Prevention of acute/severe hypoglycemia-induced neuron death by lactate administration

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Hypoglycemia-induced cerebral neuropathy can occur in patients with diabetes who attempt tight control of blood glucose and may lead to cognitive dysfunction. Accumulating evidence from animal models suggests that hypoglycemia-induced neuronal death is not a simple result of glucose deprivation, but is instead the end result of a multifactorial process. In particular, the excessive activation of poly (ADP-ribose) polymerase-1 (PARP-1) consumes cytosolic nicotinamide adenine dinucleotide (NAD⁺), resulting in energy failure. In this study, we investigate whether lactate administration in the absence of cytosolic NAD⁺ affords neuroprotection against hypoglycemia-induced neuronal death. Intraperitoneal injection of sodium L-lactate corrected arterial blood pH and blood lactate concentration after hypoglycemia. Lactate administered without glucose was not sufficient to promote electroencephalogram recovery from an isoelectric state during hypoglycemia. However, supplementation of glucose with lactate reduced neuronal death by ~80% in the hippocampus. Hypoglycemia-induced superoxide production and microglia activation was also substantially reduced by administration of lactate. Taken together, these results suggest an intriguing possibility: that increasing brain lactate following hypoglycemia offsets the decrease in NAD⁺ due to overactivation of PARP-1 by acting as an alternative energy substrate that can effectively bypass glycolysis and be fed directly to the citric acid cycle to maintain cellular ATP levels.

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Introduction

Patients with diabetes often face a troubling pair of outcomes in the management of their disease; tight blood glucose control can reduce the risk of diabetic complications, but also increases the risk of dangerous hypoglycemic episodes. Hypoglycemia, a potentially devastating cerebral insult, is usually the result of attempting tight control of blood glucose levels with insulin or other hypoglycemic agents, such as sulphonylurea (Davis and Jones, 1998; Seltzer, 1989). Furthermore, patients suffering frequent hypoglycemic

mic episodes or patients with long-standing type 1 diabetes due to loss of early warning symptoms are at elevated risk of experiencing a severe hypoglycemic episode. Previous studies have shown that the neuronal cell death that occurs after hypoglycemia is not simply a result of lack of glucose supply, but is instead the result of a cell death process that is initiated by the reintroduction of glucose after a sustained period of glucose deprivation (GD) (Auer *et al*, 1986). We have termed this process ‘glucose reperfusion-induced neuronal death’ after hypoglycemia (Suh *et al*, 2007). This hypothesis is also applicable in ischemia–reperfusion-induced neuronal death (Suh *et al*, 2008b). Currently, the only available method for preventing this hypoglycemia-induced neuronal death in the clinical setting is delivery of glucose; a treatment that paradoxically may actually exacerbate the insult. In an attempt to provide clinical recourse, we have suggested several strategies to prevent this neuronal death, including inhibition of poly (ADP-ribose) polymerase-1 (PARP-1) activation, chelation of synaptically released zinc, prevention of superoxide production, and supple-

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mentation with pyruvate during the glucose reperfusion period (Suh *et al*, 2003, 2004, 2005, 2007, 2008a).

It is a commonly held view that glucose is the only energy source usable by neurons. However, several studies have suggested that lactate can also serve as a substrate for neuronal energy metabolism (Schurr *et al*, 1997b,c; Schurr, 2006). In their astrocyte–neuron lactate shuttle hypothesis, Pellerin and Magistretti (1994) proposed that lactate can be used by neurons (Magistretti *et al*, 1999; Pellerin *et al*, 2005). In addition to astrocyte-derived lactate, Boumezbeur *et al* (2011) and van Hall *et al* (2009) proposed that plasma-borne lactate may have an important role in supporting oxidative brain metabolism, suggesting that plasma-borne lactate fuels up to 60% of all cerebral metabolism and tricarboxylic acid (TCA) cycle activity even under normal physiological conditions. Consistent with this lactate-fuel hypothesis, several lines of study have also provided evidence that lactate can support energy metabolism during ischemia, which can rescue neuronal death after ischemia or under conditions of oxygen GD (Berthet *et al*, 2009). Recently, Rinholm *et al* (2011) reported that lactate can support axonal function in white matter under conditions of energy deprivation and that exogenous L-lactate can support oligodendrocyte development and myelination. Furthermore, lactate administration after traumatic brain injury also improved neurologic outcome (Ichai *et al*, 2009).

Therefore, a growing body of evidence suggests that lactate is required to sustain neuronal recovery immediately after ischemia or brain trauma; however, the protective effect of lactate administration after hypoglycemia remains untested. Therefore, the aim of this study was to determine whether lactate, when administered as an adjuvant to glucose after hypoglycemia, could reduce neuronal death. Lactate is inexpensive, easily administered, innocuous and already used clinically. Expedient clinical trials may be warranted.

Materials and methods

The surgical and animal care procedures were in accordance with the guidelines of the Institutional Animal Care and Use Committee of the San Francisco Veterans Affairs Medical Center (animal welfare assurance number A3476-01) and of the Hallym University (Hallym 2011-44). This manuscript was written up in accordance with the ARRIVE (Animal Research: Reporting *In Vivo* Experiments) guidelines.

Acute/Severe Hypoglycemia Surgery

Hypoglycemia was induced with regular insulin as described by Auer *et al* (1984) with minor modifications (Suh *et al*, 2003). In brief, male Sprague-Dawley rats weighing 250 to 350 g were fasted overnight and hypoglycemia was induced by intraperitoneal injection of 10 U/kg of regular insulin (Novolin-R, Novo Nordisk, Clayton, NC, USA). Anesthesia was induced with 3% isoflurane in a

75:25 mixture of nitrous oxide and oxygen (Air Liquid America, Houston, TX, USA). After intubation, rats were ventilated with a small rodent respirator (Harvard Apparatus, South Natick, MA, USA). A femoral artery line was placed for blood sampling and for blood pressure monitoring. Blood pH was measured at 1-hour intervals using an I-STAT machine (I-STAT, Princeton, NJ, USA). Blood pressure and electroencephalogram (EEG) were continuously monitored and recorded (BIOPAC System, Santa Barbara, CA, USA). Core temperature was kept at 36.5°C to 37.5°C with a heating blanket. Blood glucose and lactate were measured every 30 minutes in rat with an YSI 2700 glucose analyzer (YSI, Yellow Spring, OH, USA). Mean arterial blood pressure was maintained between 160 and 200 mm Hg during the entire EEG isoelectric period by adjusting the isoflurane concentration, and bradycardia was prevented with intramuscular injection of atropine (1 mg/kg). Electroencephalogram was monitored using needle electrodes placed in the cortical surface. Burr holes were made in the skull bilaterally over parietal cortex and monopolar electrodes were inserted beneath the dura. A reference needle was placed in neck muscle. The extent of neuronal death after hypoglycemia is tightly correlated with the duration of EEG isoelectricity (iso-EEG) (Auer *et al*, 1984). To generate a reproducible neuronal death of moderate severity, hypoglycemia was terminated after 30 minutes of iso-EEG (Auer *et al*, 1984; Suh *et al*, 2003) by delivery of 25% glucose infusion for 3 hours (1.5 mL/h, intravenously) to maintain blood glucose between 5 and 10 mM. Another group of rats received an intraperitoneal injection of sodium L-lactate (500 mg/kg) 5 minutes before glucose reperfusion after 30 minutes of iso-EEG (Supplementary Figure 1). Controls received an osmolarity-matched NaCl solution (104.5 mg/kg, intraperitoneally). After recovery from hypoglycemia, the rats were ambulatory and ate normally. With severe brain injuries, there is the rare possibility of seizure and death in the posthypoglycemic interval; any animals demonstrating seizure were excluded from data analysis (<10% after hypoglycemia). Sham hypoglycemia rats were fasted overnight and received the same amount of insulin as experimental animals, then immediately injected with glucose to prevent hypoglycemia.

Histological Evaluations of Neuronal Death

To identify degenerating neurons after hypoglycemia, animals were anesthetized with isoflurane (3%) 7 days after insult and intracardially perfused with 200 mL of 0.9% saline followed by 4% FA (formaldehyde) for 5 minutes. The brains were postfixed for 24 hours in the same fixative solution and immersed in 20% sucrose. Cryostat sections (25 µm) were mounted on superfrost-coated slides (Fisher Scientific, Pittsburgh, PA, USA). Fluoro-Jade B staining was performed as described by Schmued and Hopkins (2000) (Suh *et al*, 2003). In brief, the slides were immersed in a basic alcohol solution followed by 0.06% potassium permanganate. The slides were then immersed in 0.0004% Fluoro-Jade B (Histo-Chem, Jefferson, AR, USA) for 20 minutes and washed in distilled water. Sections were photographed with a

Leica confocal laser-scanning microscope with blue (450 to 490 nm) excitation light and a barrier filter wavelength of 515 nm. Five coronal sections were collected from each animal by starting 4.0 mm posterior to Bregma, and collecting every fourth section (75 μ m intervals) until five sections were in hand. These sections were then coded and given to a second, blinded experimenter who counted the number of degenerating neurons in the hippocampal CA1, subiculum, dentate gyrus, and perirhinal cortex regions of both hemispheres. The total number of degenerating neurons in a region was averaged over the five sections from each brain.

Detection of Superoxide

For *in vivo* studies, dihydroethidium (Molecular Probes, Eugene, OR, USA) was prepared as a 1 mg/mL solution in 1% dimethyl sulfoxide and administered at 1 mg/kg through the intraperitoneal space to rat at the onset of iso-EEG. Animals were euthanized 3 hours after iso-EEG and perfusion fixed with 4% FA. The brains were sectioned on a cryostat and photographed with a confocal fluorescent microscope at excitation 510 to 550 nm and emission >580 nm for detection of ethidium (Et) (Chan *et al*, 1998). Five 25 μ m thickness sections were analyzed from each brain, taken at 75 μ m intervals to span the hippocampus. Ethidium signal intensity was expressed as the ratio of the mean fluorescence in neuronal perikaria to fluorescence in the stratum radiatum of hippocampal CA1.

Immunostaining for Evaluation of Microglia Activation

Immunostaining was performed on FA fixed and coronally sectioned brain tissue at a thickness of 40 μ m. Three sections were analyzed from each brain, taken at 200 μ m intervals to span the hippocampus. After rinsing in 1 mM phosphate-buffered saline, nonspecific protein binding was blocked by a 1-hour incubation in blocking buffer (10% goat serum and 0.1% Triton X-100 in 1 mM phosphate-buffered saline) at room temperature. The sections were then immunostained with a mouse antibody to rat CD11b (Serotec, Raleigh, NC, USA) at a 1:250 dilution. After washing, the sections were incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG secondary antibody (Molecular Probes, Invitrogen, Grand Island, NY, USA) at a dilution of 1:500 for 2 hours at room temperature. Negative controls were performed with secondary antibody alone and showed no staining. Microglial activation was evaluated by a blinded observer. Three sections from each animal were evaluated for scoring. Microglial activation criteria were based on the number of CD11b immunoreactive cells and their morphology (Kauppinen *et al*, 2008).

Cell Cultures

Glia-free neuron cultures were prepared by plating the embryonic neurons onto a polystyrene surface (Kauppinen

and Swanson, 2005). These neurons were cultured in glia-conditioned medium and used at days 10 to 14 *in vitro*, at which time the cultures contained >95% neurons, as assessed by immunostaining for the neuronal marker microtubule associated protein (MAP)2 and the astrocyte marker glial fibrillary acidic protein (GFAP) (Kauppinen and Swanson, 2005). Glucose concentration in the culture media was 5.5 mM.

Glucose Deprivation in Cell Cultures

The culture medium was exchanged with a bicarbonate-buffered, balanced salt solution containing (in mM) KCl, 3.1; NaCl, 134; CaCl₂, 1.2; MgSO₄, 1.2; KH₂PO₄, 0.25; NaHCO₃, 15.7; glucose, 0. The pH was adjusted to 7.2, while the solution was equilibrated with 5% CO₂ at 37°C. Osmolarity was verified at 290 to 310 mOsm with a Wescor vapor pressure osmometer (Logan, UT, USA). After a 5-minute period to allow complete egress of glucose from the cells, the culture medium was completely exchanged a second time and the cultures were placed in a 37°C, 5% CO₂ incubator. Glucose deprivation was terminated at the designated time points by adding 5 mM glucose back to the medium. Control wells received 5 mM glucose replacement (GR) immediately after the initial medium exchange. For cell culture studies, 5 μ M dihydroethidium (Budd *et al*, 1997) was added to the cultures 30 minutes before GD. The cultures were photographed with a fluorescence microscope 1 hour after GR, and the digitized images were analyzed and expressed as percent of neurons with an Et signal at least 50% higher than background. Prior studies have confirmed that the Et signal reflects primarily superoxide under the conditions of hypoglycemia and glucose reperfusion employed here (Suh *et al*, 2007).

Statistical Analysis

Data were shown as mean values $\pm$ s.e.m. Statistical significance was assessed by analysis of variance and *post hoc* testing was accomplished using Scheffe's test. A *P* value <0.05 was considered to be statistically significant. Microglial activation data were assessed by Kruskal-Wallis nonparametric one-way analysis of variance test followed by Dunn's test for multiple group comparison.

Results

Effects of Lactate Administration on Blood Glucose, Lactate and pH, and Electroencephalogram Before, During, and After Hypoglycemia

Blood glucose, lactate, pH, and EEG were measured for the entire duration of the hypoglycemia experimental period to observe the effects of intraperitoneal administration of lactate on these parameters. Overnight fasting-blood-glucose-level was 3.72 ± 0.15 mM, which dropped to 0.46 ± 0.06 0.5 mM after insulin

injection. A silent electroencephalographic trace (iso-EEG) typically appears ~2.5 hours after insulin injection. Iso-EEG was maintained for 30 minutes and terminated by reintroduction of glucose (femoral vein injection), reinstating blood glucose levels to 7.79 ± 0.35 mM at 30 minutes after glucose reperfusion. Arterial lactate was reduced from $0.94 \pm$

0.11 mM to 0.31 ± 0.04 mM by insulin injection, returning to 1.50 ± 0.14 mM following glucose reperfusion (Figure 1A) (Table 1). To determine if lactate administration influenced blood glucose or lactate concentrations during the glucose reperfusion period, lactate was intraperitoneally injected 5 minutes before glucose reperfusion (25 minutes after iso-EEG start). Blood lactate level was increased to more than twice its initial concentration in the glucose reperfusion with lactate injection group, compared with the glucose reperfusion with saline group (Figure 1B). Blood glucose concentration also increased in the lactate-injected group during the glucose reperfusion period (1 hour after glucose reperfusion) (Figure 1C). Arterial blood pH was measured before, during, and after iso-EEG. In fasting rats that did not receive insulin injection, blood pH was maintained at 7.43 for the duration of the experiments. In the insulin-injected group, blood pH dropped during the iso-EEG period and further decreased after blood glucose reperfusion. However, blood pH in the lactate-injected rats showed no decrease and even increased during the iso-EEG and the glucose reperfusion period (Figure 1D) (Table 2). To test whether lactate administration could rescue EEG from isoelectricity, lactate was injected 15 minutes before the end of the iso-EEG period. Interestingly, silent EEG (iso-EEG) was not rescued by lactate administration without glucose reinfusion (Figure 1E). Moreover, EEG recovery after glucose reperfusion was delayed by lactate administration (Figure 1F).

Figure 1 Effects of lactate administration on blood glucose, lactate and pH, and electroencephalogram (EEG) before, during, and after hypoglycemia. Blood glucose, lactate, pH, and EEG were measured for the duration of the hypoglycemia experimental period to determine the effects of intraperitoneal administration of lactate. (A) Arterial blood glucose level was reduced after insulin injection. During iso-EEG, blood glucose level fell below 0.5 mM; blood glucose level was then reinstated to over 5 mM after glucose reperfusion (GR) (HG (hypoglycemia) + GR). Arterial lactate level was also reduced by insulin injection and then recovered to prefasting levels by glucose reperfusion. (B) A bar graph shows the effects of lactate administration on blood lactate levels during the glucose reperfusion period. Blood lactate level was increased more than two times by glucose reperfusion with lactate injection than glucose reperfusion with saline. (C) Arterial blood glucose levels also increased in the lactate-injected group during glucose reperfusion. (D) Line graphs show the effects of lactate on blood pH changes during the hypoglycemia period. Blood pH was reduced during iso-EEG period and further decreased after glucose reperfusion. However, the blood pH in lactate-injected rats showed no reduction in pH and even increased during the iso-EEG and glucose reperfusion period. (E) EEG was recorded for the entire hypoglycemia period. (a) EEG recorded in normoglycemia before insulin injection. (b) EEG recorded 1.5 hours after insulin injection. (c) iso-EEG. (d) iso-EEG 10 minutes after lactate injection. (e) EEG recorded after glucose reperfusion. (F) EEG recovery after glucose reperfusion is delayed by lactate administration. * $P < 0.05$ compared with saline treated group.

Lactate Reduces Neuronal Death After Hypoglycemia

Hypoglycemia induces a reproducible pattern of neuronal death when evaluated at 7 days after surgery. Fluoro-Jade B staining showed widespread neuronal death in the hippocampal CA1, subiculum (Sub), and dentate gyrus area (Figure 2). Fluoro-Jade B staining provides a selective, unambiguous marker of neuronal degeneration. Compared with rats receiving glucose alone, rats that received glucose plus lactate (500 mg/kg, intraperitoneally) at the end of the iso-EEG period showed a marked reduction in neuronal death (Figure 2A). As quantified in Figure 2B, rats receiving glucose plus lactate had around 90% fewer degenerating neurons than the rats given only glucose plus vehicle (saline).

Table 1 Arterial blood glucose and lactate change before, during, and after hypoglycemia (mM)

	Before hypoglycemia	Iso-EEG	After glucose reperfusion
Glucose (n = 11)	3.72 ± 0.15	0.46 ± 0.06	7.79 ± 0.35
Lactate (n = 11)	0.94 ± 0.11	0.31 ± 0.04	1.50 ± 0.14

EEG, electroencephalogram.

Lactate Prevents Hypoglycemia-Induced Superoxide Production

To image superoxide production after hypoglycemia, we evaluated the production of reactive oxygen species with dihydroethidium (Murakami *et al*, 1998). Dihydroethidium is oxidized by superoxide and superoxide reaction products to form fluorescent Et species (Zhao *et al*, 2003), which are then trapped within cells by DNA binding. Rats subject to 30

Table 2 Arterial blood pH change before, during, and after hypoglycemia

	Before hypoglycemia	Iso-EEG 1 (before lactate)	Iso-EEG 2 (after lactate)	After glucose reperfusion
HG ± lactate (n = 5)	7.43 ± 0.01	7.46 ± 0.03	7.51 ± 0.04	7.56 ± 0.04
Normoglycemia (n = 5)	7.43 ± 0.02	7.44 ± 0.02	7.45 ± 0.01	7.44 ± 0.02
HG ± saline (n = 5)	7.44 ± 0.02	7.45 ± 0.03	7.36 ± 0.02	7.31 ± 0.02

EEG, electroencephalogram; HG, hypoglycemia.

minutes of hypoglycemia followed by a subsequent 30-minute interval of glucose reperfusion showed a several-fold increase in neuronal Et fluorescence (Figure 3A). To determine the effects of intraperitoneal administration of lactate on hypoglycemia-induced superoxide production, we examined Et fluorescent intensity in the hippocampal CA1, subiculum, and dentate gyrus. Lactate injection resulted in a much smaller increase in Et fluorescence in hippocampal neurons after glucose reperfusion, relative to the saline-injected group (Figures 3A and 3B).

Lactate Prevents Hypoglycemia-Induced Microglia Activation

After hypoglycemia, neuronal deprivation of glucose and its resulting sequelae may not only impose neuronal damage via neuron-specific effects, but may also promote neuronal injury indirectly via microglial activation. Hallmarks of microglial activation include a gradual change in morphology from a highly ramified to an amoeboid shape, proliferation, migration to the site of injury, increased expression of surface molecules, increased secretion of cytokines,

Figure 2 Hypoglycemia (HG)-induced neuron death is prevented by lactate administration. (A, C, E) Confocal fluorescence images of Fluoro-Jade B (FJB)-stained brain sections harvested 7 days after HG. FJB staining shows numerous degenerating neurons (green fluorescence) in the hippocampal CA1, subiculum, and dentate gyrus (DG). Rats given 500 mg/kg lactate (intraperitoneally) with glucose at the end of the hypoglycemic interval showed fewer degenerating neurons. Photos are representative of seven to nine rats in each treatment condition. Scale bar = 100 μ m. (B, D, F) Quantitated neuronal degeneration after HG. Compared with saline-treated group, lactate-treated group shows reduced FJB (+) neurons in the hippocampal CA1, subiculum, and DG area. Data are the mean $\pm$ s.e.m. (n = 5 to 7) *P < 0.05 compared with saline-treated group.

Figure 3 Hypoglycemia (HG)-induced reactive oxygen species (ROS) production is prevented by lactate administration. **(A)** Neuronal superoxide production in the hippocampal CA1 region was detected by ethidium (Et) fluorescence staining 3 hours after HG. HG substantially increased Et intensity in CA1 pyramidal neurons. However, lactate administration reduced Et fluorescent intensity. **(B)** Intensity of Et signal is expressed as the ratio of the mean fluorescence in neuronal perikaria to background. HG and glucose reperfusion with saline markedly increased the intensity of Et fluorescence but glucose reperfusion with lactate significantly reduced it. Data are the mean $\pm$ s.e.m. ($n = 5$ to 7) $*P < 0.05$ compared with saline-treated group. **(C)** Neuronal superoxide production in the hippocampal subiculum (Sub) was detected by Et fluorescence staining 3 hours after HG. HG substantially increased Et intensity in subiculum pyramidal neurons. However, lactate administration reduced Et fluorescent intensity. **(D)** HG and glucose reperfusion with saline markedly increased the intensity of Et fluorescence in the subiculum but glucose reperfusion with lactate significantly reduced it. Data are the mean $\pm$ s.e.m. ($n = 5$ to 7) $*P < 0.05$ compared with saline-treated group. **(E)** Neuronal superoxide production in the hippocampal dentate gyrus (DG) was detected by Et fluorescence staining 3 hours after HG. HG substantially increased Et intensity in DG neurons. However, lactate administration reduced Et fluorescent intensity. Scale bar = $100 \mu\text{m}$. **(F)** HG and glucose reperfusion with saline markedly increased the intensity of Et fluorescence in DG but glucose reperfusion with lactate significantly reduced it. Data are the mean $\pm$ s.e.m. ($n = 5$ to 7) $*P < 0.05$ compared with saline-treated group.

chemokines, free radicals and proteases, and phagocytotic activity (Kreutzberg, 1996). We investigated the degree of microglial activation after hypoglycemia with or without lactate administration. Compared with saline-treated rats, microglial activation was significantly reduced by lactate administration in the hippocampus (Figures 4A–4C).

Lactate Prevents Glucose Deprivation-Induced Superoxide Production and Subsequent Neuronal Death in Cultured Neurons

To identify the effects of lactate on superoxide production, we subjected cortical neuron cultures to 2 hours of GD followed by 1 hour of GR. As observed during *in vivo* hypoglycemia, GD alone for

3 hours caused only a negligible increase in the Et signal, but GD (2 hours) + GR (1 hour) produced a rapid increase in neuronal Et fluorescence (Figures 5A and 5B). Control cultures (5 mM glucose was maintained throughout the experiment) exhibited a negligible Et signal (Cont). However, neurons treated with lactate (5 mM) generated much less superoxide after GR than cultures treated with GD (2 hours) + GR (1 hour) (Figures 5A and 5B) and showed reduced neuronal death when evaluated 24 hours after GD/GR (Figures 5C and 5D). We found that lactate treatment alone showed no neuroprotection, which suggests that only combining lactate administration with glucose serves as a neuroprotective strategy, as seen in an additional study (Cater *et al*, 2003).

Figure 4 Hypoglycemia (HG)-induced microglia activation is prevented by lactate administration. **(A)** Fluorescent microscopic images show morphological changes and an increase in the intensity of microglial immunostaining after HG. Sodium lactate substantially decreased microglial activation in the hippocampal CA1 compared with saline-treated animals. Scale bar = 100 μ m. **(B)** Quantification of microglial activation was performed in the hippocampal CA1 area. As shown in the images, HG-induced microglial activation is strongly reduced by lactate treatment. Data are mean $\pm$ s.e.m. ($n = 5$ to 6) * $P < 0.05$ compared with saline-injected group. **(C)** Low magnification bright field microscopic images show microglial activation after HG. Lactate administration substantially decreased microglial activation in the hippocampus and cerebral cortex. Representative of $n = 5$ to 6. Scale bar = 500 μ m.

Long-Term Neuroprotective Effects of Lactate After Hypoglycemia

Assessment of the brains 8 weeks after the hypoglycemic insult revealed atrophy and loss of neurons in the CA1 region of the hippocampus (Figure 6). These changes were substantially attenuated in rats treated with lactate after hypoglycemia, suggesting that neuronal death is prevented rather than simply delayed by this intervention.

Figure 5 Neuronal superoxide production and neuronal death after glucose deprivation/glucose replacement (GD/GR) is prevented by lactate. **(A)** Ethidium (Et) fluorescence in cultured mouse neurons after GD and GR. Cont: Neuron cultures were exposed dihydroethidium with glucose for 3 hours. GD only: Neurons were subjected to GD during the entire 3 hours interval without GR. GD + GR: Neurons were subjected to 2 hours of GD followed by 1-hour glucose replacement (GR). GD + GR + lactate: Neurons were subjected to 2 hours of GD followed by glucose replacement (GR) with lactate. Representative of $n = 5$. Scale bar = 100 μ m. **(B)** Quantification of Et(+) neurons in cultures treated with 3 hours GD alone or with 2 hours GD followed by 1 hour GR with or without lactate. Controls (Cont) received 5 mM glucose after < 5 minutes of GD. Data are mean values + s.e.m; $n = 5$, * $P < 0.01$. **(C)** Phase contrast images of neuronal cultures after GD + GR with or without lactate 48 hours after GD. Representative of $n = 3$. Scale bar = 100 μ m. **(D)** Quantification of number of viable neurons in cultures treated with 3 hours GD alone or with 2 hours GD followed by 1 hour GR with or without lactate. Controls received 5 mM glucose after < 5 minutes of GD. Data are mean values + s.e.m; $n = 3$, * $P < 0.01$.

Figure 6 Long-term neuroprotective effect of lactate after hypoglycemia (HG). **(A)** Light microscopic images show viable neurons in the hippocampal CA1 region. Live neurons were detected by NeuN immunostaining. Hippocampal CA1 pyramidal neurons disappeared 8 weeks after HG. These changes were substantially attenuated in the rats treated with lactate after HG. Sections are representative of six to eight rats in each group. Scale bar = 200 μ m. **(B)** A graph represents quantified NeuN (+) neurons in the hippocampal CA1 pyramidal layer. Compared with saline-treated group, lactate-treated group shows more NeuN (+) neurons. Data are the mean $\pm$ s.e.m ($n = 6$ to 8). * $P < 0.05$ compared with saline-treated group.

Discussion

The development of clinically useful techniques to reduce the neuronal death that results from severe hypoglycemia is greatly needed. The present study demonstrates that intraperitoneal administration of lactate significantly reduced hypoglycemia-induced neuronal death in the CA1, dentate granule cell, and subiculum of the hippocampus that would occur without intervention. Furthermore, this improvement in neuronal survival was also detected 8 weeks after the hypoglycemic insult, indicating that neuronal death is fully prevented, not just delayed, by lactate administration.

The mature brain is one of the few organs that depend on glucose as its primary metabolic substrate. Once in brain, glucose may be metabolized directly by neurons and glia. Glucose may be metabolized to lactate by glia and the lactate subsequently shuttled to

neurons for oxidative metabolism (Dienel and Cruz, 2004; Pellerin and Magistretti, 1994; Schurr *et al*, 1997b; Wender *et al*, 2000). Lactate may act as a neuronal oxidative energy substrate (Schurr, 2006). While pyruvate, lactate and other ketone bodies are known to be comparable to glucose in their capacity to support neuron metabolism *in vitro*, the blood-brain barrier in the mature brain preferentially transports glucose over other energy substrates. Blood-brain barrier transport of lactate and other substrates occurs via both facilitated transport and diffusion. Therefore, increases in plasma concentration of these compounds should substantially increase their transport into the brain (Boumezbeur *et al*, 2011; van Hall *et al*, 2009). The dose of lactate used in the present studies is estimated to generate a lactate plasma concentration of ~ 2 to 3 mM, which is about twofold to threefold higher than under physiological conditions, likely increasing brain lactate concentrations accordingly. Lactate appears to have a crucial role in improving postischemic outcomes. Schurr *et al* (1997a, 2001a, b) have published several articles reporting the neuroprotective effect of lactate following hypoxia or following ischemia, which involves GD. Blockade of lactate transport into neurons prevents its utilization and, consequently, exacerbates delayed ischemic neuronal damage (Schurr *et al*, 2001b). Although previous studies demonstrated the beneficial effects of lactate on ischemia-induced neuron death, no direct study was performed to evaluate neuroprotective effects of lactate after hypoglycemia, where oxygen levels are maintained at ambient levels. Our present study shows that lactate also prevents neuronal death after severe hypoglycemia under normoxic conditions. Rather than acting as an oxidative energy substrate, several studies have suggested that lactate may also have antioxidant activities. When lactate is converted to pyruvate by mitochondrial lactate dehydrogenase (LDH) (when ATP levels are very low and thus when glucose cannot be metabolized), there is a simultaneous conversion of nicotinamide adenine dinucleotide (NAD) to reduced form of NAD (NADH), which is known to have antioxidant activity (Heinemann *et al*, 2002; Kirsch and De Groot, 2001).

Our studies indicate that a cascade of events is precipitated by the lack of energy substrate during hypoglycemia, which then leads to neuronal demise through a series of sequential pathological reactions. Key steps in this cell death pathway include glutamate release, zinc translocation, and PARP-1 activation. A major effect of this cascade is the consumption of cytosolic NAD⁺ (Engelsen *et al*, 1986; Suh *et al*, 2004; Wieloch, 1985), the depletion of which prevents metabolism of glucose. In settings where glucose is the chief metabolic substrate, this leads to mitochondrial dysfunction and cell death (Alano *et al*, 2004; Ying *et al*, 2003; Zong *et al*, 2004). Interestingly, repletion of NAD⁺ after PARP-1 activation has been shown to restore glycolytic capacity and prevent cell death (Ying *et al*, 2003, 2005) in

primary neuronal cultures. Similarly, providing nonglucose substrates such as pyruvate and α -ketoglutarate that can be metabolized without the need for cytosolic NAD⁺ also preserves cell viability, even when delivered hours after PARP-1 activation (Alano *et al*, 2004; Ying *et al*, 2002). However, to be used oxidatively, lactate must be first converted to pyruvate by lactate dehydrogenase, which also requires NAD⁺ and thus competes with glycolysis for NAD⁺. Therefore, given this fact, how can lactate administration have a neuroprotective effect in this situation? We suggest the following explanation: it has been previously suggested that PARP-1 consumes the cytosolic NAD⁺ pool, but does not consume the mitochondrial NAD⁺ pool until the point that mitochondrial permeability transition occurs (Alano *et al*, 2010). Thus, during the first few hours or so after extensive PARP-1 activation, the mitochondria remain capable of respiration but the cytosol cannot metabolize glucose. Thus, when given nonglucose substrates such as lactate, the mitochondria can make ATP (Alano *et al*, 2007, 2010; Ying *et al*, 2005). Once enough ATP is generated, glucose can again become the main energy substrate. In agreement with these cell culture studies, the present findings suggest that lactate promotes neuronal survival after hypoglycemia by circumventing a sustained impairment in glycolysis induced by PARP-1 activation. This further supports the idea that hypoglycemia causes a sustained impairment in glucose metabolism that persists after glucose reperfusion and that combining lactate administration along with glucose to terminate hypoglycemia serves as a neuroprotective strategy.

Increasing blood lactate after ischemia or hypoglycemia has traditionally been believed to be toxic through promotion of acidosis. Acid-sensing ion channels are activated after ischemia (Xiong *et al*, 2004) and their inhibition is neuroprotective (Pignatario *et al*, 2007). However, in the present study, sodium L-lactate administration induced no drop in blood pH; pH actually remained higher than 7.4. Since acid-sensing ion channels are only activated at pHs lower than 7, this lactate-induced pH elevation may close the acid-sensing ion channels. Although the mechanism by which blood pH was maintained above 7 remains unknown, the effect may possibly explain some of the neuroprotective benefits of lactate.

Using the same hypoglycemic brain injury model, very similar levels of neuroprotection in vulnerable brain regions, as well as prevention of cognitive deficits, were previously achieved by administration of PARP inhibitors at the termination of hypoglycemia (Suh *et al*, 2003, 2005). Since pathological overactivation of PARP is known to induce cell death indirectly by sequestering intracellular NAD⁺ needed to maintain glycolysis at sufficient levels, it is tempting to explain the protection afforded by lactate in terms of this relationship. While further study is needed to directly establish how lactate achieves comparable neuroprotection to PARP

inhibition, one parsimonious explanation is that a concomitant increase in brain lactate offsets the decrease in NAD⁺ due to overactivation of PARP by acting as an alternative energy substrate that can effectively bypass glycolysis and be fed directly to the citric acid cycle to maintain cellular ATP levels (Ying *et al*, 2005). Lactate is inexpensive, readily available, and unlike directly inhibiting PARP activation, does not have the theoretical limitation of potentially impairing DNA repair mechanisms (Nagayama *et al*, 2000). Results of this study suggest that lactate may be an effective intervention for patients with severe hypoglycemia.

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Author contributions

SJW, BGJ, and HKS researched the data and reviewed/edited the manuscript. BHY, MWL, BYC, and JHK researched the data. MS performed data analysis and reviewed/edited the manuscript. SWS contributed to discussion, reviewed/edited the manuscript, wrote the manuscript, and took full responsibility for the manuscript and its originality.

Disclosure/conflict of interest

The authors declare no conflict of interest.

References

- Alano CC, Garnier P, Ying W, Higashi Y, Kauppinen TM, Swanson RA (2010) NAD⁺ depletion is necessary and sufficient for poly(ADP-ribose) polymerase-1-mediated neuronal death. *J Neurosci* 30:2967–78
- Alano CC, Tran A, Tao R, Ying W, Karliner JS, Swanson RA (2007) Differences among cell types in NAD(+) compartmentalization: a comparison of neurons, astrocytes, and cardiac myocytes. *J Neurosci Res* 85:3378–85
- Alano CC, Ying W, Swanson RA (2004) Poly(ADP-ribose) polymerase-1-mediated cell death in astrocytes requires NAD⁺ depletion and mitochondrial permeability transition. *J Biol Chem* 279:18895–902
- Auer RN, Hall P, Ingvar M, Siesjo BK (1986) Hypotension as a complication of hypoglycemia leads to enhanced energy failure but no increase in neuronal necrosis. *Stroke* 17:442–9
- Auer RN, Olsson Y, Siesjo BK (1984) Hypoglycemic brain injury in the rat. Correlation of density of brain damage with the EEG isoelectric time: a quantitative study. *Diabetes* 33:1090–8
- Berthet C, Lei H, Thevenet J, Gruetter R, Magistretti PJ, Hirt L (2009) Neuroprotective role of lactate after cerebral ischemia. *J Cereb Blood Flow Metab* 29:1780–9

- Boumezbeur F, Petersen KF, Cline GW, Mason GF, Behar KL, Shulman GI, Rothman DL (2011) The contribution of blood lactate to brain energy metabolism in humans measured by dynamic ^{13}C nuclear magnetic resonance spectroscopy. *J Neurosci* 30:13983–91
- Budd SL, Castilho RF, Nicholls DG (1997) Mitochondrial membrane potential and hydroethidine-monitored superoxide generation in cultured cerebellar granule cells. *FEBS Lett* 415:21–4
- Cater HL, Chandratheva A, Benham CD, Morrison III B, Sundstrom LE (2003) Lactate and glucose as energy substrates during, and after, oxygen deprivation in rat hippocampal acute and cultured slices. *J Neurochem* 87:1381–90
- Chan PH, Kawase M, Murakami K, Chen SF, Li Y, Calagui B, Reola L, Carlson E, Epstein CJ (1998) Overexpression of SOD1 in transgenic rats protects vulnerable neurons against ischemic damage after global cerebral ischemia and reperfusion. *J Neurosci* 18:8292–9
- Davis EA, Jones TW (1998) Hypoglycemia in children with diabetes: incidence, counterregulation and cognitive dysfunction. *J Pediatr Endocrinol Metab* 11(Suppl 1): 177–82
- Dienel GA, Cruz NF (2004) Nutrition during brain activation: does cell-to-cell lactate shuttling contribute significantly to sweet and sour food for thought? *Neurochem Int* 45:321–51
- Engelsen B, Westerberg E, Fonnum F, Wieloch T (1986) Effect of insulin-induced hypoglycemia on the concentrations of glutamate and related amino acids and energy metabolites in the intact and decorticated rat neostriatum. *J Neurochem* 47:1634–41
- Heinemann U, Buchheim K, Gabriel S, Kann O, Kovacs R, Schuchmann S (2002) Cell death and metabolic activity during epileptiform discharges and status epilepticus in the hippocampus. *Prog Brain Res* 135: 197–210
- Ichai C, Armando G, Orban JC, Berthier F, Rami L, Samat-Long C, Grimaud D, Leverve X (2009) Sodium lactate versus mannitol in the treatment of intracranial hypertensive episodes in severe traumatic brain-injured patients. *Intensive Care Med* 35:471–9
- Kauppinen TM, Higashi Y, Suh SW, Escartin C, Nagasawa K, Swanson RA (2008) Zinc triggers microglial activation. *J Neurosci* 28:5827–35
- Kauppinen TM, Swanson RA (2005) Poly(ADP-ribose) polymerase-1 promotes microglial activation, proliferation, and matrix metalloproteinase-9-mediated neuron death. *J Immunol* 174:2288–96
- Kirsch M, De Groot H (2001) NAD(P)H, a directly operating antioxidant? *FASEB J* 15:1569–74
- Kreutzberg GW (1996) Microglia: a sensor for pathological events in the CNS. *Trends Neurosci* 19:312–8
- Magistretti PJ, Pellerin L, Rothman DL, Shulman RG (1999) Energy on demand. *Science* 283:496–7
- Murakami K, Kondo T, Kawase M, Li Y, Sato S, Chen SF, Chan PH (1998) Mitochondrial susceptibility to oxidative stress exacerbates cerebral infarction that follows permanent focal cerebral ischemia in mutant mice with manganese superoxide dismutase deficiency. *J Neurosci* 18:205–13
- Nagayama T, Simon RP, Chen D, Henshall DC, Pei W, Stetler RA, Chen J (2000) Activation of poly(ADP-ribose) polymerase in the rat hippocampus may contribute to cellular recovery following sublethal transient global ischemia. *J Neurochem* 74:1636–45
- Pellerin L, Bergersen LH, Halestrap AP, Pierre K (2005) Cellular and subcellular distribution of monocarboxylate transporters in cultured brain cells and in the adult brain. *J Neurosci Res* 79:55–64
- Pellerin L, Magistretti PJ (1994) Glutamate uptake into astrocytes stimulates aerobic glycolysis: a mechanism coupling neuronal activity to glucose utilization. *Proc Natl Acad Sci USA* 91:10625–9
- Pignataro G, Simon RP, Xiong ZG (2007) Prolonged activation of ASIC1a and the time window for neuroprotection in cerebral ischaemia. *Brain* 130:151–8
- Rinholm JE, Hamilton NB, Kessaris N, Richardson WD, Bergersen LH, Attwell D (2011) Regulation of oligodendrocyte development and myelination by glucose and lactate. *J Neurosci* 31:538–48
- Schmued LC, Hopkins KJ (2000) Fluoro-Jade B: a high affinity fluorescent marker for the localization of neuronal degeneration. *Brain Res* 874:123–30
- Schurr A (2006) Lactate: the ultimate cerebral oxidative energy substrate? *J Cereb Blood Flow Metab* 26:142–52
- Schurr A, Payne RS, Miller JJ, Rigor BM (1997a) Glia are the main source of lactate utilized by neurons for recovery of function posthypoxia. *Brain Res* 774:221–4
- Schurr A, Payne RS, Miller JJ, Rigor BM (1997b) Brain lactate, not glucose, fuels the recovery of synaptic function from hypoxia upon reoxygenation: an *in vitro* study. *Brain Res* 744:105–11
- Schurr A, Payne RS, Miller JJ, Rigor BM (1997c) Brain lactate is an obligatory aerobic energy substrate for functional recovery after hypoxia: further *in vitro* validation. *J Neurochem* 69:423–6
- Schurr A, Payne RS, Miller JJ, Tseng MT (2001a) Preischemic hyperglycemia-aggravated damage: evidence that lactate utilization is beneficial and glucose-induced corticosterone release is detrimental. *J Neurosci Res* 66:782–9
- Schurr A, Payne RS, Miller JJ, Tseng MT, Rigor BM (2001b) Blockade of lactate transport exacerbates delayed neuronal damage in a rat model of cerebral ischemia. *Brain Res* 895:268–72
- Seltzer HS (1989) Drug-induced hypoglycemia. A review of 1418 cases. *Endocrinol Metab Clin North Am* 18:163–83
- Suh SW, Aoyama K, Chen Y, Garnier P, Matsumori Y, Gum E, Liu J, Swanson RA (2003) Hypoglycemic neuronal death and cognitive impairment are prevented by poly(ADP-ribose) polymerase inhibitors administered after hypoglycemia. *J Neurosci* 23:10681–90
- Suh SW, Aoyama K, Matsumori Y, Liu J, Swanson RA (2005) Pyruvate administered after severe hypoglycemia reduces neuronal death and cognitive impairment. *Diabetes* 54:1452–8
- Suh SW, Garnier P, Aoyama K, Chen Y, Swanson RA (2004) Zinc release contributes to hypoglycemia-induced neuronal death. *Neurobiol Dis* 16:538–45
- Suh SW, Gum ET, Hamby AM, Chan PH, Swanson RA (2007) Hypoglycemic neuronal death is triggered by glucose reperfusion and activation of neuronal NADPH oxidase. *J Clin Invest* 117:910–8
- Suh SW, Hamby AM, Gum ET, Shin BS, Won SJ, Sheline CT, Chan PH, Swanson RA (2008a) Sequential release of nitric oxide, zinc, and superoxide in hypoglycemic neuronal death. *J Cereb Blood Flow Metab* 28: 1697–706
- Suh SW, Shin BS, Ma H, Van Hoecke M, Brennan AM, Yenari MA, Swanson RA (2008b) Glucose and NADPH oxidase drive neuronal superoxide formation in stroke. *Ann Neurol* 64:654–63

- van Hall G, Stromstad M, Rasmussen P, Jans O, Zaar M, Gam C, Quistorff B, Secher NH, Nielsen HB (2009) Blood lactate is an important energy source for the human brain. *J Cereb Blood Flow Metab* 29:1121–9
- Wender R, Brown AM, Fern R, Swanson RA, Farrell K, Ransom BR (2000) Astrocytic glycogen influences axon function and survival during glucose deprivation in central white matter. *J Neurosci* 20:6804–10
- Wieloch T (1985) Hypoglycemia-induced neuronal damage prevented by an N-methyl-D- aspartate antagonist. *Science* 230:681–3
- Xiong ZG, Zhu XM, Chu XP, Minami M, Hey J, Wei WL, MacDonald JF, Wemmie JA, Price MP, Welsh MJ, Simon RP (2004) Neuroprotection in ischemia: blocking calcium-permeable acid-sensing ion channels. *Cell* 118:687–98
- Ying W, Alano CC, Garnier P, Swanson RA (2005) NAD⁺ as a metabolic link between DNA damage and cell death. *J Neurosci Res* 79:216–23
- Ying W, Chen Y, Alano CC, Swanson RA (2002) Tricarboxylic acid cycle substrates prevent PARP-mediated death of neurons and astrocytes. *J Cereb Blood Flow Metab* 22:774–9
- Ying W, Garnier P, Swanson RA (2003) NAD⁺ repletion prevents PARP-1-induced glycolytic blockade and cell death in cultured mouse astrocytes. *Biochem Biophys Res Commun* 308:809–13
- Zhao H, Kalivendi S, Zhang H, Joseph J, Nithipatikom K, Vasquez-Vivar J, Kalyanaraman B (2003) Superoxide reacts with hydroethidine but forms a fluorescent product that is distinctly different from ethidium: potential implications in intracellular fluorescence detection of superoxide. *Free Radic Biol Med* 34:1359–1368
- Zong WX, Ditsworth D, Bauer DE, Wang ZQ, Thompson CB (2004) Alkylating DNA damage stimulates a regulated form of necrotic cell death. *Genes Dev* 18:1272–82

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