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Lactate induced excitotoxicity in hippocampal slice cultures

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Abstract

During the initial minutes of cerebral ischemia, lactic acid accumulates and acidifies brain pH to 6.0–6.7. Glutamate is also released during ischemia that activates glutamate receptors and induces excitotoxicity. While glutamate excitotoxicity is well established to induce ischemic injury, a role of lactic acidosis in ischemic brain damage is poorly understood. This study analyzes acidosis neurotoxicity in hippocampal slice cultures in the presence or absence of lactate. At pH 6.7, neuronal loss was similar whether or not lactate was present. At pH 6.4, neuronal loss was significantly greater in the presence of lactate suggesting that lactate potentiates the acidosis toxicity. At pH 6.4 in the presence of lactate, NMDA or non-NMDA receptor antagonists reduced neuronal loss, while in the absence of lactate, NMDA or non-NMDA receptor antagonists had little effect. [3H]-Glutamate uptake was inhibited by acidic pH, and the amount of inhibition was significantly greater in the presence of lactate. These findings suggest that lactate plays a role in acidosis neurotoxicity by inducing excitotoxicity. Lactic acidosis and excitotoxicity have been previously thought to be independent events during ischemia. This study suggests that during ischemia, lactic acidosis contributes to excitotoxic neuronal loss.

Keywords: pH; Stroke; Cerebral ischemia; Excitotoxicity; Glutamate

Introduction

During cerebral ischemia, brain metabolism switches from aerobic to anaerobic respiration that rapidly produces lactic acid and acidifies brain pH. The amount of acidosis differs between animal models, yet intracellular pH typically acidifies to the range of pH 6.5–6.7 (Barber et al., 2003; Li and Siesjo, 1997; Li et al., 1995; Siesjo et al., 1996). pH may acidify to as low as 6.0 if hyperglycemia precedes ischemia, or if ischemia is incomplete and glucose continues to be delivered to hypoxic cells (Li and Siesjo, 1997; Siesjo et al., 1996). In vivo studies analyzing the role of acidosis to ischemic brain injury have been inconclusive, yet induction of hypercapnea or hyperglycemia before ischemia is accompanied by severe acidosis and greater brain injury (Katsura et al., 1994; Kraig et al., 1987; Li et al., 1995; Siesjo et al., 1996). In vitro, neonatal neurons were injured only at pH 6.2 (Himmelseher et al., 1998; Kaku et al., 1993; Nedergaard et al., 1991). Some in vitro studies also suggested a neuroprotective role for acidosis since NMDA and AMPA receptors are inhibited by acidic pH (Ihle and Patneau, 2000; Low et al., 2000). Dissociated embryonic neurons and cell lines, however, are resistant to injury induced by hypoxia and hypoglycemia and may not adequately model acidosis toxicity. Unlike other in vitro systems, hippocampal slice cultures are isolated from older animals and contain neurons that are more sensitive to ischemic injury (Carpenedo et al., 2002; Laake et al., 1999; Noer et al., 2002). Hippocampal slice cultures also display acidosis toxicity (Ding et al., 2000; Shen et al., 1995). Acidosis induces synaptic depression in slice cultures. Neuronal loss ensues, regardless if synaptic responses recover (Xiang and Bergold, 2000). Neurons undergo both necro-

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sis and apoptosis following acidosis (Ding et al., 2000). This study analyzes neuronal loss in hippocampal slice cultures induced by acidosis in the presence or absence of lactate. These studies suggest that lactate potentiates acidosis toxicity in hippocampal slice cultures. One mechanism that lactate utilizes is excitotoxicity.

Materials and methods

Hippocampal slice cultures

Hippocampal slice cultures were prepared from 20- to 30-day-old Sprague–Dawley rats as previously described (Xiang et al., 2000). Rats were treated with the anesthetics ketamine and halothane to minimize any pain or discomfort during brain removal. Three 400-μm transverse hippocampal slices were plated on a Millipore-CM filter (Millipore, Woburn, MA) in 1 ml of slice culture media (SCM, 50% Basal Medium Eagle's, 25% Earle's Balanced Salt solution, 25% horse serum, 25 mM *N*-(2-hydroxyethyl) piperazine-*N*′-2-ethanesulfonic acid (HEPES), 1 mM glutamine, 28 mM glucose, pH = 7.2) and incubated at 32°C in a 5% CO_2 atmosphere. Previous studies have shown that slice cultures retain better organotypic structure when cultured for at least 1 week at 32°C (Xiang et al., 2000). After 1 week, slices were cultured in slice culture medium containing 5% horse serum. Cultures were fed every 2 days. Slice cultures were maintained for at least 2 weeks before the beginning of all experiments. One day before an experiment, the cultures were shifted to a 37°C incubator.

Measurement of intracellular pH

Slice cultures were loaded with 2*N*, 7*N*-*bis*-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM; Molecular Probes, Eugene, OR), and optical recording of intracellular pH was performed as previously described (Xiang and Bergold, 2000). BCECF fluorescent images were taken from the CA1 pyramidal cell layer, a region with a high density of pyramidal cell somata (Gahwiler et al., 1997; Xiang and Bergold, 2000). Since multiple cells were imaged simultaneously, measurements of BCECF measurements may have included glia in addition to neurons. Measurement of intracellular pH in stratum radiatum, a region of the slice culture that has few neurons, was similar to intracellular pH in the CA1 pyramidal cell layer (data not shown). Intracellular pH values between individual soma varied less than 10% (data not shown).

Assay of cell death

Neuronal loss was assayed using the fluorescent dye propidium iodide (PI) and verified in fixed, cresyl violet-stained slice cultures. Numerous reports in the last decade have confirmed the PI assay as a quantitative assay of neuronal loss in slice cultures (Ding et al., 2000; Laake et al., 1999; Newell et al., 1995a,b; Noraberg et al., 1999). PI does not identify which cells are dying; therefore, cresyl violet stain was used as a qualitative stain to show loss of neurons. Slice cultures were transferred to serum-free SCM with 4 μg/ml propidium iodide (Sigma, St. Louis, MO) and incubated in a 5% CO_2 incubator at 32°C for 30 min. Fluorescent images were taken using a PTI-intensified CCD camera mounted on a Zeiss Axiovert 100 microscope. Images were normalized using InSpeck red 100% fluorescent beads (Molecular Probes) and the mean pixel values in CA1 pyramidal layer analyzed using NIH Image v.1.59. After each image session, slices were rinsed with serum-free SCM, transferred to SCM containing 5% horse serum, and returned to the incubator. PI fluorescence was assayed one hour after the acidosis treatment and at 1-day intervals for 8 days. After the final PI analysis, slice cultures were fixed and stained with cresyl violet. Analysis of cresyl violet cultures was performed 8 days after the acidosis treatment.

Induction of acidosis

Intracellular acidosis was induced in glucose aCSF (in mM): NaCl, 144; KCl, 3; $MgCl_2$, 1.6; NaH_2PO_4, 1.2; $NaHCO_3$, 5; glucose, 11; HEPES, 20, or lactate aCSF (in mM): NaCl, 124; KCl, 3; $MgCl_2$, 1.6; NaH_2PO_4, 1.2; $NaHCO_3$, 5; glucose, 11; lactate, 20. Glucose and lactate aCSF were titrated to different pH values using HCl. Acidosis was induced by transferring slice cultures to sterile beakers that was vigorously perfused with 95% O_2, 5% CO_2 at 37°C. After a 30-min acidosis treatment, slice cultures were returned to fresh slice culture medium.

Measurement of glutamate uptake

The time course of glutamate uptake was obtained by incubating slices for 5–15 min with [3H]-L-glutamate (5 nM > 40 Ci/mmol, Amersham) in glucose or lactate aCSF, titrated to pH 7.35 or 6.4. Glutamate uptake was linear over this time interval (data not shown). To ensure that receptor-mediated uptake was measured, glutamate uptake was also assayed in the presence of the competitive glutamate uptake inhibitor threo-beta-hydroxyaspartate (THA, 500 μM, Sigma) (Balestrino et al., 1989). After exposure to [3H]-L-glutamate, cultures were chilled to 4°C, washed three times with ice-cold glucose or lactate aCSF and lysed in 0.1 M NaOH. The protein concentration of the lysate was measured by BCA assay (Pierce, Rockford, IL) and L-glutamate uptake determined by scintillation counting in Ecolume (ICN, Costa Mesa, CA) using a 6000 IC Beckman scintillation counter. All values were converted to picomoles glutamate uptake/slice culture protein (mg).

Results

Neuronal loss following acidosis in the presence or absence of lactate

To determine if lactate potentiates toxicity induced by acidosis, slice cultures were treated for 30 min with aCSF buffers containing glucose or lactate. Lactate or glucose aCSF was titrated to pH 7.00, 6.70, 6.40, 6.10, or 5.80. Control cultures were treated with lactate or glucose aCSF that had been titrated to pH 7.35. All slice cultures received a 30-min aCSF treatment. Immediate neuronal loss was assayed using PI 1 h after the aCSF treatment. After the assay of immediate neuronal loss, the PI staining solution was replaced with fresh SCM. Delayed neuronal loss was assayed using PI for the next 7 days. No increase in PI fluorescence was seen after treatment with lactate aCSF, pH 7.35 (data not shown) or 7.0 (Figs. 1A, B), suggesting that these treatments were nontoxic. PI fluorescence was not increased 1 h after lactate aCSF, pH 6.7, treatment, yet fluorescence was increased 1, 2, and 3 days later (Figs. 1A, B). PI fluorescence increased 1 h after treatment with lactate aCSF, pH 6.4, and remained elevated for an additional 8 days. A similar increase in PI fluorescence was observed after treatment with lactate aCSF, pH 6.1 and 5.8. These data suggest that the lactic acidosis treatment at pH 6.7 induced delayed cell loss, while the treatments at pH 6.4, 6.1, and 5.8 induced both immediate and delayed cell loss. The pH 6.4, 6.1, and 5.8 treatments likely induced more cell loss than at pH 6.7.

No PI fluorescence increase was observed in cultures receiving glucose aCSF, pH 7.35 (data not shown) or pH 7.0 (Figs. 2A, B). PI fluorescence did not increase 1 h following glucose aCSF, pH 6.7 or 6.4, but did increase for the following 3 days (Figs. 2A, B). PI fluorescence was elevated for all 8 days following treatment with glucose aCSF, pH 6.1 or 5.8, acidosis treatment. An increase in PI fluorescence was never observed 1 h following glucose aCSF treatment. These data suggest that glucose aCSF, pH 6.7, 6.4, 6.1, and 5.8, induced delayed but not immediate cell loss.

The presence of PI fluorescence for many days following the acidosis may result from staining of newly dying cells or to retention of PI in dead cells. The PI staining assay was altered to address this question. Slice cultures were treated with lactate aCSF, pH 6.4, returned to the incubator, and rather than assaying daily, a single assay of PI fluorescence was performed either 1, 3, or 6 days after the acidosis treatment (day 1, 39.3 ± 3.7; day 3, 48.8 ± 6.0; day 6, 33.8 ± 5.8; $n = 4$ for each day). PI fluorescence at 1, 3, or 7 days did not differ statistically in cultures that received a single PI assay or daily PI assays (Fig. 1;

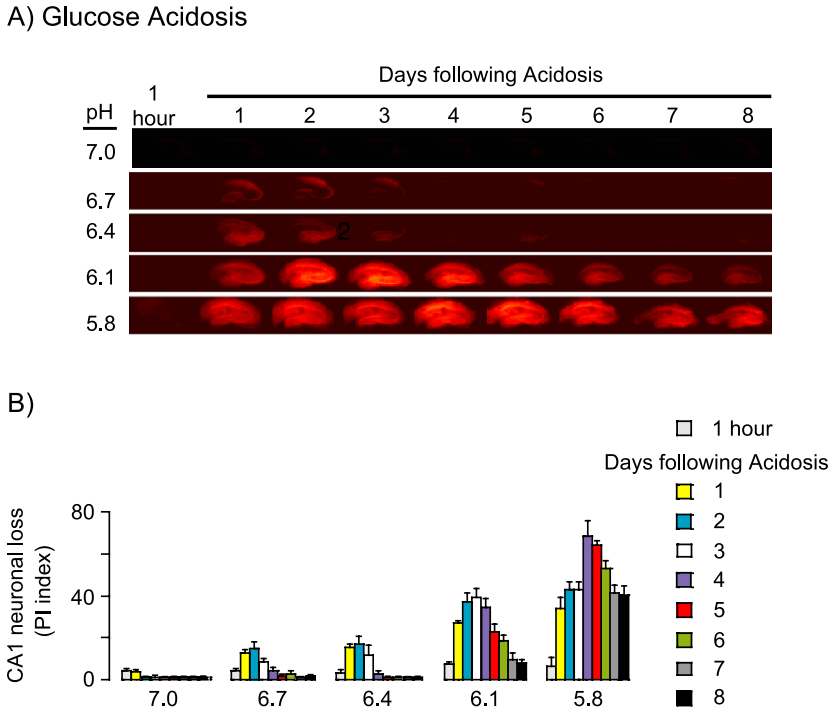

Fig. 1. PI fluorescence following acidosis in the presence of lactate. (Panel A) Slice cultures received a 30-min treatment of lactate aCSF titrated to pH 7.00, 6.70, 6.40, 6.10, or 5.80. PI fluorescence was assayed 1 h after the acidosis treatment (1 hour) and each day for the subsequent 8 days (Balestrino et al., 1989; Barber et al., 2003; Bender et al., 1997; Carpenedo et al., 2002; Ding et al., 2000; Dirnagl et al., 1999; Gahwiler et al., 1997; Himmelseher et al., 1998). Multiple video micrographs of PI fluorescence are shown for a representative slice culture for each treatment. (Panel B) Summary of the changes in PI fluorescence induced by acidosis in the presence of lactate. PI fluorescence was assayed in the CA1 pyramidal cell region ($n = 10$). PI fluorescence was significantly elevated in the CA1 pyramidal cell layer in pH 6.70, 6.40, 6.10, and 5.80 groups suggesting that neurons were lost (ANOVA, $P < 0.0001$; $F = 17.85$). PI fluorescence was significantly greater in the pH 6.40, 6.10, and 5.80 groups when compared to the pH 6.70 group (ANOVA, $P < 0.001$; $F = 13.64$). PI fluorescence was significantly greater in the pH 6.40 in the presence of lactate as compared to the pH 6.40 group in the absence of lactate (ANOVA, $P < 0.001$; $F = 8.43$).

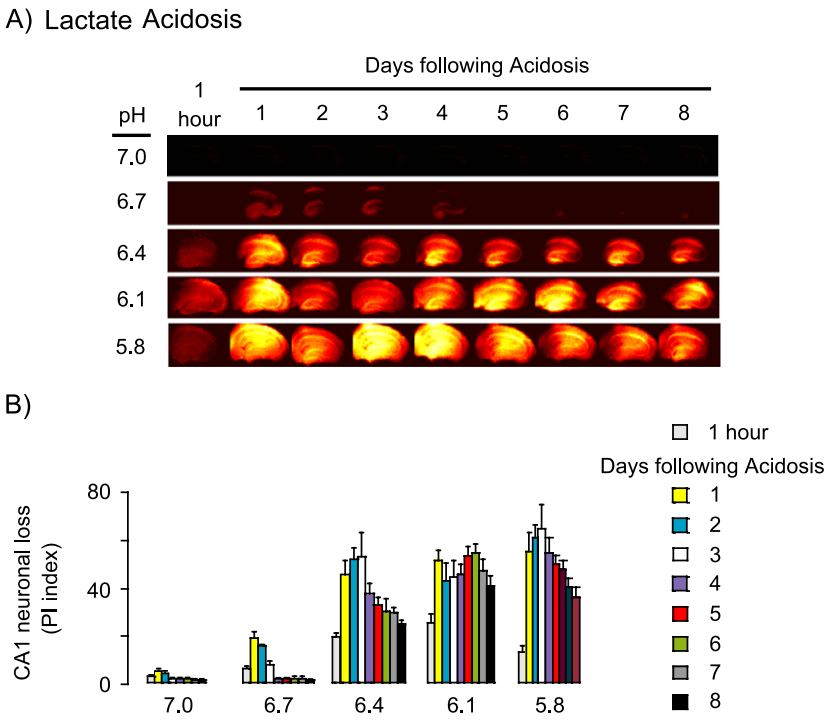

Fig. 2. PI fluorescence following acidosis in the presence of glucose. (Panel A) Slice cultures received a 30-min treatment of glucose aCSF titrated to pH 7.00, 6.70, 6.40, 6.10, or 5.80. PI fluorescence was assayed 1 h after the acidosis treatment (1 hour) and each day for the subsequent 8 days (Balestrino et al., 1989; Barber et al., 2003; Bender et al., 1997; Carpenedo et al., 2002; Ding et al., 2000; Dirnagl et al., 1999; Gahwiler et al., 1997; Himmelseher et al., 1998). Multiple video micrographs of PI fluorescence are shown for a representative slice culture for each treatment. (Panel B) Summary of the changes in PI fluorescence induced by acidosis in the absence of lactate. PI fluorescence was assayed in the CA1 pyramidal cell region. Until day 2, $n = 10$; following day 2, $n = 6$. PI fluorescence was significantly elevated in the CA1 pyramidal cell layer in the pH 6.70, 6.40, 6.10, and 5.80 groups suggesting that neurons were lost (ANOVA, $P < 0.0001$; $F = 46.88$). PI fluorescence was significantly greater in the pH 6.10 and 5.80 groups when compared to the pH 6.40 group (ANOVA, $P < 0.0001$; $F = 12.98$).

ANOVA, $F = 1.779$). These data suggest that the changes in PI fluorescence were due to the staining of newly dying cells rather than the retention of PI in dead cells. The presence of PI fluorescence many days following the acidosis treatment suggests delayed cell loss.

The increase in PI fluorescence in the CA1 pyramidal cell layer suggested that neuronal loss was being assayed. To confirm that neuronal loss was responsible for the changes in PI fluorescence, slice cultures were fixed and stained with cresyl violet after the final PI treatment (Fig. 3). Pyramidal and granule cell neurons were easily identified in cresyl violet-stained slice cultures. Cultures treated with glucose aCSF, pH 7.35 (data not shown) or 7.0, closely resembled untreated slice cultures. These data, together with the absence of an increase in PI fluorescence, further suggests that the glucose aCSF, pH 7.35 and 7.0, treatments did not induce neuronal loss. Cultures treated with glucose aCSF pH 6.7 displayed a partial loss of CA1 and CA3 pyramidal cells with a less apparent loss of dentate granule cells. These cultures displayed increased PI fluorescence in both the pyramidal and granule cell layers (Fig. 1). These data suggest that the glucose aCSF, pH 6.7, treatment induced loss of pyramidal and granule cells. A less likely alternative explanation is that dying neurons in the slice culture were replaced by new neurons. To date, we have no evidence that neurogenesis occurs in mature slice cultures (P.J.B., unpublished observations). Cultures treated with glucose aCSF, pH 6.4, showed a large loss of pyramidal cells with a smaller loss of granule cells. These data suggest that acidosis induced neuronal loss in the absence of lactate. These data also suggest that more neurons were lost with more acidic buffers. Cultures treated with lactate aCSF, pH 7.0, appeared similar to untreated cultures. CA1 and CA3 pyramidal cells were lost in cultures treated with lactate aCSF, pH 6.7, while lactate, pH 6.4, induced widespread loss of pyramidal and granule cell neurons. Cultures receiving glucose or lactate, pH 6.1 or 5.8, were not analyzed since they consistently fragmented during the fixation and staining protocol (data not shown). These data suggest that lactic acidosis is more neurotoxic as pH becomes more acidic. At pH values of 6.4 and more acidic, lactate contributes to acidosis neurotoxicity.

Lactic acidosis induced both rapid and delayed neuronal loss. Rapid neuronal loss was suggested by the increase of PI fluorescence 1 h after lactic acidosis (pH 6.4–5.8, Fig. 1). If acidosis was done in the absence of lactate, rapid neuronal loss was not observed (Fig. 2). Increased PI fluorescence 1–7 days following acidosis was suggestive of delayed neuronal loss. At pH 6.7, the amount and time course of PI fluores-

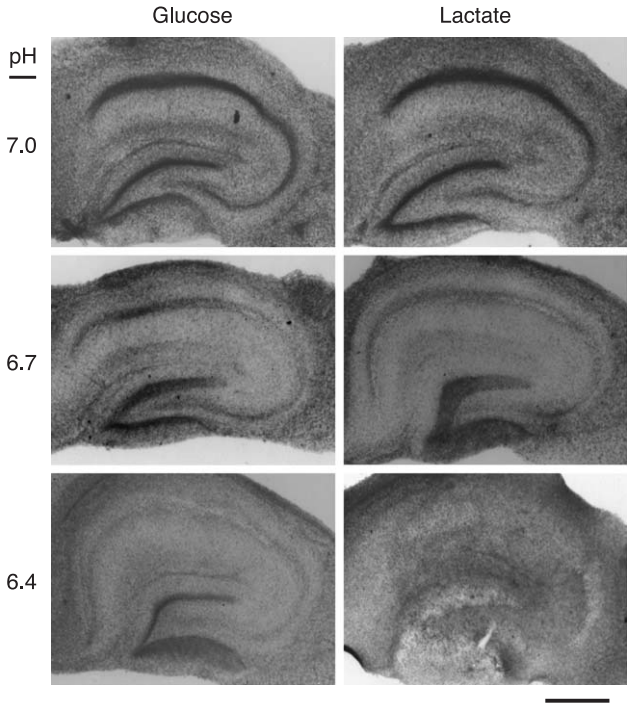

Fig. 3. Neuronal loss following acidosis in the presence of glucose or lactate. Slice cultures were fixed and stained with cresyl violet 8 days following the 30-min acidosis treatment. Slice cultures receiving acidosis in the presence of glucose (left panel, glucose) showed less neuronal loss than slice cultures in the presence of lactate (right panel, lactate). Scale bar, 1 mm.

cence was similar regardless if lactate were present (Figs. 1 and 2). There may be slight differences in the density of cresyl violet in the cultures that received the pH 6.7 acidosis treatment (Fig. 3), but these differences are due to the variation between cultures that one normally obtains with the slice culture technique. At pH values of 6.4 or more acidic, greater neuronal loss occurred in the presence of lactate.

Measurement of intracellular pH

Lactate aCSF may induce more neuronal loss than glucose aCSF of similar solution pH due to greater acidification of intracellular pH. To address this issue, we examined intracellular pH in the CA1 pyramidal cell layer in lactate or glucose aCSF (Fig. 4). At solution pH of 7.35, intracellular pH in lactate aCSF was 7.14 ± 0.12 ($n = 4$) over the 30-min recording period. In glucose aCSF, intracellular pH was recorded at 7.00 ± 0.13 ($n = 4$), this value was significantly more acidic than the intracellular pH recorded in lactate aCSF (ANOVA, $F = 4.62$, $P < 0.05$). These values agree well with previous measures of intracellular pH in the presence of lactate aCSF (Xiang and Bergold, 2000). Intracellular pH was also measured with glucose or lactate aCSF, pH 6.7 (Fig. 4). The time of onset or rate of acidification did not significantly differ between glucose and lactate aCSF, pH 6.7 (ANOVA, $F = 2.34$). In glucose aCSF, intracellular pH stabilized at pH 6.51 ± 0.03 ($n = 4$), a value that did not differ significantly from 6.48 ± 0.06 ($n = 4$, ANOVA, $F = 2.06$) in lactate aCSF (Fig. 4). A small but significantly slower rate of recovery was observed with glucose aCSF as compared to lactate aCSF (ANOVA, $F = 4.52$, $P < 0.05$). These data suggest that at acidic pH, glucose and lactate aCSF induce similar intracellular acidosis. Differences in intracellular pH or the rates of acidification or recovery were unlikely to be responsible of the potentiation of acidosis toxicity by lactate.

Glutamate receptor antagonists reduce lactate neurotoxicity

At pH 6.4, lactate aCSF induced more PI fluorescence and neuronal loss than glucose aCSF (Figs. 1–3). Excitotoxicity is a well-known mechanism of ischemic injury, we therefore tested if excitotoxicity contributed to lactate acidosis neurotoxicity (Barber et al., 2003; Sattler and Tymianski, 2001). Excitotoxic neuronal loss can result from excessive activation of either the AMPA or NMDA subtype of glutamate receptor (Barber et al., 2003; Dirnagl et al., 1999). To test for excitotoxic neuronal loss, slice cultures received 30 min of glucose or lactate acidosis, pH 6.4, in the presence of the AMPA receptor antagonist CNQX (30 μM) or the NMDA receptor antagonist MK-801 (30 μM). The concentrations of these antagonists have previously been shown to block excitotoxic neuronal loss in slice cultures (Kristensen et al., 2001). The CNQX or MK-801 was removed 4 h after the treatment. Neuronal loss was assayed using PI at 1-day intervals.

PI fluorescence was induced by glucose aCSF, pH 6.4 (Fig. 5). The amount or time course of PI fluorescence did not significantly change in the presence of MK-801 or CNQX. PI fluorescence was also increased following lactate aCSF, pH 6.4. As shown before, PI fluorescence following lactate aCSF, pH 6.4, was significantly greater

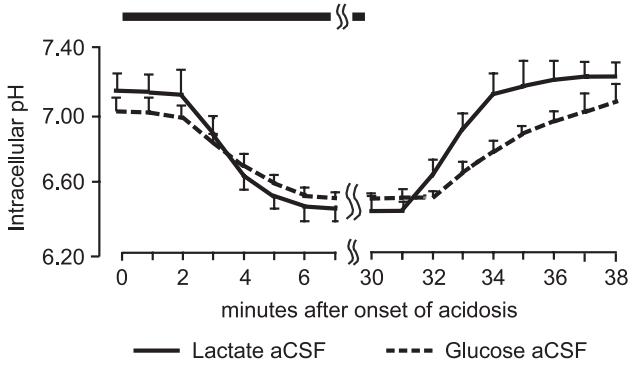

Fig. 4. Changes in intracellular pH during acidosis in the presence of glucose or lactate. Slice cultures were loaded with the pH sensitive dye BCECF-AM, and intracellular pH of multiple cells was optical recorded at 1-min intervals in the CA1 pyramidal cell layer. The cultures were initially perfused with glucose aCSF, pH 7.35. The black bar indicates when the perfusion was changed to aCSF, pH 6.70, than contained either glucose (dotted line) or lactate (solid line). The scale in the *x* axis has been expanded to highlight changes in intracellular pH following changes in the perfusion.

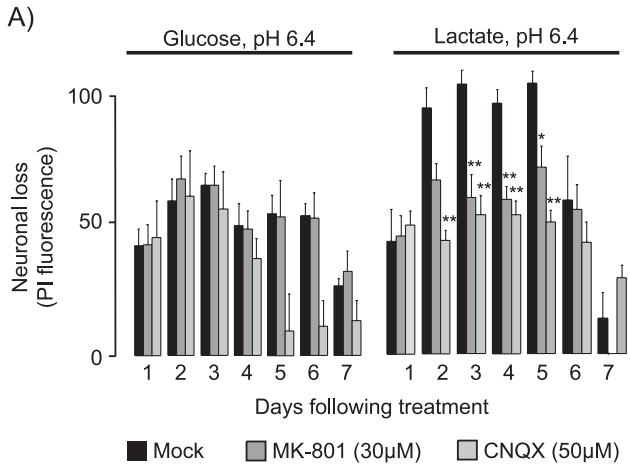

Fig. 5. Glutamate receptor antagonists reduce lactate toxicity. (Panel A) Excitotoxic neuronal loss induced by lactic acidosis. Summary of the reduction of PI fluorescence induced by lactate acidosis in the presence of glutamate receptor antagonists. Slice cultures received a 30-min treatment of pH 6.4 acidosis in the absence (glucose, left panel) or presence of lactate (lactate, right panel). Cultures were either mock-treated (black bars) or treated with the NMDA receptor antagonist MK-801 (dark gray bars, 30 μM) or the AMPA receptor antagonist CNQX (light gray bars, 50 μM). PI fluorescence was assayed in the CA1 pyramidal cell region each day for the subsequent 8 days. Glutamate receptor antagonists significantly reduced the amount of PI fluorescence in the cultures receiving lactate acidosis, pH 6.4 (ANOVA, $P < 0.0001$, $F = 14.208$). PI fluorescence was also significantly reduced on specific days (Student Neuman Keul's post-test, $*P < 0.05$, $**P < 0.01$). In contrast, PI fluorescence was not significantly reduced by glutamate receptor antagonists in the cultures receiving acidosis, pH 6.4, in the absence of lactate (ANOVA, $P > 0.1$; $F = 2.24$).

than following glucose aCSF, pH 6.4 (Figs. 1B and 2B). PI fluorescence on day 1 following lactate aCSF, pH 6.4, was unchanged in cultures treated with MK-801 and CNQX, but significantly reduced on days 2–5. These data suggest that NMDA and AMPA receptor activation was responsible, in part, for neuronal loss on days 2–5. The reduction of PI fluorescence by glutamate receptor antagonists suggests that lactate acidosis induces excitotoxicity. Examination of cresyl violet-stained cultures treated with CNQX or MK801 confirmed that the reduction of PI staining following lactic acidosis was due to a greater retention of slice culture neurons (data not shown). In contrast, excitotoxicity does not appear to be induced by glucose aCSF, pH 6.4. There is a significantly greater reduction of PI fluorescence on days 2 and 5 in cultures treated with CNQX as compared to MK-801 (Fig. 5). This suggests that AMPA receptor activation has a greater contribution to the lactate potentiation of acidosis neurotoxicity than NMDA receptor activation. This result is consistent with an earlier report showing acidosis potentiation of AMPA toxicity (McDonald et al., 1998).

Lactic acidosis blocks glutamate uptake

Excitotoxicity requires glutamate receptor activation, yet NMDA (IC_{50} = 7.3), and AMPA (IC_{50} = 6.3) receptors are inhibited at acidic pH (Ihle and Patneau, 2000; Low et al., 2000). Lactic acidosis is therefore unlikely to induce excitotoxicity by directly activating glutamate receptors. At pH values of 6.1 or lower, lactate inhibits glutamate uptake in astrocytes cultures (Bender et al., 1997; Swanson et al., 1995). Decreased uptake has been shown to activate glutamate receptors in hippocampal slice cultures when pH recovers to physiological levels (Jabaudon et al., 2000). Glutamate uptake was assayed in glucose or lactate aCSF at pH 7.35 or 6.4 (Fig. 6). To ensure that glutamate transporters mediated [3H]-glutamate uptake, some cultures were treated with the competitive uptake inhibitor THA (500 μM). THA (500 μM) inhibits all isoforms of glutamate transporters in the hippocampus. THA (500 μM) greatly diminished glutamate uptake in all four conditions tested, suggesting that uptake by glutamate transporters was being assayed (Fig. 6). In either lactate or glucose aCSF, glutamate uptake at pH 6.4 was significantly less than at pH 7.4 (Fig. 6). These data agree with previous studies that acidic pH inhibits glutamate uptake yet uptake was inhibited at pH 6.4, a value that more commonly occurs during ischemia (Barber et al., 2003; Bender et al., 1997; Swanson et al., 1995). Interestingly, uptake in lactate aCSF, pH 6.4, was significantly less than at glucose aCSF, pH 6.4, suggesting that lactate potentiates the inhibition of glutamate uptake at acidic pH. Uptake in lactate aCSF, pH 6.4, was similar to THA (500 μM)-treated cultures suggesting that there was complete inhibition of glutamate uptake.

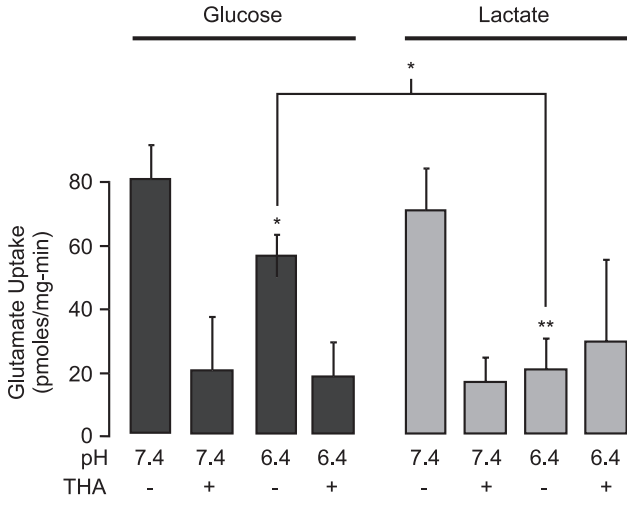

Fig. 6. Glutamate uptake during lactic acidosis. Slice cultures received a 15-min treatment with glucose aCSF (black bars) or lactate aCSF (gray bars). [3H]-Glutamate uptake was measured during the 15-min treatment. In some cultures, the glutamate uptake inhibitor THA (500 μM) was added to ensure that receptor-mediated uptake was being measured. All values are the mean of 4–5 determinations ± SEM. Glutamate uptake was significantly lower at pH 6.4 as compared to pH 7.4 in slice cultures treated with glucose or lactate aCSF (ANOVA, $F = 14.54$, $P < 0.01$; Student Neuman Keul's post-test, $*P < 0.05$, $**P < 0.01$). Glutamate uptake was also significantly lower in lactate aCSF, pH 6.4, as compared to glucose aCSF, pH 6.4 ($*P <$ 0.01, Student Neuman Keuls post-test).

Discussion

Despite a strong association between lactic acidosis and ischemic injury, the role of lactic acidosis in brain injury has remained controversial (Ding et al., 2000; Kraig et al., 1987; Schurr et al., 1997a,b; Takata et al., 2001; Tombaugh, 1994; Xiang and Bergold, 2000). In this study, we provide evidence that lactic acidosis is toxic to hippocampal neurons at conditions of pH and lactate that occur during cerebral ischemia (Figs. 1–3). Acidosis in the absence of lactate was toxic, but lactate potentiated acidosis toxicity. Lactate potentiation of acidosis toxicity was not observed at pH 6.7, but at values of pH 6.4 and more acidic, lactate potentiated acidosis neurotoxicity. The potentiation of acidosis neurotoxicity by lactate was not due to differences in intracellular pH (Fig. 4). Surprisingly, excitotoxicity was identified as a potential mechanism of lactate potentiation of acidosis toxicity (Fig. 5). Both NMDA and AMPA receptor activation were responsible for excitotoxic loss of neurons. Excitotoxicity may arise from increased inhibition of glutamate uptake that was observed at pH 6.4 in the presence of lactate (Fig. 6). This was unexpected since lactic acidosis and excitotoxicity were previously thought to be independent events during cerebral ischemia (Barber et al., 2003).

Both NMDA and AMPA receptor activation are involved in excitotoxicity induced by lactic acidosis (Fig. 5). Glutamate receptor antagonists significantly reduced neuronal loss on days 2, 3, and 4. Surprisingly, neuronal loss on day 1 was unaffected. Since the PI assay was shown to assay delayed neuronal loss, these data suggest neuronal loss resulting from glutamate receptor activation beginning 2 days following the acidosis treatment. Acidosis has been shown to potentiate AMPA toxicity (McDonald et al., 1998). During excitotoxicity, NMDA and AMPA receptors are activated by excess extracellular glutamate. Although we did not examine whether extracellular glutamate was elevated, glutamate uptake was blocked at pH 6.4 when lactate was present (Fig. 6). Glutamate uptake inhibition may be needed for excitotoxic neuronal loss in slice cultures, but it is unlikely to be sufficient. Blockade of glutamate uptake in slice cultures with THA for as long as 7 days did not induce PI fluorescence. This suggests that prolonged glutamate uptake inhibition was nontoxic (S.I. Moskowitz and P.J.B., unpublished results). Excitotoxicity was not previously not thought to contribute to acidosis toxicity since NMDA and AMPA receptors inactivate at acidic pH (Ihle and Patneau, 2000; Low et al., 2000). Inactivation of NMDA and AMPA receptors rapidly reverses when pH returns to physiological levels (Ihle and Patneau, 2000; Low et al., 2000). Slice culture pH recovers within 4 min following removal of lactate aCSF, pH 6.7 (Fig. 4). The recovery to physiological pH rapidly reactivates NMDA and AMPA receptors (Xiang and Bergold, 2000). Glutamate uptake is inhibited when intracellular pH is acidic, but it remains uncertain how long uptake remains inhibited following recovery of pH (Fig. 6). The uptake inhibition likely leads to an increase in extracellular glutamate since glutamate levels were elevated when lactate replaced glucose in hippocampal slice cultures (Jabaudon et al., 2000).

Acidosis also utilizes death mechanisms other than excitotoxicity in slice cultures. NMDA and AMPA receptor antagonists had no affect on acidosis neurotoxicity, yet were only partially effective when lactate was present (Fig. 5). Acidosis is well known to induce cell swelling and swelling could contribute to acidosis toxicity. Cell swelling could be the consequence of the opening of acid-sensing ion channels as well as disruption of the proton gradient during acidification of intracellular pH (Barber et al., 2003; Plesnila et al., 1999). Acidic pH may also directly interfere with mitochondrial function leading to inhibition of ATP synthesis and formation of reactive oxygen species (Barber et al., 2003).

The potentiation of acidosis toxicity by lactate differs from the results of earlier studies that suggest a neuroprotective role for acidosis (Kaku et al., 1993; Tombaugh, 1994; Tombaugh and Sapolsky, 1990). The issue of whether lactic acidosis is neurotoxic or neuroprotective may lie in the age of the neurons being studied. Most in vitro studies use embryonic or neonatal neurons, while this study used hippocampal slices cultured from 20- to 30-day-old rats. The maturity of the slice culture neurons may make them more susceptible to lactic acidosis toxicity (Carpenedo et al., 2002; Laake et al., 1999; Noer et al., 2002). The age dependency of the lactate potentiation of acidosis toxicity may result from the ability of neurons to utilize lactate. In addition, adult neurons also have less efficient buffering of intracellular pH than young neurons (Roberts and Chih, 1997, 1998; Roberts and Sick, 1996; Roberts et al., 1997). Neural activity was maintained with lactate as the sole energy source in immature hippocampal neurons, but not in neurons in more adult slices (Wada et al., 1997, 1998). More mature neurons may more properly model for acidosis as well as other neurotoxic insults.

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