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Metformin accelerates glycolytic lactate production in cultured primary cerebellar granule neurons 15

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Abstract

Metformin is the most frequently used drug for the treatment of type-II-diabetes. As metformin has been reported to cross the blood-brain barrier, brain cells will encounter this drug. To test whether metformin may affect the metabolism of neurons, we exposed cultured rat cerebellar granule neurons to metformin. Treatment with metformin caused a time- and concentration-dependent increase in glycolytic lactate release from viable neurons as demonstrated by the 3-to 5-fold increase in extracellular lactate concentration determined after exposure to metformin. Half-maximal stimulation of lactate production was found after incubation of neurons for 4 h with around 2 mM or for 24 h with around 0.5 mM metformin. Neuronal cell viability was not affected by acute incubations with up to 30 mM metformin nor by incubations with up to 10 mM metformin for up to 48 h, although alterations in cell morphology were observed during long term treatment with 10 mM metformin. The acute stimulation of neuronal lactate release by metformin was persistent upon removal of metformin from the medium and was not affected by the presence of modulators of adenosine-monophosphate activated kinase activity. In contrast, rabeprazole, an inhibitor of the organic cation transporter 3, completely prevented metformin-mediated stimulation of neuronal lactate production. In summary, the data presented identify metformin as a potent stimulator of glycolytic lactate production in viable cultured neurons and suggest that organic cation transporter 3 mediates the uptake of metformin into neurons.

Keywords:

Glycolysis; lactate; metformin; neurons

Introduction

The biguanide metformin has a high therapeutic potential for the treatment of type-II-diabetes [1] as it lowers blood glucose levels [2-4] and reduces insulin resistance [5, 6]. Currently, metformin is the most frequently used drug for diabetes treatment and is taken by around 150 million people worldwide [2, 7]. Metformin is considered as a save drug. As common side effects only mild gastrointestinal problems are described [8] and only a small subgroup of metformin-treated patients suffers from severe lactate acidosis [9, 10]. Metformin appears not to be metabolized in humans, but internalized metformin is removed from the body by renal excretion [11]. However, some accumulation of metformin has been reported for the renal tract [12], the small intestine and the hepatic portal vein [13] but also for brain and cerebrospinal fluid [14], demonstrating that metformin can enter the brain. Cellular uptake of metformin, at least in peripheral cells, is mediated by the organic cation transporters OCT1 and OCT3 [15-18], which are also expressed in brain cells [19, 20]. The therapeutic potential of metformin is mediated by its ability to modulate cellular metabolism by targeting different metabolic pathways including activation of adenosine monophosphate activated kinase (AMPK) [5, 15, 21, 22], inhibition of complex I [23-25] and inhibition of mitochondrial glycerol-phosphate dehydrogenase [26].

Metformin treatment has been reported to have beneficial effects on brain functions. In animal models post-stroke treatment of mice with metformin was shown to improve functional and behavioral recovery [27]. Metformin has also been reported to improve learning and memory of rodents via a reduction of oxidative stress [28] and to prevent seizures in mice suffering from induced epilepsy [29]. For cultured brain cells, metformin has been shown to prevent neurodegeneration induced by advanced glycation products (AGEs) in human neuronal stem cells [30], to prevent glutamate-induced toxicity in cultured cerebellar granule neurons [31] and to reduce neurotoxicity caused by etoposide [24] and amyloid-β [32] in cultured

hippocampal neurons. In addition, metformin has been reported to stabilize barrier functions in primary cultures of rat brain capillary endothelial cells [33].

For cultured brain astrocytes it has very recently been reported that metformin modulates the glucose metabolism as demonstrated by the metformin-induced stimulation of glycolytic lactate production and by impairing mitochondrial pathways [34, 35]. In order to test whether metformin may also affect the glucose metabolism of neurons, we have exposed cultured primary cerebellar granule neurons to metformin. Here we demonstrate that application of metformin to cultured neurons caused a time- and concentration-dependent stimulation of glycolytic lactate production in a process which is likely to involve uptake of metformin by OCT3 and an AMPK-independent cellular mechanism.

Materials and Methods

Materials

Metformin hydrochloride, cytosine β-D-arabinofuranoside (AraC), poly-D-lysine, soybean trypsin inhibitor, rabeprazole, cimetidine and 5-aminoimidazole-4-carboxyamide-1-β-D-ribofuranoside (AICAR) were obtained from Sigma-Aldrich (Steinheim, Germany). Compound C (dorsomorphin) was purchased from Tocris Biosciences (Bristol, England). NADH, NAD^+ and $NADP^+$ were from AppliChem (Darmstadt, Germany). The enzymes lactate dehydrogenase, glutamate-pyruvate transaminase, hexokinase and glucose-6-phosphate dehydrogenase were from Roche Diagnostics (Mannheim, Germany). All other chemicals and buffer ingredients were purchased from Sigma (Steinheim, Germany), Applichem (Darmstadt, Germany) or Merk (Darmstadt, Germany). Minimal essential medium (MEM) and Earle´s balanced salt solution to prepare and maintain the neuron cultures were obtained from Life Technologies-Invitrogen (Darmstadt, Germany). Fetal calf serum and penicillin/streptomycin solution were purchased from Biochrom (Berlin, Germany). Sterile cell culture dishes and unsterile 96-well plates were obtained from Sarstedt (Nümbrecht, Germany).

Primary neurons cultures

Cerebellar granule neuron-rich primary cultures were prepared from the cerebella of 7 day old Wistar rats as previously described in detail [36]. Of the harvested cells, 750.000 viable cells were seeded per well of a poly-D-lysine coated 24-well plate in 1 mL culture medium (90% MEM with 10% heat-inactivated fetal calf serum, containing 30 mM glucose, 25 mM KCl, 2 mM L-glutamine, 100 U/mL penicillin G and 100 µg/mL streptomycin sulfate). After 24 h, AraC was added from a concentrated stock solution to a final concentration of 10 µM per well to prevent proliferation of contaminating glial cells. Primary cerebellar granule neuron

cultures are strongly enriched in neurons and contain only around 1% contaminating astrocytes [36]. The neurons were cultured in the humidified atmosphere in a cell incubator (Sanyo, Japan) at 37°C with 5% CO_2 and were used for experiments after 7 to 9 days in culture.

Experimental incubations

For incubations of neurons for 24 h or 48 h, the cultures were washed with 1 mL culture medium and incubated in 1 mL (data shown in Fig. 1a,b) or 500 µL (data shown in Fig. 1c,d) culture medium containing the indicated concentrations of metformin. For acute incubations of up to 6 h, the culture medium was removed and the cells were washed ones with 1 mL in pre-warmed (37°C) incubation buffer (IB; 145 mM NaCl, 30.4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 0.8 mM Na_2HPO_4, 20 mM HEPES, 5 mM glucose, adjusted with NaOH to pH 7.4 at 37°C) before they were incubated with 200 µL IB containing metformin and/or other compounds in the concentration indicated. If not stated otherwise, test compounds were dissolved directly in IB. A concentrated stock solution of cimetidine (125 fold in DMSO) were diluted with IB to generate the final concentration of this compounds in IB. Control experiments revealed that the solvent in the final concentration present did not affect cell viability or lactate production (data not shown). After the respective incubation periods, the incubation media were collected for determining the extracellular concentrations of lactate and glucose and the extracellular LDH activity. The cells were used for morphological inspection or PI staining.

Cell viability and protein content

The viability of cultured neurons after a given treatment was analyzed by determining the activity of the cytosolic enzyme LDH in incubation media and cell lysates as described previously [36, 37]. For analysis of extracellular LDH activity after acute incubations (up to 6

h) 10 µL of the incubation media were analyzed, while for longer incubations (24 h or 48 h) 20 µL of the incubation media were used to determine LDH activity. To confirm the cell viability microscopic inspections were performed to analyze the morphology of cultured neurons that had been incubated without or with metformin. Phase contrast images were taken directly after the incubation with a Digital Sight DSL1 camera connected to a Nikon Eclipse TS100 microscope (Nikon, Düsseldorf, Germany). As an additional method to test for a potentially compromised cell viability, propidium iodide (PI) staining was performed. PI is membrane impermeable and a PI-positive nuclear staining will only be observed if the membrane integrity of cells is impaired [36]. To visualize the total number of cells the membrane permeable dye Hoechst 33342 was applied with PI. After incubation with PI and H33342 fluorescence images were taken with a Digital Sight DSL1 camera connected to a Nikon Eclipse TS100 microscope.

To determine the cellular protein content per well, the neuron cultures were washed once with 1 mL ice-cold phosphate-buffered saline (10 mM potassium phosphate buffer, pH 7.4, containing 150 mM NaCl) and lysed in 200 µL 0.5 M NaOH for 30 min on a shaker. The protein content was determined for 50 µL of lysate by the Lowry method [38] using bovine serum albumin as a standard protein.

Determination of extracellular lactate and glucose concentrations

The extracellular concentrations of lactate and glucose in the incubation media were measured before and after incubations of neurons by the coupled enzymatic assays described previously [36]. For incubation in 200 µL and 500 µL incubation media 10 µL samples we applied to the assays, while for incubations in a medium volume of 1 mL a sample volume of 20 µl was analyzed. Glucose consumption was calculated from the difference between the values determined for media harvested from the cells after the incubation and values of the

respective media that did not have contact with cells. The specific values given for lactate production and glucose consumption were calculated by normalization of the data obtained for lactate production and glucose consumption to the protein content of untreated cells.

Presentation of data

The quantitative data shown represent means ± SD of values obtained in three or more (n) independent experiments that had been performed on individually prepared neuron cultures. In the case of three or more data sets, the data were analyzed for statistical significance of differences by ANOVA followed by the Bonferroni *post-hoc* test and the level of significance is indicated by asterisks (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Significance of difference between two sets of data was analyzed by the t-test and the level of significance is indicated by hashes ($^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$). p-values lower than 0.05 were considered as not significant. Pictures showing cell morphology are from representative experiments that have been reproduced on an independently prepared neuron cultures with a similar outcome.

Results

Effects of metformin exposure on the viability of cultured neurons

To investigate potential consequences of an exposure of neurons to metformin, we incubated cultured primary cerebellar granule neurons in culture medium for up to 48 h with metformin in concentrations of up to 10 mM and tested for cell viability. During the incubation of neurons in the absence of metformin a slow increase in the extracellular LDH activity was observed that accounted after 24 h and 48 h to around 15 % and 20 % of the initial specific cellular LDH activity of the cultures (Fig. 1a). However, no obvious alteration in the cell morphology was observed during the incubations of neurons for up to 48 h in the absence of metformin (Fig. 1e,h). Incubations of neurons for 24 h or 48 h with metformin in concentrations of 0.1 mM, 1 mM or 10 mM did not significantly increase the extracellular LDH activity compared to control cells that had been incubated without metformin (Fig. 1a,c). Microscopic analysis of the incubated cultures revealed that compared to control cultures (absence of metformin, Fig. 1e,h) an incubation with 1 mM metformin did not affect the morphology of neurons as indicated by the clearly visible cell bodies and by the intact cell processes (Fig. 1f,i). In contrast, for neuron cultures that had been exposed to 10 mM metformin a gradually increasing altered morphology with broken processes was observed after incubations for 24 h and 48 h (Fig. 1 g,j). PI staining of neurons that had been exposed to metformin in concentrations of up to 10 mM for 24 h revealed no increase in the number of PI positive cell nuclei (Fig. 2b-d) in comparison to control cells (Fig. 2a), while a treatment with silver nitrate as control for a toxic condition caused a PI staining of all neurons (Fig. 2e,j).

To test for acute consequences of an exposure of neurons with metformin, the cells were incubated in the amino acid-free IB to metformin in concentrations of up to 10 mM for up to 6 h or up to 30 mM metformin for 4 h. None of these conditions caused any significant increase

in extracellular LDH activity compared to the low values (around 10% of initial cellular LDH) determined for control cultures that had been incubated without metformin (Fig. 3a,d).

Effects of metformin on the glycolytic lactate production by cultured neurons

During incubations of primary neurons in culture medium in the absence of metformin, extracellular lactate accumulated to concentrations of 1.2 ± 0.2 mM and 1.6 ± 0.5 mM after incubations for 24 h and 48 h, respectively (Fig. 1b). If metformin was present in concentrations of 1 mM or 10 mM during the incubation, the extracellular lactate concentrations were found significantly increased after 24 h and 48 h of incubations to values that were elevated 2fold (10 mM metformin) and 3-4fold (1 mM metformin) compared to the values determined for controls (incubated in the absence of metformin, Fig. 1b). To study the concentration dependency of the stimulation of the neuronal lactate production by metformin in more detail, neurons were incubated in incubation medium for 24 h with concentrations of metformin ranging between 0.1 and 10 mM (Fig. 1d). The presence of metformin caused a concentration-dependent increase in the extracellular lactate concentration from 1.4 ± 0.14 mM (absence of metformin), which was significant already for a metformin concentrations of 1 mM (4.42 ± 1.1 mM; Fig. 1d). Half-maximal and maximal stimulation of lactate production within 24 h was determined for incubation with metformin in concentrations of around 0.5 mM and 3 mM, respectively (Fig 1d).

To investigate an acute stimulation by metformin of the glycolytic flux in cultured neurons, the cells were incubated in IB for up to 6 h without or with metformin and lactate production as well as glucose consumption were determined. In the absence of metformin a slow increase in extracellular lactate concentration was observed which resulted within 6 h in an extracellular concentration of 0.82 ± 0.24 mM (Fig. 3b). In the presence of metformin the extracellular lactate accumulation was accelerated in a concentration-dependent manner.

While the presence of 0.1 mM metformin during the incubation did not significantly affect glycolytic flux, the lactate production was found significantly increased after 6 h of incubation with 1 mM metformin and already after 2 h of incubation with 10 mM metformin (Fig. 3b). After exposure of neurons for 6 h to 1 mM and 10 mM metformin, the extracellular lactate concentration had increased compared to the control incubation (0.82 ± 0.24 mM, absence of metformin) to 2.41 ± 0.60 mM (1 mM) and 4.43 ± 0.62 mM (Fig. 3b). A more detailed analysis of the concentration-dependency of the metformin-induced acute stimulation of lactate production during a 4 h incubation revealed half-maximal and maximal increases in extracellular lactate concentration by incubations with metformin in concentrations of 2 mM (1.8 ± 0.6 mM lactate) and 10 mM (3.24 ± 1.04 mM), respectively (Fig. 3e). Application of metformin in concentrations higher than 10 mM did not further increase the metformin-induced stimulation of glycolytic lactate production but was less efficient to stimulate glycolysis (Fig. 3e,f). For neuronal glucose consumption, a similar acute time- and concentration-dependent stimulation by metformin was observed (Fig. 3c,f) as for extracellular lactate accumulation (Fig. 3b,e), although the significance levels for differences in glucose consumption between controls and metformin-treated cells were low due to the problem of differences of large numbers.

For further experiments on the effects of metformin on cultured neurons we have used acute incubations for 4 h in the absence or the presence of a standard concentration of 10 mM metformin. Table 1 lists data for such incubations from 14 experiments. Compared to control incubations, the presence of metformin increased the extracellular lactate accumulation and the glucose consumption around 4-fold. In each individual experiment, metformin strongly increased the extracellular lactate accumulation, but the extent of stimulation as well as the basal lactate release from cultured neurons differed between individual experiments (Table 1). Also the ratio of lactate production to glucose consumption varied between individual

experiments, but did not differ significantly between incubations without and with metformin (Table 1).

The metformin-mediated stimulation of lactate production in cultured neurons is persistent

To test whether the stimulation of lactate release by metformin requires continuous presence of metformin in the medium, the cells were pre-incubated for 2 h without or with 10 mM metformin, washed and subsequently incubated for up to 4 h in metformin-free or metformin-containing IB. After the 2 h pre-incubations of neurons with metformin the extracellular lactate concentration was almost 4fold increased compared to that of neurons, which had been pre-incubated in the absence of metformin (none; Fig. 4a). During a main incubation of metformin-preincubated neurons in metformin-containing IB, the accelerated lactate productions continued and remained 4fold elevated during the entire main incubation period (Fig. 4a). Also if metformin-preincubated neurons were washed and incubated during the main incubation in metformin-free IB, the glycolytic lactate production remained accelerated and did not differ to that of neurons exposed to metformin during pre- and main incubation (Fig. 4a), demonstrating that presence of metformin is not required to maintain the accelerated glycolytic lactate production in metformin-treated cultured neurons. Neither the medium change nor the washing step between pre- and main incubation caused a substantial increase in extracellular LDH activity (Fig. 4b), demonstrating that the cell viability was not affected by the treatments applied.

Rabeprazole blocks the metformin-induced stimulation of lactate release in cultured neurons

Metformin has been reported to be taken up into cells by OCTs [15-18]. As OCT2 and OCT3 are functionally expressed in brain [19, 39], we have tested whether inhibitors of OCT2

(cimetidine) or OCT3 (rabeprazole) [15, 40] are able to affect the metformin-induced stimulation of lactate production by neurons. Neither an incubation of neurons for 4 h with the inhibitors alone nor their co-incubation with 10 mM metformin significantly increased the extracellular LDH activity (Fig. 5a). In addition, none of the two OCT inhibitors affected the basal lactate production of neurons, while metformin increased the extracellular lactate concentration 2.5-fold (Fig. 5b). This stimulation was not significantly lowered by the presence of 10 mM cimetidine, while already 100 µM rabeprazole completely prevented the metformin-induced extracellular accumulation of lactate (Fig. 5b).

Modulators of AMPK do not affect the metformin-induced lactate production in neurons

As metformin has been reported to activate AMPK [15], we investigated a potential role of this enzyme in the metformin-induced stimulation of lactate release from cultured neurons. AMPK-dependent processes were investigated by applying the AMPK activator AICAR [41, 42] or the AMPK inhibitor compound C [43]. Incubations of cultured neurons with these compounds in the absence or the presence of 10 mM metformin did not significantly elevate the extracellular LDH activity (Table 2), suggesting that the conditions used were not acutely toxic to the cells. The AMPK modulators, compound C and AICAR, did not affect the basal lactate release from neurons nor significantly lowered the metformin-induced stimulated lactate release (Table 2).

Discussion

Metformin is the first choice drug for the treatment of type-II-diabetes, but a treatment with metformin has also been shown to have promissing benefical effects for certain neurological complications [29, 44-46] and against cancer progression [3, 47-49]. Metformin has been reported to enter the brain and to accumulate in the cerbrospinal fluid [14], demonstrating that brain cells will encounter metformin. As metformin has been reported to strongly affect the metabolism of peripheral cells [2, 3], this drug may also modulate the metabolism of brain cells. Indeed, recently it was reported that metformin stimulates glycolysis and inhibits mitochondrial metabolism in cultured astrocytes [34, 35, 50]. Here we report that metformin also stimulates the glycolytic flux in viable cultured primary cerebellar granule neurons.

Upon incubation of cultured cerebellar granule neurons in glucose-containing media, the cells produced lactate which accumulated in the medium. The specific lactate production rates observed for incubations in the hour range in incubation buffer (around 0.52 µmol/(h x mg)) or for 24 h incubations in culture medium (around 0.45 µmol/(h x mg)) are consistent with literature data [51-54]. However, the basal lactate release from cultured neurons varied strongly between individual neuron cultures, as demonstrated by a high SD of around 50% observed for the specific extracellular lactate accumulation within 4 h (Table 1). Such variations between batches of cultured neurons have previously been reported [54, 55] and may be a consequence of inconsistencies in the cell harvest or in cell density.

Metformin appears not to be neurotoxic, as even in concentrations of up to 30 mM metformin were not acutely toxic for cultured neurons. Also during longer incubations these cells had to be exposed to 10 mM or higher concentrations of metformin for at least 24 h before some alterations in cell morphology were observed as potential first indicator for an impairment of cellular functions. These results are consistent with literature data reported for cultured

peripheral [56, 57] and brain cells [32, 34], confirming the low cell toxic potential of metformin.

During incubations with metformin the glycolytic lactate production of cultured neurons was strongly accelerated in a time- and concentration-dependent manner. For acute stimulation in the hour range concentrations higher than 1 mM of metformin had to be applied, while for longer incubations already metformin in concentrations of 1 mM significantly increased neuronal lactate production. This reflects most likely a slow cellular uptake of the strongly basic metformin. Accordingly, either the application of high concentrations of metformin for short incubations periods or longer incubation periods with low concentrations of metformin appear to be required to allow cellular accumulation of metformin to concentrations which are sufficiently high to affect cellular metabolism. As metformin is positively charged at physiological pH [58] a simple diffusion of the compound over the cell membrane is unlikely. For peripheral cells uptake of metformin has been shown to be mediated by OCTs [15, 18, 59]. For cultured neurons, the presence of the OCT3 inhibitor rabeprazole [40], but not the presence of the OCT2 inhibitor cimetidine [60], prevented the metformin-induced stimulation of neuronal lactate production. As OCT3 in expressed in cultured rat cerebellar granule neurons [20], these results suggest that OCT3 is involved in the uptake of metformin in cultured neurons.

In peripheral cells, metformin has been reported to activate AMPK [21, 22, 61], but seveal studies have reported that metformin triggers also AMPK independent pathways [62, 63]. As neither the AMPK inhibitor compound C [43] nor the AMPK activator AICAR [41] significantly affected the basal and the metformin-induced stimulation of lactate release from neurons, AMPK is unlikely to be involved in the stimulation of glycolysis observed under the conditions applied. This is consistent with recent literature data on the stimulation of glycolysis by metformin in cultured astrocytes [34].

The observed stimulation of glycolytic flux adds metformin to the list of structurally diverse compounds which have been reported to accelerate neuronal lactate production, including formaldehyde [51, 52], the uncoupler 2,4-dinitrophenol [54] or inhibitors of the respiratory chain (data not shown). Common to all these compounds is that they target mitochondrial respiration. Also metformin has been discussed as an inhibitor of complex I of the respiratory chain in peripheral cells [25, 64] and for cultured cortical neurons a partial decrease complex I activity was shown [24]. Very recently, metformin has been reported to slow mitochondrial citric acid cycle and complex I-mediated respiration in cultured astrocytes [35]. These data suggest that metformin may also in cerebellar granule neurons compromise mitochondrial functions which subsequently leads to a stimulation of glycolysis in order to compensate for an impaired mitochondrial ATP production, as shown for myoblasts [65]. Further studies are now required to confirm this hypothesis.

Slow cellular uptake of metformin is most likely the reason why metformin has frequently been applied in millimolar concentrations to study acute metformin-induced modulation of cellular functions [22, 34, 57, 59, 66]. However, it should be considered that such concentrations are substantially higher than the plasma concentrations found in treated patients [67, 68]. Those are, depending on the doses of metformin applied, mainly in the range of around 20 µM [69, 70], but can reach in some cases even concentrations of up to 400 µM [10]. The half-live of metformin in the plasma of patients is around 50 h demonstrating that renal removal of the drug from the human body is slow [12]. As metformin concentrations in CSF exceeded the concentrations in the plasma and even increase during prolonged administration and as metformin contents in cerebellum are higher than those in other brain regions [14], it appears likely that a chronic treatment of patients with high doses of metformin may lead to concentrations of metformin in the cerebellum which are sufficiently high to affect the metabolism of brain cells.

In conclusion, metformin stimulates the glycolytic lactate production in cultured neurons in a time- and concentration-dependent manner by a process that involves most likely uptake by OCT3. Whether a metformin-induced stimulation of glycolysis also occurs in the brain of metformin-treated animals or patients remains to be elucidated. A potential modulation by metformin of the metabolism of brain cells *in vivo* may be involved in the reported beneficial effects of metformin for several neurological conditions [29, 44, 45].

Conflict of interest

The authors have no conflict of interest to declare.

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Figure legends

Figure 1: Effects of metformin on the viability and the glycolytic lactate production of cultured neurons. The cells were incubated for up to 48 h (a-b) or for 24 h (c-d) in culture medium without or with metformin in the indicated concentrations and the extracellular LDH activity (a,c) and the extracellular lactate concentration (b, d) were determined. The data shown in panels a-d represent means ± SD of values obtained from experiments on three independently prepared cultures. The significance of differences of data obtained for incubations in the absence (0 mM) or the presence of metformin was calculated by ANOVA followed by the Bonferroni *post-hoc* test and is indicated by *$p<0.05$, **$p<0.01$ or ***$p<0.001$. Panels e-j show representative phase contrast pictures of the morphology of neuron cultures that had been incubated for 24 h (e-g) or 48 h (h-j) without (e,h) or with 1 mM (f, i) or 10 mM (g, j) metformin. The scale bar in panel e represents 50 µm and applies to the panels e-j.

Figure 2: Membrane integrity of metformin-treated neuron cultures. The cells were incubated for 24 h without metformin (control; a, f) or with 0.1 mM (b,g), 1 mM (c,h) or 10 mM metformin (d,i). Alternatively, as control for cell toxicity, the cells were treated for 30 min with 150 µM $AgNO_3$ (e,j). After the incubation the cells were exposed to PI and H33342 before pictures were taken for PI-staining (a-e) and H33342-staining (f-j). The scale bar in panel a (50 µm) applies to all panels.

Figure 3: Concentration- and time-dependent acute effects of metformin on the viability and the glycolytic flux of cultured primary neurons. The cells were incubated with the indicated concentrations of metformin for up to 6 h (a-c) or for 4 h (d-f). The extracellular LDH activity (a, d), the extracellular lactate concentration (b, e) and the extracellular glucose concentration

(c, f) were determined. The data represent mean ± SD of values obtained in four independently prepared neuron cultures. Significant differences (ANOVA) compared to the control condition (0 mM metformin) are indicated by *p<0.05, **p<0.01 and ***p<0.001.

Figure 4: Effects of a removal of extracellular metformin on the metformin-induced stimulation of glycolytic lactate production. The cells were pre-incubated for 2 h without (None) or with (MF) 10 mM metformin, washed and incubated for additional 4 h without (None) or with (MF) 10 mM metformin. The extracellular concentration of lactate (a) and the extracellular LDH activity (b) were determined. The data given for the main incubation represent the sum of the values obtained for the main plus the respective pre-incubation. The data presented are means ± SD of values obtained in experiments performed on three individually prepared cultures.

Figure 5: Effect of OCT inhibitors on the metformin-induced stimulation of lactate production by cultured neurons. The cells were incubated for 4 h without (None) or with 10 mM metformin in the absence (Control) or the presence of 10 mM cimetidine or 100 µM rabeprazole and the extracellular LDH activity (a) and the extracellular lactate concentration (b) were determined. The data presented are means ± SD of values obtained in experiments performed on seven (control), four (rabeprazole) and three (cimetidine) independently prepared cultures. Significance of differences compared to the respective controls was analyzed by ANOVA followed by the Bonferroni post-hoc test and is indicated by **p<0.01. Significant differences between data obtained for incubations without (None) and with metformin (as calculated by the paired t-test) are indicated by $^{\#\#}$p<0.01 and $^{\#\#\#}$p<0.001.