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Neuroprotective role of lactate after cerebral ischemia

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It is well established that lactate can be used as an energy substrate by the brain by conversion to pyruvate and a subsequent oxidation in the mitochondria. Knowing the need for readily metabolizable substrates directly after ischemia and the protective effect of lactate after excitotoxicity, the aim of this study was to investigate whether lactate administration directly after ischemia could be neuroprotective. *In vitro*, the addition of 4 mmol/L L-lactate to the medium of rat organotypic hippocampal slices, directly after oxygen and glucose deprivation (OGD), protected against neuronal death, whereas a higher dose of 20 mmol/L was toxic. *In vivo*, after middle cerebral artery occlusion in the mouse, an intracerebroventricular injection of 2 μL of 100 mmol/L L-lactate, immediately after reperfusion, led to a significant decrease in lesion size, which was more pronounced in the striatum, and an improvement in neurologic outcome. A later injection 1 h after reperfusion did not reduce lesion size, but significantly improved neurologic outcome, which is an important point in the context of a potential clinical application. Therefore, a moderate increase in lactate after ischemia may be a therapeutic tool.
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Introduction

Stroke is the first cause of disability and the third cause of death in the world (Feigin *et al*, 2003; Murray and Lopez, 1997). Approximately 80% of strokes are ischemic and most of the infarcts involve the territory of the middle cerebral artery. During an ischemic stroke, glucose and oxygen supply to the brain decreases, leading to a cascade of damaging mechanisms. Disruption of brain metabolism is clearly a key element in stroke; a better knowledge of its cellular and molecular determinants may lead to novel therapeutic targets.

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Glucose was believed to be the only energy substrate for neurons until Schurr *et al* showed 20 years ago that lactate, as a sole energy substrate, can support normal synaptic function in rat hippocampal slices (Schurr, 2006; Schurr *et al*, 1988), opening the possibility that lactate produced during ischemia might not just be an end product, but might also be used as a substrate for energy metabolism. A few years later, Pellerin and Magistretti (1994) proposed their astrocyte–neuron lactate shuttle hypothesis, in line with the idea that lactate can be used by neurons.

On the basis of this hypothesis, several studies have tested whether lactate is beneficial to *neurons* under pathologic conditions, such as ischemia, glutamate excitotoxicity, and traumatic brain injury, and showed promising results even if the use of lactate as an energy substrate by neurons is still a source of controversy (Hertz, 2008). *In vitro*, on hippocampal slices, it has been shown that lactate, either endogenously produced during hypoxia or applied exogenously, can be used after hypoxia, is

preferential to glucose during the reoxygenation period (Cater *et al*, 2003; Schurr *et al*, 1997*b*), and is protective against glutamate excitotoxicity (Schurr *et al*, 1999). *In vivo*, lactate administered to cortical superfusate during and after global ischemia in rat, allows a better recovery of the electrocorticogram during reperfusion and reduces amino-acid release (Phillis *et al*, 1999). When perfused in combination with glutamate, lactate has been shown to protect against glutamate excitotoxicity and to reduce lesion size (Ros *et al*, 2001). Another study using an inhibitor of lactate transport, which also inhibits the entry of pyruvate into the mitochondria, showed an important neuroprotective effect of endogenously produced lactate during global ischemia (Schurr *et al*, 2001). Furthermore, lactate administration in patients after traumatic brain injury improved neurologic outcome (Ichai *et al*, 2009).

There is growing evidence showing that lactate is required to sustain neuronal recovery directly after ischemia. However, the effect of lactate administration after reperfusion has never been tested. Therefore, the aim of this study was to test a potential neuroprotective effect of lactate administration at the end of ischemia or later.

Materials and methods

All animal experiments were conducted in accordance with the guidelines of the cantonal veterinary office.

Organotypic Hippocampal Slice Cultures

Hippocampal slice cultures obtained from P12 rats were subjected to *in vitro* ischemia by exposure to reduced oxygen and glucose concentrations (oxygen and glucose deprivation, OGD), as described previously (Hirt *et al*, 2004). Briefly, 350-μm-thick coronal hippocampal slices were cultured on porous membranes (Millicell; Millipore, Billerica, MA, USA) in a medium containing 25% horse serum (Oxoid, Hampshire, UK), 50% minimal essential medium supplemented with HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and sodium bicarbonate (MEM; Gibco, Paisley, UK), 25% HBSS (Hanks' balanced salt solution; Gibco), 2 mmol/L L-glutamine and 6.4 mg/mL D-glucose. Cultures were grown at 33°C, 100% humidity, and 5% CO_2 for 3 days. The medium was replaced by a fresh identical medium at day 3, and then by a medium with 15% horse serum, 60% MEM, 25% HBSS, and 2 mmol/L L-glutamine at day 6. For OGD, slices were transferred to a serum-free hypoglycemic medium, DMEM (Dulbecco's modified Eagle's medium; D5030; Sigma, St Louis, MO, USA), supplemented with 1 mmol/L D-glucose and 2 mmol/L L-glutamine, in a humidified hypoxia chamber (COY, Grass Lake, MI, USA) with a hypoxic atmosphere of 5% O_2, 5% CO_2, and 90% N_2 at 37°C for 30 mins. Sodium L-lactate (Fluka, Sigma-Aldrich, Buchs, Switzerland) diluted in 1 × phosphate-buffered saline (PBS) (stock solution of 500 mmol/L) was administered immediately after OGD (final concentration of 1, 4, 10, or 20 mmol/L). Cell death was determined in the neuronal CA1 region after incubation in 5 μg/mL propidium iodide (Sigma) for 30 mins. Propidium iodide fluorescence emission (excitation wavelength 568 nm) was measured 48 h after OGD. After subtracting background fluorescence, the results were expressed as a percentage of maximal cell death obtained by incubating the hippocampal slice cultures in 1 × PBS for 24 h at 4°C. Cell death was averaged for the four slices of each culture.

Transient Middle Cerebral Artery Occlusion in the Mouse

A total of 13 male ICR-CD1 mice (weighing 24 to 34 g, Charles River, L'Arbresle, France) were anesthetized by an intraperitoneal injection of 8 mg/kg xylazine (Rompun 2%, Bayer, Zürich, Switzerland) + 100 mg/kg ketamine (Ketanarkon 100, Streuli Pharma AG). A second dose of 4 mg/kg xylazine + 50 mg/kg ketamine was added after 40 mins of anesthesia. A total of 26 male ICR-CD1 mice (22 to 32 g, Charles River) were anesthetized and maintained under 1.5% to 2% isoflurane in 30% oxygen and 70% nitrous oxide using a face mask. At 0 h, ischemia was induced by inserting an 11-mm silicone-coated 8-0 filament through the left common carotid artery into the internal carotid artery (see Wiegler *et al* (2008) and references therein). The filament was withdrawn after 30 (for the mice anesthetized with xylazine–ketamine) or 60 mins (for the mice anesthetized with isoflurane), allowing reperfusion. Regional cerebral blood flow was measured by LDF (laser-Doppler flowmetry) (Periflux 5000, Perimed, Stockholm, Sweden) with a flexible probe fixed on the skull, 1 mm posteriorly and 6 mm laterally from the bregma. Throughout the entire operation, regional cerebral blood flow was monitored and maintained under 20% of the baseline level during ischemia and above 50% of the baseline level after reperfusion. Throughout surgery and until awaking, rectal temperature was maintained at 37 ± 0.5°C using a temperature control unit (FHC, Bowdoinham, ME, USA).

Mice were administered 0.025 mg/kg of buprenorphine subcutaneously for analgesia at the end of the operation. Once the animals were awake, they were housed in an incubator at 31°C. The mice were killed 48 h after ischemia.

To mimic the *in vitro* condition, we estimated that the intracerebroventricular injection of 2 μL of 100 mmol/L L-lactate would increase the cerebrospinal fluid lactate concentration *in vivo* by ~4 mmol/L in a mouse. L-lactate was diluted to a final concentration of 100 mmol/L in 1 × PBS (pH 7.4). Randomly, 2 μL of either the 100 mmol/L L-lactate solution or vehicle (1 × PBS, pH 7.4) was injected intracerebroventricularly (intracerebroventricular, 0.9 mm laterally, 0.1 mm posteriorly, 3.1 mm deep from the bregma) using a Hamilton syringe (Hamilton Company, Bonaduz, Switzerland) (Hirt *et al*, 2004) on the left side directly after reperfusion (at 35 or 70 mins from the beginning of ischemia) or at 120 mins from the beginning of ischemia. For this later injection (120 mins), animals were reanesthetized using an intraperitoneal injection of 4 mg/kg xylazine + 50 mg/kg ketamine. For the first group of mice,

anesthetized with xylazine–ketamine, the experimentator was not blinded to the treatment group at injection time, but lesion size measurements were blinded. For all the mice anesthetized with isoflurane, the experiment was blinded from the beginning of the first operation until the end of data analysis.

Determination of Ischemic Lesion Volumes

Animals were killed at 48 h after the onset of focal ischemia and 20-μm-thick, 720-μm-apart, coronal cryostat sections were stained with cresyl violet for histologic determination of lesion size. A digitalized image of the Nissl-stained tissue was obtained under a light stereomicroscope (Leica MZ16FA, Leica, Heerbrugg, Switzerland). The lesion area was determined by an examiner blinded for the treatment group using ImageJ software (NIH, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/) on stained sections. Direct infarct volume was calculated by multiplying the sum of the infarct areas on each section by the spacing distance. To avoid bias due to edema, an indirect lesion size was calculated as follows: indirect lesion = $\text{Volume}_{\text{contralateral}} - (\text{Volume}_{\text{ipsilateral}} - \text{direct infarct volume})$ (Swanson *et al*, 1990).

Neurologic Deficits

A neuroscore composed of three tests was assessed 48 h after ischemia for mice operated under isoflurane anesthesia. In this neuroscore, each test has a maximum of three points. The first test evaluates the neurologic deficit and is graded for severity, as described previously (Hirt *et al*, 2004) (0: no observable neurologic deficit; 1: failure to extend the right forepaw; 2: circling to the contralateral side, and 3: loss of walking or righting reflex). The second test is a beam walking test (Carter *et al*, 2001) (0: mice walk directly to the end of the beam; 1: mice can walk along the beam, but slip a few times; 2: mice cannot walk more than a few steps. 3: mice do not move). The third test is the Rotarod treadmill (UgoBasile, Comerio, Italy) (Carter *et al*, 2001) test. The mice were placed on the rotating drum, set to accelerate uniformly from 4 to 40 r.p.m., and their latency to fall from the drum was recorded. The animals were trained on 2 different days before surgery, with two trials in each training session. The test was then performed 2 days after middle cerebral artery occlusion (MCAO), with two consecutive trials for each animal. The better of the two trials was selected. Points were attributed on the basis of performances expressed as a percentage of the best performance before ischemia (0:90% to 100% and then 0.5 point for each 15% decrease).

In Vivo Magnetic Resonance Studies of Lactate-Injected Mice

To evaluate the distribution of the intracerebroventricularly injected lactate, six ICR-CD1 mice (weighing 29 to 33 g) were measured by magnetic resonance (MR) imaging and spectroscopy before and after an intracerebroventricular injection of 2 μL of 100 mmol/L L-lactate. Four additional ICR-CD1 mice (29 to 35 g) were measured after an intracerebroventricular injection of 2 μL of PBS to eliminate any possible lactate contribution due to the intracerebroventricular injection itself.

All MR studies were carried out in a horizontal, 14.1-T/26-cm magnet (Magnex Scientific, Abingdon, UK), as described previously (Lei *et al*, 2009).

Throughout the entire MR studies, including the intracerebroventricular injection, the animals were maintained in an anesthetized state with 1% to 2% isoflurane mixed with O_2 and stereotaxically fixed with two ear pieces and a bite piece in a holder (RAPID Biomedical GmbH, Rimpar, Gemany). During the mean time, the animal was simultaneously monitored for breathing and temperature using an MR-compatible monitor system (Model 1025, SA Instruments, Stony Brook, NY, USA), and rectal temperature was maintained at $\sim$37.0°C by circulating warm water.

Briefly, multislice T_2-weighted images were acquired using the fast spin-echo technique (Hennig, 1988), with TE_{eff} (effective echo time) = 50 msecs and TR (repetition time) 6,000 msecs to locate the volume of interest in the left striatum (6 to 8 μL) or cortex (2.2 to 2.5 μL). Thereafter, all first- and second-order shim terms over the volume of interest were altered accordingly, using the echo-planar version of FASTMAP (see Mlynarik *et al* (2006) and references therein), which resulted in a water line width within 25 Hz. Localized ^{1}H-MR spectrum was obtained using the SPECIAL techniques, TE/TR = 2.8/4,000 msec in combination with outer volume suppression and VAPOR water suppression (see Mlynarik *et al* (2006) and references therein). To sustain sufficient signal-to-noise ratios from such microliter volumes, 240 and 480 of such spectra were acquired for the striatum and cortex, respectively.

The *in vivo* ^{1}H-MR spectra obtained from each study were processed as in Tkac *et al*, (2007), frequency drift was corrected, summed and eddy current was compensated using the water signal from the same volume of interest. Thereafter, absolute quantification was obtained using a linear combination analysis method, LCModel, assuming 80% tissue water content (see Tkac *et al* (2007) and references therein).

Statistics

All data are presented as mean ± s.d. Statistical analyses were carried out using nonparametric tests: the Kruskal–Wallis test followed by Dunn's multiple-comparison test for *in vitro* experiments and the Mann–Whitney test for *in vivo* experiments (one-tailed *P*-value for *in vivo* experiments).

Results

In an *in vitro* model of ischemia consisting of OGD in rat organotypic hippocampal slices, the administration of 4 mmol/L L-lactate directly after OGD significantly decreased neuronal cell death in the

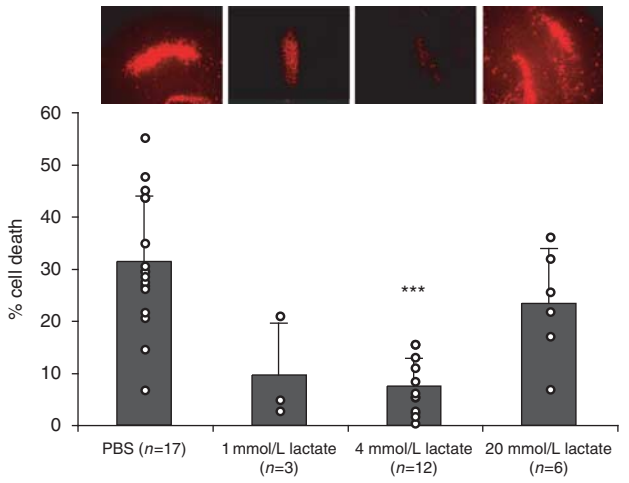

Figure 1 Administration of 4 mmol/L L-lactate after OGD is neuroprotective. Rat organotypic hippocampal slices were subjected to 30 mins of OGD. Various doses of L-lactate were administered to the medium directly after OGD. Cell death was assessed by propidium iodide staining at 48 h after OGD. $***P < 0.001$ for the Kruskal–Wallis test, followed by Dunn's multiple-comparison test. Open circles represent one well of four organotypic hippocampal slices.

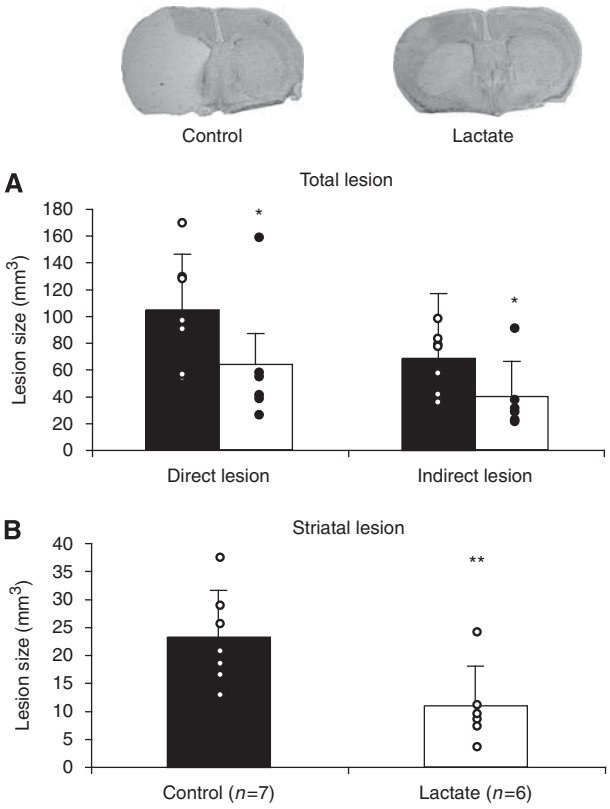

Figure 2 Injection of 2 μL of 100 mmol/L L-lactate intracerebroventricularly directly after reperfusion is neuroprotective after 30 mins of MCAO under xylazine–ketamine anesthesia. (**A**) Total direct and indirect lesion sizes were measured at 48 h after ischemia. Black bars represent control mice, and white bars represent mice injected with lactate. (**B**) Striatal lesion. $*P < 0.05$ and $**P < 0.01$ for the Mann–Whitney test, one-tailed *P*-value. Circles represent individual animals.

CA1 region of the hippocampus at 48 h, from $31 \pm 12\%$ to $7 \pm 5\%$, whereas lower (1 mmol/L) or higher (20 mmol/L) doses of L-lactate did not have a significant protective effect (Figure 1). The higher dose of 20 mmol/L L-lactate was in fact toxic without OGD, inducing 15% to 20% cell death at 48 h (data not shown). This toxicity was not due to medium acidification, as we could not detect any change in the pH of the medium after L-lactate addition at 4 or 20 mmol/L (data not shown), but could be due to increased osmolarity.

On the basis of this apparent neuroprotective effect of lactate *in vitro*, we studied the potential beneficial effect of lactate administration *in vivo* after 30 mins of MCAO under xylazine–ketamine anesthesia. A volume of 2 μL of 100 mmol/L L-lactate or $1 \times$ PBS was randomly administered intracerebroventricularly, directly after reperfusion, and L-lactate was found to significantly ($P = 0.05$) reduce lesion size from $104 \pm 42\,mm^3$ (control group) to $63 \pm 48\,mm^3$ (lactate group), 48 h after ischemia. The same result was observed when calculating indirect lesion size to avoid bias due to edema (Swanson *et al*, 1990) (Figure 2A). When lesions were measured separately in each brain structure, it appeared that lactate did not significantly protect the cortex or hippocampus, but interestingly induced a very significant decrease in lesion size in the striatum from 23 ± 8 to $11 \pm 7\,mm^3$ ($P = 0.007$) (Figure 2B).

To determine whether the neuroprotective effect of lactate *in vivo* is present under different anesthetic conditions and longer ischemia duration, we carried out a second set of experiments with 60 mins MCAO in mice under isoflurane anesthesia. This set of experiments was blinded and was carried out randomly. In addition, behavior was assessed. In these conditions, 48 h after ischemia, lesions of control mice were similar to those produced by the aforementioned 30 mins MCAO under xylazine–ketamine anesthesia. Lactate administration had a small, nonsignificant ($P = 0.06$), protective effect (from $112 \pm 30\,mm^3$ for control mice to $94 \pm 18\,mm^3$ for lactate-injected mice) (Figure 3A). Once again, when locating the lesion among the cortex, striatum, and hippocampus, no protection after lactate treatment was observed in the cortex and hippocampus. However, lactate induced a significant reduction in lesion size in the striatum (from $19 \pm 3\,mm^3$ for control mice to $14 \pm 4\,mm^3$ for lactate-injected mice, $P = 0.036$) (Figure 3B). Lactate administration significantly improved the neuroscore at 48 h from 5.3 ± 1.7 for control mice to 3.6 ± 1.3 for lactate-treated mice ($P = 0.046$) (Figure 3C).

In an attempt to delineate the therapeutic window of lactate administration, 11 mice were injected

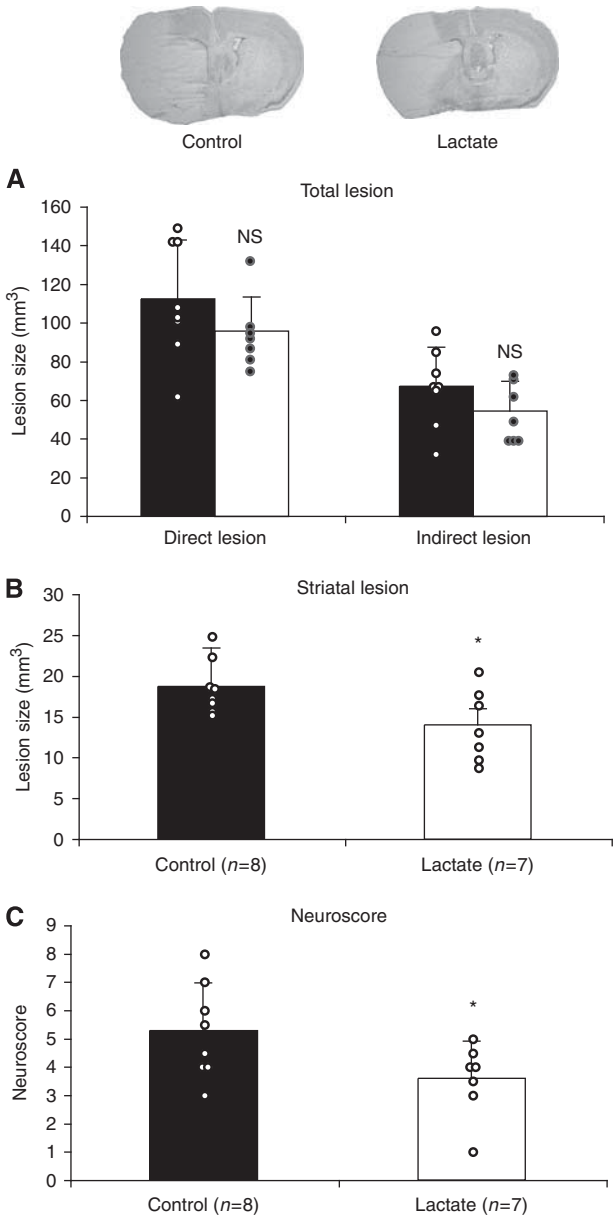

Figure 3 Injection of 2 μL of 100 mmol/L L-lactate intracerebroventricularly directly after reperfusion protects the striatum after 60 mins of MCAO under isoflurane anesthesia. (**A**) Total direct and indirect lesion sizes were measured at 48 h after ischemia. Black bars represent control mice and white bars represent mice injected with lactate. (**B**) Striatal lesion. (**C**) Neurologic deficit scores from 0 (no deficit) to 9. $*P < 0.05$ for the Mann–Whitney test, one-tailed *P*-value. Circles represent individual animals.

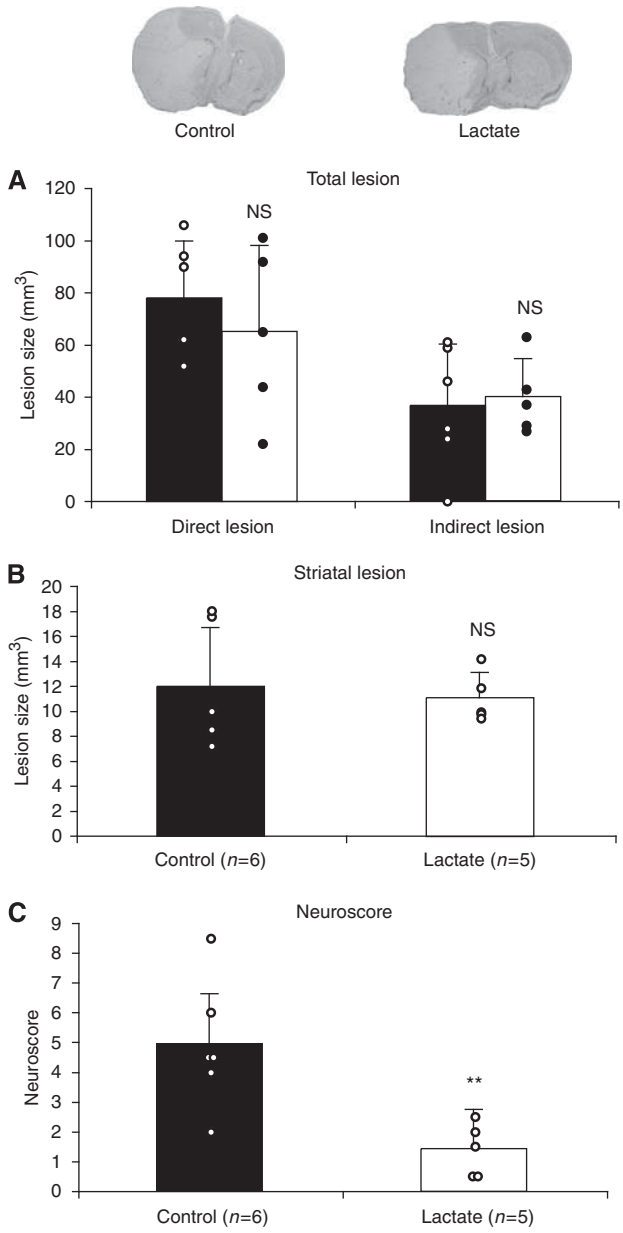

Figure 4 Injection of 2 μL of 100 mmol/L L-lactate intracerebroventricularly, 120 mins after the beginning of 60 mins of MCAO under isoflurane anesthesia, improves neurologic outcome, but does not significantly decrease lesion size. (**A**) Total direct and indirect lesion sizes were measured at 48 h after ischemia. Black bars represent control mice and white bars represent mice injected with lactate. (**B**) Striatal lesion. (**C**) Neurologic deficit scores from 0 (no deficit) to 9. $**P < 0.01$ for the Mann–Whitney test, one-tailed *P*-value. Circles represent individual animals.

intracerebroventricularly with either PBS or 100 mmol/L lactate (2 μL of each) 60 mins after the end of 60 mins MCAO under isoflurane anesthesia and were killed at 48 h. The secondary anesthesia required for the intracerebroventricular injection with xylazine–ketamine by itself had a protective effect, resulting in lesions of 78 ± 22 mm^3 compared with those of 112 ± 30 mm^3 as described in Figure 3A. Lactate injection at this later time point was not significantly neuroprotective, neither on the total lesion (Figure 4A) nor in the striatum (Figure 4B). However, it had a striking effect on the behavior of these mice, decreasing their neuroscores very significantly ($P = 0.009$) at 48 h, from 4.9 ± 2.2 in control mice to 1.4 ± 0.9 in lactate-injected mice (Figure 4C). Moreover, Table 1 shows for this set of mice that

Table 1 Effect of anesthesia and lactate on physiological parameters

	Xylasine–ketamine		*Isoflurane*		*Isoflurane (120 mins)*	
	Control	*Lactate*	*Control*	*Lactate*	*Control*	*Lactate*
CBF during ischemia (% of baseline)	19 ± 6	17 ± 8	20 ± 6	16 ± 3	18 ± 4.4	15 ± 5
CBF after ischemia (% of baseline)	80 ± 14	76 ± 17	74 ± 18	72 ± 14	72 ± 19	83 ± 23
Temperature during ischemia (°C)	37.0 ± 0.5	37.1 ± 0.3	36.8 ± 0.2	36.9 ± 0.3	36.9 ± 0.4	36.9 ± 0.1
Temperature at killing (°C)	35.5 ± 0.9	36.1 ± 1.2	35.9 ± 0.9	35.7 ± 0.7	35.6 ± 0.7	36.3 ± 0.4
Body weight loss (g)	7 ± 1.6	5.2 ± 2.1	6.4 ± 1.6	6.4 ± 1.3	7 ± 1.4	5 ± 1.6*

CBF, cerebral blood flow.
*$P = 0.04$

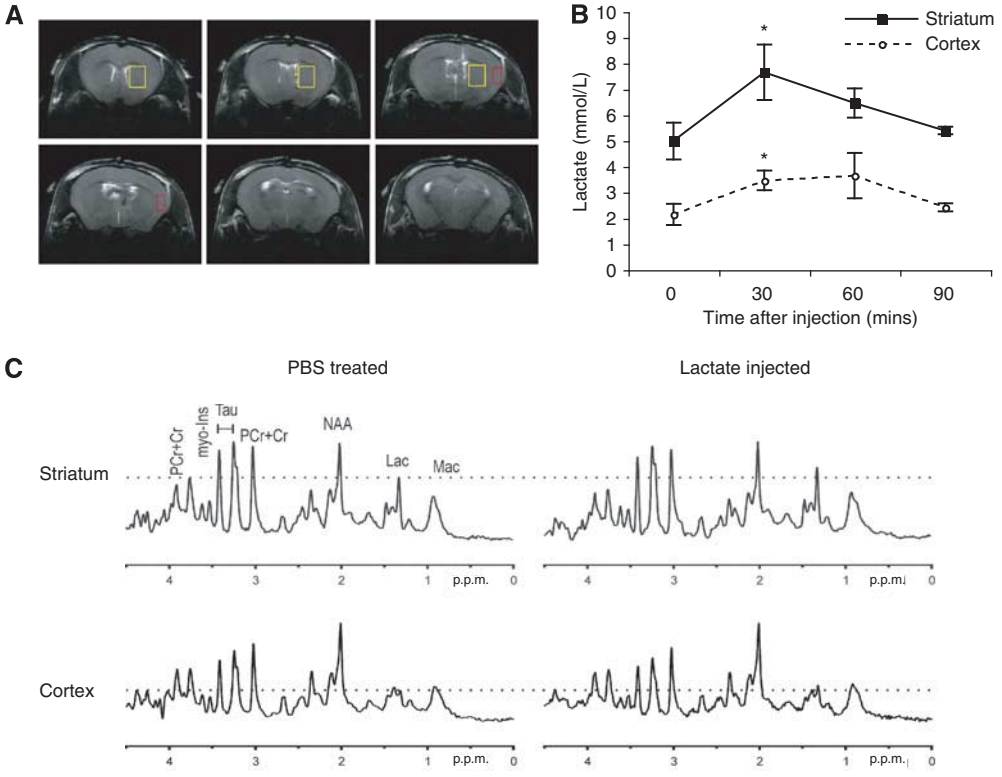

Figure 5 Intracerebroventricular injection of 2 μL of 100 mmol/L L-lactate in healthy mice significantly increased lactate concentration in the striatum and in the cortex. (**A**) MRI of one mouse injected with lactate, showing the site of injection (arrowhead) and the VOIs used for MRS measurements in the striatum (6.4 μL, yellow square) and in the cortex (2.5 μL, red square). (**B**) Evolution of lactate concentrations measured in the striatum and in the cortex of lactate-injected mice by MRS. (**C**) Typical localized ^{1}H-spectra from mouse striatum and cortex after treatments. All spectra were acquired ~30 mins after PBS injection (the left column) and lactate injection (the right column) from one of each mouse striatum (the top row) and cortex (the bottom row). Spectra were processed with Gaussian apodization (gf = 0.08 secs) and scaled relatively to the heights of both PCr + Cr and Mac, which were indicated in the spectrum of PBS-treated striatum along with other major metabolites, such as NAA, Tau, and myo-Ins. Dotted lines were drawn on the basis of the heights of lactate peaks from the spectra of PBS-treated striatum and cortex. Abbreviations: Cr, creatine; Lac, lactate; Mac, macromolecule; myo-Ins, myo-inositol; NAA, *N*-acetyl-aspartate; PCr, phosphocreatine; Tau, taurine. *$P < 0.05$ for the Kruskal–Wallis test, followed by Dunn's multiple-comparison test.

control mice lost significantly more weight ($P = 0.04$) than did lactate-injected mice, reflecting a better neurologic outcome in treated animals.

With other neuroprotective agents tested after ischemia, in the majority of cases, protection appears in the cortex, which is less severely injured by MCAO. In an attempt to understand why lactate protected mainly the striatum in this study, we measured lactate concentrations in the striatum and cortex of healthy mice by MR spectroscopy before and after injecting 2 μL of 100 mmol/L L-lactate intracerebroventricularly. At the same time, this procedure allowed us to check the localization of lactate injection by MR imaging (Figure 5A). Before

injection, lactate concentrations were consistently higher in the striatum than in the cortex. After lactate injection, lactate increased rapidly in the striatum (from 5 ± 0.7 to 7.7 ± 1.1 mmol/L) and cortex (from 2.2 ± 0.4 to 3.5 ± 0.4 mmol/L) and remained high for 1 h after injection (Figure 5B), whereas no significant change was observed after PBS injection (Figure 5C). At 90 mins after injection, lactate concentrations returned to control values (Figure 5B). Therefore, the intracerebroventricular injection of lactate rapidly increased lactate concentrations both in the striatum and in the cortex, but concentrations in the cortex increased to a lesser extent.

Discussion

The results presented in this study show, using three different models, that lactate administered at early time points after ischemia can indeed protect against cell death, decrease lesion size, and improve neurologic outcome.

In vitro, the addition of 4 mmol/L Na L-lactate to the medium directly after OGD was clearly neuroprotective. Owing to the fact that lactate concentrations in a rat brain at the p10 stage of development are approximately 1 to 1.5 mmol/L (Tkac *et al*, 2003), the addition of 1 mmol/L L-lactate will mean that the medium will have the same lactate concentration as that of the hippocampal tissue, which may not be sufficient enough to achieve neuroprotection. In contrast, the higher lactate concentration (20 mmol/L) induced cell death in these cultures, even without any OGD (data not shown), and thus could not protect against OGD-induced cell death. Lactate increase after ischemia is believed to be toxic by causing acidosis. Indeed, acid-sensing ion channels have been shown to be activated after ischemia (Xiong *et al*, 2004) and their inhibition to be neuroprotective (Pignataro *et al*, 2007). However, in our experiments, we could not detect any significant change in the pH of the medium after the addition of L-lactate (1, 4, or 20 mmol/L). In addition, the pH always remained higher than 7.2, whereas acid-sensing ion channels could only be activated at a pH lower than 7. Moreover, it has been shown in astrocyte cultures that Na-lactate, even at high concentrations (35 mmol/L), does not cause acidosis, in contrast to lactic acid (Morishima *et al*, 2008). However, we only measured pH in the medium and could not exclude an intracellular acidification due to L-lactate administration, which could have toxic effects. Another study showed that 20 mmol/L Na L-lactate can induce swelling and nucleotide release as a consequence of intracellular acidification (Phillis and O'Regan, 2002), a fact that could explain the toxicity observed in our experiments.

The conditions we used for MCAO in this study induced very large lesions covering more than half of the left hemisphere. In these conditions, the penumbra is reduced to a small rim around the ischemic core and neuroprotection might be difficult to obtain (as reviewed in Hossmann (2008)). The 40% reduction in lesion size that was observed under xylazine–ketamine anesthesia is thus very impressive. With isoflurane anesthesia (Sakai *et al*, 2007), a longer duration of ischemia resulted in a similar lesion size, which was ascribed to isoflurane protection (Sakai *et al*, 2007). Under these conditions, in which isoflurane protects, an additional beneficial effect of lactate may be more difficult to show. Moreover, isoflurane neuroprotection might already involve lactate, because lactate has been shown to increase in the medium of cells treated with isoflurane (Brabec *et al*, 1984). Although the total lesion size was not affected by lactate treatment under isoflurane anesthesia, lactate significantly reduced the striatal lesion with both types of anesthesia, when administered at reperfusion. Striatal protection was unexpected, as this structure is the first to be damaged after a milder ischemia (Lei *et al*, 2009), probably because it is less well irrigated by collateral circulation. Therefore, we speculated whether the intracerebroventricularly injected lactate reached the striatum and cortex evenly. The results presented in Figure 5 show that lactate was distributed in both structures. However, basal concentrations were significantly different between these two regions as already observed by other groups (Tkac *et al*, 2004). Thus, the 1 mmol/L increase observed in the cortex might not be sufficient enough for protection, consistent with *in vitro* experiments (Figure 1), whereas the striatal increase between 2 and 4 mmol/L might be more efficient. However, at 48 h, the cortical lesion may still be evolving in our model and thus a protection of the cortex by lactate may become apparent only at later time points.

The protective effect of lactate treatment observed in this study might be surprising, because endogenous lactate is known to increase dramatically after ischemia (Malisza *et al*, 1998). Without lactate injection, lactate has been shown to increase during ischemia because of anaerobic glycolysis, normalizing within 15 mins after reperfusion in the rat after 2 h MCAO (Higuchi *et al*, 1996) and strongly increasing from 1 h after reperfusion, from 12 mins global ischemia or 2 to 3 h MCAO in the rat (Michaelis *et al*, 1999; Thoren *et al*, 2006). One possible explanation for a lactate decrease at reperfusion could be the preferential consumption of lactate when adenosine triphosphate stores deplete. Indeed, adenosine triphosphate is required for the phosphorylation of glucose to produce glucose-6-phosphate for glycolysis, whereas lactate can directly be metabolized without an investment of adenosine triphosphate (Schurr, 2006). Moreover, Schurr *et al* (1997*a*, *b*, *c*) showed that, at least *in vitro*, lactate produced by astrocytes can be used after hypoxia and is even preferred to glucose, which our results support. At later time points, increased lactate is probably not because of a lack of blood supply and anaerobic metabolism, as glucose levels in the

ischemic core are already normalized 2 h 30 mins after reperfusion (Lei *et al*, 2009), but might be due to the metabolism of macrophages infiltrating into the brain after ischemia (Petroff *et al*, 1992) or may possibly reflect the fact that lactate transporters (Kuhr *et al*, 1988; Walz and Mukerji, 1988) have changed, so that the lactate clearance rate is altered relative to the synthesis rate. On the basis of our *in vitro* experiments, lactate seems to be neuroprotective at low doses and toxic at higher concentrations. Thus, lactate increases induced by ischemia might protect in the beginning and become toxic later when it reaches higher concentrations. After 1 h of reperfusion in the rat, lactate increases between 30 (Michaelis *et al*, 1999) and 300% (Thoren *et al*, 2006), depending on the model. Owing to technical limitations, we did not measure lactate concentration 1 h after reperfusion in our model. However, under isoflurane anesthesia, a 30-min MCAO induced a 3 mmol/L increase in lactate 2 h after reperfusion compared with that in control animals (Lei *et al*, 2009), which is similar to the increase observed after lactate injection (Figure 5). As lactate has been shown to increase rapidly during the first 20 mins of ischemia, but to be stable thereafter (Hopwood *et al*, 2005), the concentration of lactate at reperfusion should be similar in our 30- and 60-min models of MCAO. It might thus be possible that endogenous lactate is already increased at 1 h of reperfusion and available in a sufficient amount to fulfill metabolic requirements; therefore, lactate administration is not beneficial compared with endogenous lactate. This might explain why it influenced neither the total nor the striatal lesion. Conversely, Higuchi *et al* (1996) showed a decrease in lactate concentration from the end of ischemia until 15 mins of reperfusion, but no change thereafter until 1 h after reperfusion from 1 h of ischemia in the rat. If the lactate evolution in our model is similar, endogenous lactate concentration should be higher at reperfusion than 1 h later; thus, the difference in protection observed between the two time points of lactate injection could not be explained. However, these are only speculations and we will need to measure the exact early evolution of lactate after ischemia in our model and control plasma glucose to better understand a possible relationship between endogenous and injected lactate. Conversely, the expression of MCTs (monocarboxylate transporters), which are necessary for lactate internalization, has been shown to be influenced by ischemia, decreasing in some cells and increasing in others (reviewed in Pierre and Pellerin (2005)). However, MCT 1 and 2 seem to be stably expressed after ischemia, at least for 4 h (Zovein *et al*, 2004), as well as lactate dehydrogenase, which might even increase (Calvert *et al*, 2006). Thus, neither a reduction in MCTs nor a reduction in lactate dehydrogenase should be the reason for the lack of effect of lactate injected 60 mins after reperfusion in our experiments. However, the short therapeutic window we observe with lactate in this severe ischemic model is not surprising, because the length of the therapeutic window of other neuroprotective agents has been shown to decrease with severity of ischemia (e.g., D-JNKI1; Hirt *et al* (2004)).

In view of a potential clinical application, the substantial improvement in neurologic outcomes is extremely interesting. In our study, we observed a significant improvement in the neuroscore assessed at 48 h after ischemia when lactate was administered at reperfusion, and even more, when it was administered 1 h after reperfusion. In addition, with this later administration, mice lost significantly less weight than did control animals, which is another indication of improvement of the animal's health status (Modo *et al*, 2000). However, there is a discrepancy between the lesion volume and the neurologic outcome of these mice (injected at 120 mins after the onset of ischemia). This could be explained if lactate had different effects depending on the moment of administration. When injected at reperfusion, it might be metabolized and might promote the survival of neurons, as reflected in the observed decrease in lesion size. There is no effect on lesion volume 1 h after reperfusion, although lactate supply could possibly improve the performances of suffering, but surviving, neurons. However, its beneficial effect on neurologic outcome suggests that it could influence other mechanisms that still need to be discovered; this hypothetical action is beyond the scope of this study.

Knowing that lactate can be used as an energy substrate by neurons (Schurr *et al*, 1988), and that it can be used even when adenosine triphosphate stores are depleted, we believe that at least a part of the beneficial effect of lactate treatment might be due to its metabolic utilization. However, we have no experimental data to support this hypothesis and we cannot exclude the presence of another mechanism. For example, lactate might influence osmolarity, which could have beneficial effects on edema when moderate (with 4 mmol/L lactate), but could become toxic with higher concentrations of lactate. Another explanation for the *in vivo* results could be the lactate effects on CBF. Shackford *et al* (1994) showed a decrease in cerebral vascular resistance leading to increased CBF after lactate infusion. Moreover, peri-infarct depolarizations after ischemia, which are increased with hypoglycemia, have been shown to lead to vasoconstriction (Strong *et al*, 2007). Therefore, addition of lactate may not have a direct survival effect, but may give energy to reduce the number of peri-infarct depolarizations and reduce the associated vasoconstriction. Our *in vitro* data, however, show that the neuroprotection observed after lactate treatment is not only due to CBF changes. In any case, understanding the mechanism underlying the protective effect of lactate injection is of great interest and will be a subject for further studies.

In conclusion, lactate administration directly to the brain after reperfusion can effectively protect

against ischemia-induced cell death and disability. In view of a potential clinical application, the next step will be to test whether lactate can have the same beneficial effects when injected intravenously, which can be expected knowing that blood lactate is an important energy source for the human brain (van Hall *et al*, 2009).

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Conflict of interest

The authors declare no conflict of interest.

References

Brabec MJ, Bedows E, Davidson BA, Knight PR (1984) Effect of general anesthetics and pressure on aerobic metabolism of monkey kidney cells. *Anesthesiology* 61:43–7

Calvert JW, Cahill J, Yamaguchi-Okada M, Zhang JH (2006) Oxygen treatment after experimental hypoxia-ischemia in neonatal rats alters the expression of HIF-1alpha and its downstream target genes. *J Appl Physiol* 101:853–65

Carter RJ, Morton J, Dunnett SB (2001) Motor coordination and balance in rodents. *Curr Protoc Neurosci* Chapter 8, Unit 8.12

Cater HL, Chandratheva A, Benham CD, Morrison 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

Feigin VL, Lawes CM, Bennett DA, Anderson CS (2003) Stroke epidemiology: a review of population-based studies of incidence, prevalence, and case-fatality in the late 20th century. *Lancet Neurol* 2:43–53

Hennig J (1988) Multiecho imaging sequences with low refocusing flip angles. *J Magn Reson* 78:397–407

Hertz L (2008) Bioenergetics of cerebral ischemia: a cellular perspective. *Neuropharmacology* 55:289–309

Higuchi T, Fernandez EJ, Maudsley AA, Shimizu H, Weiner MW, Weinstein PR (1996) Mapping of lactate and N-acetyl-L-aspartate predicts infarction during acute focal ischemia: in vivo 1H magnetic resonance spectroscopy in rats. *Neurosurgery* 38:121–9

Hirt L, Badaut J, Thevenet J, Granziera C, Regli L, Maurer F, Bonny C, Bogousslavsky J (2004) D-JNKI1, a cell-penetrating c-Jun-N-terminal kinase inhibitor, protects against cell death in severe cerebral ischemia. *Stroke* 35:1738–43

Hopwood SE, Parkin MC, Bezzina EL, Boutelle MG, Strong AJ (2005) Transient changes in cortical glucose and lactate levels associated with peri-infarct depolarisations, studied with rapid-sampling microdialysis. *J Cereb Blood Flow Metab* 25:391–401

Hossmann KA (2008) Cerebral ischemia: models, methods and outcomes. *Neuropharmacology* 55:257–70

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

Kuhr WG, van den Berg CJ, Korf J (1988) In vivo identification and quantitative evaluation of carrier-mediated transport of lactate at the cellular level in the striatum of conscious, freely moving rats. *J Cereb Blood Flow Metab* 8:848–56

Lei H, Berthet C, Hirt L, Gruetter R (2009) Evolution of the neurochemical profile after transient focal cerebral ischemia in the mouse brain. *J Cereb Blood Flow Metab* 29:811–9

Malisza KL, Kozlowski P, Peeling J (1998) A review of in vivo 1H magnetic resonance spectroscopy of cerebral ischemia in rats. *Biochem Cell Biol* 76:487–96

Michaelis T, Wick M, Fujimori H, Matsumura A, Frahm J (1999) Proton MRS of oral creatine supplementation in rats. Cerebral metabolite concentrations and ischemic challenge. *NMR Biomed* 12:309–14

Mlynarik V, Gambarota G, Frenkel H, Gruetter R (2006) Localized short-echo-time proton MR spectroscopy with full signal-intensity acquisition. *Magn Reson Med* 56:965–70

Modo M, Stroemer RP, Tang E, Veizovic T, Sowniski P, Hodges H (2000) Neurological sequelae and long-term behavioural assessment of rats with transient middle cerebral artery occlusion. *J Neurosci Methods* 104:99–109

Morishima T, Aoyama M, Iida Y, Yamamoto N, Hirate H, Arima H, Fujita Y, Sasano H, Tsuda T, Katsuya H, Asai K, Sobue K (2008) Lactic acid increases aquaporin 4 expression on the cell membrane of cultured rat astrocytes. *Neurosci Res* 61:18–26

Murray CJ, Lopez AD (1997) Mortality by cause for eight regions of the world: Global Burden of Disease Study. *Lancet* 349:1269–76

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

Petroff OA, Graham GD, Blamire AM, al-Rayess M, Rothman DL, Fayad PB, Brass LM, Shulman RG, Prichard JW (1992) Spectroscopic imaging of stroke in humans: histopathology correlates of spectral changes. *Neurology* 42:1349–54

Phillis JW, O'Regan MH (2002) Evidence for swelling-induced adenosine and adenine nucleotide release in rat cerebral cortex exposed to monocarboxylate-containing or hypotonic artificial cerebrospinal fluids. *Neurochem Int* 40:629–35

Phillis JW, Song D, Guyot LL, O'Regan MH (1999) Lactate reduces amino acid release and fuels recovery of function in the ischemic brain. *Neurosci Lett* 272:195–8

Pierre K, Pellerin L (2005) Monocarboxylate transporters in the central nervous system: distribution, regulation and function. *J Neurochem* 94:1–14

Pignataro G, Simon RP, Xiong ZG (2007) Prolonged activation of ASIC1a and the time window for neuroprotection in cerebral ischaemia. *Brain* 130:151–8

Ros J, Pecinska N, Alessandri B, Landolt H, Fillenz M (2001) Lactate reduces glutamate-induced neurotoxicity in rat cortex. *J Neurosci Res* 66:790–4

Sakai H, Sheng H, Yates RB, Ishida K, Pearlstein RD, Warner DS (2007) Isoflurane provides long-term protection against focal cerebral ischemia in the rat. *Anesthesiology* 106:92–9

Schurr A (2006) Lactate: the ultimate cerebral oxidative energy substrate? *J Cereb Blood Flow Metab* 26: 142–152

Schurr A, Miller JJ, Payne RS, Rigor BM (1999) An increase in lactate output by brain tissue serves to meet the energy needs of glutamate-activated neurons. *J Neurosci* 19:34–9

Schurr A, Payne RS, Miller JJ, Rigor BM (1997*a*) 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, Rigor BM (1997*b*) 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 (1997*c*) 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, Tseng MT, Rigor BM (2001) Blockade of lactate transport exacerbates delayed neuronal damage in a rat model of cerebral ischemia. *Brain Res* 895:268–72

Schurr A, West CA, Rigor BM (1988) Lactate-supported synaptic function in the rat hippocampal slice preparation. *Science* 240:1326–8

Shackford SR, Schmoker JD, Zhuang J (1994) The effect of hypertonic resuscitation on pial arteriolar tone after brain injury and shock. *J Trauma* 37:899–908

Strong AJ, Anderson PJ, Watts HR, Virley DJ, Lloyd A, Irving EA, Nagafuji T, Ninomiya M, Nakamura H, Dunn AK, Graf R (2007) Peri-infarct depolarizations lead to loss of perfusion in ischaemic gyrencephalic cerebral cortex. *Brain* 130:995–1008

Swanson RA, Morton MT, Tsao-Wu G, Savalos RA, Davidson C, Sharp FR (1990) A semiautomated method for measuring brain infarct volume. *J Cereb Blood Flow Metab* 10:290–3

Thoren AE, Helps SC, Nilsson M, Sims NR (2006) The metabolism of C-glucose by neurons and astrocytes in brain subregions following focal cerebral ischemia in rats. *J Neurochem* 97:968–78

Tkac I, Dubinsky JM, Keene CD, Gruetter R, Low WC (2007) Neurochemical changes in Huntington R6/2 mouse striatum detected by in vivo 1H NMR spectroscopy. *J Neurochem* 100:1397–406

Tkac I, Henry PG, Andersen P, Keene CD, Low WC, Gruetter R (2004) Highly resolved in vivo 1H NMR spectroscopy of the mouse brain at 9.4 T. *Magn Reson Med* 52:478–84

Tkac I, Rao R, Georgieff MK, Gruetter R (2003) Developmental and regional changes in the neurochemical profile of the rat brain determined by in vivo 1H NMR spectroscopy. *Magn Reson Med* 50:24–32

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

Walz W, Mukerji S (1988) Lactate release from cultured astrocytes and neurons: a comparison. *Glia* 1:366–70

Wiegler K, Bonny C, Coquoz D, Hirt L (2008) The JNK inhibitor XG-102 protects from ischemic damage with delayed intravenous administration also in the presence of recombinant tissue plasminogen activator. *Cerebrovasc Dis* 26:360–6

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

Zovein A, Flowers-Ziegler J, Thamotharan S, Shin D, Sankar R, Nguyen K, Gambhir S, Devaskar SU (2004) Postnatal hypoxic-ischemic brain injury alters mechanisms mediating neuronal glucose transport. *Am J Physiol Regul Integr Comp Physiol* 286:R273–82