuclei was significantly lowered compared to that of untreated cultures (Table 1). However, the specific cellular LDH activity remained almost constant during the entire incubation and almost identical to the values of untreated cells (Fig. 4b). In addition, the extracellular LDH activity remained very low throughout the incubation (Fig. 4b) and the specific WST1 reduction capacity of astrocytes, determined after application of glucose, was not altered (Fig. 4c). These data demonstrate that the viability of most of the cells in the cultures was not compromised for the incubation condition used. Also the specific activity of the mitochondrial enzyme citrate synthase remained almost constant at around 100 nmol/(min \times mg) during the 7 days of incubation (Fig. 4d).

The specific cellular contents of NAD^+ and NADP^+ were not significantly altered during the incubation and remained at about 2 nmol/mg protein (Fig. 4e) and 0.5 nmol/mg protein (Fig. 4f), respectively. In contrast, the amount of NADH (Fig. 4e) and the ratio of NADH to NAD^+ (Fig. 4g) decreased within the first day of incubation, but remained almost constant during longer incubations (Fig. 4e, g). Also the specific NADPH content (Fig. 4f) and the ratio of cellular NADPH to NADP^+ (Fig. 4h) appear to decline during the first day of incubation, but the differences observed did not reach the level of significance (Fig. 4f, h).

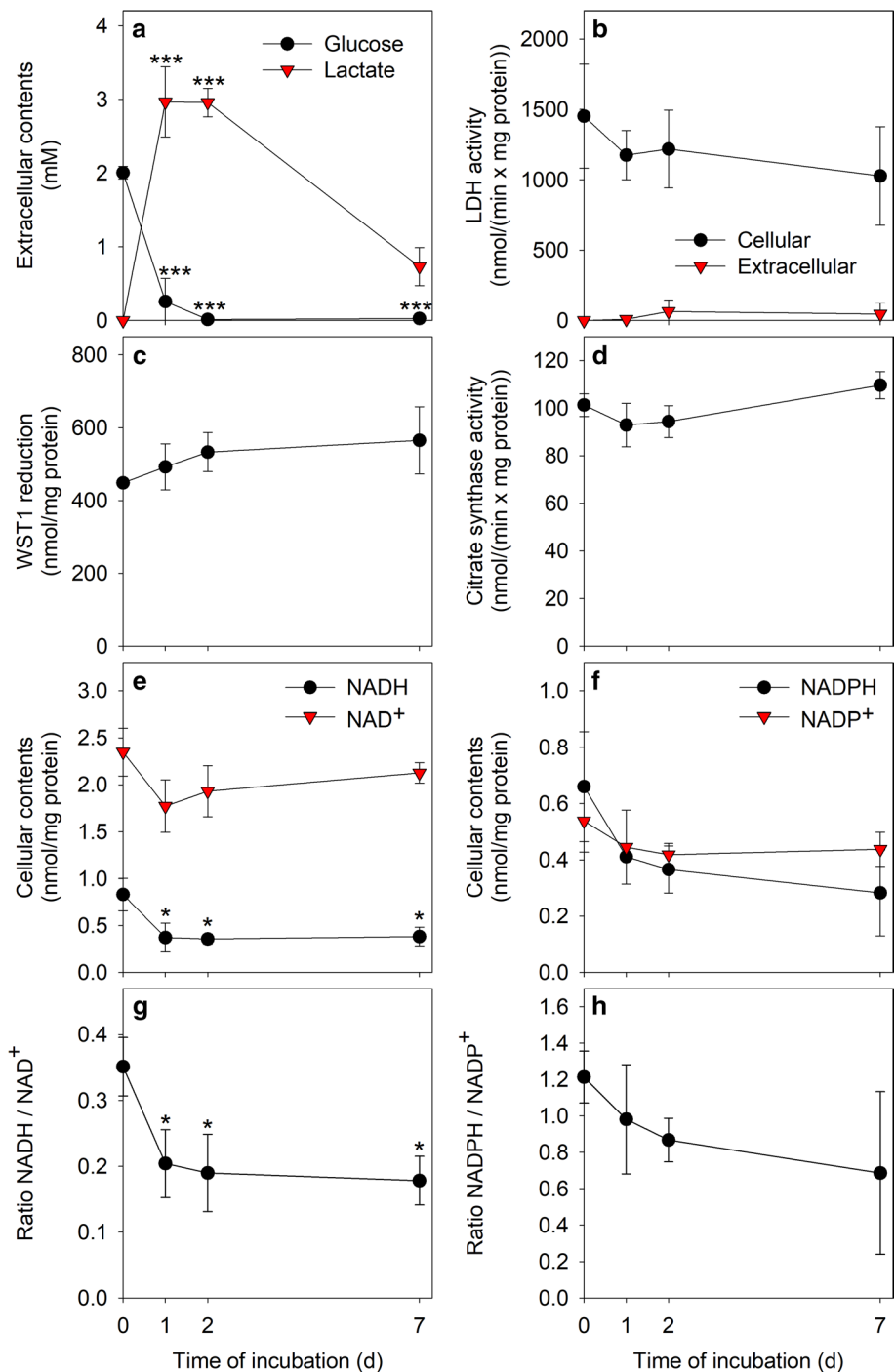
Staining for Mitochondrial Mass and Membrane Potential

To monitor potential changes in mitochondrial mass and/or membrane potential during the glucose consumption and the lactate consumption period, the treated cultures were stained with TMRE to monitor mitochondrial membrane potential [45] or with MTG as indicator for mitochondrial mass [44]. Both staining protocols allowed visualization of mitochondria in cultured astrocytes (Fig. 5). The overall fluorescence intensity after staining of untreated cultures with TMRE was high but seemed to decrease during the first 2 days of incubation (Fig. 5a, c, e), while after 7 days the fluorescence intensity was found higher (Fig. 5g) than that recorded after 2 days of incubation (Fig. 5e). In contrast, the fluorescence signal obtained after MTG staining appeared to remain constant or even slightly increased during the 7 days of incubation (Fig. 5b, d, f, h). However, for both types of mitochondrial stainings a change in the cellular localization of mitochondria was observed from a mainly perinuclear staining of high intensity (Fig. 5a, b) to a more outspread localization of the stained mitochondria throughout the cytoplasm of the cells (Fig. 5g, h).

Discussion

Cultured rat astrocytes consumed glucose that had been applied in a concentration of 2 mM almost completely within the initial 2 days of incubation, while substantial amounts of lactate were produced and released from the cells during this glucose consumption phase. This observation is consistent with the reported high glycolytic capacity of cultured astrocytes to metabolize glucose to lactate [7, 50], even if only low concentrations of glucose are available [51]. After the applied glucose had been completely consumed by the cells, the released lactate was taken up and consumed during the following 10 days (lactate consumption phase), thereby keeping the cells viable. This separation of a glycolytic glucose consumption phase and a subsequent lactate consumption phase has previously

Fig. 4 Cell viability and metabolic parameters of cultured astrocytes during and after metabolic glucose depletion. Primary astrocyte cultures were exposed to 2 mM glucose and then incubated for up to 7 days. For the given incubation periods the extracellular concentrations of glucose and lactate (a), the cellular and extracellular LDH activities (b), the specific cellular WST1 reduction capacity after application of glucose as substrate (c), the specific cellular citrate synthase activity (d), and the specific cellular contents of NAD⁺ and NADH (e) as well as the contents of NADP⁺ and NADPH (f) were determined. Furthermore the cellular ratios of NADH to NAD⁺ (g) and of NADPH to NADP⁺ (h) were calculated. The significance of differences (ANOVA) of the data obtained in comparison to the initial values determined for untreated cultures (time point 0 day) was calculated and is indicated by *p<0.05 and ***p<0.001



been reported for cultured primary mouse fibroblasts and astrocytes [34] as well as for some cell lines of peripheral origin [35, 36]. The transporters involved in the observed transport processes are well known for astrocytes. Glucose uptake in astrocytes is mediated by the glucose transporter GluT1 [52], while the monocarboxylate transporters MCT1 and MCT4 are likely to contribute to the export and reuptake of lactate by astrocytes [53, 54].

The majority of cells in astrocyte cultures survived both the glucose consumption phase and the long lactate consumption phase, as indicated by the unaltered specific cellular LDH activity, the low extracellular LDH activity and the high specific cellular WST1 reduction capacity. Nevertheless, a loss in total protein content was observed during the lactate consumption phase and some morphological alterations were seen during the incubation, at least

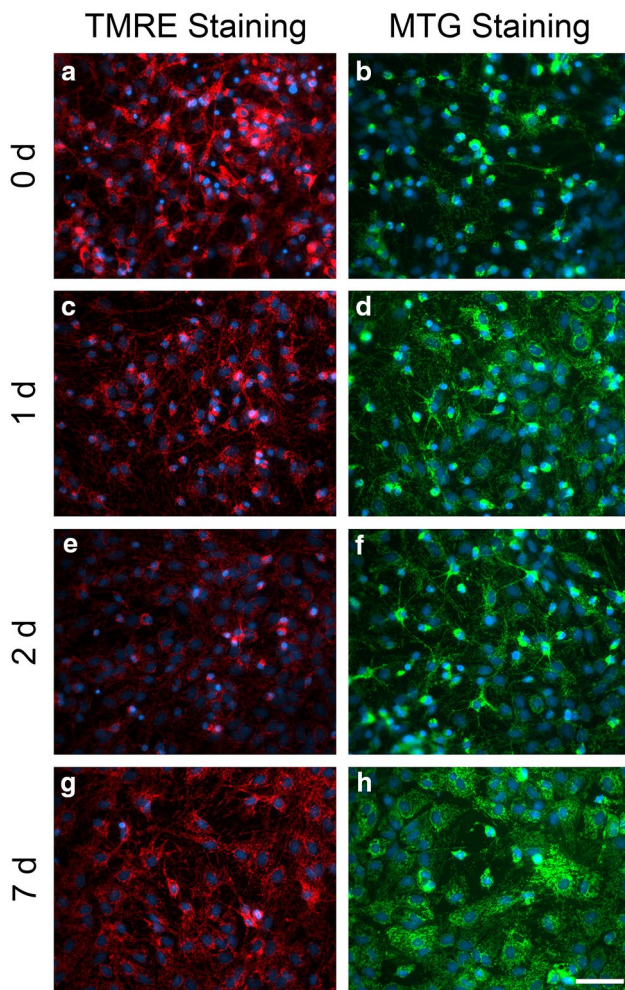


Fig. 5 Staining for mitochondrial membrane potential and mitochondrial mass of cultured astrocytes during and after metabolic glucose depletion. Primary astrocyte cultures were exposed to 2 mM glucose and then incubated for up to 7 days. After the given incubation periods, the cells were stained with TMRE (**a**, **c**, **e**, **g**) to indicate mitochondrial membrane potential or with MTG (**b**, **d**, **f**, **h**) to visualize the presence and the cellular localization of mitochondria. In addition, the cultures were stained with H33342 (blue) to visualize all cell nuclei. The scale bar in panel **h** represents 50 μm and applies to all panels

for some cells in the cultures. This observation may reflect the presence of a minor population of cells in the astrocyte cultures which is more vulnerable towards glucose deprivation than the majority of astrocytes populating the cultures. A vulnerable subtype of astrocytes may be present in untreated glucose-fed cultures, as the astrocytes populating primary cultures may be as heterogeneous in morphology and function [55, 56], as astrocytes in the brain [57–59]. Alternatively, microglial or oligodendroglial cells which are present in low number in the primary cultures used [8, 37] may be more vulnerable to the depletion of glucose than astrocytes, as reported at least for oligodendrocytes [60] but

not for microglia [61]. The loss of some cells in the cultures appears not to be compensated by proliferation of remaining viable cells, as for the serum-free incubation conditions applied cell proliferation in astrocyte cultures is expected to be strongly limited [62, 63]. The gradual loss of some cells during the incubation could also contribute to the gradual slowing of lactate consumption during extended incubations for up to 2 weeks. Nevertheless, the majority of cells in the astrocyte cultures survived the lactate consumption phase, at least as long as sufficient amounts of extracellular lactate were present, as demonstrated by the unchanged specific activities of various enzymes. However, as soon as most of the extracellular lactate had been utilized, the viability of the cells in the cultures was severely compromised, suggesting that the depletion of the extracellular substrate lactate will lower mitochondrial ATP production which will in turn lead to the observed cell death.

The importance of mitochondrial metabolism for the survival of astrocytes during the lactate consumption phase was confirmed by the application of modulators of mitochondrial metabolism, including the complex III inhibitor antimycin A [64], the antiretroviral drug efavirenz and its metabolite 8-hydroxy efavirenz [48, 65–68], the antidiabetic drug metformin [69, 70] and the protein tyrosine kinase inhibitor T23 [71]. Compared to control cells (absence of modulators) all these substances accelerated the initial glucose consumption and increased the initial extracellular lactate accumulation. This increase in glycolytic flux is thought to meet the cellular energy demand during compromised mitochondrial ATP production and confirms literature data on the alteration of mitochondrial functions and on the stimulation of glycolytic lactate production in astrocytes after exposure to antimycin A [46, 72], efavirenz [47], 8-hydroxy efavirenz [47, 48], metformin [16, 49] or T23 [14, 17].

After exposure of astrocytes to 2 mM glucose in presence of antimycin A the glucose consumption was accelerated and the ratio of extracellular lactate to glucose consumed was two, as expected for a situation that prevents mitochondrial ATP production [16]. Glucose depletion in presence of antimycin A was accompanied with severe cell toxicity, suggesting that the absence of extracellular glucose that is essential as substrate for glycolytic ATP production under these conditions is the reason for the observed toxicity. Indeed, the rapid toxicity of antimycin A was completely prevented in the presence of 20 mM glucose, demonstrating that a continuous excess of the substrate glucose which is efficiently metabolized to lactate is sufficient to keep cultured astrocytes alive in a situation of impaired mitochondrial ATP production. This is consistent with the reported ability of astrocytes *in vivo* to survive despite of a defective respiratory chain [73].

The metabolism of extracellular lactate is thought to involve lactate uptake, cytosolic oxidation of lactate to

pyruvate, uptake of pyruvate into mitochondria and subsequent mitochondrial oxidation of pyruvate [74]. However, for some cells lactate appears to be taken up and oxidized directly in mitochondria for energy production. Such a mitochondrial lactate oxidation complex has been reported for rat neurons [75, 76] and mitochondrial LDH activity has been at least reported for human astrocytoma cells [77]. However, whether mitochondrial lactate oxidation takes place in astrocytes remains to be elucidated.

Of the applied inhibitors of mitochondrial metabolism, antimycin A as well as 8-hydroxy efavirenz caused rapid and severe toxicity already during the first day of the incubation, most likely due to their high potential to inhibit the mitochondrial respiratory chain [48, 64]. In contrast, the presence of efavirenz, metformin or T23 did not immediately prevent but rather slowed down the lactate consumption that followed the glucose consumption phase. Thus, these substances appear to be less efficient to affect mitochondrial metabolism in the concentrations applied, as indicated by the detectable lactate consumption and by the ability of the cells to survive at least for some days following metabolic glucose depletion.

For cultured fibroblast, the metabolic switch from the glycolytic glucose consumption phase to the mitochondrial ATP production during the lactate consumption phase was accompanied by an increase in mitochondrial membrane potential and by an increased expression of mitochondrial proteins, including citrate synthase [34]. However, such alterations were not observed for astrocytes. The mitochondrial membrane potential was already very prominent for untreated astrocytes, confirming literature data [78–80] and was not found to be increased during or after glucose depletion. Also the specific citrate synthase activity remained unchanged during the incubation with a specific activity of approximately 100 nmol/(min × mg protein), a value consistent with literature data [38]. Moreover, no obvious increase for mitochondrial mass was detected by MTG staining for cultured astrocytes in the lactate consumption phase. The absence of any obvious upregulation of mitochondrial mass during or after glucose depletion suggests that the principal metabolic capacity of the mitochondria which are present in untreated astrocyte cultures is sufficiently high, even if the cultures had been cultured continuously in high-glucose-containing medium, to meet the energy demand of the cells by mitochondrial ATP production that is fueled by the metabolism of the lactate that had been released during the glucose consumption phase.

Comparison of the initial glucose consumption during the first day of incubation (1249 ± 131 nmol/d) and the lactate consumption in the first 3 days of the lactate consumption phase between 2 and 5 days of the incubation (488 ± 20 nmol/d), revealed that the initial glucose consumption was 2.5 times higher than the lactate consumption

after glucose depletion. Glucose is consumed quickly within 2 days and the lactate produced from glucose during this time serves the cells well to keep them alive for around 2 weeks. This is consistent with the known higher efficiency for ATP production of oxidative lactate consumption in comparison to ATP synthesis during glycolytic glucose metabolism to lactate. As the glycolytic metabolism of 1 mol glucose to 2 mol lactate yields only 2 mol ATP, while mitochondrial metabolism of 1 mol lactate generates around 15 mol of ATP, the potential maximal rates of ATP generation from extracellular substrates appear to be around 2.5-times higher during the lactate consumption phase compared with the initial glucose consumption phase. This suggests that during the initial glucose consumption phase the mitochondrial oxidative phosphorylation contributes already substantially to the overall ATP production of the cultures. This view is strongly supported by the low ratio for lactate produced versus glucose consumed of 1.5 after the glucose consumption phase, which was found increased to the maximal value of two for incubations with inhibitors of mitochondrial metabolism.

Mitochondria can be directly recruited to sites of elevated metabolic load or activity in various brain cell types [19, 81, 82]. Also for astrocyte cultures it has been demonstrated that mitochondria are moved to sites of high activity in order to provide locally the energy required [19]. Although no indication for an increase in mitochondrial mass was observed during or after glucose depletion, the mitochondrial staining revealed evidence for alterations in the cellular localization of mitochondria in astrocyte cultures from a mainly perinuclear localization at the onset of the incubation to a spread localization throughout the cytoplasm, confirming the high dynamics of mitochondrial localization in astrocytes [83]. Interestingly, the high intensity of staining of the mitochondrial membrane potential of untreated cultures was found lowered during the glucose consumption phase, but the intensity of this staining recovered at least partially in the lactate consumption phase. This observation may be a consequence of the ongoing reorganization of the mitochondrial network, which is required for the redistribution of mitochondria in the cells. Such fission events are characterized by an initial depolarization of the mitochondrial membrane potential and by a recovery of the membrane potential after reorganization of the mitochondrial network [24]. The more spread localization of mitochondria in astrocytes may facilitate a more efficient utilization of the substrate lactate for mitochondrial ATP production under conditions of glucose deprivation, as previously discussed for other experimental conditions [19].

During the glucose consumption phase the overall fluorescence intensity of the TMRE staining appeared to be weakened during the first 2 days of incubation. Potential reasons for this observation could be the high glucose

concentration applied to the cells 24 h before the onset of the experiment as well as the absence of serum during the incubation. However, control experiments revealed that neither the fluorescence intensity for MTG and TMRE nor the cellular distribution of mitochondria was obviously altered by omitting the medium renewal before the onset of the experiment nor by serum-deprivation (data not shown).

The cellular redox pairs NADH/NAD^+ and NADPH/NADP^+ are crucial for astrocytic metabolism and the maintenance of their redox states is important to ensure proper cellular functions [84]. Cultured rat astrocytes contained an initial total concentration of NADH plus NAD^+ of approximately 3.4 nmol/mg protein which is close to the values reported for cortical mouse astrocytes and also the initial NADH/NAD^+ ratio of approximately 0.35 for untreated rat cultures was similar to reported data [40], demonstrating the oxidized status of the NADH/NAD^+ redox pair to accept electrons that are generated for example by the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [40, 84]. The initial content of NADPH plus NADP^+ of approximately 1.5 nmol/mg protein and the reduced status of the NADPH/NADP^+ redox pair confirm literature data [85], consistent with the view that NADPH has to be available for reductive biosynthesis reactions and glutathione regeneration [84, 86].

For the incubation conditions studied here, the specific cellular contents of NAD^+ and NADP^+ remained almost constant during the 7 days of incubation, while a quick loss in the contents of NADH and NADPH was found already after the first day of incubation. Such a loss in the content of NADH , while the NAD^+ content remains unaltered, has previously been reported for cultured mouse astrocytes incubated with low concentrations of glucose [40], but the reason for this loss in NADH (and NADPH) that is not accompanied by an increase in the levels of the oxidized cofactors remains to be elucidated. During the first day of incubation, the cells efficiently consumed glucose and lowered the extracellular glucose concentration from the initial 2 mM to around 0.7 mM. A likely explanation for the loss in cellular NADH and NADPH contents during this first day of incubation is that the available extracellular glucose concentration becomes too low to maintain the initial high rates of glucose uptake and metabolism that is required to foster maximal NAD^+ reduction via the glycolytic GAPDH reaction [84, 87] and efficient reduction of NADP^+ by the oxidative part of the pentose phosphate pathway [86].

In conclusion, glucose depletion experiments revealed that astrocytes in culture efficiently metabolize glucose to lactate via glycolysis and subsequently utilize the lactate released for mitochondrial energy production. This metabolic capacity of astrocytes prevents cell toxicity after glucose depletion and keeps the cells viable as long as sufficient lactate is available as energy fuel. The switch from glycolytic

glucose metabolism to mitochondrial lactate oxidation was not accompanied by an alteration of mitochondrial mass, but a redistribution of mitochondria was observed after glucose depletion from a perinuclear to a more distributed localization in the cytoplasm. These data demonstrate the remarkable ability of astrocytes to survive glucose depletion and provides evidence that an alteration of mitochondrial localization in astrocytes may be a consequence of the need for an accelerated mitochondrial metabolism. Further studies are now required to elucidate whether the observed alteration in the cellular localization of mitochondria after glucose depletion is correlated with altered functions and/or an altered metabolic potential of astrocytic mitochondria.

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Compliance with Ethical Standards

Conflict of interest The authors have no conflict of interest to declare.

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