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The Neuroprotection of Kappa Opioid Receptor Agonist BRL52537 is Partly Through Enhancing Endogenous GABA Function

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Abstract

Objective: We previously demonstrated that pretreatment with selective κ-opioid agonist BRL52537 hydrochloride [(κ)-1-(3,4-dichlorophenyl) acetyl-2-(1-pyrrolidinyl) methylpiperidine] provides ischemic neuroprotection following transient focal ischemia in rats. The present study was undertaken to determine whether the neuroprotection of BRL52537 attenuates ischemia-evoked efflux of GABA in the striatum *in vivo* following transient focal ischemia.

Materials and Methods: Using the intraluminal filament technique, halothane-anesthetized male Wistar rats ($n=20$) were subjected to 2 hours of middle cerebral artery occlusion (MCAO). In a blinded, randomized fashion, rats were treated with saline (vehicle) or 1 mg/kg/hr BRL52537 started 30 minutes before MCAO and continued at 0.5 mL/hr until the 22nd hour of reperfusion. We also utilized *in vivo* microdialysis to measure extracellular levels of amino acids including glutamate and GABA in the striatum during the 2 hours of MCAO and 3 hours of reperfusion. Data are presented as mean±standard error of the mean. Statistical analysis was performed using the unpaired Student's *t* test.

Results: Infarct volume of 26.2±3.6% in the cortex and 42.9±4.2% in the striatum were significantly attenuated in the BRL52537 group when compared with the control subjects (42.8±5.7% in cortex; 74.0±3.7% in striatum). Pretreatment with BRL52537 significantly increased microdialysate levels of GABA in the striatum during MCAO and early reperfusion, when compared with the control subjects. However, other amino acids did not show significant changes.

Conclusion: The data demonstrated that BRL52537 provided robust ischemic neuroprotection with altering ischemia-evoked efflux of GABA in the striatum during ischemia and early reperfusion. [*Tzu Chi Med J* 2008;20(4):280–285]

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1. Introduction

The opioidergic system in the brain has been implicated in the pathophysiology of cerebral ischemia [1–4]. Opioid receptors in the central nervous system have been divided into various subtypes, including mu (μ), kappa (κ) and delta (σ). In addition to playing a role in antinociception, several experimental studies have demonstrated that kappa opioid receptor (KOR) agonists attenuate histological brain injury [5–7] as well as improve functional recovery in animal models of global and focal cerebral ischemia [8,9].

Excessive activation of excitatory amino acid receptors is widely accepted as being integral to the pathogenesis of brain damage following ischemia. There is a marked elevation in the extracellular concentrations of glutamate in the neocortex [10–13] and striatum [10,14] during cerebral ischemia, which correlates with the resultant degree of ischemic brain damage [10,13]. However, it has been argued that glutamate in the extracellular fluid may be a consequence rather than a cause of progressive neuronal injury [15]. Of the neurotransmitters, glutamate has been studied the most often in relation to the development of cerebral ischemia-induced cell death. While many neurotransmitter systems play roles in ischemia-induced neuronal death, the γ-aminobutyric acid (GABA)-ergic system may be of particular importance because it functions in opposition to that of glutamate. Compared with glutamate, GABA has received relatively little attention in the area of cerebral ischemia-induced neuronal death and it may be an attractive pathway for neuroprotection after cerebral ischemia.

There is good evidence that GABA exerts an inhibitory tone on glutamate mediated neuronal activity [16] and several different approaches have revealed that enhanced GABA function can inhibit N-methyl-D-aspartic acid (NMDA) receptor-mediated response [17–19]. Thus, enhancing GABAergic inhibitory mechanisms *in vivo* might also be expected to attenuate the excitotoxic process and provide neuroprotection.

We previously demonstrated that the selective KOR agonist, BRL52537 hydrochloride ((±)-1-(3,4-dichlorophenyl) acetyl-2-(1-pyrrolidinyl) methylpiperidine), provides significant neuroprotection when given as a pretreatment as well as when started at the onset of reperfusion in the rat model of middle cerebral artery occlusion (MCAO) [20]. We also demonstrated that BRL52537 provides a long therapeutic opportunity (at least 6 hours) for ischemic neuroprotection without altering ischemia-evoked efflux of dopamine and its metabolites in the striatum during ischemia and early reperfusion [21]. However, the signaling mechanisms utilized by KOR under normal conditions or during neuroprotective actions are not well established *in vivo*. Therefore, we sought to use *in vivo* microdialysis to test the hypothesis that BRL52537 provides neuroprotection by limiting excessive glutamate release while preserving an adequate amount necessary for neurotrophic and neurotransmitter function or the GABA neurotransmission system.

2. Materials and methods

2.1. Animal preparation

The experimental protocols were approved by the Tzu Chi General Hospital Animal Care and Use Committee. All techniques were as previously described. Briefly, adult male Wistar rats (250–300 g) that had fasted from food overnight were anesthetized with halothane 1.0–2.0% for 3–5 minutes in oxygen-enriched air (inspiratory oxygen concentration, 25–30%) and spontaneous breathing was allowed. Using aseptic surgical techniques, the right femoral artery was cannulated with PE50 tubing to monitor arterial blood pressure and sample arterial blood gases; meanwhile, the femoral vein was cannulated and tunneled subcutaneously as well as exteriorized using a swivel for venous access and drug administration. Rectal temperature was maintained using a heat lamp throughout the surgical procedures, during MCAO and during early reperfusion.

2.2. Local cerebral blood flow monitoring

Laser Doppler flowmetry (LDF Model MBF3D; Moor Instruments Ltd., Devon, UK) was used for laser cerebral blood flow (LCBF) measurements. The scalp was incised along the midline, and the skull was thinned using a high-speed drill over the right parietal cortex; coordinates were 2 mm posterior and 6 mm lateral to the bregma for the placement of the LDF probe. To allow for continuous monitoring of the LDF, the headpiece of a specially designed cradle was modified to allow for free rotation around the longitudinal axis of the rat and was equipped with a snout mask for spontaneous ventilation as well as a holder for the LDF probe. The probe was then positioned over an area devoid of large cortical blood vessels, and the position was not changed throughout the experiment. LCBF was serially measured at 5-minute intervals prior to, during cerebral ischemia and until 120 minutes after reperfusion to collect the microdialysates. The incision was stapled, and the animals were then returned to their cages. The LCBF values were calculated and expressed as percentages of the baseline values.

2.3. Transient focal ischemia model

Transient focal ischemia (2 hours) is produced by MCAO by using an intraluminal suture technique as

previously described [20,21], with some modifications. Briefly, the right common carotid artery was exposed through a paramidline incision, and the external carotid artery was ligated. The occipital artery branch of the external carotid artery was coagulated, and the internal carotid artery (ICA) was separated from the vagus nerve. The pterygopalatine artery was ligated with a 4-O silk suture close to its origin. Ischemia was produced by advancing a 4-O monofilament nylon suture, with its distal tip rounded by application of heat, into the ICA through a puncture in the common carotid artery until the LDF signal displayed a significant reduction. After placement, the intraluminal suture was secured with a 4-O silk suture tied around the ICA. Reperfusion was produced by withdrawal of the intraluminal suture; this was associated with rapid restoration of the LDF signal. Our previously reported data demonstrated that a reduction to <40% of the baseline LDF signal was critical in producing consistent infarction volume in our model of MCAO.

2.4. Animal grouping and assessment of infarction volume

We used a blinded randomized method to divide the rats into: Group I—saline (vehicle); and Group II—KOR agonist BRL52537 ($1\,mg \cdot kg^{-1} \cdot hr^{-1}$; from Research Biochemical International, Natick, MA, USA). All infusions start at 30 minutes before MCAO and continued at 0.5 mL/hr until the 22nd hour of reperfusion. Following the closure of the operative sites, the animal was allowed to awaken from the anesthesia. After 22 hours of reperfusion, the rats were deeply anesthetized with 5% halothane and decapitated. The brain was sliced into seven 2-mm thick coronal sections from the frontal pole for staining with 1% triphenyltetrazolium chloride (TTC) in saline at 37°C for 30 minutes [1]. Each slide was drawn using a computer-based image analysis system using the OS/2 operating system (MCID, Imaging Research, Ontario, Canada) and the infarct area was numerically integrated across each section and over the entire ipsilateral hemisphere. Infarct volumes were measured separately in the cerebral cortex and caudoputamen (CP) complex and expressed as percentages of the ipsilateral structure volume as described previously [21,22].

2.5. In vivo cerebral microdialysis

We placed each rat's head in a Kopf stereotaxic frame, and the microdialysis cannulas were placed into the striatum bilaterally. The cannula was advanced to predetermined coordinates of 0.5 mm anterior and 2.5 mm lateral to the bregma at a depth of 6 mm from the dura using a micromanipulator and they were fixed in position using dental cement [21]. Each dialysis probe used in the study was a microdialysis probe (CMA/11; CMA/Microdialysis AB., Stockholm, Sweden) which possessed greater spatial resolution and caused less tissue damage. It consisted of a cuprophane membrane with an outside diameter of 0.24 mm. The outer steel shaft diameter was 0.38 mm. The dialysis membrane diameter was 300 μm and had a molecular mass cut-off of 6000 Daltons. Two hollow silica perfusion tubes were inserted into the dialysis fiber so that the ends were 3 mm apart (for the striatum) and 2 mm apart (for the cortex). The distance between the tips constituted the effective dialyzing area of the cannula. A 2-hour postsurgical equilibration period was given before the experiment began. Microdialysis cannula was perfused with artificial cerebrospinal fluid at 1 μL/min. The *in vitro* recovery from the 3-mm microdialysis probes was 10–15%. Rats were randomized to receive either intravenous infusions of 1 mg/kg/hr BRL52537 or saline (0.5 mL/hr) that were initiated 30 minutes prior to MCAO. Microdialysates were collected in epochs of 15 minutes from equilibration to 2 hours after reperfusion and stored at –80°C. The brains were harvested at 22 hours of reperfusion, sectioned for confirmation of microdialysis probe position and analysis of infarction volume using TTC staining.

2.6. High performance liquid chromatography measurements

An ESA (ESA Inc., Chelmsford, MA, USA) Coulochem high performance liquid chromatography pump, ESA electrochemical detector, and an automatic integrator were used with a reverse phase column. Microdialysates (15 μL samples) were derivated using o-phthaldehyde (OPA) via an ESA or Gilson (Gilson Inc., Middleton, WI, USA) autosampler. The mobile phase, 0.1 M Na_2HPO_4, 0.13 mM Na_2EDTA and 28% methanol, was continuously recycled every 2 weeks and then changed with a fresh solution. The total area under the peaks were integrated and compared with the homoserine internal standard. The full chromatograms of the amino acid profiles were obtained. Peaks for excitotoxic and inhibitory amino acids including glutamate, glycine, taurine, alanine and GABA were well resolved.

2.7. Statistical analysis

Physiological parameters and mean LDF measurements between the groups were subjected to unpaired Student's *t* test. Differences in the infarct volume were determined using one-way ANOVA. *Post hoc* analysis comparisons were made with the Newman-Keuls test. Data are presented as mean±standard error of the mean. For microdialysis experiments, GABA in the

microdialysates between the groups were analyzed using unpaired Student's *t* test.

3. Results

There were no physiologically relevant differences between the two groups for all the monitored variables such as mean arterial blood pressure, pH value, $PaCO_2$, PaO_2, hemoglobin level, rectal and temporalis muscle temperature during the pre-ischemic, ischemic and 30 minutes post-ischemic experimental period (data not shown). Pericranial temperature was not significantly different between the two groups during the experiment (saline 35.6±0.3°C *vs.* BRL 36.0±0.2°C). There were also no significant daily body weight loss differences at 24 hours after the procedures between the two groups (saline 11.0±0.9% *vs.* BRL 13.4±1.0%). LDF-determined cerebral perfusions were not different between the groups during MCAO. Similarly, LDF was promptly restored on withdrawal of the intraluminal sutures during reperfusion in both groups.

TTC-determined infarct volumes were significantly attenuated in both the cortex (26.2±3.6%) and CP complex (42.9±4.2%) in the BRL group as compared with the control subjects (42.8±5.7% in cortex; 74.0±3.7% in CP complex) (Table 1). In the microdialysis experiments, *in vitro* probe recovery for GABA, prior to implantation of the probe, was 26±5% (n=8 probes). A total of 10 animals per group were included in the final analysis. There was almost a time-dependent significant increase in GABA concentration from pre-ischemic values during MCAO when we compared the BRL group with the saline group ($p<0.05$ at 60, 90 and 120 minutes, but $p=0.05$ at 75 and 105 minutes). These values gradually returned to pre-ischemic baseline values at reperfusion. However, there were no differences in the variables of glutamate (Fig. 1) and the other amino acids (glycine, taurine, alanine) in the dialysates with MCAO between the rats treated with saline and BRL.

4. Discussion

The study results demonstrated the following important findings: first, the pre-30 minute intravenous administration of a selective KOR agonist, BRL52537, can provide neuroprotection as seen in previous

Table 1 — Infarction volume between two groups*

Infarct volume (% of ipsilateral structure)	Cerebral cortex	Caudoputamen complex	Hemisphere
Control group	42.8±5.7	74.0±3.7	24.8±2.9
BRL group	26.2±3.6†	42.9±4.2†	15.1±1.7†

*Data presented as mean±standard error of the mean; †$p<0.05$ *vs.* control group, unpaired Student's *t* test.

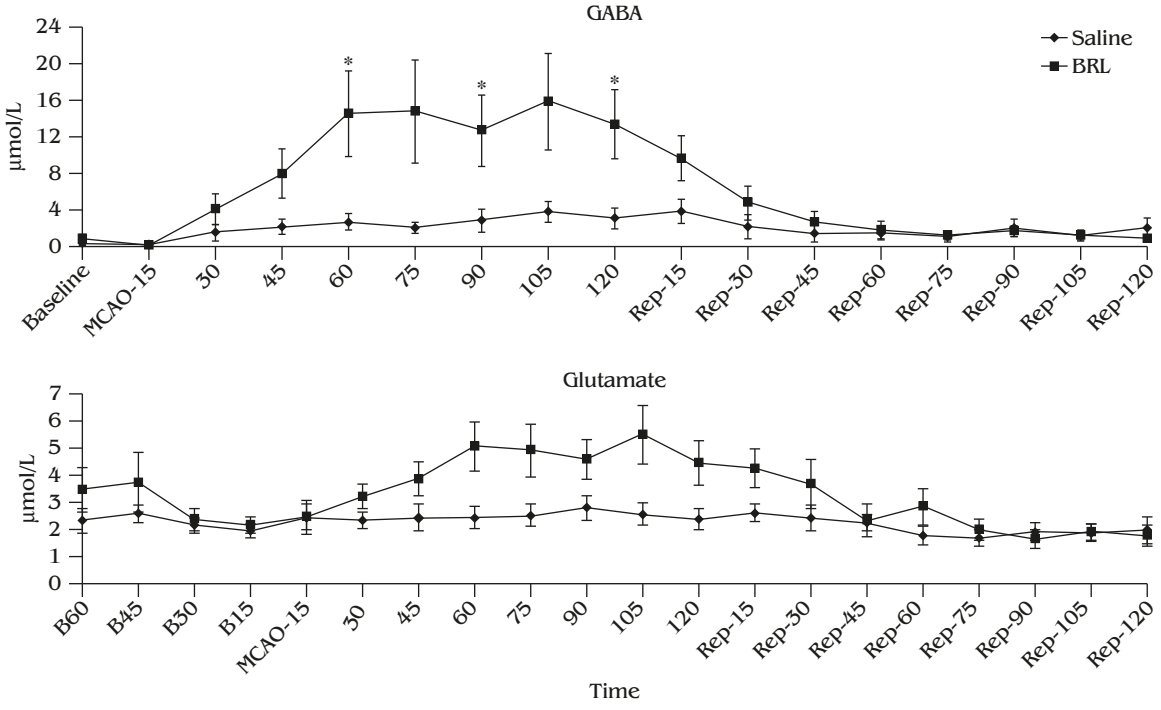

Fig. 1 — Variable values of GABA and glutamate by high performance liquid chromatography during the experiment in both groups. *$p<0.05$ when comparing the two groups. Data presented as mean±standard error of the mean.

studies [20–22]. Secondly, BRL52537, when given as a pretreatment, significantly increased the GABA concentration in the BRL52537-treated group when compared with the control group. However, there were no significant differences in the increases in glutamate during the MCAO period between the groups. The data demonstrated that BRL52537 may provide robust ischemic neuroprotection with altering ischemia-evoked efflux of GABA but not glutamate in the striatum during ischemia and early reperfusion.

KOR agonists as a potential therapy for ischemic neuroprotection have been of interest for several years [5–7]. As seen in previous studies, we have shown that BRL52537 hydrochloride is highly specific for the KOR [20] and provides significant ischemic neuroprotection even when delayed for up to 6 hours of reperfusion after 2 hours of MCAO in rats [21]. In this BRL52537 pretreatment study, we demonstrated that there was neuroprotection without alterations in core body and pericranial temperatures as well as no significant effects on the physiological parameters evaluated within this study.

We did not observe modulation of the ischemic-evoked acute release of dopamine or its metabolites in our previous study [21], though some *in vivo* studies demonstrated that the KOR agonist modulated dopaminergic neurotransmission in the substantia nigra, neostriatum, and the mesolimbic systems [23–25]. Goyagi et al demonstrated that BRL52537 attenuated ischemic-evoked nitric oxide production in the striatum *in vivo* and postulated that this may account for the neuroprotective effects [26], but the precise signaling of this interaction remains unclear. Therefore, several researchers support the hypothesis that the antiexcitotoxic mechanism may be important in the neuroprotection provided by KOR agonists in cerebral ischemia [7,20–22]. It has been demonstrated that KOR agonist modulated glutamate toxicity via inhibition of presynaptic glutamate release, possibly by closing N-type Ca^{2+} channels and also inhibiting excitatory postsynaptic potentials by attenuating presynaptic Ca^{2+} influx [26]. Other researchers reported that the attenuation of glutamate was released with graded ischemia in experimental stroke with a KOR agonist, as well as the modulation of the inhibitory neurotransmitter GABA [27]. There is substantial evidence linking excessive glutamate neurotransmission to the development of neuronal death following ischemic events [28,29]. While many neurotransmitter systems play roles in ischemic neuronal death, the GABAergic system may be important due to its functions as opposition to that of glutamate [29]. GABA has received relatively little attention in the area of ischemic neuroprotection when compared with glutamate. As our study showed in the BRL group, GABA significantly accumulated in the extracellular space during transient cerebral ischemia but glutamate did not significantly increase. In addition, both amino acids gradually returned to normal levels after reperfusion. Extracellular GABA accumulation has been shown using *in vivo* microdialysis and cortical cup techniques with the hippocampus, striatum and cerebral cortex during global cerebral ischemia [30–34]. These results are compatible with our results using the microdialysis technique at the ischemic striatum to extract the GABA concentration.

GABA function may be decreased following an ischemic insult and then presents evidence that some compounds that increase GABAergic function are neuroprotective. Several mechanisms have been proposed for the increased extracellular GABA during ischemia [29,32]. The mechanisms include: (1) depolarization-induced Ca^{2+}-dependent vesicular release; (2) depolarization-induced Ca^{2+}-dependent reversal of GABA transporters; and (3) leakage of GABA from injured, permeable terminals. In addition to depolarization, other factors generated by ischemia can cause accumulation of extracellular GABA like arachidonic acid and reactive oxygen species [29]. There are some questions with regard to the consequences of extracellular GABA accumulation. It may be that accumulated GABA can downregulate GABA synthesis transiently [28] and also be opposite to glutamate function. From our study results, we believe that the KOR agonist, BRL52537, provided neuroprotection, at least partially, by accentuation of the function of the inhibitory neurotransmitter GABA.

In conclusion, the data demonstrated that pretreatment of continuous intravenous infusion of the potent and selective KOR agonist BRL52537 attenuated brain damage in rats subjected to transient focal ischemia. The neuroprotection of BRL52537 is partially through the enhancement of GABA function.

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