Pyruvate Protects the Brain Against Ischemia–Reperfusion Injury by Activating the Erythropoietin Signaling Pathway

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Background and Purpose—Pyruvate is known to be cytoprotective through antioxidant and anti-inflammatory mechanisms. We tested the hypothesis that pyruvate protects the brain against ischemia–reperfusion injury by inducing endogenous erythropoietin (EPO) expression.

Methods—Pyruvate’s protective effect was evaluated in C6 glioma cells and HT22 neuronal cells subjected to transient oxygen glucose deprivation. Cell viability (calcein AM assay) and expression of hypoxia-inducible factor-1α, EPO, Akt and Erk (immunoblot), and EPO receptor (reverse transcription–polymerase chain reaction) were analyzed. Transient focal cerebral ischemia in rats was induced by 2 hours middle cerebral artery occlusion followed by 24 hours reperfusion. Pyruvate or saline was infused from 60 minutes occlusion until 30 minutes reperfusion. Lesion volume and DNA fragmentation were assessed by 2,3,5-triphenyltetrazolium staining and terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling assay, respectively. Immunoblots were conducted to determine cerebral EPO contents.

Results—Pyruvate increased cell viability, hypoxia-inducible factor-1α, EPO, and Akt phosphorylation. Small interfering RNA suppression of hypoxia-inducible factor-1α and EPO abolished pyruvate-induced cytoprotection. In the rat stroke model, pyruvate reduced lesion volume by 84% and DNA fragmentation by 77% versus controls; increased EPO content paralleled these cerebroprotective actions of pyruvate.

Conclusions—Pyruvate activation of the hypoxia-inducible factor-1α–EPO signaling cascade in neurons and glia could protect the brain from ischemia–reperfusion injury. (Stroke. 2012;43:00-00.)

Key Words: brain recovery ■ erythropoietin ■ focal ischemia ■ hypoxia-inducible factor ■ pyruvate ■ stroke care

Despite decades of research and promising results from animal studies, effective treatments to protect the brain from ischemic stroke have proven elusive. Erythropoietin (EPO) has been found to be neuroprotective in stroke models.1 However, because the blood–brain barrier (BBB) limits EPO entry into the brain parenchyma,2 high doses are required that can produce untoward side effects including hypercoagulability and polycythemia in clinical practice.3,4 Strategies to lower the EPO dosages necessary to protect the ischemic brain merit investigation.

Preclinical studies have shown that the mammalian brain can synthesize substantial amounts of EPO.5,6 Astrocytes6 and neurons5 are the major sources of EPO in the brain. Moreover, EPO’s membrane receptors (EPOR) are expressed in the brain.8 Therefore, alternative approaches that induce endogenous EPO production in the brain might obviate the detrimental side effects of EPO.

Pyruvate has proven to be protective in cerebropathological conditions,9,10 but its mechanisms remain unclear. We demonstrated that pyruvate-enriched cardioplegia induced EPO mRNA and protein expression in swine myocardium undergoing cardiopulmonary bypass.11 Moreover, monocarboxylate transporters in the BBB12 and plasma membranes13 efficiently deliver circulating pyruvate to the brain parenchyma.

Erythropoietin is the first identified target gene induced by hypoxia inducible factor-1 (HIF-1), a key transcription factor for cellular responses to hypoxia.14 Pyruvate stabilizes the O2-regulated subunit of HIF-1, HIF-1α, by inhibiting its proteosomal degradation.15 Accordingly, pyruvate-enriched cardioplegia increased myocardial HIF-1α.13

This study tested the hypotheses that pyruvate induces EPO signaling, and interference with EPO’s signaling pathways abrogates pyruvate-induced protection in neuronal and glial cells, thereby implicating EPO as a pivotal mediator of pyruvate cerebroprotection. Additionally, we determined whether exogenous pyruvate can protect the rat brain from ischemic stroke and whether this protection parallels EPO induction within the brain.
Materials and Methods

Hypoxia–Reoxygenation of Neuronal and Glial Cells

Glia and neurons are the major cell types in the brain; therefore, delineating cytoprotective signaling cascades in these cells can provide crucial information regarding pyruvate’s protective mechanisms against ischemic stroke. Mouse neuronal HT22 cells were a gift from Dr David Schubert, Salk Institute, San Diego, CA, and glial fibrillary acidic protein-positive rat C6 glioma cells were obtained from ATCC (Rockville, MD). For hypoxia/reoxygenation (H/R), cells were switched to glucose- and fetal bovine serum-free Dulbecco modified Eagle medium containing 0, 5, or 10 mmol/L pyruvate and incubated in a hypoxic atmosphere (0.5% O2) for 12 hours followed by 24 hours of reoxygenation and renewed D-glucose availability.

Cell Viability

Cell viability was determined with calcein AM assay. C6 glial and HT22 neuronal cells were assigned to control or pyruvate groups of 6 independent trials, each in sextuplicate. Cells were incubated with 1 μmol/L calcein AM at 37°C for 10 minutes. Fluorescence was measured with excitation/emission at 485/530 nm.

Immunoblot of Hypoxia-Responsive Proteins

Contents of HIF-1α, EPO, Erk/p-Erk, Akt/p-Akt, and actin (Santa Cruz Biotechnology). Goat antimouse secondary antibody was obtained from Jackson Immunoresearch (West Grove, PA). Protein bands were quantified (Ultraviolet Products, Upland, CA) and normalized to actin.

Real Time Reverse Transcription–Polymerase Chain Reaction Measurements of mRNA

Real-time reverse transcription–polymerase chain reaction was used to assess abundances of EPOR and actin mRNA in HT 22 neuronal cells. Total RNA was isolated using Trizol Reagent (Invitrogen). cDNA was synthesized from total RNA with Taqman reverse transcriptase (Applied Biosystems, Foster City, CA). EPOR and β-actin cDNA were amplified in a Smart Cycler II (Cepheid, Sunnyvale, CA) by a SYBR Green Polymerase Chain Reaction Master Mix (Promega) and an Applied Biosystems 7300 Real-Time Polymerase Chain Reaction System. Two-step real-time polymerase chain reaction was performed (95°C for 15 seconds, 60°C for 60 seconds extension and detection, 40 cycles) with specific primers for EPOR (forward: 5'-GTAGGGCCGTACAGATGT-3’; reverse: 5’-CCCTTTCCCTGCTCTGTGG-3’) and β-actin (forward: 5’-TCTTTTCCAGCTTCCTCTGTG-3’; reverse: 5’-GCACTGTGGCAGCAGGTC-3’). Abundances of amplified genes were assessed by analysis of cycle threshold.

Small Interfering RNA Silencing of HIF-1α

HIF-1α small interfering RNA (siRNA) transfection was performed with siRNA transfection reagent (Santa Cruz Biotechnology) according to the manufacturer’s protocols. HIF-1α siRNA (2 nmol/L) was

Figure 1. Viability of glial and neuronal cell lines during reoxygenation with pyruvate. A, Five and 10 mmol/L pyruvate dose-dependently protected posthypoxic C6 glioma cells vs control. B, Representative calcein AM images under each treatment in C6 glioma cells. C, Ten but not 5 mmol/L pyruvate preserved posthypoxic HT22 neuronal cells. *P<0.05 vs normoxia; †P<0.05 vs control.
mixed with a transfection reagent added to neuronal cells (n=6/group) and equivalent concentrations of scrambled sequence siRNAs were transfected for negative control. Silencing of HIF-1α transcription was confirmed by immunoblot.

EPO Pathway Inhibition
To interrogate the involvement of EPO signaling in pyruvate-induced cytoprotection, soluble EPOR (Sigma) was applied to HT22 neuronal (n=15/group) and C6 glial (n=10/group) cells and pre-treated for 6 hours. After H/R, cell viability was determined as described previously.

Surgical Preparation for Rat Stroke Experiments
Animal experimentation was approved by the Institutional Animal Care and Use Committee of University of North Texas Health Science Center. Male Sprague-Dawley rats (275–300 g; Charles River, Wilmington, MA) were housed under 12-hour light:dark cycles and consumed food and water ad libitum. Rats were anesthetized with 1.5% isoflurane and temperature was maintained at 37.0±0.5°C with an electric heating pad. Ventilatory frequency, heart rate, and SpO2 were monitored. The right jugular vein was cannulated for pyruvate or normal saline infusion.

Pharmacokinetics of Infused Pyruvate
Circulating pyruvate concentrations during pyruvate infusion and washout were determined colorimetrically in a Shimadzu dual-wavelength spectrophotometer (337/417 nm, ε=5.65 mol/L cm⁻¹). Plasma (300 µL) was sampled at 10-minute intervals before, during, and after 30 minutes pyruvate infusion (0.05 mmol·kg⁻¹·min⁻¹), flash-frozen in liquid N₂, and extracted in 1 vol 0.6 N HClO₄.

Middle Cerebral Artery Occlusion–Reperfusion Protocol
Transient focal cerebral ischemia was induced by middle cerebral artery occlusion, in which the left middle cerebral artery was occluded by a 3-0 monofilament suture for 2 hours and then reperfused for 24 hours by withdrawing the suture. Rats were randomly assigned to control (normal saline, n=8) or pyruvate (n=12) treatments. Sodium pyruvate (1 mol/L) prepared in sterile water was infused at 0.05 mmol/kg/min for 90 minutes from 60 minutes occlusion until 30 minutes after reperfusion.

At 24 hours postreperfusion, brains were harvested, divided into slices at 3, 5, 7, 9, 11, 13, and 15 mm posterior to the olfactory bulb, stained with 2% 2,3,5-triphenyltetrazolium chloride, and planimetrically analyzed (Image-Pro Plus 4.1; Media Cybernetics, Silver Spring, MD) to determine lesion volume. In another 18 rats (9 control, 9 pyruvate), the cerebral hemispheres were harvested at 24 hours reperfusion, and the cortex and subcortex were separated, snap-frozen in liquid nitrogen, and extracted in buffer containing phosphatase/protease inhibitor cocktail. Proteins were analyzed by immunoblot as described previously.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling Detection of DNA Fragmentation
Paraffin-embedded brain sections (8 µm) were stained for terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling-positive cells with the Dead END Fluorescent kit (Promega, Madison, WI) according to the manufacturer’s instructions and then counterstained with 4’,6-diamidino-2-phenylindole dihydrochloride nuclear stain. The fluorescent signal was visualized and photo-
graphed on a Zeiss fluorescence microscope. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling-positive cells were counted in 20 microscope fields from a single section for each animal using a CAST-Grid system (Nikon, Inc.). Seven microscope fields were selected within frontoparietal cortices and subcortex as previously described.20

Statistical Analysis
Data are expressed as mean values ± SEM. Single comparisons between 2 groups used Student t test. Multiple comparisons between hypoxia groups and with the normoxic control was accomplished by 2-way analysis of variance combined with Tukey multiple comparison test to identify statistically significant differences. Plasma pyruvate concentrations before and after pyruvate infusion were compared by single-factor analysis of variance followed by Tukey multiple comparison test. Probability values < 0.05 were taken to indicate statistically significant effects.

Results
Cytoprotection by Pyruvate Against Hypoxia–Reoxygenation
Pyruvate-induced cytoprotection was tested in C6 glioma and HT22 neuronal cell lines subjected to H/R. Twelve hours of hypoxia and 24 hours reoxygenation killed >50% of C6 cells (Figure 1A). Five and 10 mmol/L pyruvate increased viability of these cells by 39% and 81%, respectively (Figure 1A–B). A similar response was detected in HT22 cells: 10 mmol/L pyruvate increased viability by 40% versus control (Figure 1C).

Pyruvate-Induced EPO Signaling
Components of the EPO signaling cascade were examined in HT22 neuronal and C6 glioma cells to determine if pyruvate can activate cytoprotective signaling. Relative to control, 10 mmol/L pyruvate increased HIF-1α nearly 3- and 2-fold in HT22 and C6 cells, respectively. Pyruvate increased EPO contents 2-fold in HT22 cells (Figure 2A–B) and 2.5-fold in C6 cells (Supplemental data; http://stroke.ahajournals.org). Because EPO primary antibody reportedly crossreacts with heat shock protein,21 EPO mRNA expression was analyzed. Pyruvate increased EPO mRNA abundance in HT22 cells approximately 20-fold both before and after H/R (Figure 2C). Pyruvate increased Akt phosphorylation 4- and 2.5-fold in HT22 neuronal and C6 glioma cells, respectively (Figure 2A–B; Supplemental Figure I) but did not alter Erk-1/2 phosphorylation (Figure 2A–B).

To interrogate the roles of HIF-1α and EPO in pyruvate-induced neuroprotection, HT22 cells were transfected with HIF-1α siRNA, which lowered H/R-induced HIF-1α expression 50% (Figure 3A). HIF-1α siRNA abrogated the cytoprotection of 5 and 10 mmol/L pyruvate (Figure 3B). To further define EPO’s role in protection, HT22 neuronal and C6 glioma cells were incubated with soluble EPO receptor (sEPOR) to bind EPO originating in the cells. This strategy blunted pyruvate-induced protection of HT22 neuronal (Figure 3C–D) and C6 glioma (Supplemental Figure II) cells subjected to H/R.

Pyruvate Cerebroprotection in an In Vivo Stroke Model
The impact of pyruvate infusion on circulating pyruvate was evaluated. Venous pyruvate concentration rapidly increased at the onset of infusion, stabilized at approximately 0.7 mmol/L within 10 minutes, and then subsided after pyruvate infusion was discontinued (Figure 4A). Next, the cerebroprotective effects of pyruvate infusion were examined in rats subjected to 2 hours occlusion and 24 hours reperfusion of the middle cerebral artery occlusion. Pyruvate or normal saline was infused intravenously for 90 minutes, from 60 minutes middle cerebral artery occlusion until 30 minutes reperfusion. Pyruvate sharply attenuated lesion volume by 84% versus control (Figure 4B).
The effect of pyruvate on ischemia–reperfusion-induced DNA fragmentation was examined. In control rats, DNA fragmentation in the subcortex and cortex was sharply increased versus the nonischemic contralateral hemisphere. Pyruvate decreased DNA fragmentation by 80% versus the control (Figure 4C). A significant correlation ($r^2=0.80$) emerged between DNA fragmentation and lesion volume at 24 hours reperfusion (Figure 4D).

**Effect of Pyruvate on EPO Content in the Brain After Stroke**

Erythropoietin contents in the ischemic cortex and subcortex were approximately 4-fold increased versus contralateral nonischemic regions (Figure 5). Pyruvate appreciably increased EPO in the ischemic and contralateral cortex and subcortex versus the respective controls. Interestingly, pyruvate did not increase renal EPO content (Supplemental Figure III).

![Figure 4.](image-url) Pyruvate reduces lesion volume and DNA fragmentation after middle cerebral artery occlusion–reperfusion. A, Plasma pyruvate concentration plateaued during pyruvate infusion and subsided within 20 minutes postinfusion ($P<0.05$ vs preinfusion). Pyruvate treatment during cerebral ischemia–reperfusion sharply lowered brain lesion volume (B) and TUNEL-positive cells (C, percent of DAPI-stained cells) in the cortex and subcortex 24 hours later ($P<0.05$ vs control). D, Linear correlation between TUNEL-positive cells and lesion volume. TUNEL indicates terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; DAPI, 4',6-diamidino-2-phenylindole dihydrochloride.

![Figure 5.](image-url) Cerebral EPO content after transient cerebral ischemia. Ischemia and pyruvate augmented EPO contents separately and more effectively in combination. $^*P<0.05$ vs nonischemic control; $^\dagger P<0.05$ vs ischemic control. EPO indicates erythropoietin.
Discussion

This study tested the hypothesis that pyruvate treatment protects the brain from ischemia/reperfusion injury by evoking EPO synthesis and cytoprotective signaling. In vivo and in vitro results demonstrated that pyruvate treatment (1) increased HIF-1α and EPO; (2) activated Akt; (3) enhanced cell viability; (4) decreased lesion volume; and (5) suppressed DNA fragmentation.

A pivotal transcription factor, HIF-1, mediates cellular adaptations to hypoxia. Transcriptional activity of HIF-1 is controlled by regulating cellular content of its α subunit. In the presence of α-ketoglutarate, Fe²⁺, and normal intracellular O₂ concentrations, prolyl residues in HIF-1α are hydroxylated by prolyl hydroxylase, leading to proteosomal degradation and, thus, preventing assembly of HIF-1 heterodimers. Pyruvate inhibits prolyl hydroxylase by competing with α-ketoglutarate for access to the enzyme’s catalytic domain. Recently, we demonstrated pyruvate-enriched cardioplegia increased HIF-1α, EPO, and EPOR contents and suppressed postbypass inflammation and edema in the myocardium of pigs maintained on cardiopulmonary bypass. In the brain, astrocytes and neurons can synthesize EPO. EPOR are present in the brain parenchyma, and pyruvate readily traverses the BBB. Because preclinical studies have shown exogenous EPO to be cerebroprotective, pyruvate induction of endogenous cerebral EPO production could be powerfully cerebroprotective. In accordance with this hypothesis, pyruvate afforded significant protection in the rat stroke model, manifest as marked reductions in ischemic lesion volume and DNA fragmentation. Moreover, pyruvate sharply increased EPO contents in both the ischemic and nonischemic brain.

Two strategies were used to interrogate the cytoprotective actions of the pyruvate-induced HIF-1/EPO axis. First, transfection of cultured neuronal cells with siRNA against the HIF-1α gene abrogated pyruvate-induced cytoprotection against oxygen–glucose deprivation. Second, incubation with soluble EPOR to bind EPO attenuated pyruvate’s cytoprotective effect, although some residual cytoprotection was noted. These results substantiate the hypothesis (Figure 6) that HIF-1α stabilization and, consequently, EPO induction were pivotal to the pyruvate-induced cerebroprotection.

Soluble EPOR did not completely block pyruvate-induced cytoprotection, suggesting the involvement of additional, EPO-independent mechanisms. Indeed, HIF-1 activates expression of a variety of protective factors, that enable cells to survive hypoxic or ischemic insults. Conceivably, these proteins may confer some cytoprotection even when EPO signaling is disabled. Furthermore, a neuronal EPOR isoform, which forms heterodimers with CD131, has been shown to mediate cytoprotection but not hematopoiesis. Because EPO is a ligand of both classical and neuronal EPOR isoforms, the use of soluble EPOR to bind EPO cannot differentiate the potential contributions of the 2 EPOR isoforms to pyruvate’s cytoprotection.

Although mounting evidence of EPO’s cerebroprotection has been documented in stroke models, EPO’s limited ability to cross the BBB necessitates high dosages for stroke treatment, which might impose hypercoagulability and increased blood viscosity. Pyruvate readily traverses the BBB and enters the brain parenchyma through monocarboxylate transporters in the BBB and plasma membranes. In rats, middle cerebral artery occlusion induced a rapid and substantial increase of monocarboxylate transporters in neurons, astrocytes, and vascular endothelium, which could increase the capacity for pyruvate uptake. On entering the brain parenchyma, pyruvate may stabilize HIF-1α in both neurons and astrocytes, thereby driving endogenous EPO synthesis. The endogenous EPO could then activate EPOR in a paracrine or autocrine fashion, avoiding the rheological consequences of high doses of exogenous EPO.

Induction of EPO signaling might not be pyruvate’s sole neuroprotective mechanism. A natural intermediary metabolite, pyruvate could function as an energy-yielding fuel for adenosine triphosphate production and as an antioxidant that neutralizes reactive oxygen and nitrogen intermediates and enhances reducing power of the glutathione antioxidant system. Given the tight coupling between cerebral blood flow and cerebral metabolism, it is conceivable that the vascular component of the neurovascular unit could also be involved in the protective mechanisms of pyruvate against ischemic stroke. Other pharmacological antioxidants that traverse the BBB also have been found to decrease lesion volume in rodent focal cerebral ischemia. Nevertheless, the fact that HIF-1α siRNA and soluble EPOR abrogated

Figure 6. Proposed pyruvate-induced cerebroprotective mechanisms. See text for details. ROS indicates reactive oxygen species; PHD, prolyl hydroxylase; α-KG, α-ketoglutarate; VHL, von Hippel-Lindau tumor-suppressor protein; (Ub)ₙ, polyubiquitinylation.
pyruvate cytoprotection implicates EPO as an essential component of the protective mechanism and suggests that, without EPO, pyruvate’s energy-yielding and antioxidant properties may not be sufficient to defend the brain from severe ischemia or hypoxia.

Limitations
In this study, the longer-term effects of pyruvate on neuroprotection were not examined. In this regard, intravenous pyruvate administration during cardiopulmonary resuscitation afforded substantial neuroprotection 3 days later and protracted pyruvate-induced protection against focal cerebral ischemia was reported. Other HIF-1-activated cytoprotective proteins could act independently of or in collaboration with EPO to protect the brain from ischemia–reperfusion.

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Disclosures
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References