A multinuclear NMR study of glucose metabolismin thiamine-deficient cerebellar granule cells: new mechanistic insights

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Introduction. Wernicke's encephalopathy (WE) is a serious neurological disorder which is characterized by ataxia, nystagmus, ophtalmoplegia, disturbances in consciousness and region-selecticve brain lesions [1]. In most cases, brain damage in WE is associated with chronic alcoholism as a result of a deficiency in thiamine which is essential in the metabolic turnover of carbohydrates. Thiamine-deficiency (TD) leads to region-selective neuronal death and irreversible structural damages in advanced stages, which is suggested to be the result of impaired pyruvate oxidation and focal lactate accumulation. Furthermore, it is known that hyperglycemic conditions aggravate developing secondary brain damage in TD *in vivo*. In the present study, multinuclear NMR spectroscopy combined with the administration of [U-^{13}C]glucose was used to assess in more detail changes in carbon fluxes and cellular energy state of thiamine-deficient cultured rat cerebellar granule neurons previously shown to manifest reduced α-KGDH activities, lactate accumulation and cell death.

Methods. Cerebellar granule cells (CGCs) were prepared from 7-day-old Sprague-Dawley rats [2]. Cerebella were cleaned of vascular debris, and mechanically dissociated by trypsinization in DMEM. The cells were plated onto 100-mm dishes precoated with poly-L-lysine and cultured in DMEM containing specific supplements for these cells. After 24 h, 20 μM cytosine arabinoside was added to inhibit the growth of nonneuronal cells. Cells were cultivated for 6 days. Pyrithiamine (Pyr) was used as an inhibitor of thiamine metabolism. For Pyr-treated and control groups, 50 μM Pyr and 8 mg/L thiamine were added, respectively. Controls and six experimental groups were used: Cells treated for 2 h, or 3 or 5 days, with TD media in the absence or presence of Pyr. Additional experiments were carried out using control- or Pyr-containing TD media containing 100 mM instead of 25 mM glucose. After removal of incubation media, cells were washed with saline, frozen in liquid nitrogen and extracted with perchloric acid [3]. After lyophilization, the samples were redissolved in 0.5 ml D_2O, centrifuged and neutralized. ^{1}H-, ^{13}C- and ^{31}P-NMR spectra were recorded on Bruker spectrometers DRX 600/WB 360. Metabolite concentrations were calculated from ^{1}H-NMR spectra; flux of ^{13}C was followed up by ^{13}C isotopomer analysis [4]. Data are represented as means of n = 4-6. Data between individual groups were analyzed using ANOVA and post-hoc Tukey`s test.

Results

Cell viability. Prolonged Pyr treatment for 3 days led to altered cellular morphology, shrinkage of the cell body and loss of neurites, compared to treatment of cultures with TD media without addition of Pyr. No cell death of CGCs was observed after one day in culture, neither under control conditions nor after treatment with Pyr in thiamine-deficient media. Only marginal cell death has been observed at day 3 of in thiamine-deficient media by 11% (from 3.58 ± 0.23% to 3.98 ± 0.47%; ns). After 3 d pre-incubation in media containing 100 mM glucose, the number of cells decreased compared to pre-incubation with 25 mM glucose, to 85% ± 11.4% in thiamine-containing media ($p < 0.05$).

Energy state. Analysis of ^{31}P-NMR spectra showed that 2 h incubation of CGCs in TD-media produced no changes in adenosine triphosphate (ATP) and phosphocreatine (PCr) in the absence of Pyr. The addition of 50 μM Pyr, however, led to a 19% decrease of PCr already within 2h, whereas ATP was unchanged. After 3 days incubation in Pyr-containing TD-media ATP levels decreased non-significantly, while ATP declined to 40% of control after 5 days. PCr concentrations declined significantly already after 3 days treatment to 37% of control and decreased below NMR detectability after 5 days incubation in Pyr-containing TD-media. These effects were significantly deteriorated under hyperglycemic conditions (ATP and PCr were below NMR detectability).

Lactate synthesis. The addition of Pyr to TD-media caused an elevation in extracellular [U-^{13}C]lactate to 148%. After 3 d pre-incubation in TD-media + Pyr, extracellular [U-^{13}C]lactate increased to 159% of control. 5 days pre-incubation in TD-media + Pyr caused a marked increase of intracellular and extracellular [U-^{13}C]lactate to 171% and 223% of control, respectively. Conversely, after 3 d pre-incubation under the same conditions, but in media containing 100 mM instead of 25 mM glucose (unlabelled), synthesis of lactate and its total amounts during the subsequent 2 h incubation with [U-^{13}C]glucose were the same as after 3 d pre-incubation in normoglycemic media, either intracellularly and extracellularly. Interestingly, after 3 d incubation in hyperglycemic TD-media + Pyr, the subsequent incubation with [U-^{13}C]glucose (TD-media + Pyr) caused no changes in synthesis, total amount, or release of lactate.

Metabolite concentrations. Treatment of CGCs with TD-media caused an increase of intra- and extracellular glutamine concentrations. Apart from approximately 70% elevated extracellular glutamine after 2 h incubation in TD-media without Pyr, glutamine increase showed a general dependence on the duration of Pyr exposure, and was highest after 5 d of treatment (increase to 153% and 142% of control intra- and extracellularly, respectively). Intra- and extracellular glutamine increased also after incubation with media containing 100 mM instead of 25 mM glucose, which further increased extracellularly in Pyr-containing TD media. Intracellular glutamate declined to 80% and 75% after incubation of CGCs with Pyr-containing TD-media for 2 h and 3 days, respectively. No changes, however, were detected after 5 d treatment. Interestingly, acetate, which decreased considerably intracellularly, increased several-fold extracellulary, whereby this increases corresponded to the amounts lost intracellularly. NAA (N-acetyl-aspartate), a neuronal marker molecule, decreased in relation to the duration of Pyr exposure up to 57% of control after 5 d treatment.

[U-^{13}C]glucose metabolism. Acute (2 h) treatment with TD-media in the absence of Pyr caused no changes in the percentage ^{13}C-enrichment in glutamate C4 or C3. After chronic treatment for 3 and 5 days, however, the relative ^{13}C-label incorporation from [U-^{13}C]glucose into glutamate decreased to 89% and 72% ($p<0.05$), respectively, for the C4 position and to 87% and 70% ($p<0.05$), respectively, for the C3 position. As a measure of the TCA cycling ratio, the ratios between the glutamate isotopomers formed during the first TCA cycle turn and all glutamate isotopomers (∑C4 or ∑C3) as well as those formed after subsequent turns ([1,2,4,5-^{13}C]-, [1,3,4,5-^{13}C]-, [U-^{13}C]-, or [2,3,4,5-^{13}C]glutamate), were calculated. 2 h treatment of CGCs with TD-media with or without Pyr caused no decrease or even an increase in the TCA cycling ratio. However, the TCA cycling ratio decreased slightly (to 81% of control, $p<0.05$) after treatment for 3 days and considerably to 58% ($p<0.01$) after 5 days treatment of CGCs in Pyr-containing TD media – in parallel to decreased total entry of [1,2-^{13}C]acetyl-CoA into the TCA cycle and ^{13}C-label incorporation into glutamate ($p<0.01$). After incubation in control media containing 100 mM instead of 25 mM glucose, the TCA cycling ratio during the 2 h incubation with [U-^{13}C]glucose decreased to 44% of control. The lower relative decrease after treatment with Pyr in high glucose-containing media was due to a higher decrease of the glutamate isotopomers formed during the first TCA cycle turn, i.e. decreased formation and/or entry of acetyl-CoA into the TCA cycle.

Conclusions. Early changes in neuronal glucose metabolism may determine the progression of neurological signs in TD, while impaired flux through PDH and lactate accumulation appears to play a key role for selective neuronal cell death in late stages of the disease. In particular, neuronal energy failure and death likely result from a primary impairment of neuronal □-KGDH causing subsequently impaired carbon flux from glucose/pyruvate through PDH as well as decreased catabolism of glutamine and finally accumulation and release of lactate by these cells. These data also provide some new hints into the effects of hyperglycemia. Pre-treatment under hyperglycemic conditions, which aggravate developing secondary brain damage in TD *in vivo,* led to considerably deterioration of the cellular energy state, but this was not associated with impaired mitochondrial glucose metabolism, increased intra- or extracellular lactate levels or enhanced cell death of cerebellar granule cells. Thus, under hyperglycemic conditions alternative explanations to the lactic acidosis hypothesis have to be considered as factors leading to further neuronal degeneration.

References. [1] Butterworth RF et al., Alcoholism: Clinical and Experimental Research 1993, 11:893-894. [2] Thangnipon W et al., Brain Res. 1983, 313:177-189. [3] Zwingmann C et al. , Glia 2000, 32:286-303. [4] Zwingmann et al. Hepatology 2003, 37:420-428.