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Inhibition of lactate export by quercetin acidifies rat glial cells in vitro

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Abstract

The relationship between glial lactate release and glial intracellular pH (pH_i) regulation is studied using C6 glioma cells and rat astrocytes in vitro, and the lactate transport inhibitors quercetin and α-cyano-4-hydroxycinnamate (CHC). pH_i is measured using 2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF). The results show that lactate release is mediated partly by a specific lactate transport system inhibitable by quercetin (50 μM), but not by CHC (5 mM). Inhibition by quercetin results in a significant 3–4-fold increase of intracellular lactate and a decrease of intracellular pH to 6.9. A participation of quercetin-inhibitable lactate transport in glial pH_i-regulation is suggested by the observation that pH_i-homeostasis after acidification by diffusion of undissociated lactic acid into the cell is inhibited by quercetin. The existence of a system controlling lactate release in glial cells may also reflect a function of astrocytes to supply neurons with lactate. © 1997 Elsevier Science Ireland Ltd.

Keywords: Lactate transport; pH Regulation; Glial cells; Quercetin; α-Cyano-4-hydroxycinnamate; 2′,7′-Bis(carboxyethyl)-5,6-carboxyfluorescein

In vitro studies of cerebral ischemia often employ glial cells [11,17]. Under these conditions glial cells as well as neurons produce vast amounts of lactic acid by anaerobic glycolysis. Moreover, glial cells in vitro generate more lactate than is metabolized by oxidative pathways [18]. To avoid intracellular lactate accumulation which would lead to increased osmotic pressure and feedback inhibition of glycolysis, lactate has to be released from the cells. Special lactate transport mechanisms were observed in several cell types, such as Ehrlich ascites tumor cells [2], erythrocytes [4], hepatocytes [7] and some others (for review see [14]). Since in these cells lactate is transported together with one proton, lactate transport mechanisms may also affect intracellular pH (pH_i) regulation [15]. Although a significant role of lactate transport in pH_i regulation as been suggested [21], and the existence of a specific lactate transport system has been shown for astrocytes [13], no further information is available clarifying the interactions of lactate transport and glial pH_i homeostasis. This, therefore, was the purpose of the current study. C6 glioma cells were used as a model cell line for glial cells [9–11,17]. Important results were confirmed using rat astrocytes.

C6 glioma cells were cultivated in petri dishes using Dulbecco's modified Eagle's medium (DMEM) with 100 IU Penicillin G, 50 μg/ml Streptomycin and 10% fetal calf serum. Astrocytes were prepared from 2–3 day old Wistar rats [17]. In brief, cerebral hemispheres were isolated and freed from cerebellum, brain stem, meninges and choroid plexus. The tissue was minced and repeatedly exposed to dispase. Suspended cells were harvested from the supernatant and seeded into petri dishes. Cells obtained showed the morphology of astrocytes and were positive for glial fibrillary acidic protein to more than 90%. For studies of lactate transport C6 glioma cells or confluent rat astrocytes at passages 2 and 3 were incubated in HEPES-buffered medium for 2 h. This experimental medium contained 130 mM NaCl, 3 mM KCl, 0.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM glucose and 40 mM HEPES. The pH was adjusted with HCl and NaOH. Osmolality was measured by freezing-point depression and adjusted to 300 mmol using NaCl. The concentration of lactate in the medium was measured as described by HOHORST [8]: lactate was converted to pyruvate by lactate dehydrogenase, and the NADH produced was measured. The cells

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were extracted by disintegrating the membranes using 12% $HClO_4$ and ultrasound to quantify intracellular lactate. To calculate the amount of lactate released or stored per cell, the cell density in the petri dishes was determined before lactate measurements; cells were counted in 16 random visual fields using a CCD camera mounted on the microscope, a genlock interface and an AMIGA 2000 computer (Commodore). Counting and calibration were performed using self-programmed BASIC software. Results are expressed as means ± SEM and were tested for statistical significance by the Kruskal–Wallis test for non-parametric one-way analysis of variance (ANOVA) and multiple comparisons on ranks for unpaired samples.

The rate of lactate release was found to be 0.33 ± 0.06 pmol lactate/h per cell (n = 12). When cells had been pre-incubated with 50 μM quercetin (n = 10), a concentration found to cause maximal swelling of C6 cells [20], lactate release was significantly reduced to 0.18 ± 0.03 pmol/h per cell ($P < 0.05$). To make sure that this reduction was due to an inhibition of lactate transport and not primarily to a reduction of lactate production, e.g. by inhibition of glycolysis, intracellular lactate content was also determined after cell extraction. In the control group without quercetin intracellular lactate amounted to 7.47 ± 0.30 fmol/cell after 2 h incubation. After exposure to 50 μM quercetin the intracellular lactate content was 27.1 ± 8.02 fmol/cell ($P < 0.05$ vs. baseline, n = 5).

These data confirm that glial cells possess a specific transport system to release lactate, similar to the one described for Ehrlich ascites tumor cells [2]. This transporter can be inhibited by the flavonoid quercetin, as shown here by the increased intracellular lactate content and the reduced lactate release after quercetin application. Lactate release is not completely inhibited by quercetin [2]. An explanation might be that some lactate can cross the cell membrane by other ways, e.g. diffusion as undissociated lactic acid.

The occurrence of a lactate export system in glial cells could be a consequence of the specific function of astrocytes, which are responsible for homeostasis of the extracellular conditions of the brain, including nutrient supply. Lactate is a preferred nutrition of neurons [6]. As astrocytes store glycogen [3], and are able to metabolize it to lactate [5], a controlled lactate export from astrocytes would improve the supply of the neurons and could therefore participate in the protection of brain function, e.g. under hypoglycaemic conditions.

To study the interaction of lactate transport and pH_i regulation, pH_i had to be assessed. Confluent dishes were inserted into a measurement chamber allowing for control of temperature (37°C) and gas supply. Within this chamber petri dishes were superfused with fresh media. The intracellular pH-sensitive fluorescent dye 2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF; Molecular Probes, USA) was used for pH_i measurements. Adherent cells were incubated for 15 min with 4 μM BCECF-acetoxymethylester, which diffuses into the cells and is hydrolyzed to BCECF by cytosolic esterases. Cells were alternately illuminated at 442 and 492 nm (50 Hz) using an AMKO LTI Multiscan 2 system. At 442 nm excitation the intensity of the emitted fluorescence is nearly independent of pH, at 492 nm it is strongly pH-dependent. The emitted light was quantified by a photomultiplier and online averaging. All measurements were corrected for background activity as determined with BCECF-free cultures. pH_i was then calculated from the ratio of the emitted fluorescences at the two excitation wavelengths, thus correcting for inhomogeneous distribution of the dye, loss of dye, or photobleaching. For calibration of the measurements the ionophore nigericin was used, which adjusts pH_i to the extracellular pH (pH_e) if the potassium concentration of the medium is set equal to the cytosolic concentration. Calibration measurements were performed at 11 different

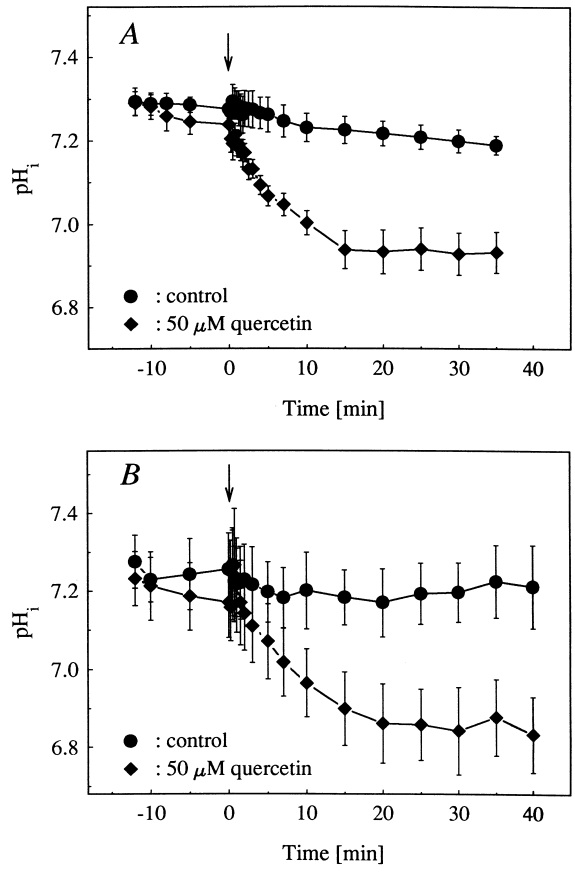

Fig. 1. (A) pH_i of C6 glioma cells begins to decrease immediately after addition of 50 μM quercetin at time 0 (arrow), while the control group without application of quercetin is able to maintain pH_i, a difference which is statistically significant ($P < 0.05$). (B) Addition of 50 μM quercetin at time 0 (arrow) causes intracellular acidification of cultured rat astrocytes, whereas without application of quercetin baseline pH_i is maintained ($P < 0.05$). All data are means ± SEM, HEPES-buffered media. The data were tested for statistical significance by the Kruskal\tq-Wallis test for non-parametric ANOVA and multiple comparisons on ranks for unpaired samples.

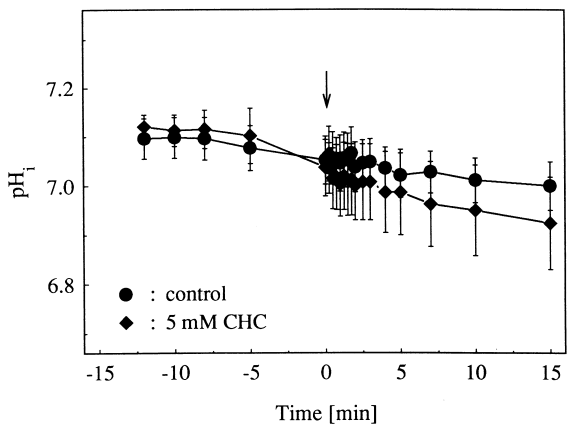

Fig. 2. There was no significant effect on pH_i of 5 mM CHC at time 0 (arrow) in HEPES-buffered media in cultured rat astrocytes. Nearly identical data were obtained using C6 glioma cells (not shown).

pH_e values over a range between pH 6.0 and 8.0, i.e. with 0.2 pH unit intervals.

The inhibition of lactate export by quercetin was accompanied by changes of pH_i. During a 15–20 min control phase at a pH_e of 7.4, C6 glioma cells and rat astrocytes were able to maintain their pH_i. When 50 μM quercetin was added, this caused a reduction in pH_i of C6 glioma cells as well as of rat astrocytes. In C6 glioma cells, pH_i began to decrease directly after application of quercetin (Fig. 1A). After 15 min pH_i was reduced from 7.24 ± 0.03 to 6.94 ± 0.05 ($P < 0.05$, $n = 6$). At that time point a plateau had been reached which remained constant during the further experiment. In contrast, the control group without quercetin was able to maintain pH_i during the experiment (Fig. 1A). Similar observations were made in cultured rat astrocytes (Fig. 1B; $n = 5$). Here pH_i decreased from 7.17 ± 0.09 to 6.86 ± 0.09 after 25 min of quercetin exposure ($P < 0.05$). Again, this plateau was maintained during the further measurements. The acidifying effect of quercetin indicates that lactate is transported together with a proton, most likely by a lactate$^-$/H^+ cotransporter as has been suggested for neutrophils [15]. The plateau which was reached at a pH_i of 6.8–6.9 might be caused by two consequences of intracellular acidification. First, an inhibition of glycolysis by intracellular acidification has been described [2], and second, this acidification might activate pH_i regulatory mechanisms, especially the Na^+/H^+ exchanger, which then would prevent further acidification. Na^+/H^+ exchange in glia is nearly inactive at physiological pH_i, and becomes activated by cytosolic acidification [10]. The kinetics of quercetin-induced acidification correspond very well with those of quercetin-induced glial swelling [20]. C6 swelling in that study depended on the quercetin concentration employed and had reached a plateau at 50 μM [20]. The swelling has been explained by the intracellular accumulation of lactate which leads to increased osmotic pressure and by the activation of pH_i-regulating systems, e.g. Na^+/H^+ exchange, that would also increase the intracellular osmotic load, and therefore enhance cell swelling [9,11]. Assuming a cell volume of 800 fl [11,17] an increase from 7.47 to 27.1 fmol/cell lactate is equivalent to an increase of the intracellular lactate concentration from 8.8 to 33.9 mM, i.e. a 50.2 mmol osmotic load which is in good agreement to the 15–20% swelling found before [20].

In contrast to quercetin, α-cyano-4-hydroxycinnamate (CHC), which has been used for the inhibition of lactate transport in several other studies [7,16], showed no significant effect on pH_i. C6 glioma cells (data not shown, $n = 8$) as well as rat astrocytes (Fig. 2; $n = 4$) maintained their pH_i after application of 5 mM CHC. The observation that CHC had no significant effect on pH_i and, concluded from that, on lactate transport is in contrast to investigations with neutrophils [16], hepatocytes [7] or neurons [6], where CHC inhibited lactate transport [6,7,16], or pH_i regulation [1]. The absence of inhibition by CHC might be a distinct property of the glial lactate transporter, a hypothesis which is supported by the lack of an inhibitory effect of CHC on lactate transport [13]. On the other hand, the above mentioned studies [6,7,16] measured lactate uptake. Different mechanisms might be responsible for lactate uptake and release, with only the uptake mechanism being CHC-sensitive. Another explanation might be an asymmetry of the lactate transporter between efflux and uptake conditions, as has been shown in the case of erythrocytes [4] or cardiac myocytes [19].

The observation that inhibition of the lactate transporter leads to intracellular acidification moreover might indicate that lactate transport is also involved in pH_i regulation. This is of particular interest, because glial pH_i regulation is not completely understood yet. Although numerous studies have been performed in this context, some results

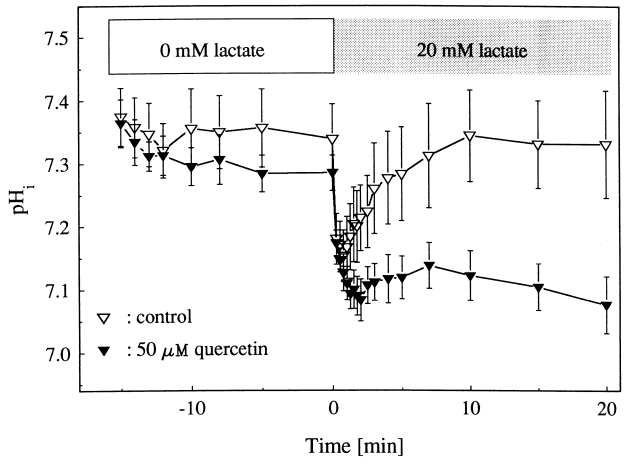

Fig. 3. Effect of exposure to 20 mM lactate on pH_i of C6 glioma cells. Isotonicity was maintained by replacing lactate for chloride ions. Extracellular pH was kept at 7.4 throughout the experiment. An immediate pH_i drop was observed, followed by a complete recovery of pH_i. This recovery was inhibited in the presence of 50 μM quercetin (means ± SEM, HEPES-buffered media).

have been reported [12,21], which could not be explained by the common pH_i regulating systems, i.e. the Na^+/H^+ exchanger, Na^+/HCO_3^- cotransport, and the Cl^-/HCO_3^- exchangers, which most of the studies concerning glial pH_i regulation have focused on so far. A participation of the lactate transporter could explain some of the open questions [21]. Results supporting an involvement in pH_i-regulation are shown in Fig. 3: C6 cells were exposed to 20 mM lactate while extracellular pH was maintained at 7.4. As a consequence undissociated lactic acid entered the cell and pH_i dropped from 7.34 ± 0.06 until a new equilibrium at 7.15 ± 0.04 was reached within 45 s ($P < 0.05$, $n = 4$). Thereafter, pH_i began to recover, and after 10 min had again reached its initial level (7.34 ± 0.07) although extracellular lactate concentration was still 20 mM. In the presence of 50 μM quercetin, a similar drop in pH_i from 7.29 ± 0.03 to 7.09 ± 0.02 was observed after lactate application, but in contrast to controls pH_i recovery was absent. These experiments suggest that transport of lactic acid may contribute to pH_i homeostasis in cases where acidosis is caused by an accumulation of lactic acid as it can be expected in hypoxia or low flow conditions of the brain. Taken together glial cells appear to exhibit a specific lactate export system which on the one hand side may support the role of astrocytes to supply neurons with lactate, but also may participate in pH_i regulation in pathophysiological conditions.

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[1] Amos, B.J. and Richards, C.D., Intrinsic buffering capacity in rat CNS neurones maintained in culture, Exp. Physiol., 81 (1996) 261–271.
[2] Belt, J.A., Thomas, J.A., Buchsbaum, R.N. and Racker, E., Inhibition of lactate transport and glycolysis in Ehrlich ascites tumor cells by bioflavonoids, Biochemistry, 18 (1979) 3506–3511.
[3] Cataldo, A.M. and Broadwell, R.D., Cytochemical identification of cerebral glycogen and glucose-6-phosphatase activity under normal and experimental conditions. 1. Neurons and glia, J. Electron. Microsc. Techn., 3 (1986) 413–437.
[4] Deuticke, B., Monocarboxylate transport in erythrocytes, J. Membr. Biol., 70 (1982) 89–103.
[5] Dringen, R., Gebhardt, R. and Hamprecht, B., Glycogen in astrocytes: possible function as lactate supply for neighboring cells, Brain Res., 623 (1993) 208–214.
[6] Dringen, R., Wiesinger, H. and Hamprecht, B., Uptake of l-lactate by cultured rat brain neurons, Neurosci. Lett., 163 (1993) 5–7.
[7] Edlund, G.L. and Halestrap, A.P., The kinetics of transport of lactate and pyruvate into rat hepatocytes. Evidence for the presence of a specific carrier similar to that in erythrocytes, Biochem. J., 249 (1988) 117–126.
[8] Hohorst, H.-J., l-(+)-Lactate. Bestimmung mit lactate-dehydrogenase und DPN. In H.U. Bergmeyer (Hg.), Methoden der enzymatischen Analyse, Verlag Chemie, Weinheim, 1962, pp. 266–270.
[9] Jakubovicz, D.E. and Klip, A., Lactic acid-induced swelling in C6 glial cells via Na^+/H^+ exchange, Brain Res., 485 (1989) 215–224.
[10] Jean, T., Frelin, C., Vigne, P. and Lazdunski, M., The Na^+/H^+ exchange system in glial cell lines. Properties and activation by hyperosmotic shock, Eur. J. Biochem., 160 (1986) 211–219.
[11] Kempski, O., Staub, F., Jansen, M., Schödel, F. and Baethmann, A., Glial swelling during extracellular acidosis in vitro, Stroke, 19 (1988) 385–392.
[12] Mellergard, P.E., Ouyang, Y.-B. and Siesjö, B.K., The regulation of intracellular pH in cultured astrocytes and neuroblastoma cells, and its dependence on extracellular pH in a HCO3-free solution, Can. J. Physiol. Pharmacol., 70 (1992) S293–S300.
[13] Nedergaard, M. and Goldman, S.A., Carrier mediated transport of lactic acid in cultured neurons and astrocytes, Am. J. Physiol., 265 (1993) R282–R289.
[14] Poole, R.C. and Halestrap, A.P., Transport of lactate and other monocarboxylates across mammalian plasma membranes, Am. J. Physiol., 264 (1993) C761–C782.
[15] Simchowitz, L. and Textor, J.A., Lactic acid secretion by human neutrophils: evidence for an $H^+ + lactate^-$ cotransport system, J. Gen. Physiol., 100 (1992) 341–367.
[16] Simchowitz, L. and Vogt, S.K., Substrate and inhibitor specificity of the lactate carrier of human neutrophils, J. Membr. Biol., 131 (1993) 23–34.
[17] Staub, F., Baethmann, A., Peters, J., Weigt, H. and Kempski, O., Effects of lactacidosis on glial cell volume and viability, J. Cerebral Blood Flow Metab., 10 (1990) 866–876.
[18] Walz, W. and Mukerji, S., Lactate production and release in cultured astrocytes, Neurosci. Lett., 86 (1988) 296–300.
[19] Wang, X., Levi, A.J. and Halestrap, A.P., Kinetics of the sarcolemmal lactate carrier in single heart cells using BCECF to measure pH_i, Am. J. Physiol., 267 (1994) H1759–1769.
[20] Weigt, H., Staub, F., Baethmann, A. and Kempski, O., Inhibition of lactate transport causes glial swelling. In J. Grote and E. Witzleb (Eds.), Funktionsanalyse Biologischer Systeme 22, Gustav Fischer Verlag, Stuttgart, 1994, pp. 249–256.
[21] Wuttke, W.A. and Walz, W., Sodium- and bicarbonate-independent regulation of intracellular pH in cultured mouse astrocytes, Neurosci. Lett., 117 (1990) 105–110.