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Pyruvate protects against experimental stroke via an anti-inflammatory mechanism

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Abstract

Pyruvate, a key intermediate in glucose metabolism, was explored as a potential treatment in models of experimental stroke and inflammation. Pyruvate was administered to rodents after the onset of middle cerebral artery occlusion (MCAO). Since the extent of inflammation is often proportional to the size of the infarct, we also studied a group of animals given lipopolysaccharide (LPS) to cause brain inflammation without cell death. Following MCAO, pyruvate did not affect physiological parameters but significantly reduced infarct volume, improved behavioral tests and reduced numbers of neutrophils, microglial and NF- κ B activation. Animals given LPS showed increased microglial and NF- κ B activation which was almost completely abolished by pyruvate. Lactate, a major metabolite of pyruvate, was increased after pyruvate administration. However, administration of lactate itself did not have any anti-inflammatory effects. Pyruvate protects against ischemia possibly by blocking inflammation, but lactate itself does not appear to explain pyruvate's anti-inflammatory properties.

Keywords

cerebral ischemia; inflammation; pyruvate; stroke; neuroprotection

Introduction

Pyruvate, a final metabolite in glycolysis, has recently been shown to have salutary effects in brain ischemia (Lee et al. 2001; Yi et al. 2007; Yu et al. 2005). The precise mechanism of pyruvate's protective effect is unclear, but has been correlated to reduction in microglial activation and suppression of proinflammatory cytokines following focal cerebral ischemia. In related models of cardiac ischemia, pyruvate was also shown to have both cardioprotective and anti-oxidant properties (Woo et al. 2004). At the in vitro level, ethyl pyruvate was found to inhibit nuclear factor kappa B (NF κ B) activation in both cultured microglia (Kim et al. 2005) and RAW 264.7 cells (Han et al. 2005; Ulloa et al. 2002) through a modification of its

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p65 subunit. In models of lethal sepsis, ethyl pyruvate also reduced mortality and reduced circulating levels of HMBG1 (Ulloa et al. 2002). Post ischemic inflammation, including activation of NFkB and inhibition of downstream immune mediators, has been established to contribute to ischemic progression (Wang et al. 2007). Thus, this anti-inflammatory property of ethyl pyruvate may be an important factor in its protective effect.

However, the ethyl group of ethyl pyruvate may itself have pharmacological actions which could have influenced prior observations. Whether pyruvate itself, an endogenous metabolic intermediate, also has anti-inflammatory effects is less clear. In order to study pyruvate in its more endogenous state, we study similar properties in sodium pyruvate. Further, whether sodium pyruvate has similar protective and anti-inflammatory properties is less clear, and the existing literature is conflicting (Gonzalez-Falcon et al. 2003; Kim et al. 2005; Lee et al. 2001). We explore here whether sodium pyruvate protects against experimental stroke, and whether it does so via an anti-inflammatory mechanism using a model of brain inflammation that does not cause brain cell death. Since pyruvate can be rapidly metabolized to lactate, we also explored whether any anti-inflammatory effect of may be mediate through this metabolite.

Materials and Methods

Stroke Model

All experiments were performed following an institutionally approved protocol in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Measures were taken, in accordance with the above guidelines to minimize pain and discomfort. Male Sprague-Dawley rats (Charles River) weighing between 270-300 g were anesthetized using isoflurane with a face mask and maintained with 1.5% isoflurane in 150ml/min oxygen and 850ml/min air. Rectal temperature and other physiological parameters were recorded every 15 min and maintained in the normal range. The left femoral artery was cannulated for monitoring mean arterial blood pressure (MAP) and to sample blood for blood gas, glucose, pyruvate and lactate levels. Middle cerebral artery occlusion (MCAO) was performed as previously described by our group (Han et al. 2003). Briefly, after exposing the right common carotid, external carotid, internal carotid, and pterygopalatine arteries, MCAO was induced by introducing an uncoated 30 mm segment of 3-0 nylon monofilament suture (Harvard Apparatus, Holliston, Massachusetts) with an enlarged tip into the stump of the common carotid artery, and advanced into the internal carotid artery to 18-20 mm from the bifurcation of the internal and external carotid arteries to occlude the ostium of the MCA. The suture was left in the place for 2 hours. Animals were treated with sodium pyruvate 500 mg/kg IP (Sigma) or an equivalent volume of vehicle 10 min before the onset of reperfusion. After the ischemic period, the suture was removed and the animal was allowed to recover. The Bederson score (see below) was assessed at the time of recovery from anesthesia. Animals scoring 0 (normal) at this timepoint, were excluded, as it was likely that these animals had no ischemic injury. Mortality was also documented and recorded. Animals that died before the end of the experiment were not used for assays.

Behavior Tests

Ischemic and sham-operated animals were subjected to the following behavioral tests 72 hours after MCAO. Forelimb-placement, elevated body swing test, cylinder test, and Bederson score were conducted, and scoring was performed by investigators blinded to the experimental conditions.

Elevated Body Swing Test (EBST)

The EBST was conducted to evaluate asymmetrical motor behavior (Borlongan et al. 1995). Animals were held by the tail and elevated approximately 10 cm above the bench top. The

direction of the body swing, defined as an upper body turn of >10 degrees to either side, was recorded for each of 30 trials. The numbers of left and right turns were counted, and the percentage of turns made to the side contralateral to the ischemic hemisphere (left sided bias) was determined.

Cylinder Test

The cylinder test based on that developed by Hua et al. (Hua et al. 2002) was also performed with some modifications. The animal was placed in its home cage with transparent walls, and videotaped for forelimb use during exploratory activity. We place the animal in its home cage, rather than an actual cylinder as originally described. We have found that this causes less stress to the animal, but provides us with similar results. One trial was observed for 3 to 10 minutes depending on the degree of activity. Scoring was done through viewing recorded tapes of the animal activity by two experimenters blinded to the experimental conditions of the animal. After the rat was placed in its home cage, the first contact by the forelimb against the wall after rearing and during lateral exploration was scored according to the following criteria: (1) independent use of either forelimb to contact the wall during a full rear to initiate a weight-shifting movement or to regain center of gravity while moving laterally in a vertical posture and (2) simultaneous use of both the left and right forelimbs by contacting the wall of cylinder during a full rear and for alternating lateral movements along the wall.

Behavior was quantified by determining the number of times the unimpaired (I, ipsilateral) and impaired (C, contralateral) forelimbs contacted against the wall, as well as when both forelimbs were contacted simultaneously (B). A single overall limb use asymmetry score was calculated as follows: Forelimb Use Asymmetry Score = $(I - C) / (I + C + B)$. Thus, higher percentages reflect more impairment of the contralateral (C) forelimb.

Forelimb Placement Test

The forelimb placing test was used as described previously (De Ryck et al. 1989; Yu et al. 2005). Each animal was brought laterally towards the bench top to allow for spontaneous forelimb placing. The limb was then gently pulled down and away from the bench top edge and observed for retrieval and placing. Normal rats immediately place forelimbs on the bench top followed by brisk retrieval and placing. Scales were graded as follows: 0, immediate and complete placing; 1, delayed or incomplete placing (>2 s); and 2, no placing.

Bederson score

A neurological score to evaluate the motor function was also assigned to rats based on a scoring system first described by Bederson et al (Bederson et al. 1986), and previously used by us (Zheng et al. 2008). Scores were graded on a 4-point scale: 0, no observable deficit; 1, paretic forelimb weakness and torso turning to the ipsilateral side on lifting the animal by the tail; 2, circling to the contralateral (paretic) side but normal posture at rest; 3, reclinination to the contralateral side at rest; and 4, absence of spontaneous locomotor activity or barrel rolling.

2,3,5-Triphenyl Tetrazolium Chloride Staining

Brains were quickly removed and sliced into 2-mm thick coronal sections. Slices were incubated immediately by immersing in 2% 2,3,5-triphenyl tetrazolium chloride (TTC) in saline at 37°C for 15 minutes and then fixed in 4% paraformaldehyde overnight. Infarct size from each section was measured followed by a correction for cerebral edema was measured according methods previously used by our group (Tang et al. 2007; Zheng et al. 2008). Infarct volume was determined by summing the infarction areas of all sections and multiplying by the slice thicknesses.

Brain Inflammation Model

Male Sprague-Dawley rats weighing between 280–300 g were anesthetized as described above. Inflammation was induced by administration of 5 mg/kg (intraperitoneally, I.P.) bacterial LPS in sterile normal saline (*Escherichia coli* serotype 055; B5; Sigma). The experimental group was injected with pyruvate (500 mg/kg, IP) or lactate (500 mg/kg IP, Sigma) at the same time as LPS, while the control group received normal saline. Animals were sacrificed after 24 hours with a CO₂ overdose and then perfused intracardially. Brains were quickly removed and prepared as for immunohistochemistry, NF-κB DNA binding activity, and western blot measurement.

Serum measurement of pyruvate and lactate

Rats were anesthetized as described above. The femoral artery was exposed and cannulated, in order to collect blood samples. Animals were injected with either pyruvate (500 mg/kg, IP) or lactate (500 mg/kg, IP) in sterile normal saline, while the control group received only normal saline. Blood was collected at different timepoints (5, 10, 15, 30, 60, 120 and 180 min) and lactate levels were assessed using a YSI 2700 Select automat (Rotech).

In order to measure the pyruvate levels, the collected blood sample was centrifuged (1000g, 3 min, 4°C) and the supernatant was collected into a new tube. Perchloric acid (0.4 N) was added in a 1:1 ratio and the sample was centrifuged at 5000g for 10 min. The supernatant was collected and neutralizing buffer (0.6 N NaOH / 120 mM K₂HPO₄ / 80 mM NaH₂PO₄ / 500 mM KCl) was added in a 1:3 ratio. The samples were then frozen at –20°C and centrifuged at 5000g for 10 min just before being assayed. The pyruvate levels were measured in a 96-well plate by mixing the samples with LDH 0.1 U/ml and NADH 400 μM. The decrease in OD was measured at 340 nm using a kinetic plate reader for a 5 min period, during the linear phase, and compared to a standard curve.

Immunohistochemistry

Immunohistochemistry was performed to identify microglia, neutrophils, and macrophages as well as NFκB. These methods have been published by us previously (Deng et al. 2003; Han et al. 2003; Han et al. 2002; Wang et al. 2002). Brains were cryoprotected in 20% sucrose, then fixed in 2% paraformaldehyde, cut into 25 μm thick coronal sections on a cryostat, and treated for endogenous peroxidases with 0.03% hydrogen peroxide. To identify neutrophils, macrophages and phagocytic microglia, sections were blocked with 5% normal serum, then incubated for 1 hour in antibodies against myeloperoxidase (MPO; A0398; 1:500; Dako, to identify neutrophils), ED1 antibody (MCA341R; 1:400; Serotec), a marker for macrophages and microglia. Sections were washed, then incubated with a biotinylated secondary antibody that had been preabsorbed to rat serum (Elite Vectastain ABC Kit, Vector Labs), followed by a tertiary binding complex (ABC), and visualized with diaminobenzidine (Sigma Fast Diaminobenzidine, Sigma). Lectin histochemistry was used to identify microglia. Brain sections were incubated for 3 hours with 10 μg/mL *Griffonia simplicifolia* isolectin B4 (IB4; L5391; Sigma), and visualized with diaminobenzidine. In order to detect NFκB, sections were reacted with primary antibody against NFκB p65 (1:200, sc-109; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). Negative controls were run in parallel using adjacent sections incubated with *isotype controls* instead of the primary antibody. Methyl green was used as a counterstain for ED1, while hematoxylin and eosin (H & E) was used for all other stains.

Immunostains for OX-42 and OX-6 were performed on floating sections. Brains were cut in 50 μm-thick sections and stored at 4°C in 0.04% sodium azide solution. Sections were blocked for non-specific binding with 3% BSA and 0.1% Triton X-100 in PBS. For OX-42 staining, these sections were then exposed to a primary antibody against CD11b (Mouse anti rat CD11b; MCA275R; 1:100; AbD Serotec) followed by a biotinylated secondary antibody (Elite

Vectastain ABC Kit, Vector), and a tertiary binding complex (ABC), and visualized with Vector SG (Vector). For OX-6 staining, the slices were exposed to a primary antibody against RT1B (Mouse anti rat MHC class II RT1B; MCQ46GA, 1:100, AbD Serotec) followed by Alexa Fluor 488 conjugated anti mouse antibody (A-11017; 1:500; Molecular probes).

Cell counting

Densities of positive cells from the above immunostains were counted using methods previously published (Deng et al. 2003; Wang et al. 2002; Zheng et al. 2008). Briefly, brain regions corresponding to the infarct border were identified from the H & E stains. This peri-infarct region, though not necessarily anatomically identical between brains, was chosen because it represents ischemic regions of similar pathophysiology. Also, the peri-infarct region is an area where we consistently observe high numbers of positive inflammatory cells following focal cerebral ischemia (Wang et al. 2002). These peri-infarct regions were sample from the 3rd brain slice corresponding to the level containing the striatum, and where maximal and consistent ischemic injury is observed. Five non-overlapping high power fields were sampled from the ventral and dorsal aspects of the infarct and compared to similar counts from homotypic regions from the contralateral non-ischemic side.

For the LPS model, anatomically similar fields from the lateral temporal lobes (a region where consistent microglial activation could be observed) were selected. A rater blinded to the experimental details counted the numbers of positive cells per high power field over 5 adjacent, non-overlapping fields. For the neutrophil and ED1 stains, only counts were made from the MCAO studies, since very little neutrophils or ED1 staining was observed in the LPS model (Deng et al. 2003). Myeloperoxidase positive cells were only counted if the cell size and nuclear morphology were consistent with a leukocyte. For the p65 stains, all positive cells and cells with only cytosolic staining were counted.

Microwell colorimetric nuclear factor- κ B assay

A commercially available kit (Trans-AMTM NF κ B p65, 40096; Active Motif) was used to measure the binding ability of NF κ B in tissue lysates to bind DNA consensus sequences (Han et al. 2003; Zheng et al. 2008). This method was reported to be more sensitive and quantitative than EMSA and is used here as an alternative to electrophoretic mobility shift assay (EMSA) (Shen et al. 2002). Tissue lysates were tested for their ability to bind to a double-stranded oligonucleotide probe containing the consensus binding sequence for NF κ B. The assay was run following the manufacturer's directions, modified from that of Renard et al. [Renard, 2001, 11160941]. Briefly, samples were homogenized in 3 mL ice-cold lysis buffer (20 mmol HEPES, pH 7.5; 350 mmol NaCl; 20% glycerol; 1% Igepal-CA630; 1 mmol MgCl₂; 0.5 mmol 5EDTA; 0.1 mmol EGTA) per gram tissue. Lysates were centrifuged at 10,000 g for 10 minutes at 4°C. The Bradford assay (Bio-Rad) was used to measure the protein concentration in the supernatant. NF κ B activity was determined by the sample's ability to bind to consensus sequences (5'-GGGACTTCC-3') in a 96-well plate. A primary antibody that recognizes an epitope on p65 and is accessible only when NF κ B is activated and bound to its target DNA was added to the wells, followed by a secondary horseradish peroxidase-conjugated antibody. Developing solution (tetramethylbenzidine) was added and the colorimetric reaction was halted by adding stop solution (0.5-mol/L H₂SO₄). Absorbance was measured at 450 nm with a reference wavelength of 655 nm on a spectrophotometer within 5 minutes. HeLa whole-cell extract was used as positive control for NF κ B activation. The NF κ B wild-type and mutated consensus oligonucleotides were used in order to monitor the specificity of the assay. A wild-type oligonucleotide should compete with NF κ B for binding to the probe immobilized on the plate, whereas the mutated consensus oligonucleotide should have no effect on NF κ B binding.

Western blot

Western blots were performed for MMP9 as described before (Lee et al. 2005). Tissue samples were prepared from ischemic and non ischemic brain (brain regions sampled corresponded to similar areas where cell counts were made), as well as hemispheres of animals given LPS. Tissue extracts were subjected to electrophoresis in 15% polyacrylamide gels and transferred to nitrocellulose membranes (Millipore, Bedford, MA), which were pre-incubated in methanol for 5 minutes and then in transfer buffer for 15 minutes. Membranes were stained with Ponceau Red to ensure equal protein loading. After 2 hours incubation in blocking solution of 5% nonfat milk in phosphate-buffered saline-Tween, the membranes were incubated in primary antibodies against MMP-9 (rabbit polyclonal antibody, 1:1000 dilution; Chemicon) which “recognize” both pro- and active forms, followed by incubation in horseradish peroxidase-conjugated secondary antibody for 1 hour (1:2000; Amersham Pharmacia Biotech). Signals were detected with a chemiluminescence kit enzyme chemiluminescence (ECL)-plus Western Blotting Detection System (Amersham Pharmacia Biotech). The membranes were first stripped and then reprobed with a murine monoclonal antibody against β -actin (C-2, 1:1000 dilution; Santa Cruz Biotechnologies, Santa Cruz, CA) as a loading control, and developed using the enhanced chemiluminescence system. Densitometric measurements were made from the protein bands by using Gelscope (Imageline, Gardena, CA).

Results

Pyruvate did not affect physiologic parameters or mortality in MCAO

No significant differences were found among any of the physiological parameters monitored between control and pyruvate treated groups (Table). Mortality was less than 15% in each group, and not statistically different between groups. One animal died before the end of the experiments in the vehicle treated control group, and none among those given pyruvate.

Serum pyruvate& lactate levels after systemic administration

Blood pyruvate and lactate were evaluated at serial time points after the i.p. pyruvate injections. Blood pyruvate peaked 15 minutes after pyruvate i.p. injections, with the peak pyruvate level roughly 10-fold higher than the basal level, and returned to baseline by 3 hours, (Fig 1A). Since pyruvate can be metabolized to lactate, blood lactate levels were also measured, and found to have a similar time course (Fig 1B). The peak blood lactate level following pyruvate injections was roughly 5-fold higher than the basal level. Blood pyruvate and lactate were also evaluated at serial time points after i.p. lactate injections. As expected, the lactate injections produced a larger increase in blood lactate but smaller increase in blood pyruvate than the equimolar pyruvate injections (Fig 1A,B).

Pyruvate reduced infarct sizes of MCAO using TTC staining

To investigate the neuroprotective effect of pyruvate in cerebral ischemia, pyruvate (500mg/kg, IP) was administered 10 minutes before the onset of reperfusion. Infarct volume was evaluated 24 hours later. The administration of pyruvate reduced the infarct volume by 71.25 % compared with vehicle treatment (Fig 2 A-B). Similar reductions were observed by 72 h (*data not shown*).

Pyruvate improved sensorimotor function and neurological deficits following MCAO

Neurological deficits evaluated by the Bederson score were significantly reduced in pyruvate treated groups (2.85 ± 0.37 vs 2.0 ± 0.53 , $n = 8$; $P < 0.05$, Fig. 2C) compared with the vehicle treated group. This effect was still evident 72 h post insult (pyruvate: 1.66 ± 0.51 vs control: 2.33 ± 0.51 , $n = 6$; $P < 0.05$). The elevated body swing test also showed that in vehicle treated rats, the percentage of left biased swing increased to $87 \pm 4\%$ compared with $49 \pm 4\%$ among

shams. Pyruvate treatment reduced this bias to $66\pm 2\%$ ($P < 0.05$, Fig 2D). Rats treated with pyruvate also performed better on the cylinder test (Fig. 2E) and forelimb placement tests (Fig. 2F) compared with vehicle treated controls.

Pyruvate reduced neutrophil infiltration and microglial activation after MCAO

Within peri-infarct regions, pyruvate decreased densities of inflammatory cells compared to vehicle treated controls. Compared to shams, numbers of MPO positive cells with nuclear morphology consistent with neutrophils increased 24 h after MCAO, and this increase was significantly reduced by pyruvate treatment (Fig. 3A). ED1 positive cells, representing macrophages and phagocytic microglia (We do not attempt to differentiate.), were observed in the ischemic brain at 24 h, and this was also decreased by pyruvate treatment (Fig. 3B). Reduction of ED1 positive cells was also observed at 72 h, but few neutrophils were observed in the brain at this timepoint (*data not shown*).

Pyruvate suppresses microglial activation after LPS exposure independent of lactate

Since the immune reaction may reflect the extent of ischemic injury, any decreases in immune cells could simply reflect a reduction in infarct size due to any number of upstream mechanisms. As a positive control, we studied groups of rats given LPS to stimulate an inflammatory response in the brain without causing detectable brain cell death. Using this model, we observed several immune cells/ activated microglia following LPS exposure, and this was decreased by pyruvate (Fig. 4). Since ED1 staining is not observed in our LPS model, we used similar markers to identify monocyte lineage cells, OX 42 to identify CD11b and OX6 to identify MHC Class II molecule. Since pyruvate is metabolized to lactate, and lactate levels were actually higher than pyruvate after pyruvate administration, we also explored the possibility that lactate might be mediating some of the observed anti-inflammatory effects. LPS led to increased OX42 and OX6 staining compared to sham injected controls. Pyruvate markedly suppressed microglial activation, but interestingly, lactate had no effect.

Pyruvate reduces nuclear factor- κ B staining following MCAO and LPS exposure

Immunostaining for NF κ B's p65 subunit appeared faint, but present within the cytosol in uninjured brains. Following ischemia, a variety of different cell types showed increased NF κ B staining in both the cytoplasm and nuclei (Fig. 5). Pyruvate treatment significantly decreased overall NF κ B staining, as well as numbers of cells with nuclear staining. Similar patterns were observed in the LPS model, with pyruvate treatment resulting in NF κ B staining largely confined to the cytoplasm.

Pyruvate reduces nuclear factor- κ B's DNA binding capacity in MCAO and LPS-induced brain inflammation

NF κ B activity was estimated using an ELISA based assay. Activity, as measured by the ability of a tissue sample to bind to DNA kappa B consensus sequences, was found to be increased 24 h after MCAO and LPS exposure. Treatment with pyruvate in both models led to statistically significant reductions in its DNA binding capacity (Fig. 6). In order to monitor the specificity of the assay, the NF κ B wild-type and mutated oligonucleotides were used.

Pyruvate decreases MMP9 expression after pyruvate treatment

Western blots of MMP9, a gene regulated by NF κ B, showed increased expression after both MCAO and LPS administration. Pyruvate decreased MMP9 in both models (Fig. 7).

Discussion

We show here that treatment of experimental stroke with systemically administered sodium pyruvate leads to reduction in infarct size and improved neurological deficits. This effect was associated with less inflammation and decreased activation of the inflammatory transcription factor, NFkB. Furthermore, we suggest a direct anti-inflammatory property of pyruvate, as administration led to similar decreases in microglial and NFkB activation in a model of pure brain inflammation that does not cause brain cell death. The protective and anti-inflammatory effect of pyruvate does not appear to be through its metabolism to lactate, as lactate itself does not appear to suppress inflammation.

Prior work in this area has shown the beneficial effects of pyruvate in the acute treatment of experimental stroke and related brain insults. Both sodium pyruvate and ethyl pyruvate have been shown to improve outcome in rat global cerebral ischemia (Lee et al. 2001), hypoglycemia (Suh et al. 2005) and focal cerebral ischemia (Gonzalez-Falcon et al. 2003; Kim et al. 2005; Yi et al. 2007; Yu et al. 2005). The studies of focal cerebral ischemia are notable for somewhat varying results. Two studies (Yi et al. 2007; Yu et al. 2005) showed robust protection against 1h transient MCAO (tMCAO) followed by reperfusion using ethyl pyruvate. Sodium pyruvate was also protective in a similar model of tMCAO (Yi et al. 2007), but only showed modest results in one model of permanent MCAO without reperfusion (pMCAO) (Gonzalez-Falcon et al. 2003) and robust results in a different model of pMCAO (Yi et al. 2007). Small discrepancies in the published literature might be due to the use of ethyl pyruvate compared to sodium pyruvate, short duration in the tMCAO model, and lower dose used in the pMCAO model of robust protection. Regardless, the collective literature indicates that pyruvate may have a wide temporal therapeutic window, as one group showed neuroprotection when administered as late as 12 h following 1 h tMCAO (Yu et al. 2005), which was maintained 14 d later (Yi et al. 2007). In the current study, we show robust protection using a moderately high dose of sodium pyruvate in a severe tMCAO model (2 h occlusion).

Pyruvate can scavenge hydrogen peroxide and other reactive oxygen species (Andrae et al. 1985; Desagher et al. 1997). Pyruvate can also prevent energy failure induced by zinc and PARP-1 activation (Sheline et al. 2000; Ying et al. 2002; Zeng et al. 2007). These properties have been suggested as mechanisms by which pyruvate reduces cell death after brain ischemia. Results of the present studies further suggest that pyruvate can suppress inflammation at the transcriptional level. Not only did we document fewer infiltrating neutrophils and activated microglia within ischemic brain, but we observed similar patterns in our brain inflammation model. In both ischemic brain and brain exposed to LPS, pyruvate reduced numbers of immune cells in the brain. Since inflammation due to ischemia can be proportional to the amount of injury, observing a similar anti-inflammatory effect in a model that does not cause brain cell death serves as a positive control. In addition to seeing fewer active immune cells in the brain with pyruvate treatment in both models, we also saw less activation of the inflammatory transcription factor NFkB. This is in line with 2 prior reports which correlated reduced stroke-induced inflammation by pyruvate treatment (Kim et al. 2005; Yu et al. 2005). These same investigators showed more direct evidence of pyruvate's anti-inflammatory effect by studying cultured microglia exposed to LPS. They found decreased inflammatory mediator expression and reduced NFkB activation, findings similar to what we present here, except that our evidence is at the in vivo level. This is not to suggest that pyruvate may have other mechanisms of protection, as pyruvate has also been shown to protect cultures of primary cortical neurons against ischemia-like insults (Kim et al. 2005), and has also been correlated to reduced TUNEL positive cells (Yi et al. 2007).

Pyruvate has also been shown to reduce mortality in murine models of inflammation and sepsis (Ulloa et al. 2002). In this study, the investigators produced endotoxemia or severe

inflammation in mice by peripheral LPS administration of cecal puncture. Pyruvate treatment in this setting increased survival even when administered 24 h later. NFkB inhibiting effects of pyruvate have been described in macrophage cell lines (RAW 264.7) exposed to LPS (Han et al. 2002). In this study, ethyl pyruvate decreased NFkB activation and binding, but had no effect on the degradation of NFkB's inhibitor proteins, I κ B α or I κ B. By using a cell free system, the authors found that pyruvate could prevent DNA binding by NFkB's p65 subunit, but not p50.

The precise mechanism of this protective and anti-inflammatory effect is still unclear in our in vivo models. While we were able to document increased pyruvate levels presumably due to parenteral administration, whether the administered pyruvate enters the brain cannot be established here. For technical reasons, we were not able to reliably measure pyruvate levels in the brain. Thus, we cannot make any conclusions as to where it acts. The blood-brain barrier normally transports pyruvate at a rate much slower than glucose, but prior work suggests that significant pyruvate entry to the brain can be achieved with elevated plasma pyruvate concentrations (Lee et al. 2001; Suh et al. 2007) as achieved in the current study. Pyruvate crosses the blood-brain barrier by both facilitated transport and diffusion (Conn et al. 1982; Oldendorf 1973), and in the absence of a blood-brain barrier pyruvate movement into brain will occur in the direction of concentration gradients. Once in brain, pyruvate can be taken up by neurons, astrocytes, and microglia through a family of monocarboxylate transporters (Enerson et al. 2003).

Regardless of whether peripherally administered pyruvate enters the brain or not may be less important, since biological effects were observed. In fact, the spin trap agent NXY-059, which showed robust protection in animal models, and early promising results in clinical trials of stroke (Shuaib et al. 2007), did not significantly penetrate the blood brain barrier (Fong et al. 2006). Since circulating leukocytes are now known to significantly contribute to ischemic brain pathology (Wang et al. 2007), it is possible that pyruvate's actions interfere with peripheral immune cell function. However, the anti-inflammatory effect of pyruvate does not appear to be mediated through lactate itself, as direct administration of lactate had no effect in our brain inflammation model. The rise in pyruvate levels following lactate injection is not surprising since these two metabolites are kept in equilibrium by lactate dehydrogenase. Thus, elevations in pyruvate would be predicted after exogenous administration of lactate.

Since the collective literature to date in stroke models suggests that the benefit of pyruvate lies largely in models of ischemia followed by reperfusion, this would limit its broad application to stroke patients. However, since treatment with recombinant tissue plasminogen activator (rt-PA) and mechanical embolectomy is often incomplete, or may be complicated by pro-inflammatory processes brought on by reperfusion, pyruvate may be a useful adjunct to recanalization strategies. Thus, combinatorial approaches with pyruvate should be investigated.

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Figure 1.

Pyruvate injection increases blood pyruvate as well as blood lactate. Rats were injected either with sodium pyruvate (500 mg/kg, IP, n=3) or sodium lactate (500 mg/kg, IP, n=3) and saline (IP, n=2) as a vehicle control. Blood was withdrawn from the femoral artery just before injection (0 min) and after 15, 30, 60 and 180 min. Blood levels of (A) pyruvate and (B) lactate were then assayed.

Figure 2.

Pyruvate reduced infarct size after middle cerebral artery occlusion (MCAO). Rats were exposed to 2 h MCAO followed by 24 h reperfusion. Representative TTC-stained brain sections (A) show smaller infarcts among rats treated with pyruvate (Pyr) compared to vehicle (Con). Pyruvate significantly reduced infarct size by ~70% (B, $n=8$ / group; $**P<0.01$). Pyruvate also improves neurological outcome following MCAO. Four different tests of sensorimotor function were administered. The Bederson neurological scores (C), elevated body swing test (D), cylinder test (E), and the elevated forelimb-placing test (F) all showed more favorable parameters in rats treated with pyruvate (Pyr) compared to vehicle (Con). (C-F, $*P<0.05$ vs. Con/ Sham, $**P<0.001$ vs. Con/ Sham, $n=6-8$ rats/ condition)

Figure 3.

Pyruvate inhibits inflammatory cell infiltration and activation following MCAO. Brain sections of sham operated (Sham), rats exposed to MCAO and given vehicle (Con) and rats exposed to MCAO and treated with pyruvate (Pyr) were immunostained with various immune cell markers, then counterstained (H&E for myeloperoxidase, and methyl green for ED1) to delineate infarct borders. Counts were made within the ipsilateral ischemic cortex at the infarct border. A. Pyruvate reduces tissue neutrophil infiltration after stroke. Immunostains for myeloperoxidase (MPO), a neutrophil marker, show increased tissue neutrophils within the ipsilateral ischemic cortex (peri-infarct region) following MCAO (Con). Pyruvate reduces numbers of neutrophils within a comparable peri-infarct ischemic region (** $P < 0.001$ vs. Con/Sham, arrows indicate positive staining cells with nuclear morphology consistent with neutrophils). B. Pyruvate reduces numbers of ED1 positive cells after stroke. Immunostains for ED1, a marker of activated monocyte lineage cells, show increased numbers by 1 and especially 3 d post stroke. This was reduced by pyruvate treatment at both timepoints, but especially 3 d (* $P < 0.01$, ** $P < 0.001$ vs. Con, scale bar=50 μm).

Figure 4. Pyruvate, but not lactate, decreased LPS-induced microglial activation

Brain inflammation not associated with brain cell death was induced by administration of LPS (5mg/kg, IV) followed by IP injections of either normal saline as a control (LPS), pyruvate (500mg/kg, LPS+Pyr) or lactate (500mg/kg, LPS+Lac). Sham controls were given only saline (Sham). Adjacent brain sections were stained for OX-42 (A, to identify CD11b on immune cells) or OX6 (B, MHC Class II antigen). LPS clearly increased the number of positive staining cells as well as the intensity of staining for each immune marker. Positive cells all possessed processes, and would thus be consistent with microglia rather than circulating leukocytes. This was markedly suppressed by pyruvate, but not lactate. To quantify these observations, 3 different fields from the cortex were counted for positive cells at 20 X magnification. 3 to 4 animals were studied for each condition. No positive staining was observed in sham animals, but LPS increased numbers of both OX 42(C) and OX6 (D) positive cells which were decreased by pyruvate, but not lactate treatment. Scale bar = 50μm, *P<0.05, **P<0.001.

Figure 5.

Pyruvate reduces NFκB nuclear translocation following MCAO and LPS exposure. In the sham brain which is served as control, NFκB-stained cells were hardly detectable (Sham). In the ischemic (MCAO-Con) and LPS exposed (LPS-Con) brains, increased NFκB staining, both cytoplasmic and nuclear, were markedly increased. Pyruvate treatment (MCAO-Pyr and LPS-Pyr) markedly reduced overall staining. The insert in the LPS-Pyr image shows a single cell with very faint immunostaining restricted to the cytosol. Graph shows the percentage of cells with nuclear immunostaining compared to all positively stained cells. (** $P < 0.001$ vs. respective control; $n=3-4$ animals/group)

Figure 6.

Both cerebral ischemia and LPS increased DNA binding of nuclear factor-κB (NFκB) p65, and pyruvate attenuated this increase. Rats were subjected to 2 h of middle cerebral artery occlusion (MCAO) or LPS injection at 5mg/kg, and brains were taken 24 h later. Peri-infarct cortical regions (for MCAO group) or cortices (for LPS group) were harvested and prepared for the NFκB-binding activity assay. Tissue samples from sham-operated rats were taken from the same regions as those from ischemic or inflammation brains and served as controls. 24 hours after ischemia or brain inflammation, NFκB-binding activity increased and this increase was significantly suppressed by pyruvate treatment. (** $P < 0.001$, Con=sham control, MCAO=vehicle treated animals subjected to MCAO, M-Pyr=pyruvate treated animals subjected to MCAO, LPS=animals given LPS to induce brain inflammation, L-Pyr=animals given LPS and treated with pyruvate, $n=3-4$ animals/group).

Figure 7.

Both ischemia and LPS increased MMP9, an NFkB regulated gene, protein expression by 4 and 3.70 fold, respectively compared with shams (9.36 ± 0.73 % for shams vs. 37.39 ± 11.97 % and 34.86 ± 8.21 % for LPS and MCAO, respectively). Pyruvate treatment reduced MMP9 increases due to LPS administration (10.78 ± 1.32 % for LPS+ pyruvate) and MCAO (11.54 ± 1.27 % for MCAO + pyruvate). ($n=3-4$ /group, * $P<0.01$ and ** $P<0.01$ vs. no treatment, S=sham controls, M or MCAO= animals subjected to MCAO and given vehicle, MPy or M-Pyr= animals subjected to MCAO and treated with pyruvate, L or LPS= animals administered LPS and vehicle, LPy or L-Pyr= animals administered LPS and pyruvate).

Table
Pyruvate does not affect physiological parameters following MCAO.

	Pyruvate			Vehicle			P-value	
	Pre	During	Post	Pre	During	Post	Pre	Post
MABP (mmHg)	71±1	71±2	67±1	71±2	69±2	67±1	0.85	0.72
Temp (C)	37±0.1	37±0.1	37±0.2	37±0.1	37±0.1	37±0.2	0.32	0.66
Glu (dl/ml)	198±4	170±10	165±17	202±13	169±11	167±16	0.64	0.86
Hct (%)	36±3	32±5	28±4	35±3	31±4	25±2	0.69	0.77
pH	7.3±0.1	7.3±0.1	7.3±0.1	7.3±0.1	7.3±0.3	7.3±0.1	0.86	0.10
pO2 (mmHg)	135±5	153±5	156±5	137±9	164±3	167±2	0.83	0.14
pCO2 (mmHg)	48±4	55±7	58±12	48±4	60±4	57±13	0.89	0.46

MABP=mean arterial blood pressure, Temp=rectal temperature, Glu=Glucose, Hct= hematocrit