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# GLUTAMATE DEHYDROGENASE AS A NEUROPROTECTIVE TARGET AGAINST BRAIN ISCHEMIA AND REPERFUSION

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**Abstract—Deregulation of glutamate homeostasis is associated with degenerative neurological disorders. Glutamate dehydrogenase (GDH) is important for glutamate metabolism and plays a central role in expanding the pool of tricarboxylic acid (TCA) cycle intermediate alpha-ketoglutarate (α-KG), which improves overall bioenergetics. Under high energy demand, maintenance of ATP production results in functionally active mitochondria. Here, we tested whether the modulation of GDH activity can rescue ischemia/reperfusion-induced neuronal death in an *in vivo* mouse model of middle artery occlusion and an *in vitro* oxygen/glucose depletion model. Iodoacetate, an inhibitor of glycolysis, was also used in a model of energy failure, remarkably depleting ATP and α-KG. To stimulate GDH activity, the GDH activator 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid and potential activator beta-lapachone were used. The GDH activators restored α-KG and ATP levels in the injury models and provided potent neuroprotection. We also found that beta-lapachone increased glutamate utilization, accompanied by a reduction in extracellular glutamate. Thus, our hypothesis that mitochondrial GDH activators increase α-KG production as an alternative energy source for use in the TCA cycle under energy-depleted conditions was confirmed. Our results suggest that increasing GDH-mediated glutamate oxidation represents a new therapeutic intervention for neurodegenerative disorders, including stoke. © 2016 Published by Elsevier Ltd on behalf of IBRO.**

**Key words: neuroprotection, focal ischemia, reperfusion, energy metabolism, glutamate dehydrogenase.**

## INTRODUCTION

During pathological conditions, such as ischemic stroke, the mismatch between energy consumption and energy production leads to complex energy-dependent cellular damage. ATP levels are depleted within minutes, and when blood flow is restored, it triggers irreversible post-ischemic mitochondrial dysfunction if it does not occur within a certain time frame (Lust et al., 2002; Pundik et al., 2012). In addition, a secondary energy depletion occurs with reperfusion. This event is accompanied by decreased glucose utilization, despite normal blood flow; thus, ATP depletion during reperfusion may be not related to glucose availability (Sims and Muyderman, 2010).

Energy dysfunction induces adaptations in cerebral metabolism, including the utilization of alternative energy sources. For example, intracellular amino acids (e.g., glutamate and glutamine) can be used as metabolic fuel to recover from mitochondrial failure (Pascual et al., 1998; Sonnewald and McKenna, 2002; Stelmashook et al., 2011). Tight control of glutamate handling in the brain is crucial to the maintenance of mitochondrial integrity (Frigerio et al., 2008). Abnormalities in glutamate metabolism are associated with neurodegenerative disease (Malthankar-Phatak et al., 2006; Owen et al., 2009).

Under both normal conditions and high energy demand, glutamate can be converted to alpha-ketoglutarate (α-KG) by glutamate dehydrogenase (GDH) and/or aspartate aminotransferase (AAT) (Yu et al., 1982; McKenna et al., 1996; Sonnewald et al., 1997). GDH is an essential enzyme in the oxidative catabolism of glutamate for enlargement of the pool of tricarboxylic acid (TCA) cycle intermediates and ATP production (Skytt, 2012; Karaca et al., 2015). In brain-specific GDH-null mice (CnsGlud1$^{-/-}$), a reduction in GDH-dependent glutamate oxidation induces a state of central energy deprivation, which changes the whole-body energy balance (Karaca et al., 2015). In contrast, mice over-expressing GDH exhibit smaller infarction lesions and reduced edema volume in an ischemia–reperfusion

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*Abbreviations:* AAT, aspartate aminotransferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GDH, glutamate dehydrogenase; I/R, ischemia and reperfusion; IOA, iodoacetate; OGD, oxygen and glucose deprivation; TCA, tricarboxylic acid; α-KG, alpha-ketoglutarate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PBS, phosphate-buffered saline; FBS, fetal bovine serum; Ara-C, cytosine arabinofuranoside; PMSF, phenylmethylsulfonyl fluoride; βLA, beta-lapachone; BCH, 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid; TTC, 2,3,5-triphenyltetrazolium chloride.

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(I/R) model compared to wild-type (Badawi et al., 2015). In a previous study using proteomics also, an ischemia induced by middle cerebral arterial occlusion increases the GDH expression level (Datta et al., 2011). These findings suggest that the modulation of GDH can be a therapeutic target for neurodegeneration, including stroke. However, elevations in GDH levels and activity are accompanied by increases in depolarization-evoked glutamate release (Michaelis et al., 2011).

Although the oxidative metabolism of glutamate is mainly mediated by GDH, AAT can also produce α-KG from glutamate (Mckenna et al., 2006; Abbrescia et al., 2012; Cooper and Jaitner, 2016). However, in contrast to GDH, AAT would simultaneously drain oxaloacetate from the pool of TCA cycle intermediates (Nissen et al., 2015). Therefore, GDH is an anaplerotic enzyme catalyzing the net synthesis of α-KG and an increase in the total pool of TCA cycle intermediates. Because both enzymes are physiologically reversible, they may not have a preferred direction of reaction *per se*; thus, the direction of the reaction may be driven by the environmental conditions at a particular moment. Intracellular GDH activity is allosterically controlled by numerous metabolic intermediates, including ADP, GTP, and $NAD^+$/NADH (Li et al., 2012; Spanaki et al., 2014). Although the GDH reaction is freely reversible, the direction in the brain *in vivo* is mainly toward glutamate catabolism rather than glutamate synthesis (Skytt et al., 2012; Nissen et al., 2015; Cooper and Jeitner, 2016).

An activator of GDH, leucine analog 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH), can increase glutamate oxidation upon anaplerotic flux (Erecinska and Nelson, 1990). BCH replenishes the pool of TCA cycle intermediates by increasing α-KG production from glutamate (Erecinska and Nelson, 1990). This activity strongly protects cells by increasing ATP production, when beta-cells exhibit impaired energy-producing metabolism (Han et al., 2012). Another potential GDH activator, beta-lapachone (βLA), is derived from natural naphthoquinones isolated from the lapacho tree (*Tabebuia avellanedae*), though no evidence indicates it has regulatory effects on GDH. βLA possesses pharmacological activity as a substrate of NADH:quinone oxidoreductase 1, which increases the $NAD^+$/NADH ratio by accepting two electrons from NADH (Hwang et al., 2009). By regulating the intracellular $NAD^+$/NADH ratio, βLA has a positive effect on mitochondrial functions, rescuing cells with mitochondrial diseases, including mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) (Jeong et al., 2014a). In addition, βLA protects against lipotoxic cardiomyopathy, oxygen-induced retinopathy, spontaneous hypertension, alcoholic fatty liver, and renal I/R injury (Park et al., 2014; Shin et al., 2014; Gang et al., 2014; Oh et al., 2014; Jeong et al., 2014b; Kim et al., 2015) and promotes wound healing (Kung et al., 2008).

In the present study, we investigated whether the activation of GDH improves the intracellular state of metabolic energy and protects against cerebral I/R-induced energy failure. We also investigated whether βLA can rescue I/R-induced neuronal death by activating GDH. We found that increased oxidative glutamate catabolism by GDH activators provides α-KG for the TCA cycle, which increases ATP production and neuronal survival.

## EXPERIMENTAL PROCEDURES

### Focal cerebral ischemia–reperfusion injury

All animal experiments were performed in accordance with the guidelines on the use and care of laboratory animals set forth by the Animal Care Committee at Ajou University. We induced focal cerebral ischemia in 8-week-old male ICR mice (weight 32–33 g) by occluding the left middle cerebral artery using the intraluminal filament technique as described previously (Jang et al., 2009). For occlusion, we inserted a 6-0 nylon monofilament blunted at the tip with an open flame. Sixty minutes after middle cerebral artery occlusion, we achieved reperfusion by carefully withdrawing the monofilament.

Animals were randomly assigned to one of the following five experimental groups: (1) normal, (2) vehicle for βLA-treated group, (3) 50 or 100 mg/kg βLA, (4) vehicle for BCH-treated group, (5) 350 mg/kg BCH (< 0.5% $NH_4OH$ in saline; Sigma, USA). Each group ($n$ = 5–8) was compared to the control group and evaluated for histochemical or biochemical measurements. βLA was administered orally by gavage 3 h before the ischemia period. BCH was injected intraperitoneally 1 h prior to the operation.

### Assessment of cell death

For 2,3,5-triphenyltetrazolium chloride (TTC; Sigma) staining, mice were euthanized 24 h after reperfusion and decapitated. The brains were removed and coronally sectioned into five 2-mm slices using a brain matrix. The slices were immediately stained with 2% TTC for 30 min. Borders were drawn around regions of non-infarcted (red-stained) and infarcted (unstained) tissue and the areas measured using Photoshop and TINA software (KAIST Biotech, Korea).

For Nissl staining, animals were re-anesthetized 6 h after reperfusion and transcardially perfused with a saline solution, followed by fixation solution of 4% paraformaldehyde in phosphate-buffered saline (PBS) for 24 h. The brains were removed and cryoprotected for 2 days in PBS with 30% sucrose. The isolated brains were frozen in an optimal cutting temperature compound and 30-μm-thick coronal sections serially cut on a cryostat. Serial sections were stained with 0.25% Cresyl Violet (pH 3.6; Sigma) and dehydrated in a graded ethanol series (Kim et al., 2008). The stained viable neurons were evaluated in a blinded manner. Neuronal damage was semi-quantitatively estimated using the following grades: grade 0, normal; grade 1, scattered ischemic neurons; grade 2, moderate ischemic damage; grade 3, whole pyramidal cell damage; and grade 4, extensive cell damage in hippocampal regions (Lee et al., 2009).



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