**Astrocyte Glucose Metabolism under Normal and Pathological Conditions in vitro**

**Key Words**
Acidosis  
Adenosine triphosphate  
Astrocyte  
2-Deoxyglucose  
Glutamate uptake  
Glycolysis  
Hypoxia  
Lactate

**Abstract**
Astrocytes in primary culture produce lactate. The net production of lactate from glucose requires that the carbon flux through glycolysis exceed the carbon flux to CO₂. This study investigates the control and function of this 'excess' glycolysis in astrocyte cultures. Blockade of glycolysis was found to have minimal effects on astrocyte ATP and function if other substrates for oxidative metabolism were available. In contrast, selective blockade of oxidative metabolism reduced adenosine triphosphate (ATP) levels and slowed glutamate uptake despite a marked increase in glycolytic rate. Acidosis suppressed both glucose utilization and lactate production but had minimal effects on ATP levels. Acidosis in combination with blockade of oxidative metabolism blunted the increase in glycolytic rate and accentuated ATP depletion relative to oxidative blockade alone. These studies suggest that glycolysis in astrocyte cultures is regulated by factors other than energy demand, and that the capacity of glycolysis to support astrocyte metabolism during hypoxia is markedly pH dependent.

The brain derives energy almost exclusively from glucose, and glucose utilization is nearly matched by CO₂ production [Siesjö, 1978]. Studies using diverse approaches have shown, however, that local glycolytic rate can exceed local oxidative metabolic rate and increase brain lactate content, at least over short periods of time [Fellows et al., 1993; Fox et al., 1988; Korf et al., 1993; Uecki et al., 1988; Sappey-Marinier et al., 1992]. Elevations in glycolytic rate also occur during pathological conditions such as ischemia. In this setting, anaerobic metabolism of residual glucose supply can increase brain lactate by more than tenfold [Folbergrová et al., 1992].

Lactate production during hypoxia and ischemia reflects anaerobic energy production from glucose. However, the significance of net lactate production under normoxic conditions remains unsettled. One possibility is that increased energy demand in brain requires a disproportionate increase in glycolytic rate because of the more limited responsiveness of oxidative metabolism [Lear and Ackerman, 1989]. Second, ATP production specifically by glycolysis, rather than by oxidative phosphorylation, could be important for certain compartmentalized astrocyte functions [Kauppinen, 1988]. In smooth muscle, for example, contraction appears to be driven preferentially by glycolytically-derived ATP [Ishida et
al., 1994]. Third, astrocytes might produce lactate as a substrate for oxidative metabolism in neurons [Dringen et al., 1993; Tsacopoulos and Magistretti, 1996]. Lactate can support synaptic activity in the hippocampal slice [Schurr et al., 1988], and lactate is preferred over glucose for oxidative metabolism in isolated chick ganglia [Larrabee, 1995].

Astrocytes in culture exhibit high rates of glucose utilization and lactate production [Walz and MUKerji, 1988; Hertz and Peng, 1992; Dringen et al., 1993]. It is unknown whether astrocytes also produce excess lactate in situ, in accord with the possibilities mentioned above, or whether rapid lactate production is an artifact of cell culture. In the present study the following experiments were performed to distinguish among these possibilities: (1) Glucose utilization, lactate production, and ATP levels were assessed under hypoxic and acidic conditions to determine whether glycolysis in astrocyte cultures was responsive to these factors and to changes in cellular ATP content. (2) Glycolytic and oxidative ATP production were selectively inhibited in order to assess their relative importance for maintaining ATP levels and astrocyte function. (3) Glucose utilization and lactate production were measured after addition of lactate to the culture medium to determine if lactate produced a feedback inhibition on astrocyte glycolytic rate.

**Methods**

All reagents were obtained from Sigma Chemicals, St. Louis, Mo., USA, except where noted.

Primary cortical astrocyte cultures were prepared as described by Hertz et al. [1985] with minor modifications [Swanson et al., 1995]. Forebrain cortices were dissected from 1-day-old Sprague-Dawley rats and the meninges carefully removed. Dissociation was accomplished by incubation in papain/DNAase followed by trituration. The dissociated cells were washed, suspended in Eagle’s minimal essential medium (MEM) with 10% fetal bovine serum (FBS) (HyClone, Ogden, Utah, USA) and glutamine (2 mM), and plated in Falcon 24-well tissue culture plates at an approximate density of 5 x 10^4 cells/cm^2. The medium was exchanged with fresh medium at day 5. At confluence (days 12–15), 10 μM cytosine arabinoside was added to prevent proliferation of other cell types. This medium was removed after 48 h and replaced with medium containing 3% FBS and 0.15 mM dibutyryl cyclic AMP to induce differentiation. This medium was exchanged with fresh medium weekly. Cells prepared in this manner displayed stellate morphology and were uniformly positive for glial fibrillary acidic protein (GFAP) (ICN; Costa Mesa, Calif., USA). The cells were used at 25–30 days in vitro.

**Experimental procedures:** Before each experiment, cells were washed and preincubated for 30 min in Hank’s balanced salt solution (HBSS) modified to contain 6 mM PIPES adjusted to the desired pH. Glucose was adjusted to 1 mM except where indicated. Additions to this medium were made from 10 x iso-osmolar stock solutions. Experiments were performed at 37°C in room air with the exception of the 24-hour incubation in glucose-free medium. This study was done in a 5% CO2 atmosphere in bicarbonate-buffered medium. The final media volumes in all experiments were 300–400 μl.

**Glucose utilization** was assessed by an adaptation of the Sokoloff 2-deoxyglucose (2DG) method [Sokoloff et al., 1977] as previously described [Swanson et al., 1990]. After 30 min of preincubation under the test conditions, 2.5 μCi of [14C]2-deoxyglucose (340 Ci/mmol, St. Louis, Mo., USA) was added to each culture well. After incubation with 2DG for 45 min the media was exchanged 3 times, 5 min each, with ice-cold HBSS to allow egress of unphosphorylated 2-deoxyglucose. Following the third wash the cells were lysed in 0.01 N NaOH/0.5% SDS and the lysates taken for scintillation counting. Blanks prepared by shortening the 2DG incubation period to less than 15 s had negligible 14C content.

**Lactate production:** Cultures were preincubated for 30 min in the designated test conditions, then placed in fresh test medium for 45 min. Incubations were terminated by the addition of 30 mM HCl (final concentration) directly to the culture wells. After waiting 10 min for the cells to lyse, aliquots of the acidified media were taken for lactate assay using the enzymatic method of Lowry and Passoneau [1972]. The values thus obtained reflect the total lactate content of the culture wells: lactate released into the medium by the astrocytes plus lactate that had been intracellular prior to lysis. Some of the cultures were lysed at the beginning of the 45-min incubations to determine lactate present in the cells at that time. These values were subtracted from the values obtained after the 45-min incubations to yield net lactate production.

**ATP content** was determined by lysing the cells in ice-cold 0.1 N NaOH containing 0.01% lauryl sulfate and 1mM EDTA. Proteins were precipitated with perchloric acid and centrifugation. Supernatants were neutralized with KOH/K2HPO4, and again centrifuged. ATP levels were measured fluorometrically according to the method of Lowry and Passoneau [1972] and compared with ATP standards run in parallel with each assay.

**Protein determinations** were made by the Lowry et al. [1951] method using bovine serum albumin standards.

**Glutamate uptake** was assessed at 37°C by adding 0.025 μCi/mL L-[2,3-3H]glutamate (American Radiochemicals, St. Louis, Mo., USA) plus 50 μM unlabeled glutamate to the culture wells. Uptake was terminated after 7 min by two ice-cold washes with 500 μl modified HBSS followed by immediate lysis in ice-cold 0.1 N NaOH/0.01% lauryl sulfate. Aliquots of the lysates were taken for scintillation counting. Previous studies have determined the K_d for glutamate uptake to be 24 μM in this preparation and uptake to be linear over 10 min [Swanson et al., 1995].

**Statistical differences** between experimental groups were determined by performing one-way ANOVA followed, where applicable, by Dunnett’s test for multiple comparisons against the control groups.

**Results**

The mean rates of lactate production in three experiments using three different astrocyte preparations were 6.0 ± 0.7, 7.0 ± 1.3 and 10.5 ± 2.3 nmol/min/mg protein (n = 4–6) under the control conditions of 1 mM glucose,
Fig. 1. Lactate production was independent of medium glucose concentrations over the physiological range. Blockade of oxidative ATP production with azide caused an approximately 2-fold increase in lactate production. n = 4; results from 1 of 3 similar experiments.

Fig. 2. Under normoxic conditions lactate production and 2DG accumulation were reduced during media acidification without change in cellular ATP content (a). Addition of 5 mM azide to produce chemical hypoxia accelerated lactate production and 2DG accumulation, but ATP levels were not maintained. Media acidification under these conditions attenuated the increased lactate production and 2DG accumulation and further lowered ATP content (b). n = 10, **p < 0.001 vs. pH 7.2, no azide values.

pH 7.2, 37°C. The mean protein content among wells from these dissections varied from 36.8 ± 3.0 to 47.8 ± 3.2 μg/well (n = 4). These lactate values represent the net increase in lactate from both intracellular and extracellular compartments over the experimental interval. As shown in figure 1, lactate production by the astrocyte cultures was insensitive to medium glucose concentrations between 0.25 and 5 mM, with or without addition of sodium azide. This confirms that measured lactate production at 1 mM glucose was not limited by medium glucose concentrations.

Astrocyte ATP levels were 26 ± 1.5 nmol/mg protein under the control conditions. Acidification of the medium reduced both lactate production and glycolytic rate (as estimated by the 2-deoxyglucose method), but had no measurable effect on ATP (fig. 2a). Blockade of oxidative ATP production with azide increased both lactate production and the apparent glycolytic rate, but these increases were insufficient to maintain ATP at control values (fig. 2b). Previous studies have established that the concentration of azide used in these studies is supra-maximal [Swanson, 1992]. Acidosis attenuated the rise in glycolytic rate and lactate production caused by azide, and further exacerbated ATP depletion. ATP levels fell below 20% of control values during exposure to azide at pH 6.2. These changes do not reflect cell lysis, as the fraction of cells staining with propidium iodide during these studies remained less than 5% (results not shown).

The observation of normal ATP levels during normoxic acidosis despite marked reductions in glycolytic rate and lactate production suggests that the high glycolytic rate exhibited at normal pH serves a function other than ATP production. This issue was further examined using cultures in which glycolysis was prevented by glucose deprivation. Both ATP and glutamate uptake were maintained at 80% of control values after 24 h incubation in glucose-free medium containing glutamine as an oxidative substrate (fig. 3). Glutamate uptake is driven by the ion and charge gradients established by the plasma membrane Na⁺/K⁺ ATPase activity [Nichols and Attwell, 1990], such that uptake assessed at near saturating glutamate concentrations serves as a measure of the cells' capacity to maintain these gradients.

The 24-hour preincubations in glucose-free medium were used to ensure depletion of glycogen stores [Dringen et al., 1993]. This prolonged interval without glu-
Fig. 3. Glutamate uptake (a) and ATP content (b) were maintained near control values following the elimination of glycolytic ATP production by 24 h of glucose deprivation. n = 6, *p<0.05.

Fig. 4. Glutamate uptake (a) and ATP levels (b) were maintained near control values during inhibition of glycolysis with 2DG. In contrast, inhibition of oxidative ATP production with 5 mM azide reduced glutamate uptake by nearly 50%. The additive effect of azide and 2DG on both glutamate uptake and ATP levels suggest that 2DG was effective in blocking glycolytic flux under these conditions. Medium [glucose] = 1 mM; n = 6, *p<0.05, **p<0.001.

cose may, however, affect cell metabolism in other ways that could confound these results. A second approach to this question employed short incubations with metabolic inhibitors. As shown in figure 4, inhibition of glycolysis with 10 mM 2-deoxyglucose (2DG) similarly caused little or no decrease in ATP content or glutamate uptake. In contrast, blockade of oxidative metabolism with azide caused significant reductions in both glutamate uptake and ATP content. Combined treatment with 2DG and azide reduced glutamate uptake and ATP content by more than 90%.

The additive effects of 2DG combined with azide suggest that 2DG was present at concentrations high enough to block glycolytic flux. Alternatively, azide could reduce intracellular glucose content and thereby potentiate the effects of 2DG at 2DG concentrations that would otherwise be ineffective. This possibility was examined with the study shown in figure 5. Reduction in media glucose to 0 did not potentiate the effect of 2DG. 5 mM fluoride, which blocks glycolysis in a manner not competitive with glucose [Shahed et al., 1980], had effects on glutamate uptake that were similar in magnitude to those of 2DG. Moreover, the effect of azide was significantly increased by either glucose removal, addition of fluoride, or addition of 2DG. Taken together, the studies presented in figures 3–5 suggest that glycolysis can partially compensate for loss of oxidative metabolism, but under aerobic conditions elimination of glycolytically produced ATP has only minor effects on the total ATP pool and on the transmembrane charge and ion gradients that drive glutamate uptake.

The above findings suggest that the high glycolytic flux observed in astrocyte cultures is not driven by demand for ATP. An alternative possibility is that the lactate produced is not simply a byproduct of anaerobic ATP production, but instead serves to fuel oxidative metabolism of neurons in brain [Dringen et al., 1993; Tsacopoulos and Magistretti, 1996]. If production and release of lactate is a regulated process serving this function, then the presence of lactate or other substances that can fuel neuronal oxidative metabolism should reduce glycolytic rate and lactate production. Addition of either lactate or glutamine to the medium did reduce glucose utilization in a dose dependent manner over the range these compounds are normally present in brain extracellular fluid (fig. 6a), but the degree of inhibition achieved
Fig. 5. Fluoride and glucose deprivation have effects on glutamate uptake similar to those of 2DG. Glucose-free medium, 2DG, fluoride, and 2DG or fluoride combined with 2DG all reduced glutamate uptake to between 68 and 80% of control values in 5 mM glucose. Each of these conditions also exhibited a similar additive effect with 5 mM azide. n = 6, *p<0.05 vs. control at 5 mM glucose; †p<0.05 vs. 0 mM glucose.

by lactate concentrations as high as 10 mM were less than those observed with acidosis. Repeated measurements of media lactate concentrations over several days showed that lactate levels rose above 6 mM (data not shown). Lactate production showed a similar partial reduction in response to glutamine additions to the medium (fig. 6b).

Discussion

As previously reported [Walz and Mukerji, 1988; Hertz and Peng, 1992; Dringen et al., 1993] astrocyte cultures exhibited a rapid net production of lactate under control conditions. This rate was not immutable, however. Acidosis under normoxic conditions caused a marked reduction of both glucose utilization and lactate production. These reductions did not affect astrocyte ATP content, suggesting that glycolytic ATP production is not required for astrocyte energy homeostasis under aerobic conditions. This is further supported by the negligible reductions in ATP levels and glutamate uptake observed during glucose deprivation or glycolytic block-

Fig. 6. Astrocyte glycolytic rate is reduced by the addition of physiological concentrations of lactate or glutamine to the culture medium (a). Glutamine also reduces lactate production (b), n = 8, **p<0.001 vs. controls.

ade. Hypoxic conditions caused a striking increase in lactate production, but ATP levels fell below normal levels despite this increase. The inhibitory effect of acidosis on glycolytic rate was also observed during hypoxia, and the combination of acidosis plus hypoxia exacerbated the depletion of ATP.

The net production of lactate requires a mismatch between the rates of carbon flux through the glycolytic and oxidative phases of glucose metabolism. While lactate can be produced by other routes [Hassel and Sonnewald, 1995], these fluxes must be negligible when glucose is the only carbon source available. The intracellular ratio of pyruvate/lactate will change with pH or redox state, but pyruvate concentrations are always low relative to lactate [Erecinska et al., 1996] and negligible relative to lactate production over 45 min.

Both glucose utilization and lactate production were reduced by medium acidification (fig. 2). Relative glucose utilization rates were assessed by measuring the accumulation of [14C]2-deoxyglucose-6-phosphate, which is trapped in the cells. Absolute values for glucose utilization cannot be obtained by this technique without knowledge of the kinetic rates of transport, phosphoryla-
tion, and dephosphorylation for both glucose and 2-deoxyglucose [Sokoloff et al., 1977]. In addition, the intracellular ratio of [14C]2DG: glucose must be constant (or known) to permit comparisons between treatment groups. Intracellular glucose is not known to be altered by changes in pH, but may be decreased during azide exposure. As applied to figure 2, these considerations suggest that values obtained by the 2DG method should not be compared between the 0 azide and 5 mM azide treatment groups. Comparisons within each treatment group suggest that increasing azidosis causes a fall in glycolytic rate roughly in parallel to the fall in lactate production. In other words, azidosis does not appear to alter the proportion of glucose that is metabolized oxidatively.

The fall in glycolytic rate and lactate production during azidosis is not a surprising result. Astrocyte pHi falls during media acidification [Mellergård et al., 1991], and a rate-limiting glycolytic enzyme, phosphofructokinase, is inhibited by increased [H+] [Dobson et al., 1986]. Inhibition of PKF indirectly results in an accumulation of glucose-6-phosphate (G6P) [Erecińska et al., 1996], which in turn leads to a feedback inhibition of hexokinase. Acidosis therefore raises the steady state G6P concentration but lowers the net accumulation of [14C]2-deoxyglucose-6-phosphate (2DG6P). Stated differently, acidosis causes a rise in the G6P concentration measured at any single point in time, but causes a decrease in the amount of 2DG6P that accumulate over time. 2DG6P accumulation provides an integral of hexokinase rate over the period of observation, and for observation periods of several minutes this is equal to the rate of glycolytic flux [Sokoloff et al., 1977].

The rate of lactate production at all pH values examined was increased during exposure to azide (fig. 2). This illustrates that the inhibition of glycolysis by acidosis can be overcome under conditions of altered energy charge [Dobson et al., 1986]. These findings suggest that the glycolytic rate under the control conditions of normoxia, pH 7.2, far exceeds that needed to produce adequate ATP. Erecińska et al. [1996], using synaptosomes and C6 glioma cells, have also shown that H+ inhibition of glycolysis can be reversed by a fall in energy charge.

Like acidosis, blockade of glycolytic ATP production with fluoride, high concentrations of 2-deoxyglucose, or prolonged glucose deprivation caused only modest reductions in glutamate uptake rate and ATP content. These studies further suggest that the high glycolytic rate of these cells is not required to maintain energy homeostasis. Moreover, the failure of glycolytic blockade to significantly reduce glutamate uptake at near-saturating glutamate concentrations argues against a special role for glycolytically produced ATP in fueling membrane Na+/K+ ATPase function.

Could the primary function of nonoxidative glucose utilization in astrocytes be the production of lactate? In its strongest form, this hypothesis implies that lactate production and release is a regulated process, involving feedback inhibition. Addition of lactate to the medium did reduce the glycolytic rate (fig. 6), but the degree of reduction was less than observed at pH 6.6 suggesting that net lactate production continued. Repeated measurements of media lactate concentrations over several days showed that lactate levels rose above 6 mM rather than plateauing at levels typical of brain extracellular space (1–2 mM). Similar results have previously been reported by Walz and Munker [1987] and by Alves et al. [1995]. Moreover, addition of glutamine to the medium had effects nearly identical to those of lactate on glucose utilization. It is possible that the reduction in glucose utilization observed with both lactate and glutamine addition to the medium resulted from the use of these compounds as alternative carbon sources [Hertz and Peng, 1992], rather than true feedback inhibition.

These studies confirm that astrocytes in culture exhibit a rapid net production of lactate under a variety of physiologic and pathologic conditions. The lactate production does not appear to be driven by a requirement for glycolytic ATP production, as ATP levels and energy dependent-glutamate uptake were little affected by selective blockade of glycolysis. It is tempting to speculate that the net lactate production by astrocytes in culture reflects their activity in brain, where several lines of evidence suggest that lactate produced by astrocytes could be used for oxidative metabolism by neurons [Schurr et al., 1988; Dringen et al., 1993; Larabee, 1995; Tsacopoulos and Magistretti, 1996]. It should be emphasized, however, that astrocytes grown in isolation from neurons and endothelia may develop metabolic differences from astrocytes in brain. Moreover, astrocytes prepared by different methodologies may also exhibit different characteristics [Juurlink and Hertz, 1985].

While glycolytic ATP production appears to have only minor significance under aerobic conditions, it becomes the primary energy source during hypoxia. The combined actions of hypoxia and acidosis on astrocyte energy metabolism are relevant to the setting of cerebral ischemia because residual glucose delivery to ischemic brain results in anaerobic glycolysis and, consequently, progressive acidosis [Folbergrová et al., 1992]. The pre-
sent results suggest a mechanism for the well recognized deleterious effect of acidosis on outcome from brain ischemia. At neutral or mildly acidic pH, anaerobic glycolysis is capable of maintaining glial survival and at least partially maintaining ATP levels and glutamate uptake [Swanson, 1992]. With increasing acidosis, however, glycolytic rate is depressed, resulting in marked depletion of astrocyte ATP, failure of glutamate uptake, and ultimately astrocyte death. These factors may explain why ischemia of sufficient severity to cause only selective necrosis of vulnerable neurons at neutral or mildly acidic pH can cause pan-necrosis of both neurons and glia (infarction) with more severe acidosis [Plum, 1983].

Acknowledgments

The authors would like to thank K. Farrell for technical assistance. This work was supported by NIH grant NS31914 and by a Department of Veterans Affairs Merit Review Award.