

Copper Accelerates Glycolytic Flux in Cultured Astrocytes

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Abstract Astrocyte-rich primary cultures were used to investigate the consequences of a copper exposure on the glucose metabolism of astrocytes. After application of CuCl_2 (30 μM) the specific cellular copper content increased from initial 1.5 ± 0.2 nmol/mg to a steady state level of 7.9 ± 0.9 nmol/mg within about 12 h. The copper accumulation was accompanied by a significant increase in the extracellular lactate concentration. The stimulating effect of copper on the lactate production remained after removal of extracellular copper. Copper treatment accelerated the rates of both glucose consumption and lactate production by about 60%. The copper induced acceleration of glycolytic flux was prevented by inhibition of protein synthesis, and additive to the stimulation of glycolysis observed for inhibitors of respiration or prolyl hydroxylases. A copper induced stimulation of glycolytic flux in astrocytes could have severe consequences for the glucose metabolism of the brain in conditions of copper overload.

Keywords Astrocytes · Copper · Glucose · HIF · Lactate

Introduction

Copper is an essential trace metal that is required as catalytic co-factor or as structural component of several enzymes [1–4]. However, an excess of copper is harmful to cells, most likely by the copper-catalysed generation of hydroxyl radicals in a Fenton-like reaction [5, 6]. To avoid both copper deficiency and copper toxicity, cellular uptake, distribution and excretion of copper are tightly regulated. However, disturbances of these homeostatic mechanisms in copper metabolism have been considered to contribute to the development and progression of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease and prion disorders [7–9].

Astrocytes fulfil many important functions for the brain. These cells modulate synaptic transmission, synaptic plasticity and extracellular ion homeostasis [10–12]. In addition, astrocytes are metabolically coupled to neurons and have been discussed to supply neurons with metabolites for the synthesis of the antioxidant glutathione (GSH) and with substrates for energy production [13–15]. Thus, compromised astrocytic functions will also affect neighbouring neurons in brain.

Astrocytes have been shown to efficiently accumulate copper in vitro [16–18]. One transporter that is likely to be involved in this copper accumulation by astrocytes is the copper transport receptor 1 (Ctr1), but also the divalent metal transporter 1 (DMT1) has been considered to mediate uptake of copper ions into cultured astrocytes [17]. Efficient copper uptake by astrocytes may be important for the protection of neighboring neurons in brain. At least in co-cultures, astrocytes protect cerebellar neurones against copper toxicity [16]. In addition, astrocytes prevent the copper catalysed extracellular loss of GSH [19]. However, excess of copper has been reported to damage astrocytes. Exposure

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of cultured astrocytes to copper increases membrane permeability and mitochondrial permeability transition, induces a mild oxidative stress that leads to lipid peroxidation and alterations in the GSH/GSSG ratio, and inactivates enzymes such as glutathione reductase [18, 20, 21].

Astrocytes are considered as rather glycolytic cell type that produces substantial amounts of ATP by glycolysis, as indicated by the high rate of lactate production compared for example to neurons [22, 23]. However, the stimulation of glycolysis by inhibitors of mitochondrial respiration in cultured astrocytes demonstrates that these cells produce ATP also by respiration and that they do not depend exclusively on glycolytic ATP production [22, 24]. In addition to inhibitors of the respiratory chain, lactate production in cultured astrocytes is stimulated by a variety of substances such as glutamate, cytokines, ammonia, noradrenalin or pioglitazone [15, 25–28]. Furthermore, compounds that stabilise the transcription factor hypoxia inducible factor (HIF)-1 α increase the glycolytic rate in cultured astrocytes by an upregulation of glycolytic enzymes [29].

To study the consequences of subtoxic concentrations of cellular copper on the glucose metabolism of astrocytes, we have investigated lactate release and glucose consumption of astrocyte-rich primary cultures after application of micromolar concentrations of CuCl₂. Here we show that copper treatment stimulated glycolytic flux in a time- and concentration- dependent manner, in a process that depends on protein synthesis and that is additive to the expected effects of inhibitors of mitochondrial respiration and of stabilisers of HIF-1 α .

Experimental Procedures

Materials

Fetal calf serum and penicillin/streptomycin solution were obtained from Biochrom (Berlin, Germany). Dulbecco's modified Eagle's medium (DMEM, without or with 25 mM glucose) was from Gibco (Karlsruhe, Germany). Saccharose and dimethyl sulfoxide (DMSO) were purchased from Janssen Chimica (Geel, Belgium). Bovine serum albumin, NAD⁺, NADH and NADP⁺ were purchased from AppliChem (Darmstadt, Germany). Antimycin A, cycloheximide, deferoxamine mesylate (DFx), EDTA, rotenone and Tris were purchased from Sigma (Steinheim, Germany). Glucose-6-phosphate dehydrogenase (G6PDH), glutamate pyruvate transaminase (GPT), hexokinase and lactate dehydrogenase (LDH) were purchased from Roche Diagnostics (Mannheim, Germany). CoCl₂, disodium hydrogen phosphate, Folin-Ciocalteu phenol reagent, HNO₃, neutral red (NR; 3-amino-7-dimethylamino-2-methyl-phenazine hydrochloride), Pd(NO₃)₂,

potassium sodium tartrate, sodium azide, sodium dihydrogen phosphate, sodium pyruvate and triethanolamine hydrochloride were obtained from Merck (Darmstadt, Germany). Acetic acid, glutamine, MgCl₂ and NaOH were purchased from Fluka (Buchs, Switzerland). Argon was from Linde (Hamburg, Germany). HCl was purchased from Fisher Scientific (Schwerte, Germany). CuCl₂ was from Riedel-de-Haen (Seelze, Germany) and glucose from Serva (Heidelberg, Germany). Ethanol was purchased from Roth (Karlsruhe, Germany) and dimethylallyl glycine (DMOG) from Cayman Chemical (Ann Arbor, USA). Microtiter plates and sterile cell culture dishes were from Sarstedt (Nümbrecht, Germany).

Cell Cultures

Astrocyte-rich primary cultures were prepared from brains of newborn Wistar rats according to the method of Hamprecht and Löffler [30]. Specific lactate release and glucose consumption rates were determined for cell cultures on 6-well plates, while all other experiments were performed on cultures in 24-well plates. Total numbers of 300,000 and 1,500,000 viable cells were seeded in 1 ml culture medium (90% DMEM containing 25 mM glucose and 1 mM pyruvate, 10% foetal calf serum, 20 U/ml penicillin G, 20 μ g/ml streptomycin sulfate) per well of 24-well culture plates and in 2.5 ml culture medium per well of 6-well culture plates, respectively. The cultures were maintained at 10% CO₂ in the humidified atmosphere of a Sanyo (Osaka, Japan) incubator and were used for experiments at an age between 14 and 20 days. The culture medium was renewed every 7 days and 24 h prior to the individual experiment.

Experimental Incubation with Copper

Cells were washed twice with 1 ml (24-well plates) or 2.5 ml (6-well plates) of prewarmed (37°C) incubation medium (DMEM containing 25 mM glucose, 1 mM sodium pyruvate, 20 U/ml penicillin G and 20 μ g/ml streptomycin sulfate) and then incubated at 37°C for up to 24 h in 1 ml (24-well plates) or 2.5 ml (6-well plates) incubation medium with CuCl₂ in the concentrations given in the legends of the figures and the table. The experiments were stopped by washing the cells once with 1 ml (24-well plates) or 2.5 ml (6-well plates) of ice-cold phosphate buffered saline (PBS; 10 mM potassium phosphate buffer, pH 7.4, containing 150 mM NaCl) and twice with 1 ml (24-well plates) or 2.5 ml (6-well plates) of ice-cold PBS containing 10 mM EDTA. Dry cells were stored at -20°C for the quantitation of copper and of protein. For determination of other cellular parameters the cells were processed as described below. For the determination of

extracellular lactate and LDH activity, the incubation medium was collected and processed as described below.

Determination of Lactate Release Rates and Glucose Consumption Rates

If not stated otherwise, cells on 6-well culture plates were preincubated for 16 h at 37°C in 2.5 ml incubation medium without or with CuCl₂ in the concentrations given in the legends of the figures or the table, washed twice with 2.5 ml prewarmed (37°C) DMEM (containing 5 mM glucose and 1 mM pyruvate) and then incubated at 37°C for up to 4 h in 1 ml DMEM (containing 5 mM glucose and 1 mM pyruvate). The rates of glucose consumption and lactate production were calculated from the almost linear decreases and increases of the extracellular glucose and lactate concentrations, respectively. Lactate and glucose contents of the media were determined as described previously [31–33].

Determination of Cellular Copper and Protein Contents

The cells were lysed in 1 ml 50 mM NaOH and this lysate was used to determine cellular protein contents according to the Lowry method [34], using bovine serum albumin as standard, and the cellular copper content as previously described in detail [17].

Determination of Cell Viability

Compromised cell viability and functions were determined by quantifying the release of the cytosolic enzyme LDH and the accumulation of NR into lysosomes as indicators. The activity of cellular and extracellular LDH was determined as described previously [35]. Neutral red (NR) retention was investigated using a modification of a published assay [36]. Briefly, cells in wells of 24-well plates were washed twice with 1 ml of prewarmed (37°C) incubation medium and were subsequently incubated for 1 h at 37°C in 500 µl of DMEM (containing 25 mM glucose and 1 mM pyruvate) with 16 µg/ml NR. Cells were then washed twice with 1 ml PBS and lysed in 500 µl of lysis solution (50% (v/v) ethanol, 1% (v/v) acetic acid, 49% (v/v) H₂O). Of these lysates, 250 µl were transferred into wells of a microtiter plate and the absorbance at 540 nm was measured.

Presentation of Data

If not stated otherwise, results are presented as means ± SD of data obtained in at least three experiments that were performed on independently prepared astrocyte-rich primary cultures. Significance of differences between two sets of data were analysed by the *t* test and differences between groups of data were analysed by ANOVA followed by the

Bonferroni post hoc test, with **P* < 0.05, ***P* < 0.01 and ****P* < 0.001. *P* > 0.05 was considered as not significant.

Results

Copper Accumulation and Lactate Production by Copper-Exposed Cultured Astrocytes

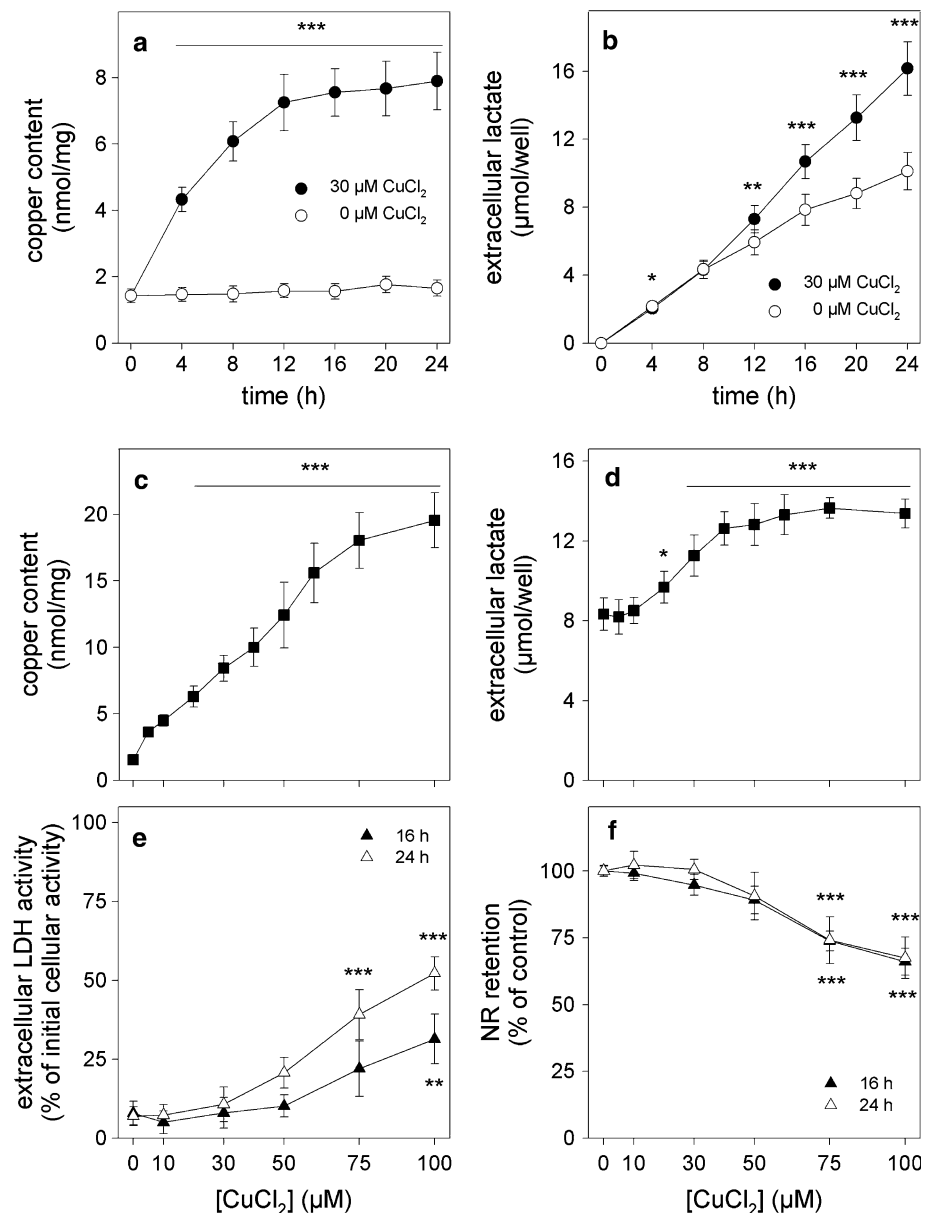
Treatment of astrocyte-rich primary cultures with 30 µM CuCl₂ resulted in a time dependent increase in the cellular copper content from a basal value of 1.5 ± 0.2 to 7.9 ± 0.9 nmol/mg within 24 h. The initial strong increase of the cellular copper content within the first hours after copper application slowed down after 8 h and reached a plateau after about 12 h of incubation (Fig. 1a). To investigate the concentration dependency of the copper accumulation, the cells were incubated with up to 100 µM CuCl₂ for 16 h and the contents of cellular copper were determined (Fig. 1c). The specific cellular copper content increased almost proportional to the concentration of copper applied. However, concentrations above 60 µM did not further increase the cellular copper content (Fig. 1c), but significantly compromised cell viability and functions as indicated by the increase in extracellular LDH activity (Fig. 1e) and by the significantly lowered NR retention after 16 and 24 h of incubation (Fig. 1f).

To determine whether exposure to CuCl₂ alters the glycolytic flux in cultured astrocytes, the lactate content in the medium was determined during incubation of the cells with or without copper for up to 24 h (Fig. 1b). During the first 8 h of incubation in the absence or presence of 30 µM CuCl₂, lactate accumulated in the medium proportional to time, reaching within 8 h a concentration of 4.0 ± 0.4 mM. However, longer incubations revealed significantly elevated concentrations of lactate in the media of cells that had been exposed to CuCl₂ compared to control cells (Fig. 1b). The extracellular lactate content strongly increased with the concentration of CuCl₂ applied, showing a half-maximal effect after 16 h exposure of the cells to about 30 µM CuCl₂ (Fig. 1d). A maximal increase in extracellular lactate concentration to about 160% of controls (absence of copper) was observed after incubation of astrocytes for 16 h with 60 µM CuCl₂ (Fig. 1d).

Lactate Production and Glucose Consumption Rates After Removal of Extracellular Copper

To investigate whether presence of extracellular copper is required to maintain the increased lactate release, lactate production and glucose consumption rates were determined for astrocytes that had been preincubated for various periods with 30 µM CuCl₂. The specific lactate release and

Fig. 1 Consequences of a copper treatment on cultured astrocytes. The cells were incubated without (*open circles*) or with (*filled circles*) 30 μM CuCl_2 for up to 24 h (**a, b**) or with CuCl_2 in the indicated concentrations for 16 h (**c, d**). The contents of cellular copper (**a, c**) and of extracellular lactate (**b, d**) were determined. The panel's **e** and **f** show the extracellular LDH activity and the NR retention, respectively, of cells that were exposed to the indicated concentrations of CuCl_2 for 16 h (*filled triangles*) or 24 h (*open triangles*). The significance of differences of the data compared to those of the control condition (absence of CuCl_2) is indicated



glucose consumption rates of cultured astrocytes were not significantly altered, if the cells were preincubated without copper for up to 24 h (Fig. 2a). In contrast, the lactate release rate increased almost linearly during the first 12 h of preincubation with CuCl_2 from a basal rate of 19.2 ± 0.8 $\text{nmol}/(\text{mg} \times \text{min})$ to 28.1 ± 2.5 $\text{nmol}/(\text{mg} \times \text{min})$ (Fig. 2a). This increase was accompanied by a similar increase in the glucose consumption rate from 10.0 ± 0.5 $\text{nmol}/(\text{mg} \times \text{min})$ to 15.7 ± 1.3 $\text{nmol}/(\text{mg} \times \text{min})$ within 12 h (Fig. 2b). Longer preincubations than 12 h with CuCl_2 did not lead to any further increase of these rates (Fig. 2a, b).

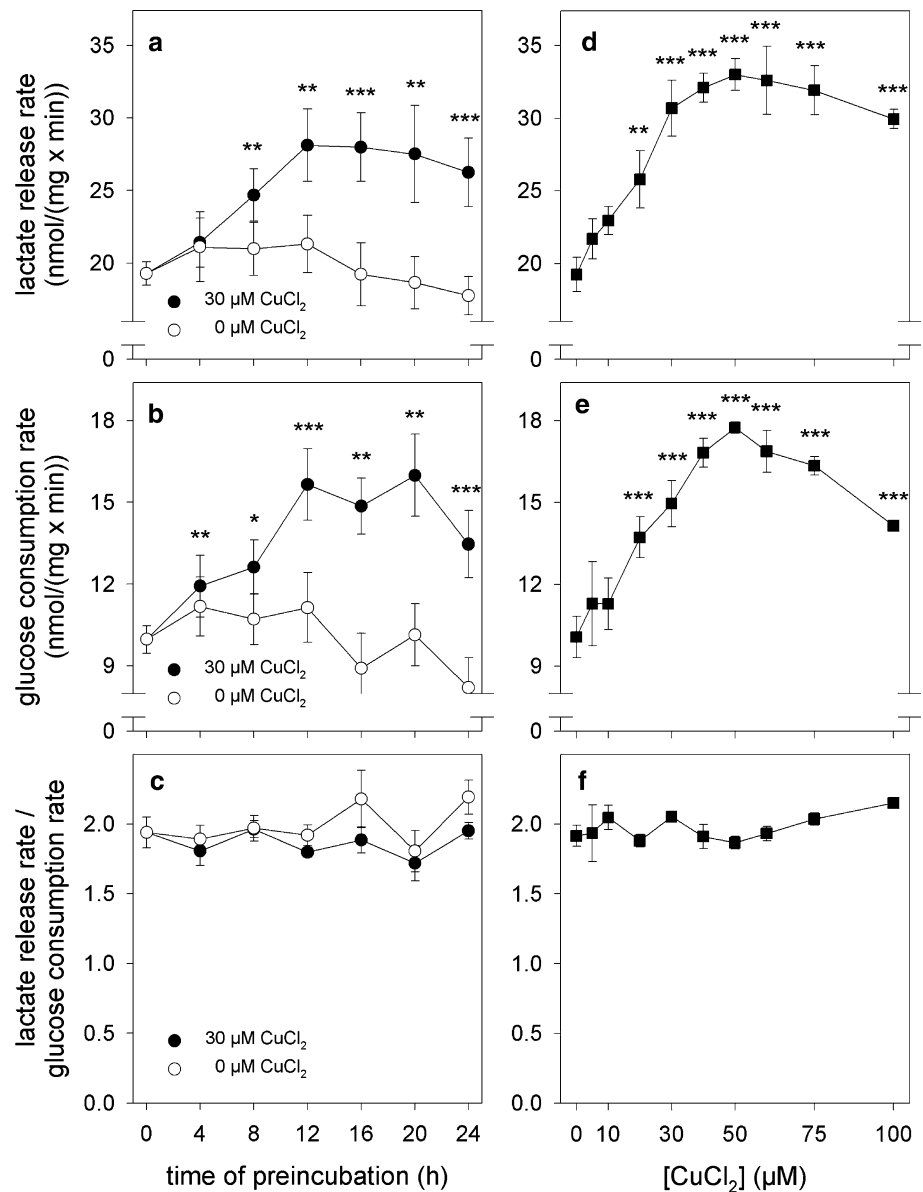
Both, lactate release rate (Fig. 2d) and glucose consumption rate (Fig. 2e) increased almost proportional to the copper concentration that had been present during a 16 h preincubation, at least for copper concentrations of up to

50 μM . Higher CuCl_2 concentrations yielded lower rates (Fig. 2d, e), most likely due to copper-induced toxicity at high CuCl_2 concentrations (Fig. 1e, f). The ratio of the lactate production rate to the glucose consumption rate was almost two for all conditions used and was not affected by the time of preincubation with CuCl_2 (Fig. 2c) nor by the concentration of CuCl_2 that had been present during preincubation of the cells (Fig. 2f).

Effects of Cycloheximide on Copper Accumulation and Lactate Production

To determine whether the copper-induced increase in lactate production and glucose consumption requires the synthesis of proteins, the cells were preincubated for 16 h

Fig. 2 Effect of copper on the glycolytic flux in cultured astrocytes. The cells were preincubated without (*open circles*) or with (*filled circles*) 30 μM CuCl_2 for up to 24 h (**a–c**) or with CuCl_2 in the concentrations indicated for 16 h (**d–f**). After removal of the extracellular copper, the rates of lactate release (**a, d**) and of glucose consumption (**b, e**) as well as the ratio of lactate release rate to glucose consumption rate (**c, f**) were determined. The significance of differences of data compared to those of controls (absence of CuCl_2 during preincubation) is indicated



without or with 30 μM CuCl_2 in the absence or the presence of 10 μM of the protein synthesis inhibitor cycloheximide, before the main incubation was performed to determine glycolytic flux rates. As described above, preincubation with copper for 16 h increased the specific cellular copper content 5fold, did not affect cell viability, but increased the extracellular lactate concentration by 37% (Table 1). Presence of cycloheximide during preincubation without or with CuCl_2 did not increase the extracellular LDH activity, but lowered significantly the cellular protein content and the NR retention by 15 and 25% of the respective controls (absence of cycloheximide), respectively (Table 1). Under these conditions, the specific cellular copper content was slightly elevated by 20%, but the copper-induced increase in extracellular lactate

concentration during the preincubation was completely prevented (Table 1). In addition, both the copper-induced increases in glucose consumption and lactate production rates of copper treated cells were abolished, if the cells had been preincubated with CuCl_2 plus cycloheximide (Table 1).

Effects of Respiratory Chain Inhibitors and of HIF-1 α -stabilising Compounds

Inhibition of respiratory chain complexes has been reported to increase glucose consumption and lactate release of cultured astrocytes [37]. To test whether such a process is involved in the observed copper-induced acceleration of glycolytic flux, astrocytes were preincubated for 16 h

Table 1 Effects of copper and cycloheximide on cultured astrocytes

	0 μM CuCl_2 0 μM CHx	30 μM CuCl_2 0 μM CHx	0 μM CuCl_2 10 μM CHx	30 μM CuCl_2 10 μM CHx
<i>Values after 16 h preincubation</i>				
Copper content (nmol/mg)	1.6 \pm 0.2	7.6 \pm 0.7***	1.9 \pm 10.3	9.1 \pm 1.2*** [#]
Protein content (mg/well)	0.49 \pm 0.03	0.54 \pm 0.03*	0.42 \pm 0.02 ^{##}	0.42 \pm 0.02 ^{###}
Extracellular lactate content ($\mu\text{mol/well}$)	7.8 \pm 0.9	10.7 \pm 1.0***	4.8 \pm 0.5 ^{###}	5.1 \pm 0.5 ^{###}
Extracellular LDH activity (% of initial cellular activity)	8 \pm 4	8 \pm 5	12 \pm 4	12 \pm 5
NR retention (% of control)	100 \pm 2	95 \pm 4	78 \pm 2 ^{###}	70 \pm 2 ^{*,###}
<i>Main incubation</i>				
Specific lactate release rate (nmol/(min \times mg))	19.2 \pm 2.1	28.0 \pm 2.1**	22.1 \pm 1.6	19.4 \pm 2.2 [#]
Specific glucose consumption rate (nmol/(min \times mg))	8.9 \pm 1.4	14.9 \pm 1.2***	10.0 \pm 1.4	9.1 \pm 1.2 ^{###}

Cultured astrocytes were preincubated for 16 h without or with CuCl_2 in the absence or presence of cycloheximide (CHx). The significance of differences of data obtained for cells that were preincubated with and without copper is indicated by * $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$. Significance of differences between data sets for cells that were treated without and with CHx is indicated by [#] $P < 0.05$, ^{##} $P < 0.01$ or ^{###} $P < 0.001$

without or with 30 μM CuCl_2 , before the rates of glucose consumption and lactate production were assessed in the absence or presence of rotenone, antimycin A or sodium azide. Inhibition of the oxidative phosphorylation by each of these inhibitors increased significantly ($P < 0.001$) both lactate release (Fig. 3a) and glucose consumption rates (Fig. 3b) 2–3 fold, whereas copper-preincubation increased lactate production and glucose consumption by only around 60% (Fig. 3). However, the effects observed for copper-preincubated cells on lactate release and glucose consumption rates were additive to those observed by each of the inhibitors (Fig. 3).

Copper is known to stabilise the transcription factor HIF-1 α , which induces increased expression of genes that are involved in glucose uptake and glycolysis during hypoxia [38]. To test whether copper-induced stabilisation of HIF-1 α is involved in the observed copper-induced stimulation of glycolytic flux, cultured astrocytes were preincubated for 16 h without or with 30 μM CuCl_2 in the presence of compounds that stabilise HIF1 α . No differences in the cellular copper content were observed after pretreatment of cells with 30 μM CuCl_2 alone or in combination with DFX or DMOG, whereas the presence of CoCl_2 resulted in a significantly higher ($P < 0.001$) cellular copper content (Fig. 4a). The presence of each of the three stabilisers of HIF-1 α , as well as presence of CuCl_2 alone, led to significantly elevated rates of lactate release and glucose consumption ($P < 0.01$ or $P < 0.001$) to about 150% of the values obtained for control cells (absence of stabilisers and copper; Fig. 4b, c). However, co-incubation of cells with both copper plus stabiliser of HIF-1 α caused additive increases in lactate production and glucose consumption rates, thereby almost doubling both rates (Fig. 4b, c).

Discussion

The present study investigated the consequences of copper accumulation on the glycolytic flux of cultured astrocytes. Elevated cellular copper levels accelerated lactate release and glucose consumption by these cells in a process that was prevented by inhibition of protein synthesis and that was additive to the effects observed for inhibitors of the respiratory chain and for stabilisers of HIF-1 α .

Cultured astrocytes accumulated copper efficiently from DMEM in a time- and concentration-dependent manner, by mechanisms that involve most likely Ctr1-dependent and Ctr1-independent transport [16, 17]. After 12 h of incubation with 30 μM CuCl_2 , the specific cellular copper content of astrocytes had reached a steady state level. This is likely to be a consequence of changes in the activity or the localisation of the transporters that are involved in copper uptake and efflux. For example Ctr1 has been demonstrated to be rapidly internalised in many cell types when extracellular copper levels rise [39, 40]. Also the level of DMT1 is decreased, at least in CaCo-2 cells, following exposure to copper [41]. In addition, a rising cellular copper content induces the translocation of the copper transporting ATPase ATP7A [42] into the plasma membrane of chinese hamster ovary cells, human fibroblasts and HeLa cells, thereby facilitating copper export [43, 44]. Similar alterations of the location of these proteins, which are involved in copper transport and are expressed in astrocyte cultures [17, 45], would lower copper accumulation and increase copper export, thereby generating a steady state level of cellular copper.

Exposure of cultured astrocytes to 30 μM CuCl_2 in DMEM caused a 5 fold increase in cellular copper content within 12 h. This copper accumulation was much slower

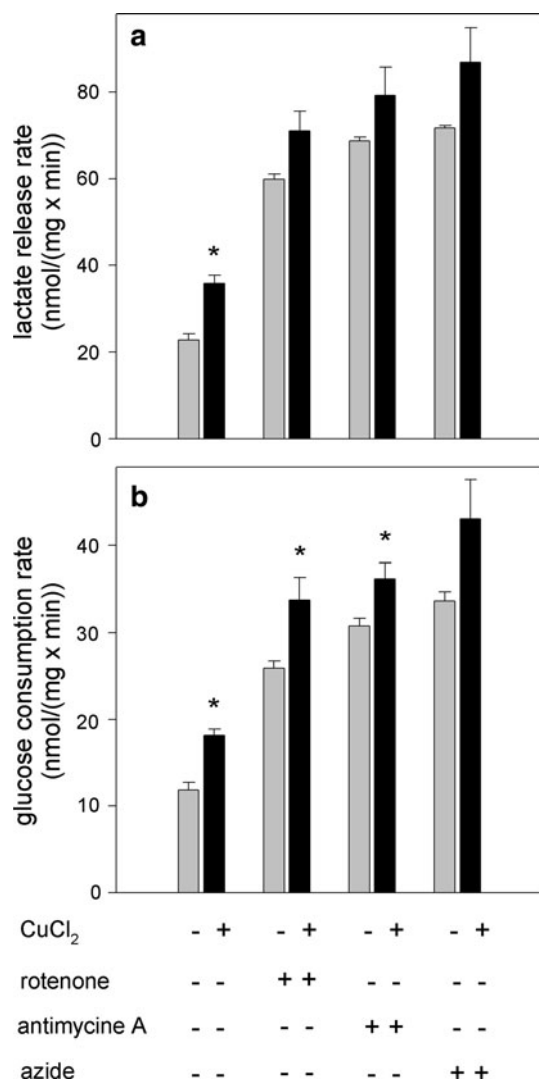


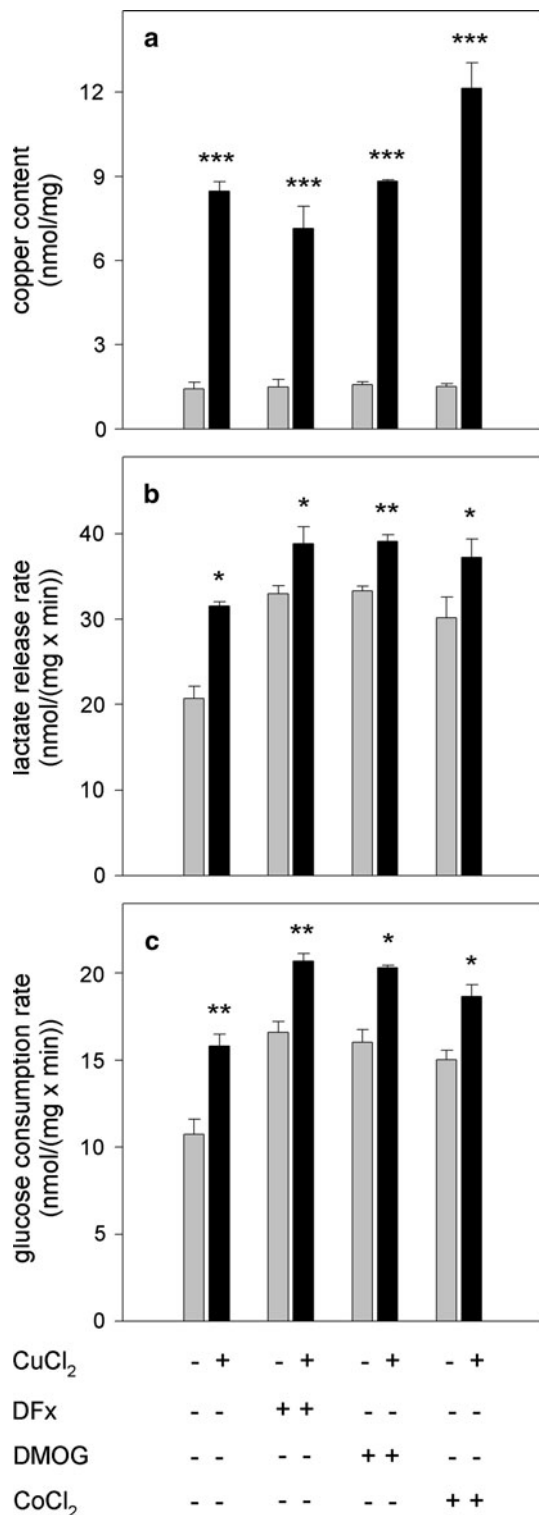
Fig. 3 Effects of inhibitors of mitochondrial respiration on the copper induced stimulation of lactate release (a) and glucose consumption (b). Cultured astrocytes were preincubated without or with 30 μ M CuCl₂ for 16 h. The specific copper contents of cells that were preincubated without and with CuCl₂ were 1.3 ± 0.1 and 10.9 ± 1.8 nmol/mg, respectively. The rates of lactate release and glucose consumption were determined in the absence or the presence of 10 μ M rotenone, 10 μ M antimycin A or 10 mM sodium azide. The difference of the data for inhibitor-treated cells compared to the respective controls (absence of respiratory chain inhibitors) was highly significant for all inhibitors ($P < 0.001$, not indicated in the figure). Indicated by stars is the significance of differences between the data obtained for cells that were preincubated without or with CuCl₂

than that observed for astrocytes that were incubated in amino acid free incubation buffer (8 fold increase within 1 h; [18]). A similar dependency of the rates of copper accumulation from the composition of the incubation media has been reported for HEK293 cells [46]. Most likely amino acids in the DMEM such as histidine form copper complexes that are known to be taken up into cells much slower than copper ions in non-complexed form [47].

During incubation of astrocytes with copper in DMEM the cells were remarkably resistant against copper induced toxicity. Even exposure to CuCl₂ in concentrations of up to 50 μ M for 24 h did not lead to cell toxicity, confirming literature data for astrocytes [20, 48, 49], while higher concentrations compromised to some low but significant extent cell viability and NR retention within 16 or 24 h. This contrasts the situation of astrocytes that have been exposed to CuCl₂ in amino acid free medium which leads to a very rapid accumulation of copper by the cells and to severe toxicity within 2 h [17, 18]. Thus, the speed of cellular copper accumulation appears to determine the extent and the velocity of copper-mediated cell damage in astrocytes. The toxicity observed for astrocytes that had been exposed to copper in amino acid free medium [17, 18] is most likely the consequence of the rapid accumulation of large amounts of copper which can under the conditions used not be compensated by a sufficient storage capacity for copper and/or by sufficient detoxification capacity.

Cultured astrocytes are known for their high rate of lactate production, although the reported specific rates for lactate release and glucose consumption vary strongly [50]. With basal rates of 19.2 ± 0.8 nmol/(mg \times min) and 10.0 ± 0.5 nmol/(mg \times min) for lactate release and glucose consumption, respectively, the values reported in the present study are similar to literature data [51, 52]. The high ratio of lactate production to glucose consumption of almost two demonstrates that cultured astrocytes are highly glycolytic under the conditions used. This predominant glycolytic ATP production is most likely a direct consequence of the strong inactivation of the pyruvate dehydrogenase complex by phosphorylation of the pyruvate dehydrogenase α -subunit in cultured astrocytes [53].

The time- and concentration-dependent increases in cellular specific copper content correlated well with the increases in extracellular lactate concentration as well as with the stimulation of the specific rates of cellular glucose consumption and lactate release, suggesting that an elevated cellular copper content stimulates glycolytic flux in astrocytes. Detectable differences in extracellular lactate concentrations of copper-treated cells compared to control cells were only observed after exposure to copper for at least 8 h. This delay in the cellular response suggests that the intracellular copper level has to reach a given threshold value to accelerate glycolytic flux and/or that protein synthesis is involved in this process. Both explanations would be supported by the observation that the copper-effect is maintained after removal of extracellular copper. The copper-induced stimulation of glycolytic flux was prevented by the presence of the protein synthesis inhibitor cycloheximide, demonstrating that indeed the time required for activation of transcription and protein synthesis contributes to the delay of the upregulation of



◀**Fig. 4** Effects of stabilisers of HIF-1 α on copper accumulation and on the copper-induced stimulation of lactate release and glucose consumption in cultured astrocytes. The cells were preincubated for 16 h without or with 30 μ M CuCl₂ in the presence or absence of 1 mM DFx, 1 mM DMOG or 200 μ M CoCl₂. Panel **a** shows the cellular specific copper content after the preincubation. The rates of lactate release (**b**) and glucose consumption (**c**) were determined as described in “Experimental Procedures”. In panels **b** and **c**, the differences of the data for cells that were treated with one of the indicated stabilisers compared to the respective controls (absence of stabilisers) was highly significant for all inhibitors used ($P < 0.01$ or $P < 0.001$), while in panel **a** only the specific copper content of cells treated with CuCl₂ in the presence of CoCl₂ was significantly elevated ($P < 0.001$) (not indicated in the figure). Indicated with stars is the significance of differences between the data obtained for cells that were preincubated without or with CuCl₂

survival after exposure to copper. In addition, the synthesis of copper sequestering proteins such as metallothioneins [54, 55] appears not to be essential for cell survival under the conditions investigated. However, copper can also be detoxified by binding to the tripeptide GSH [56] which is present in cultured astrocytes in a specific cellular content (about 35 nmol/mg [18]) that is 4 fold higher than the specific cellular copper content found after exposure to 30 μ M CuCl₂.

Inhibitors of the respiratory chain are known to stimulate glycolysis in cultured astrocytes [22, 37]. In addition, stabilisation of HIF-1 α under normoxic conditions induces the expression of genes involved in glucose uptake and glycolysis in astrocytes [57–60] and accelerates glycolytic flux [29]. However, although inhibitors of the different respiratory chain complexes as well as HIF-1 α stabilisers stimulated glucose consumption and lactate production in astrocytes, these compounds showed effects that were additive to those observed for copper-treatment. Thus, the mechanism that is responsible for stimulated glycolytic flux by elevated cellular copper levels appears not to involve inhibition of respiration and stabilisation of HIF-1 α .

In conclusion, we show for the first time that copper exposure modulates the cellular glucose metabolism of astrocytes by accelerating the glycolytic flux. Already a 5 fold increased cellular copper content accelerated glycolytic flux by about 60%. Protein synthesis is required for this effect, while contributions of copper-induced inhibition of mitochondrial respiration or stabilisation of HIF-1 α are unlikely to be involved. So far we were unable to identify the mechanism involved. Thus, further studies are required to analyse the copper-induced stimulation of glycolytic flux in astrocytes in more detail. Assuming that astrocytes in brain react in a similar way to an excess of copper as cultured astrocytes do, the consequence of a copper accumulation in astrocytes, for example in copper overload conditions such in Wilsons disease [61, 62], could

glycolytic flux in copper-treated astrocytes. The co-incubation of astrocytes with CuCl₂ plus cycloheximide did not lower the specific copper accumulation and was not accompanied by an increased toxicity, suggesting that the increased glycolytic flux is not required to maintain cell

be a lactic acidosis in brain. However, since lactate has been considered as energy rich fuel that is delivered from astrocytes to neurons [13, 15], an elevated lactate release during copper-exposure could also deliver additional substrate for the energy metabolism to neurons.

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