

ORIGINAL ARTICLE

A probable dual mode of action for both L- and D-lactate neuroprotection in cerebral ischemia

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Lactate has been shown to offer neuroprotection in several pathologic conditions. This beneficial effect has been attributed to its use as an alternative energy substrate. However, recent description of the expression of the HCA1 receptor for lactate in the central nervous system calls for reassessment of the mechanism by which lactate exerts its neuroprotective effects. Here, we show that HCA1 receptor expression is enhanced 24 hours after reperfusion in an middle cerebral artery occlusion stroke model, in the ischemic cortex. Interestingly, intravenous injection of L-lactate at reperfusion led to further enhancement of HCA1 receptor expression in the cortex and striatum. Using an *in vitro* oxygen-glucose deprivation model, we show that the HCA1 receptor agonist 3,5-dihydroxybenzoic acid reduces cell death. We also observed that D-lactate, a reputedly non-metabolizable substrate but partial HCA1 receptor agonist, also provided neuroprotection in both *in vitro* and *in vivo* ischemia models. Quite unexpectedly, we show D-lactate to be partly extracted and oxidized by the rodent brain. Finally, pyruvate offered neuroprotection *in vitro* whereas acetate was ineffective. Our data suggest that L- and D-lactate offer neuroprotection in ischemia most likely by acting as both an HCA1 receptor agonist for non-astrocytic (most likely neuronal) cells as well as an energy substrate.

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INTRODUCTION

Stroke is a frequent and often disabling disease whose early identification and treatment within a few hours from symptom onset are essential. In ischemic stroke, the most common stroke subtype resulting from an arterial occlusion, energy failure because of lack of oxygen and glucose supply to the brain is the central mechanism leading to neural damage. Brain metabolism therefore has a critical role in the pathophysiologic mechanisms underlying neuronal damage in stroke and for recovery. Even though glucose is known as the brain energy substrate *par excellence*, other metabolic intermediates such as monocarboxylates including lactate, pyruvate, acetate, and ketone bodies have been shown to be oxidized for energy production^{1–3} under certain conditions. Brain energy metabolism is a compartmentalized process involving neurons as well as astrocytes and oligodendrocytes, with metabolic interactions between different cell types. Monocarboxylates can be transported to and from different cell types by monocarboxylate transporters (MCTs) with different degrees of affinity. Monocarboxylate transporters are a family of proton-dependent carriers where MCT1, MCT2, and MCT4 are the main MCTs in the central nervous system (CNS). Under physiologic conditions, MCT1 is expressed in endothelial cells, astrocytes, and oligodendrocytes, MCT4 in astrocytes, and MCT2 is the main transporter in neurons. Under hypoxic conditions, MCT1 and MCT2 are also expressed in activated microglia.^{4–6}

Beside its role as an energy substrate, lactate also acts as a signaling molecule via the HCA1 receptor in adipose tissue and skeletal muscle.⁷ Interestingly, recent work revealed the presence of the HCA1 receptor in different brain structures, including cortex, hippocampus, and cerebellum,⁸ and it has been shown that lactate administration to cortical neurons induced specific modulations in calcium transients frequencies in the same manner as a specific HCA1 receptor agonist.⁹ HCA1 receptor involvement has also been suggested in neurologic disorders such as cerebral malaria.¹⁰

We have previously shown that L-lactate administration is neuroprotective in models of cerebral ischemia both *in vitro* and *in vivo*: it attenuated neuronal death *in vitro* in organotypic hippocampal slice cultures subjected to oxygen and glucose deprivation.¹¹ Both, intracerebroventricular and intravenous administration of L-lactate after transient middle cerebral artery occlusion (tMCAO) lead to a reduction in lesion size and improved neurologic outcome.^{11,12} Although it was assumed so far that lactate exerts its neuroprotective effect by acting as a metabolic substrate for energy-deprived neurons, the mode of action is so far not characterized and recent data on the presence and role of the lactate receptor HCA1 in the CNS challenges this assumption. In the present study, we have explored both possible modes of action, as a metabolic substrate and signaling molecule. Understanding the mechanisms by which lactate exerts its

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neuroprotective effect is essential to adequately translate this promising preclinical neuroprotection strategy to a clinical setting.

MATERIALS AND METHODS

All experiments were conducted in accordance with ordinance 455.163 of the Swiss Federal Veterinary Office and approved by the Service Cantonal des Affaires Vétérinaires (license number VD2017.4b to LH) and the Kanton Zürich Gesundheitsdirektion Veterinäräm (license number ZH53/2007 to BW) (cantonal veterinary authority). The animal studies are reported according to the ARRIVE guidelines.

Transient Middle Cerebral Artery Occlusion in the Mouse

A total of 55 male CD1 mice (body weight 26 to 35 g, Charles River, L'Arbresle, France) were housed in the animal facility of the Department of Fundamental Neuroscience of the Université de Lausanne with 12:12 light/dark cycle with normal illumination, in groups of 5 per cage during 1 week for acclimatization before surgery. For experiments, mice were maintained anesthetized with isoflurane (1.5% to 2% in nitrous oxide/oxygen 70%/30%) using a face mask. Body temperature was maintained at $37.0 \pm 0.5^\circ\text{C}$ throughout surgery (FHC, Bowdoinham, ME, USA). Regional cerebral blood flow (rCBF) was measured and continuously recorded throughout the operation in all animals by laser-Doppler flowmetry (Perimed AB, Stockholm, Sweden) with a flexible probe fixed on the skull (1 mm posterior and 6 mm lateral from bregma). Transient focal cerebral ischemia (30 or 45 minutes depending on the set of experiments) was induced by occlusion of the left middle cerebral artery (MCA) with an intraarterial suture as described elsewhere.¹¹ Briefly, the left common carotid artery and the left external carotid artery were exposed and ligated after a ventral midline neck incision. Ischemia was induced by inserting a silicon-coated nylon monofilament (0.17 mm diameter) through the left common carotid artery into the internal carotid artery until mild resistance was felt and a drop to less than 20% of initial rCBF was registered. Regional cerebral blood flow was monitored and maintained below 20% of the baseline level during ischemia. Reperfusion was considered successful if the rCBF rose above 50% of baseline. Only mice that had a drop of 80% of rCBF and accomplished a reperfusion of above 50% of baseline in the rCBF were included in the study. They received 5 μL per gram of body weight of either sodium D-lactate solution (200 mmol/L), pyruvate solution (200 mmol/L), or vehicle solution (phosphate-buffered saline, PBS) injected randomly in the tail vein at reperfusion using a 1-mL syringe with a 25-gauge needle and a mouse restrainer (Baintree Scientific, Baintree, MA, USA). All surgeries were performed during day-light, between 9 am and 5 pm. The surgeon was not masked. Each mouse was considered as an experimental unit.

At the beginning of the surgery, mice were administered 0.025 mg/kg of buprenorphine subcutaneously for post-surgery analgesia. Once the animals were awake, they were housed overnight in an incubator at 28°C .

Behavioral Evaluation

The neurologic deficit was evaluated after reperfusion and before euthanasia, with a composite neuroscore graded for severity after surgery (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) as previously described¹¹ and motor performance assessed on the Rotarod treadmill (UgoBasile, Milan, Italy). In this test, mice were placed on the rotating cylinder, set to accelerate uniformly from 4 to 40 rpm, and their latency to fall was recorded before 600 seconds. The animals were trained on two different days before surgery, with two trials in each training session. The test was then performed 24 and 48 hours after 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 decrease of 15% until a maximum of 5 points for the highest neurologic deficit). The behavior evaluation was assessed in a masked manner.

Determination of Ischemic Lesion Volumes

Animals were killed 48 hours after the onset of focal ischemia and 20- μm -thick, 720- μm -distant, coronal cryostat sections were stained with cresyl violet for histologic determination of lesion size. Digitalized images of the Nissl-stained tissue were acquired under a light stereomicroscope (Leica MZ16FA, Heerbrugg, Switzerland) and the lesion area was measured using

ImageJ software (ImageJ 1.36b, National Institute of Health). Infarct volume was calculated by multiplying the sum of the lesion areas on each section by the distance between sections.¹³

Immunohistochemistry

Mice were injected intraperitoneally with a lethal dose of pentobarbital (10 mL/kg, Sigma, Buchs, Switzerland) and then perfused with 150 mL of 4% paraformaldehyde (Sigma-Aldrich, St Louis, MO, USA) dissolved in $1 \times \text{PBS}$ at pH 7.4. Brains were dissected, postfixed overnight at 4°C , cryoprotected 24 hours in 30% sucrose solution (Sigma-Aldrich), and rapidly frozen. Twenty micrometer thick coronal microtome-cryostat (Leica MC 3050S) sections were stored in cryoprotectant (30% ethylene glycol and 25% glycerol in $1 \times \text{PBS}$) at -20°C . For immunostaining, sections were washed three times in $1 \times \text{PBS}$ and blocking of non-specific binding was achieved by incubating in $1 \times \text{PBS}$ containing 1% bovine serum albumin, 0.1% Triton X-100, and 10% normal goat serum during 1 hour. Double labeling was performed overnight at 4°C in the $1 \times \text{PBS}$ solution without normal goat serum and different antibodies: monoclonal mouse anti-neuronal nuclear antigen (NeuN) antibody (1:500 dilution, Sigma) or monoclonal mouse anti-microtubule associated protein (MAP2) antibody (1:300 dilution, Sigma) and polyclonal rabbit anti-hydroxycarboxylic acid receptor (HCA1) (1:500 dilution; Sigma). After washing three times with $1 \times \text{PBS}$, brain sections were incubated for 2 hours at room temperature with the following fluorescent secondary antibodies: donkey anti-rabbit Alexa-594 (1:250, Invitrogen, Eugene, OR, USA), donkey anti-mouse Alexa-488 (1:250, Invitrogen). After immunostaining, brain sections were incubated for 10 minutes with 4,6 diamidino-2-phenylindole (Sigma) dissolved in $1 \times \text{PBS}$ (1/100,000) to reveal nuclei. Preparations were then maintained at 4°C until observation with a Zeiss LSM 710 Quasar Confocal Microscope (Zeiss, Feldbach, Switzerland).

Western Blotting

For protein expression experiments, young adult male mice were subjected to 30 minutes MCAO as described above and killed either at 1, 3, 8, 24, or 48 hours after reperfusion. Striatum (caudate and putamen), primary motor, and somatosensory cortex as well as hippocampus were collected using a rodent brain matrix (adult mouse, coronal sections, ASI Warren, MI, USA).

Total proteins were extracted from brain tissues by cellular lysis in ice-cold RIPA buffer (#9806, Cell Signaling, Beverly, MA, USA) supplemented with a mixture of protease inhibitors (Complete 11257000; Roche, Basel, Switzerland). Protein quantification was performed with the Pierce BCA Protein Assay kit (#23227, Thermo Fisher Scientific, Pierce, Lausanne, Switzerland) and approximately 20 μg of proteins were denatured (95°C) for 5 minutes in sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer (60 mmol/L Tris-HCl pH 6.8, 5% SDS, 6.6% glycerol, 5 mmol/L EDTA, 5% β -mercaptoethanol, and 0.1% bromophenol blue). Samples were separated on a 10% acrylamide gel with a 4% stacking gel using an Electrophoresis Unit (Bio-Rad, Cressier, Switzerland). Proteins were then electroblotted onto nitrocellulose membranes (0.45 μm ; #162-0115, Bio-Rad) using the Electrophoresis Unit. Nonspecific binding sites were blocked for 1 hour at room temperature with a solution of Tris-Buffered-Saline (50 mmol/L Tris-HCl pH 7.5, 150 mmol/L NaCl) supplemented with 0.1% Tween-20 and containing 10% (wt/vol) skimmed milk. Blots were then incubated overnight at 4°C with specific primary antibodies in Tris-Buffered-Saline 0.1% containing 1% skimmed milk: rabbit anti-mouse GPR81-S296 (HCA1) (1:500 dilution; #SAB1300790, Sigma). Blots were washed three times in Tris-Buffered-Saline 0.1% and subsequently incubated 2 hours at room temperature with horseradish peroxidase-conjugated donkey anti-rabbit IgG (#NA9340V, 1:10,000 dilution; GE Healthcare, Glattbrugg, Switzerland). After being washed three times in Tris-Buffered-Saline 0.1%, blots were processed using Immobilon-StarTM WesternCTM Chemiluminescent Kit (#170-5070, Bio-Rad). Chemiluminescence detection was performed with the ChemiDoc XRS System (#170-8070, Bio-Rad). Total protein content assay was performed with the Pierce Reversible Protein Stain kit (#24580, Thermo Fisher Scientific, Pierce) and revealed with the ChemiDoc XRS System (#170-8070, Bio-Rad). Both types of labeling were quantified with the ImageLab 3.0 software (Bio-Rad) and the HCA1 (GPR81) protein expression was normalized to the total protein content.

Organotypic Hippocampal Slice Cultures

Hippocampal slice cultures were prepared as described elsewhere¹¹ from P8 to P10 rats (OFA Sprague Dawley, Janvier, France). Coronal hippocampal

sections (350 μm) were cultured on sterile porous membrane units (Millicell, Millipore, Billerica, MA, USA) in wells containing 1 mL of culture medium with D-glucose 36 mmol/L, 25% horse serum, 50% minimal essential medium (supplemented with HEPES and sodium bicarbonate; Gibco, Paisley, UK), 25% HBSS (Hank's balanced salt solution; Gibco), and L-glutamine 2 mmol/L (Sigma-Aldrich). Cultures were kept at 33 °C, 5% CO₂, 100% humidity for 4 days. The medium was replaced by fresh identical medium at day 4, and then by culture medium with 15% horse serum, 60% minimal essential medium, 25% HBSS, and L-glutamine 2 mmol/L at day 7 and 10. Experiments were conducted after 10 days of culture.

Oxygen and Glucose Deprivation on Organotypic Hippocampal Slice Cultures

Oxygen and glucose deprivation (OGD) experiments were performed in serum-free low glucose medium, Dulbecco's modified Eagle's medium (D5030, Sigma-Aldrich) supplemented with D-glucose 1 mmol/L and L-glutamine 2 mmol/L and equilibrated for 1 hour at 37 °C, in a humidified hypoxic chamber (COY, Grass Lake, MI, USA) with an atmosphere of 5% O₂, 5% CO₂, completed by N₂. Hippocampal slices were transferred into this medium and placed into the hypoxic chamber for 1 hour. Control cultures were kept in 60% minimal essential medium, 15% horse serum, 25% HBSS, and L-glutamine 2 mmol/L for 1 hour at 37 °C in a humid normoxic atmosphere.^{11,14} For recovery, cultures were then transferred into fresh culture medium at 33 °C for 48 hours. Cultures were randomly treated with either D-lactate, pyruvate, acetate (Sigma-Aldrich; diluted in 1 × PBS, pH 7), 3,5-dihydroxybenzoic acid (Sigma-Aldrich; diluted in ethanol) or an equal volume of culture medium. Sodium D-lactate at a final concentration of 4 mmol/L, pyruvate at 10 mmol/L, sodium acetate at 0.13, 0.2, 4, or 8 mmol/L, or 3,5-dihydroxybenzoic acid at 4 mmol/L was administered immediately after OGD.

Assessment of Cell Death in Hippocampal Slices

Cell death was determined in the CA1 region using the fluorescent viability indicator propidium iodide. Propidium iodide was applied in each dish (50 $\mu\text{g}/\text{mL}$) 1 hour before measurement. Propidium iodide fluorescence emission (excitation wavelength 536 nm, emission wavelength 617 nm) was measured 48 hours after hypoxia using an epifluorescence microscope with a ×5 lens coupled to a camera (Leica). Propidium iodide images were acquired with standardized camera settings and signal intensity was measured with ImageJ software. After subtracting the background fluorescence on each slice, the results were expressed as a percentage of maximal cell death obtained by submerging slices in PBS for 24 hours at 4 °C. Cell death was averaged for the four slices of each culture well. The experimenter was not masked.

Experiments Using ¹¹C-D-lactate in Sprague Dawley Rats

Radiotracer. The D- and L-enantiomers of 1-¹¹C-lactic acid were produced by a previously described radiosynthetic method, which includes separation of racemic 1-¹¹C-lactic acid by preparative chiral ligand exchange high-performance liquid chromatography (HPLC). Before use, the fractions corresponding to 1-¹¹C-D- or 1-¹¹C-L-lactic acid were further purified and finally formulated as sodium salts in physiologically acceptable sodium phosphate-buffered saline (pH 7). The chemical and enantiomeric purity of each isolated radiolabeled enantiomer was routinely monitored by analytical chiral ligand exchange HPLC as part of the radiosynthesis quality control and typically revealed more than 99% enantiomeric excess of the final product. The specific activity at the end of the synthesis was approximately 400 GBq/ μmol . Both preparative and analytical chiral ligand exchange chromatography were performed on reversed-phase C₁₈ columns (octadecyl-silica), coated with a D- or L-penicillamine-derived chiral selector. The preparation of the chiral selectors, the coating procedures for the HPLC columns, and the HPLC separation conditions have been described in detail earlier.¹⁵

Surgical preparation. Surgery was performed under isoflurane anesthesia (2% to 3% in air/oxygen 70%/30%) and involved the placement of an arteriovenous shunt from the right femoral artery to the right femoral vein, tracheotomy for mechanical ventilation, and craniotomy for the placement of the beta scintillator. The actual experiments were performed under α -chloralose anesthesia (44 mg/kg subcutaneously). The arteriovenous shunt was run through a coincidence scintillator (GE Medical Systems, Waukesha,

WI, USA). The online arterial sampling procedure is described in detail elsewhere.¹⁵ In short, total radioactivity in arterial blood was continuously recorded and was then corrected for (1) a different tracer concentration in whole blood and plasma and (2) the build-up of labeled metabolites.

D-Lactate kinetics in the somatosensory cortex. For the measurement of the time-course of 1-¹¹C-D-lactate in the brain, an intracortical beta probe was used.^{16,17} Twenty minutes of data were acquired at baseline condition after injection of 200 to 300 MBq of radiotracer in 5 animals. In one of these animals, baseline cerebral blood flow was determined before 1-¹¹C-D-lactate acquisition using ¹⁵O-H₂O and the methodology described previously.¹⁶ The first pass extraction fraction of 1-¹¹C-D-lactate was then calculated using the relationship $EF = K_1/\text{CBF}$ where K_1 is the transport parameter describing (1-¹¹C-D-lactate) transport from blood to tissue (Figure 4A).

Kinetic modeling. Acquired radioactivity data were analyzed using the software package PMOD (PMOD Technologies GmbH, Zürich, Switzerland). The investigated methods consisted of standard compartmental modeling using the arterial input function and the one-tissue compartment model. The parameters are as follows: K_1 describes the transport of the tracer across the blood brain barrier and k_2 represents the back-diffusion of label from tissue to the blood system. Carbon is labeled as 'C.' Label exchange between compartments is described by the following differential equation:

$$\frac{dC_{tiss}}{dt} = K_1 C_{plasma}(t) - k_2 C_{tiss}(t)$$

The basis of the calculation of the CBF measurement was also the one-tissue compartment model including a partition coefficient for ¹⁵O-H₂O.¹⁶

Metabolite analysis in the blood. Samples (approximately 400 μL) were collected at different time points after tracer injection, with a maximum of 4 to 5 blood samples per animal, to determine the time-course of the ratio of the ¹¹C activity in plasma to whole blood and for analysis of authentic tracer and metabolites. These samples were first centrifuged for 3 minutes at 270 g. Proteins were then precipitated with 75 μL acetonitrile in 50 μL plasma. After centrifugation for 3 minutes at 270 g, the composition of the ¹¹C-derived radioactivity in the supernatant (80 μL) was analyzed by HPLC on a polymeric column (PRP-1, 5- μm , 250 × 4.1 mmol/L intradermally, Hamilton, Franklin, MA, USA) with 3 mmol/L phosphoric acid in water (pH 2.67) as the mobile phase (1 mL/minute). The retention times of ¹¹C-HCO₃⁻ (3.3 minutes) and lactic acid (5.1 minutes) were determined by using aqueous solution of NaHCO₃ and DL-lactic acid as reference compounds, detected by ultraviolet absorption at 220 nm. The amount of authentic tracer was expressed as a fraction of total plasma counts.

Metabolite analysis in brain. At the end of the experiment, the rats were perfused with PBS, brains were removed and prepared for measurements with the HPLC system. Each brain was first homogenized before adding acetonitrile (150%, i.e., 1.5 × volume/weight of brain sample). The subsequent procedure was the same as with the blood samples described above, except that the amount of supernatant injected into the HPLC system was 200 μL .

Statistical Analysis

All data are presented as mean ± s.d. Statistical analyses were performed using non-parametric tests; one-way analysis of variance (Kruskal-Wallis followed by Dunn's multiple comparison test) was used for analysis of *in vitro* experiments and the Mann-Whitney test was used for comparison between two groups in the *in vivo* experiments and for the behavioral experiments. Paired *t*-test was used to compare protein expression. $P < 0.05$ was considered statistically significant.

RESULTS

HCA1 Receptor Expression and a Possible Neuroprotective Role in Ischemia

Recent studies revealed the presence and mechanism of action of the HCA1 receptor (formerly known as GPR81) in the CNS.⁸⁻¹⁰ Because L-lactate acts as an endogenous ligand for this receptor, it was of interest to describe the cellular expression of this receptor in several brain regions affected by ischemic damage in the

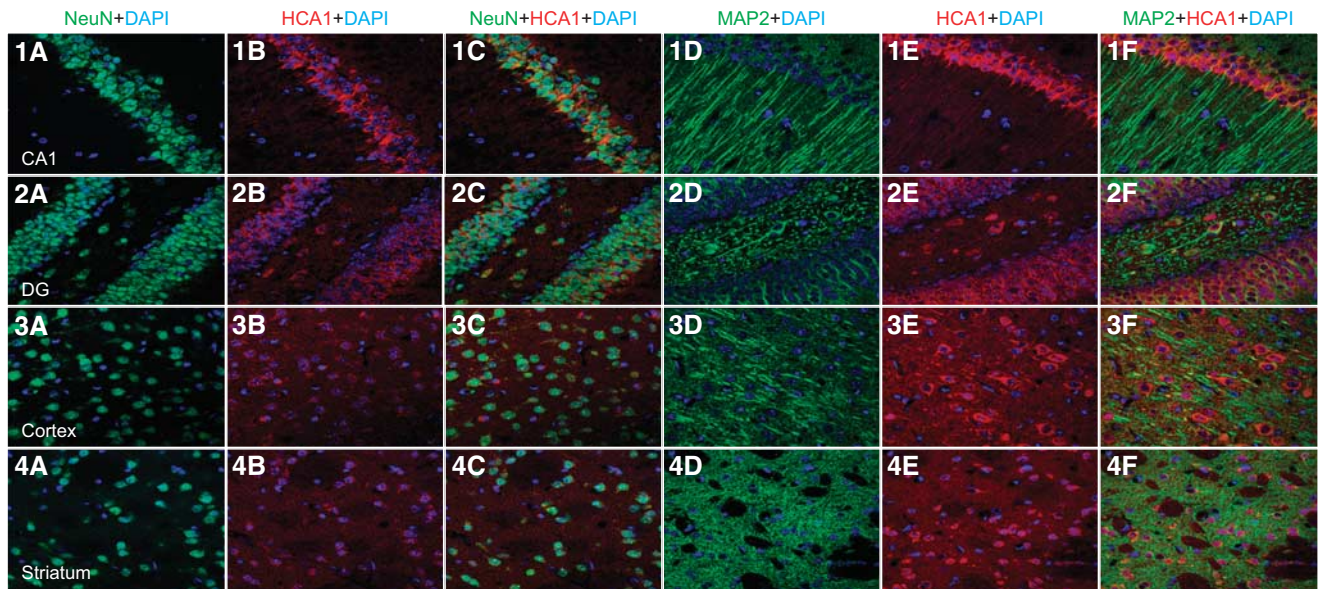


Figure 1. Confocal visualization of HCA1 receptor expression. Using two different antibodies against neuronal cells, we confirmed with immunohistochemistry the localization of the HCA1 receptor is in neurons. The brain structures analyzed were hippocampus (CA1 (1a–1f) and Dentate Gyrus (DG) (2a–2f) regions) primary cortex (3a–3f) and Striatum (4a–4f). Color code: NeuN (green, a and c), MAP2 (green, d and f), HCA1 (red, b and c and e and f), 4,6 diamidino-2-phenylindole (blue). Magnification: 40 μm .

mouse tMCAO model. We confirmed a strong neuronal expression of the HCA1 receptor in the hippocampus (CA1 and dentate gyrus), the cortex, and the striatum as revealed by double labeling immunofluorescence for the HCA1 receptor and the neuronal markers NeuN or MAP2 (Figure 1: NeuN (green), MAP2 (green), HCA1 (red), 4,6 diamidino-2-phenylindole (blue)). The next step was to evaluate whether such HCA1 receptor expression was altered at different time points after 30 minutes tMCAO. For this purpose, the level of HCA1 receptor expression was evaluated on Western blots in protein extracts from hippocampus, cerebral cortex and striatum at 1, 3, 8, 24, and 48 hours after 30 minutes of tMCAO. Results at 1, 3, and 8 hours did not reveal significant differences between the three brain structures investigated (see Supplementary Table 1). However, a significant increase in HCA1 receptor expression was seen in the ischemic cortex after 24 hours (1.2 ± 0.2 , $P < 0.05$ vs. contralateral cortex; Figure 2A). Even though there is neuronal loss in the lesion core (ischemic striatum), we did not observe a significant decrease in HCA1 receptor expression in this structure (0.8 ± 0.3 , n.s. vs. contralateral cortex) which may reflect a compensatory increase in HCA1 receptor expression in remaining neurons (Figure 2A). At 48 hours after ischemia, no difference could be observed between ischemic and contralateral tissues (see Supplementary Table 1). On the basis of this result and the possibility that lactate regulates HCA1 receptor expression, we injected L-lactate intravenously at the beginning of the reperfusion period and monitored any changes in HCA1 receptor expression at 24 hours after the ischemic episode. We found an increase in HCA1 receptor expression in both the ischemic cortex (1.57 ± 0.34 , $P < 0.04$ vs. contralateral cortex) and ischemic striatum (1.7 ± 0.4 , $P < 0.05$ vs. contralateral striatum) while no significant difference was observed for the hippocampus (Figure 2B). These observations suggest that the expression of the HCA1 receptor may be modulated by ischemia and potentially by lactate injected at reperfusion.

We went on to further investigate the role of HCA1 receptors under ischemic conditions in an *in vitro* model. For this purpose, we used rat organotypic hippocampal slices exposed to an OGD protocol. We observed a significant increase in HCA1 receptor expression 48 hours after OGD (1.45 ± 0.16 , $P < 0.01$ vs. control

slices; Figure 2C). To determine whether the enhanced HCA1 receptor expression could have a role in the neuroprotective effect of L-lactate in this model, organotypic hippocampal slices were exposed to the HCA1 receptor agonist 3,5-dihydroxybenzoic acid after OGD. Results show that 3,5-dihydroxybenzoic acid produced significant protection as it reduced neuronal cell death in the CA1 region of the hippocampus 48 hours after insult from $13.5 \pm 7.2\%$ to $7.4 \pm 5.2\%$ ($P < 0.05$; Figure 2D).

D-Lactate Neuroprotection in Ischemia Models

The D enantiomer of lactate has been described to act at least as a partial agonist of the HCA1 receptor.^{9,18} For this reason, we tested the neuroprotective effect of D-lactate both *in vitro* and *in vivo*. The administration of 4 mmol/L D-lactate to rat organotypic hippocampal slices directly after OGD also resulted in significantly reduced neuronal cell death in the CA1 region of the hippocampus 48 hours after OGD, from $29.2 \pm 14.3\%$ to $11.3 \pm 7.3\%$ ($P < 0.05$; Figure 3A). It is noteworthy that the neuroprotective effect approaches that previously reported for L-lactate in this model.¹¹ We then tested D-lactate *in vivo* by intravenous administration of 1 $\mu\text{mol/g}$ D-lactate after 45 minutes tMCAO. Mice with satisfactory ischemia (rCBF $< 20\%$ of baseline) and reperfusion (rCBF above 50% of baseline) received a single intravenous administration of D-lactate or vehicle, 10 to 15 minutes after the silicon-coated monofilament was removed allowing reperfusion. A total of nine mice were not included in the study as they did not fulfill the rCBF inclusion criteria. Single administration of D-lactate significantly decreased the infarct volume from $81.8 \pm 40.1 \text{ mm}^3$ (control group) to $45.5 \pm 32 \text{ mm}^3$ (D-lactate group) measured 48 hours after ischemia ($P < 0.05$; Figure 3B). Again, the magnitude of neuroprotection was comparable with that reported previously for L-lactate *in vivo*.¹² Behavioral performances on motor task assessment showed a clearly milder neurological deficit at 48 hours ($P < 0.05$) in the D-Lactate group, from a median of 2.5, with scores ranging from 1 to 5 (control group), to a median of 1, with scores ranging from 0 to 1 (D-lactate group, $P < 0.05$, Figure 3C). One mouse from the control group and two mice from the D-lactate group died during the first night after surgery. Three

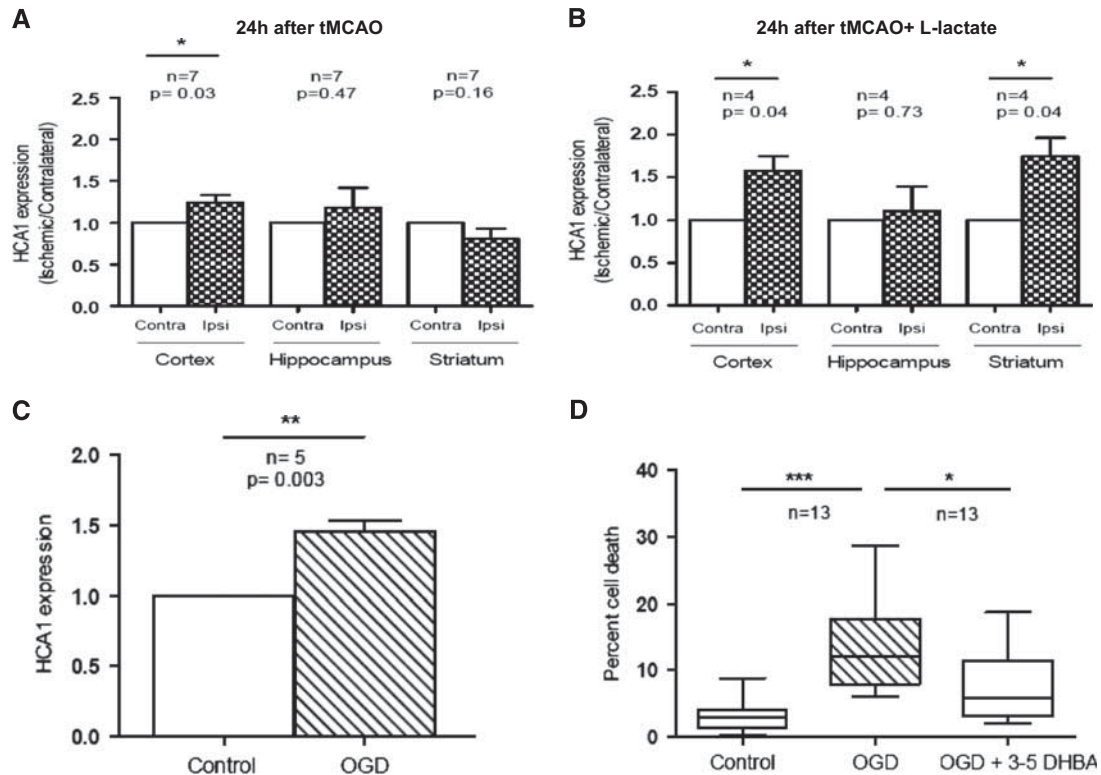


Figure 2. The HCA1 receptor expression is promoted by ischemia and has a role in the neuroprotective effect. **(A)** Western blot analysis showed an increase in the HCA1 receptor expression at 24 hours after transient middle cerebral artery occlusion (tMCAO) in the region surrounding the lesion site (primary motor and somatosensory cortex), in comparison with the contralateral hemisphere. **(B)** Intravenous L-lactate injection prompted an increase also in the lesion site (striatum) at the same time point ($*P < 0.05$; paired *t*-test ischemic vs. contralateral hemisphere). **(C)** Western blot analysis showed an increase in the HCA1 receptor expression at 48 hours after oxygen and glucose deprivation (OGD) ($**P < 0.01$ paired *t*-test). **(D)** Administration of 4 mmol/L 3,5-dihydroxybenzoic acid (3,5-DHBA), an HCA1 receptor agonist, attenuated cell death when administered directly to the medium after OGD ($*P < 0.05$, $***P < 0.001$ one-way analysis of variance (ANOVA) Kruskal–Wallis test followed by Dunn’s multiple comparison test).

mice per group were killed within the first 6 hours after surgery because of the presence of repeated seizures as required by the veterinary authority.

Cerebral D-Lactate Metabolism

Different *in vivo* and *in vitro* studies have suggested that D-lactate is not metabolized in cerebral tissue^{6,19,20} based on the apparent lack of expression of the specific enzyme D-lactate dehydrogenase in mammals. To clarify this issue in the rodent brain, we set up a series of experiments using radiolabeled D-lactate to trace its metabolism and kinetics (Figure 4A). To evaluate the brain uptake of D-lactate, we determined the first pass extraction fraction, which was 16%. The tissue kinetics of $1\text{-}^{11}\text{C}$ -D-lactate displayed an increase of more than 5 minutes and slow washout thereafter on tracer injection (Figure 4B). The average respective first-order kinetic rate constants obtained by kinetic modeling were 0.059 ± 0.01 mL/minute/mL tissue (K_1) and 0.063 ± 0.01 per minute (k_2). Average lactate levels in the blood were 2.2 ± 0.93 mmol/L.

^{11}C -CO₂ turned out to be the only metabolite in blood and brain. The time course of the fraction of authentic tracer in arterial plasma is shown in Figure 4C. The pooled fraction data of all animals were approximated by a fit of a quadratic polynomial. This function was subsequently used to convert the total plasma activity to the time-course of authentic ^{11}C -lactate (= input curve). At 40 minutes, the fraction of true tracer in plasma dropped to approximately 30%. Measurements in brain tissue at the end of the experiment revealed approximately one-third CO₂ and two-thirds lactate (Figure 4D).

Effect of Pyruvate and Acetate in Ischemia Models

Results obtained on D-lactate metabolism suggest that in addition to a possible activation of the HCA1 receptor, the use of D-lactate as an energy substrate may also contribute to its beneficial effect in ischemia. Therefore, we explored the specificity of its metabolic mode of action by testing the neuroprotective effect of two other monocarboxylates. Pyruvate is the immediate downstream product of either L- or D-lactate and is the main substrate entering the Krebs cycle to provide energy. Other groups have previously described the neuroprotective effect of pyruvate in different models of cerebral ischemia.^{2,21} In agreement with these observations, administration of 10 mmol/L pyruvate directly after OGD significantly decreased cell death in the hippocampal CA1 region at 48 hours from $25.4 \pm 14.5\%$ to $13.9 \pm 10.3\%$ ($P < 0.05$; Figures 5A and 5B). In contrast, we did not observe a significant benefit *in vivo* after tMCAO. In our hands, pyruvate administration at reperfusion after 30 minutes tMCAO induced only a slight reduction in lesion size ($P = 0.4$), from 91.9 ± 53.6 mm³ (control group, $n = 7$) to 64.6 ± 53.4 mm³ (pyruvate, $n = 5$) 48 hours after ischemia. A total of four mice were not included because they did not fulfill rCBF inclusion criteria. There was no mortality in this experiment. Because of the inherent variability of the model, our experiment was, however, not powered to detect a significant neuroprotective effect (the sample size calculation using an alpha error of 5% and a beta error of 50% resulted in a sample size of 21 per group, www.dssresearch.com).

As both L- and D-lactate could be taken up and metabolized in principle by either neurons or astrocytes, we wanted to determine

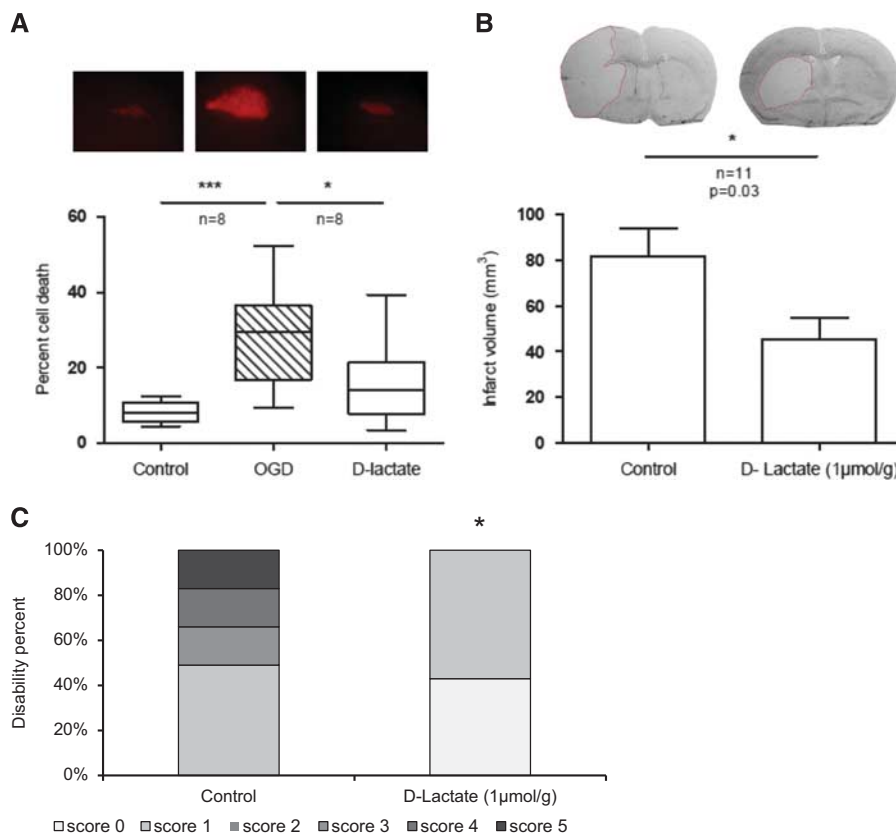


Figure 3. Neuroprotection by D-lactate. **(A)** *In vitro*, administration of 4 mmol/L D-lactate induced a significant reduction in cell death, assessed by propidium iodide (PI) staining, 48 hours after treatment ($*P < 0.05$, one-way analysis of variance (ANOVA) Kruskal–Wallis followed by Dunn's multiple comparison test). *In vivo*, intravenous injection of 1 $\mu\text{mol/g}$ D-lactate after 45 minutes transient middle cerebral artery occlusion (tMCAO) decreased total infarct volumes measured 48 hours after ischemia **(B)** and improved the neurologic outcome **(C)**, neurologic deficit scores from 0, no deficit—white, to 5 worst outcome—black; $*P < 0.05$ Mann–Whitney test, two-tailed P -value.

whether the beneficial metabolic effect of lactate *in vitro* could be exerted via its use by astrocytes. For this purpose, we made use of acetate which is known to be specifically taken up and used as energy substrate by astrocytes.²² However, the addition of different concentrations of acetate (0.13, 0.2, 4, and 8 mmol/L) to organotypic hippocampal slices 1 hour after OGD did not provide significant neuroprotection (Figures 5A and 5C).

DISCUSSION

After being considered for decades as a waste-product of metabolism, lactate has been shown to be able to support synaptic activity^{1,23} and is nowadays recognized as a prominent energy substrate in the CNS.²⁴ Lactate is produced under physiologic conditions,^{25,26} and it is suggested to have an important role in the metabolic support of long axons^{6,27} as well as in long-term memory formation.²⁸ Lack of adequate lactate supply has also been implicated in some neurodegenerative diseases such as amyotrophic lateral sclerosis.²⁹ Moreover, exogenous lactate supplementation seems beneficial after traumatic brain injury.^{30–32} In ischemic conditions, our group and others have shown neuroprotection after L-lactate administration at reperfusion after tMCAO.^{11,12,33} Similarly, brain lactate was found to be essential to improve post-ischemic outcome after cardiac arrest.³⁴ Lactate can be transported from one cell type to another by MCTs that are selectively expressed in different cell types and which can transport monocarboxylates with different affinities.³⁵ More recently, lactate has been described to act as a signaling molecule

in the CNS, either through the HCA1 receptor^{8,9} or through an unknown receptor yet to be specified.³⁶

Our initial characterization of the distribution of the HCA1 receptor in three brain regions affected after tMCAO confirmed a prominent neuronal expression in the CNS.^{8,9} Interestingly, our data also suggested that the cerebral expression of HCA1 can be modulated under certain conditions. Lactate itself seems to be a signal for the induction of its own receptor. However, whether lactate accumulation after ischemia is responsible for the enhancement of the HCA1 receptor observed after 24 hours of reperfusion remains to be directly shown. Nonetheless, our results provide the first evidence that activation of HCA1 receptors alone is sufficient to provide neuroprotection, at least in an *in vitro* ischemic model. Our demonstration that neuroprotection was also achieved with D-Lactate confirms this observation in that D-lactate was shown to exhibit at least partial agonist activity on HCA1/GPR81 receptor^{18,37} and that D-lactate was considered as a non-metabolizable lactate enantiomer and often used as a negative control to show the necessity of L-lactate use as an alternative energy substrate. However, unexpectedly, we found that D-lactate is extracted by the rodent brain and oxidized. In fact, one third of D-lactate was metabolized to CO₂, which is only marginally less than the previously reported fraction of CO₂ produced from L-lactate (42 \pm 8% CO₂ after 40 minutes¹). This observation suggests that the rodent brain may possess a D-lactate dehydrogenase activity. In accordance, the isolation of human and mouse transcripts encoding a homolog of the yeast D-lactate dehydrogenase was reported and its expression was found in various

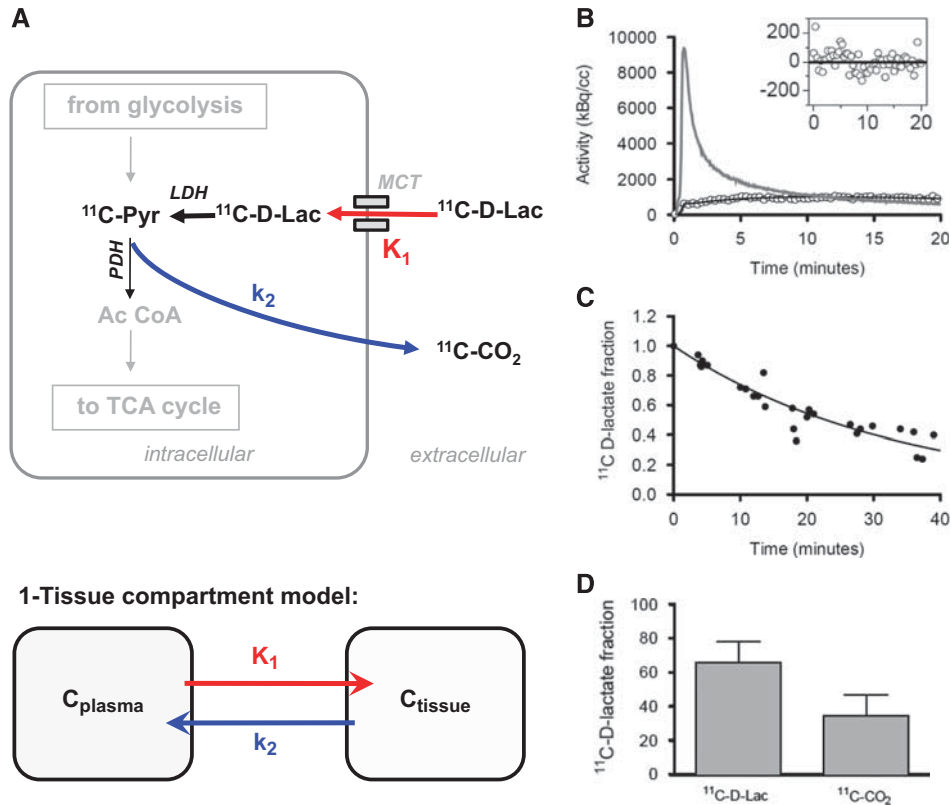


Figure 4. D-lactate metabolism. **(A)** Schematic of biochemical pathways involved in the degradation of lactate and the proposed interpretation of the rate constants K_1 and k_2 which are mathematically defined by the one-tissue compartment model used for $1\text{-}^{11}\text{C-D-lactate}$ data analysis. **(B)** Measured radioactivity concentration in the brain (open circles), model fit (black line), and arterial input curve (gray line). The inset displays the residuals of the fitting to the one-tissue compartment model. Equal distribution around the neutral line supports the adequacy of the applied model. **(C)** Fraction of native radiolabeled lactate more than 40 minutes after intravenous injection of $1\text{-}^{11}\text{C-D-lactate}$ in the blood. The filled circles represent data points from individual measurements and the solid line is the corresponding fit. **(D)** Percentage of identified metabolites in brain tissue after 40 minutes.

tissues, including the brain.³⁸ However, it remains to be determined whether the presence of a cerebral D-Lactate dehydrogenase enzyme is sufficient to explain our results or whether the activity is performed by another *a priori* unrelated enzyme.

In the CNS, pyruvate is transported mainly by MCT1 and MCT2 in the direction of the concentration gradient, and these MCTs are located in astrocytes and neurons, respectively.^{4,39} Possible explanations for the neuroprotection against ischemic damage induced by either L- or D-lactate are the fact that lactate is converted to pyruvate by the mitochondrial lactate dehydrogenase to fuel the Krebs cycle and therefore used as energetic substrate and that this conversion produces reduced nicotinamide adenine dinucleotide that will in turn act as a reactive oxygen species scavenger.⁴⁰ Evidence pointing towards this metabolic explanation is the protection exerted by the administration of 10 mmol/L pyruvate *in vitro*. Protection is not seen *in vivo* though. However, in support of a metabolism-mediated neuroprotective effect, others^{2,21} have shown protection *in vivo* using an extended intravenous mode of administration of pyruvate.²¹ This difference in the mode of administration may explain the different *in vivo* results, although we cannot exclude that lactate may prove to be a more efficient neuroprotective agent, notably *in vivo*, in particular because of the ability of both L- and D-lactate to activate the HCA1 receptor.

It has been shown that acetate is preferentially transported by MCT1 and metabolized in astrocytes.^{17,22} During ischemia, it was observed that ^{14}C -acetate uptake is decreased as early as 3 minutes after the onset of ischemia. The degree of reduction in

^{14}C -acetate uptake correlates with the severity of ischemia which, according to Hosoi *et al*,⁴¹ relates to the depression of glial metabolism. Even though the reduction in ^{14}C -acetate uptake was reversible and reached full recovery 3 hours after 30 minutes MCAO, Hosoi *et al* did not evaluate the functional outcome of acetate administration. We showed here that the administration of acetate did not exert protection against neuronal death in hippocampal slices subjected to oxygen and glucose deprivation. Even though there is an increased energy demand in neurons and astrocytes subjected to ischemic insult, this result suggests that energy supply to astrocytes is not enough to exert neuroprotection. It should be noted that using an *in vitro* assay of ligand-induced ^{35}S -GTP γ S binding with membranes from CHO cells expressing human HCA1 receptor, Cai *et al*¹⁸ described that neither sodium pyruvate nor sodium acetate showed binding activity. As neither acetate nor pyruvate binds to the HCA-1 receptor, the results shown here cannot be related to HCA1 signaling pointing to the use of pyruvate and acetate as metabolic substrates.

Some limitations to consider in the present study are that (1) our *in vivo* stroke model involves a transient occlusion with removal of the silicon filament, therefore our results are limited to be translated to patients with either a spontaneous reperfusion or successful intravenous thrombolysis or intrarterial recanalisation. Evaluating the effect of lactate administration in permanent ischemia is an important step before aiming for translation in clinical practice. (2) Ischemia is induced under isoflurane anesthesia and this compound has been described as

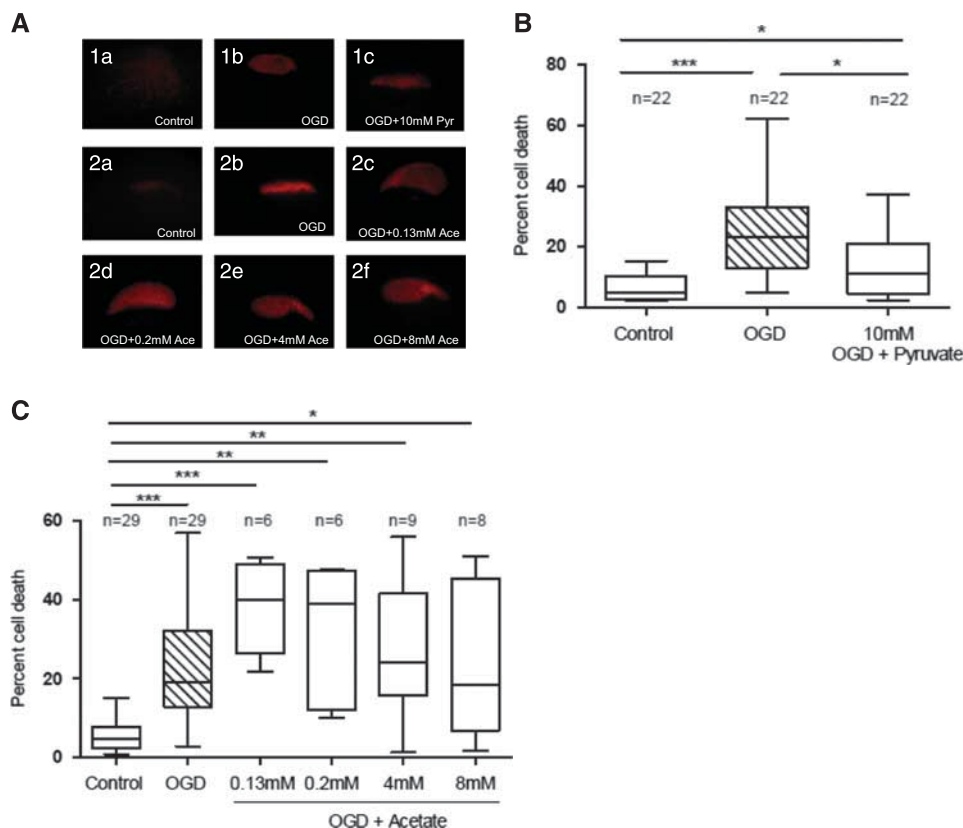


Figure 5. Administration of pyruvate and acetate after OGD. **(A)** Administration of 10 mmol/L pyruvate directly to the medium after 1 hour oxygen-glucose deprivation (OGD) in rat organotypic hippocampal slices was able to protect against ischemic damage. **(B)** Administration of various doses of acetate did not protect against ischemic damage. Cell death was assessed by propidium iodide (PI) staining 48 hours after treatment. **(C)** Representative images of PI staining among each group can be observed in panel (* $P < 0.05$; one-way analysis of variance (ANOVA) Kruskal–Wallis test followed by Dunn's multiple comparison test).

neuroprotective by itself. Therefore, we may not be observing the neurologic damage to its fullness. In organotypic hippocampal slice cultures the tissue architecture is only partially preserved and while some neuronal connections are maintained many are interrupted. Furthermore, there is no blood circulation. Despite these limitations, both models complement each other and strengthen our observations. (3) It should be considered as well that lactate was shown to increase regional CBF in physiologically activated but not resting human brain.⁴² Although we cannot rule out a contribution from CBF changes, the effect observed in the *in vitro* model, independently from blood flow, shows that lactate exerts a neuroprotective effect independently from possible effects on cerebral blood flow.

Taken together, these data point to the possibility that lactate, independently of the enantiomer type, may confer neuroprotection after ischemia by two distinct mechanisms: One, through the classic metabolic pathway providing an alternative energy supply to deprived neurons and the other through a receptor-mediated signal transduction mechanism in neurons. In both cases, the exact targets within the cell still need to be identified.

AUTHOR CONTRIBUTIONS

XC, KR, MW, KD, and AB designed and performed experiments; LP, BW, and LH designed experiments. XC, MW, LP, and LH wrote the paper.

DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Journal of Cerebral Blood Flow & Metabolism website (<http://www.nature.com/jcbfm>)