Characterization of Neuroprotective Effects of Biphalin, an Opioid Receptor Agonist, in a Model of Focal Brain Ischemia

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ABSTRACT

Approximately 795,000 people experience a new or recurrent stroke in the United States annually. The purpose of this study was to assess the protective effect of a nonselective opioid receptor agonist, biphalin, in brain edema and infarct damage by using both in vitro and in vivo models of stroke. In an in vivo model of ischemia, biphalin significantly decreased edema (66.6 and 58.3%) and infarct (52.2 and 56.4%) ratios in mouse transient (60-min occlusion/24-h reperfusion) and permanent (6 h) middle cerebral artery occlusion models, respectively. Biphalin administration also showed decreased neurodegeneration in hippocampal, cortical, and striatal brain tissue after ischemia, evidenced by reduced Fluoro-Jade C staining. In addition, bi-

phalin improved neurological function after stroke injury evidenced by neurological score and locomotor activity evaluation. Biphalin significantly decreased penumbral expression of Na^+ , K^+ , 2Cl^- cotransporter (NKCC) and the translocation of the conventional isoforms of protein kinase C (PKC). It also reversed the activation of PKC-induced cell volume increase during ischemia in primary neuronal cell cultures exposed to 1 h of oxygen glucose deprivation. These data suggest that opioid receptor activation provides neuroprotection during stroke, and a possible explanation of this mechanism could be the inhibition of NKCC function via the regulation of PKC-dependent cell signaling.

Introduction

Stroke has the third highest mortality rate of 160,000 individuals per year in the United States (Writing Group Members et al., 2010). Despite considerable preclinical and clinical research efforts, tissue plasminogen activator is the only Food and Drug Administration-approved treatment for stroke. However, tissue plasminogen activator has a short therapeutic time window of ~3 to 4.5 h, and limited number of patients qualify for this lifesaving therapy (3–5%) (Writing Group Members et al., 2010). More than 700 drug candidates have been investigated and shown to be effective in preclinical stroke studies; however, the majority have failed at different phases of clinic trials (Durukan and Tatlisumak, 2007). Because previous stroke trials have demonstrated

that drugs targeting one or only a few signaling pathways fail to improve clinical outcome after stroke, drugs with multimodal actions have been suggested to overcome this challenge (Minnerup and Schäbitz, 2009).

In stroke, the affected brain parenchyma develops discrete regions known as ischemic core and penumbra. At the core of the stroke lesion, cell death starts within minutes, resulting in energy store depletion, leading to ionic imbalance and severe metabolic failure (Durukan and Tatlisumak, 2007). Penumbra surrounds the core area with limited collateral blood flow. As the injury progresses in penumbral tissue, additional cell death occurs, leading to neurological functional deficits or death. Therefore, it is important to have a complete understanding of the changes in biochemical cascades initiating cell death in penumbra to identify viable therapeutic targets for reducing injury progression and promoting recovery (Pestalozza et al., 2002).

For this study we chose to target multiple opioid receptors (ORs), which consist of three major types: δ -opioid receptor (DOR), μ -opioid receptor (MOR), and κ -opioid receptor (KOR) (Lord et al., 1977). Among a number of important functions,

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ABBREVIATIONS: OR, opioid receptor; DOR, δ -opioid receptor; MOR, μ -opioid receptor; KOR, κ -opioid receptor; NKCC, Na^+ , K^+ , 2Cl^- cotransporter; OGD, oxygen glucose deprivation; TTC, 2,3,5-triphenyltetrazolium chloride; CCA, common carotid artery; MCA, middle cerebral artery; MCAO, MCA occlusion; tMCAO, transient MCAO; pMCAO, permanent MCAO; DAPI, 4'-6-diamidino-2-phenylindole; FJC, Fluoro-Jade C; DHE, dihydroethidium; PKC, protein kinase C; PVDF, polyvinylidene difluoride; TBS, Tris-buffered saline; TBST, TBS containing 0.1% Tween 20; PMA, phorbol 12-myristate 13-acetate; HBSS, Hanks' balanced salt solution; U-50488, 2-(3,4-dichlorophenyl)-N-methyl-N-[(1R,2R)-2-pyrrolidin-1-ylcyclohexyl]acetamid.

a great deal of interest has been focused on understanding the neuroprotective effects of different OR agonists during stroke. KOR agonists have been shown to modulate glutamate toxicity by inhibiting presynaptic glutamate release in vitro (Bradford et al., 1986) via closure of N-type Ca^{2+} channels and restriction of Ca^{2+} entry into presynaptic terminals (Gross and Macdonald, 1987). In addition, some studies suggested that κ -receptor activation decreases release of nitric oxide by inhibiting N-methyl-D-aspartate-evoked nitric-oxide synthesis in ischemic brain in vivo (Goyagi et al., 2003). Other reports have demonstrated that DOR agonists also exhibit neuroprotection by decreasing the release of glutamate (Zhang et al., 2000) or attenuating oxidative damage (Yang et al., 2009) during ischemia.

The opioid system has been reported to be differentially expressed after cerebral ischemia (Boutin et al., 1999). The density of DOR has been shown to decrease between 3 and 12 h, and the density of MOR and KOR has been shown to decrease after 12 h of permanent middle cerebral artery occlusion (pMCAO) in mice (Boutin et al., 1999). We hypothesize that during early ischemic injury neuroprotective effects can be achieved through the activation of DOR, MOR, and KOR. Our previous observations supported the above notion indicating that a nonselective OR agonist, biphalin, exhibited enhanced antiedematous effects, compared with selective DOR, MOR, and KOR agonists in hippocampal slices subjected to oxygen-glucose deprivation (OGD) and these effects were reversed by naloxone (Yang et al., 2011).

Biphalin is a dimeric enkephalin analog (Tyr-D-Ala-Gly-Phe-NH)₂ with potent, nonselective OR agonist activity. It has high affinity for MOR and DOR and low affinity for KOR (Lazarczyk et al., 2010), with the ability to reach both spinal and supraspinal sites expressing MOR and DOR (Abbruscato et al., 1997). Based on these pharmacologic and kinetic features, we hypothesize that biphalin can decrease edema formation and exhibit neuroprotective effects after ischemic stroke.

The mechanism of OR agonist neuroprotection in stroke is still not clear. Acute activation of OR can cause inhibition of protein kinase C (PKC) in neurons exposed to the OGD condition (Liu et al., 2008), affecting the phosphorylation state and activity of various proteins including ion transporters. One such ion transporter, NKCC, has been shown to play an important role in edema formation during stroke (Kahle et al., 2009). We and others have shown PKC-dependent regulation of NKCC activity during OGD conditions in brain endothelial cells (Vigne et al., 1994; Yang et al., 2006). Thus, we construe that biphalin may also decrease ischemia-induced edema formation through the inhibition of NKCC function by regulating PKC activity in neurons because NKCC has been shown to play a key role in cellular brain edema (Kahle et al., 2009).

In summary, the purpose of this study was to identify the neuroprotective effect of biphalin and elucidate its possible mechanisms via a well characterized in vivo focal ischemic stroke model, MCAO, and an in vitro mouse primary neuronal cell culture model.

Materials and Methods

Animals and Surgical Procedures. All studies were approved by the Institutional Animal Care and Use Committee of Texas Tech

University Health Sciences Center and were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996). CD-1 male mice (body weight in the range of 25–30 g), obtained from Charles River Laboratories, Inc. (Wilmington, MA), were kept under standardized light and dark conditions (12 h), humidity (70%), and temperature (22°C).

In Vivo MCAO Model. MCAO was performed using CD-1 male mice (25–30 g) based on previously reported procedures (Yang et al., 2011) with a slight modification. In brief, surgery was performed using a Zeiss OPMI pico I surgical microscope (Carl Zeiss GmbH, Jena, Germany) on an S100 suspension system with a 3CCD Toshiba (Tokyo, Japan) digital camera, connected to a high-definition LCD television monitor via a movie box. Temperature was maintained at 37°C, controlled by the thermostatic blanket (TCAT-2DF; Physitemp Instruments, Inc., Clifton, NJ). The mice were anesthetized with 4% isoflurane in the induced phase by inhalation and maintained at 1 to 1.5% isoflurane in a nitrous oxide/oxygen 70/30 mixture using a face mask and a SurgiVet vaporizer (Smiths Medical North America, Waukesha, WI). First, the skull was exposed by a midline incision on the head, and a needle Doppler probe (Moor Instruments, Wilmington, DE) was placed on the skull directly over the territory of the left MCA perfusion area of the cortex (1 mm posterior and 3 mm lateral to bregma), and baseline cerebral blood flow was measured. A midline incision was made at the neck approximately 1.5 cm long, and the left carotid bifurcation and common carotid artery (CCA) were isolated from the adjacent tissue. After the careful isolation of external carotid artery branches of the occipital and the superior thyroid arteries, as well as the internal carotid artery branch (the pterygopalatine artery), these arteries were exposed, electrocoagulated, and cut. The left CCA and internal carotid artery were carefully separated from the adjacent vagus nerve and connective tissue to prepare for clip application. After occlusion of CCA by a microclip, the left external carotid artery was ligated, coagulated, and cut distally to the cranial thyroid artery, and a 5-0 nylon monofilament suture for permanent and 6-0 for transient model with a round tip (0.2–0.25 mm) of 13 mm 5-0 nylon monofilament was introduced gently up to ~8.5 to 9 mm toward the origin of the MCA. Successful occlusion was verified by laser Doppler flowmetry, and the local cerebral blood flow in the left MCA territory declined to approximately 10 to 15% of the baseline. After the successful occlusion, the nylon monofilament was secured in the place by ligation. Then the incision was closed by microsurgical clips.

For the pMCAO model, the monofilament was kept in the vessel for 6 h. For the tMCAO model, after 60 min of occlusion, the incision was opened. The suture was withdrawn up to the left carotid bifurcation to allow the brain to be reperfused. Animals were excluded from the experimental group if cerebral blood flow did not recover up to 70% of baseline within 10 min after the start of reperfusion. Reperfusion lasted for 24 h with cages placed under a heating lamp for the first 2 h to prevent hypothermia.

Biphalin (5.7 $\mu\text{mol/kg}$ i.p.) or saline was administered 60 min after occlusion in the pMCAO model and 10 min after reperfusion in the tMCAO model.

2,3,5-Triphenyltetrazolium Chloride Staining and the Evaluation of Infarct and Edema Ratios. 2,3,5-Triphenyltetrazolium chloride (TTC) staining was carried out to identify viable and non-viable brain tissue. Because TTC stained with the intact mitochondrial, the dark red area indicated the living tissue, whereas infarct tissue areas remained unstained (white) (Gorgulu et al., 2000). The animals were anesthetized with overdoses of isoflurane (4%) and euthanized through cervical dislocation. The brain was quickly removed after 6-h occlusion or 24-h reperfusion in pMCAO and tMCAO, respectively, and sectioned into 1-mm-thick slices. Then slices were incubated in a 1% solution of TTC in phosphate-buffered saline at 37°C for 10 min. After TTC staining, slices were scanned, and areas of the infarcted regions were quantified for each slice using image analysis software (Image J 1.30 and Scion Image version Beta

4.0.2; National Institutes of Health, Bethesda, MD; downloadable from <http://rsb.info.nih.gov/ij/download.html>). We carried out the measurement of the damaged (ischemic) hemisphere, compared with the contralateral (nonischemic) hemisphere (Sydserff et al., 1996). This comparison of both hemispheres provided the chance to use every mouse as its own control to minimize the interexperimental error described previously (Sydserff et al., 1996). Six to eight mice were used for each experiment group. To avoid the overestimation of infarcted area because of brain swelling, three measurements were made on each slice for calculation of the size of lesion to correct the error. The infarct area (I) (mm^2), corrected for swelling, was calculated by using the following equation: $I = X - S$, where X is the area of infarct (mm^2), S is brain swelling (mm^2), $S = Y - Z$, Y is the area of the infarcted (ipsilateral) hemisphere slice (mm^2), and Z is the area of the noninfarcted (contralateral) hemisphere slices (mm^2). The infarct and edema ratio are presented by the percentage of the ipsilateral to the contralateral hemisphere.

Histological Staining. Fluoro-Jade C (FJC) is an anionic dye that specifically stains the soma and neurites of degenerating neurons by binding to a currently unknown basic substance in neuronal cells (Schmued et al., 2005). Dihydroethidium (DHE) is a lipophilic cell-permeable dye that is rapidly oxidized to ethidium in the presence of free radical superoxide. Ethidium is fixed by intercalation into DNA. Hence, it is an indication of oxidant stress. Because 4'-6-diamidino-2-phenylindole (DAPI) is a fluorescent stain that binds strongly to DNA, we used it to detect nuclei.

DHE (1 mg/kg) was administered in 1% dimethyl sulfoxide by intraperitoneal injection 1 h before sacrificing the animals (Suh et al., 2008). Mice were anesthetized with an overdose of isoflurane, followed by intracardiac perfusion with 30 ml of cold normal saline and 40 ml of 4% paraformaldehyde in phosphate-buffered saline, pH 7.4. The brains were removed by decapitation and kept overnight in 4% paraformaldehyde solution at 4°C. Brains were cut into 40- μm coronal sections by a Leica VT1000S vibratome (Leica Microsystems Inc., Bannockburn, IL). The slices were mounted on Golden Plus slides (Thermo Fisher Scientific, Waltham, MA) and dried at room temperature in the dark. Then the slides were stained with 0.06% potassium permanganate for 5 min. After the slices were washed with distilled water for 5 min, slices were incubated in the solution with FJC (0.0001% solution) and DAPI (0.5 $\mu\text{g}/\text{ml}$) for 10 min, followed by 3×1 -min wash with distilled water. Slices dried naturally at room temperature without light. The images were acquired with a MacroView Microscope (Olympus America Inc., Center Valley, PA) using Slidebook software (version 4.0; Intelligent-Imaging, Denver, CO).

Neurological Score Evaluation. Neurologic examinations were carried out just after 24-h reperfusion in tMCAO. The neurologic findings were scored on a four-point scale (Hara et al., 1996). Mice were held gently by the tail, suspended 50 cm above the desk and monitored for forelimb flexion. A score of 0 indicated no neurologic deficit, with mice extending both forelimbs toward the floor. Score 1 indicated mild focal neurologic deficit (animal showed forelimb flexion), mice flexed the forelimb contralateral to the injured hemisphere without other abnormality. Then mice were put on a large sheet of soft, plastic paper, which they gripped firmly by their claws. Holding the tail, gentle lateral pressure was used to push the mice behind their shoulders until the forelimbs slid several inches. The same maneuver was performed several times in each direction. Score 2 indicated moderate focal neurologic deficit (decreased resistance to lateral push and forelimb flexion without circling); after this, mice were released and then allowed to move freely to observe the circling behavior. Score 3 indicated severe focal deficit (animal showed the same behavior as in score 2, plus circling) (Hara et al., 1996).

Locomotor Activity Measurements. After treatments, behavioral activity assays were carried out using VersaMax animal monitors (Accuscan Instruments Inc., Columbus, OH), which have been used to monitor stroke behavioral outcome using several locomotor parameters and behavioral deficits that are predictive of ischemic

damage (Vendrame et al., 2004). The chamber cage was $42 \times 42 \times 30$ cm and made of clear Plexiglas glass with holes for ventilation. Infrared monitoring sensors were located every 2.5 cm along the perimeter and 2.5 cm above the bottom. Another two sets of 16 sensors were located 8.0 cm above the bottom on the opposite sides. Data were collected and input into a VersaMax analyzer (Accuscan Instruments Inc.), which transferred the signals to the computer that ran the VersaMax and Versadata programs. Those programs tabulated and processed the variable parameters related to locomotor behavior. In our study, eight different locomotor parameters were collected every 4 min for 20 min, which have been proven to reflect behavioral changes associated with neurological deficits induced by MCAO previously (Vendrame et al., 2004). Before the experiment, mice were put into the chamber to acclimate to the environment. All behavioral assays were carried out between 12:00 noon and 5:00 PM. Every group had six to eight mice.

Sample Preparation for Western Blot. At the end of experiment, the 6-h pMCAO animals were anesthetized with overdoses of isoflurane and euthanized by cervical dislocation. Brains were quickly removed after 6 h of MCAO and sectioned into 3-mm-thick slices, as first, second, and third from rostral to caudal direction. Then first and third slices were incubated in a 1% TTC solution in phosphate-buffered saline to determine the ischemic lesion described as above. The ring sample of 30 mm^2 was chosen around the core area and identified as the penumbra area. The brain was homogenized with a glass homogenizer with seven volumes of homogenization buffer (20 mM Tris-HCL, pH 7.5, 2 mM EDTA, 10 mM EGTA, 250 mM sucrose, and 0.7% protease and phosphatase inhibitor cocktails; Sigma-Aldrich, St. Louis, MO) (Shimohata et al., 2007). The homogenate was centrifuged at 100,000g for 30 min at 4°C. The supernatant was collected as the cytosolic protein fraction. The remaining pellet was resuspended in the same homogenization buffer containing 1% Triton X-100 and centrifuged at the same speed (100,000g) for 30 min at 4°C. The supernatant was collected and treated as the particulate (membrane) fraction.

PKC Translocation and NKCC Expression Assay by Western Blots. Protein concentration of isolated cytosolic and particulate samples was determined using a bicinchoninic acid assay (Thermo Fisher Scientific). Exactly 15 μg of protein of sample was loaded and separated using an 8% Tris-glycine polyacrylamide gel (Novex, San Diego, CA). This method has been used previously for the analysis of Western blot immunoreactivity (Yang et al., 2006). Protein samples were then transferred to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) and then membranes were incubated in blocking buffer [Tris-buffered saline (TBS) containing 5% nonfat dry milk] to block the non-specific protein bands for 2 h at room temperature. Rabbit peptide-specific antibodies (Ig G fraction), PKC α , PKC β I, PKC β II, and PKC γ isoforms were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The PVDF membranes were incubated with the PKC polyclonal antibody at a dilution of 1:1000 in TBS containing 0.1% Tween 20 (TBST) with 1% bovine serum albumin for 2 h at room temperature or 4°C overnight. After $3 \times$ washing with TBST, the membranes were incubated with anti-rabbit Ig G-horseradish peroxidase secondary antibody (1:5000) in TBST with 1% milk for 2 h at room temperature. After 3×10 -min wash with TBST, PKC isoform signals were detected by enhanced chemiluminescence-detecting reagents, Western Lightning (PerkinElmer Life and Analytical Sciences, Waltham, MA).

We used T4 monoclonal antibody to detect NKCC. This antibody was developed against the carboxyl-terminal 310 amino acids of the human colonic NKCC and purchased from the Developmental Studies Hybridoma Bank (Iowa City, IA), and the PVDF membranes were probed with NKCC antibody at a dilution of 1:200 in TBST with 5% milk. The secondary antibody used was anti-mouse Ig G-horseradish peroxidase secondary antibody. The incubation, washing, and detection steps were the same as above in the detection of PKC.

The density of the chemiluminescence band was quantified using Bio-Rad Quantity One software (version 4.5.2) (Bio-Rad Laboratories, Hercules, CA).

Mouse Primary Cortical Neuronal Cultures. Mouse primary cortical neurons were isolated and cultured essentially as described previously (Yang et al., 2011). In brief, cerebral cortices were obtained from embryonic day 16 or 17 embryos (CD-1 mice; Charles River Laboratories, Inc.) and dissected in Hanks' balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+} supplemented with 10 $\mu\text{g}/\text{ml}$ gentamycin. Dissected pieces of cortices (free of meninges) were digested in trypsin (+deoxyribonuclease in HBSS) for 15 to 20 min at room temperature, neutralized with trypsin inhibitor, and washed three times with HBSS. Dissociated cell suspensions were transferred into 60-mm Petri dishes ($0.5\text{--}0.7 \times 10^4$ per cm^2 surface area) coated with polyethylenimine and cultured in Neurobasal medium (Invitrogen, Carlsbad, CA) supplemented with 1.3 mM L-glutamine, 25 $\mu\text{g}/\text{ml}$ gentamicin, and 2% B27 (Invitrogen) at 37°C in a humidified atmosphere of 5% CO_2 in air. Half of the medium was replaced with fresh Neurobasal medium after overnight incubation (+supplements) and refreshed likewise twice a week.

Cell Volume Measurements. For OGD treatment, 7- or 8- in vitro-day-old primary cortical neurons were exposed to the Earle's balanced salt solution (140 mM NaCl, 5.36 mM KCl, 0.83 mM MgSO_4 , 1.8 mM CaCl_2 , 1.02 mM NaH_2PO_4 , and 6.19 mM NaHCO_3) to create an aglycemic condition. Hypoxia was induced as described previously (Yang et al., 2011) by placing the cells in a custom-made hypoxic polymer glove box (Coy Laboratories, Grass Lake, MI), which was infused with 95% N_2 and 5% CO_2 , and the temperature was maintained at 37°C . Biphalin with or without the nonselective opioid receptor antagonist naloxone (0.1 mM) (Sigma-Aldrich) were added to the Earle's balanced salt solution at the start of OGD at a concentration of 0.01 nM.

Primary neurons grown in normal conditions (specified above) served as normoxic controls. Biphalin (0.01 nM) with or without the nonselective opioid receptor antagonist naloxone (0.1 mM) were incubated in the neurobasal medium for 1 h. PKC activator phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) was incubated in medium during the last 30 min.

Intracellular volume of cells was determined by the radio isotopic evaluation method described previously (O'Donnell, 1993), using [^{14}C] urea to measure the total intracellular water space and [^{14}C] sucrose as a marker of extracellular water space. In brief, neuronal cells cultured on 60-mm Petri dishes were equilibrated with HEPES-buffered minimal essential medium (140 mM NaCl, 5.36 mM KCl, 0.81 mM MgSO_4 , 1.27 mM CaCl_2 , 0.44 mM KH_2PO_4 , 0.33 mM Na_2HPO_4 , 4.4 mM NaHCO_3 , 5.55 mM glucose, and 20 mM HEPES) (Schomberg et al., 2001) at 37°C for 15 min in a shaking water bath with or without the NKCC1 inhibitor bumetanide (20 μM) (Sigma-Aldrich). The cells were then incubated with HEPES-buffered minimal essential medium containing either [^{14}C]urea (1 $\mu\text{Ci}/\text{ml}$) or [^{14}C]sucrose (0.1 $\mu\text{Ci}/\text{ml}$) for 5 min, followed by aspiration of the medium and rapid triple rinse by 0.1 M ice-cold MgCl_2 . Next, the cells were lysed with 1 ml of 1% Triton X-100 in 10 mM NaOH, and 500- μl lysates were used to measure cell-associated radioactivity by a liquid scintillation counter. The total protein level in each Petri dish was measured using the bicinchoninic acid assay (Thermo Fisher Scientific). Cell volume was calculated by subtracting the extracellular space value (determined by [^{14}C]sucrose) from the intracellular water space value (determined by [^{14}C]urea) (O'Donnell, 1993).

Statistical Analysis. All data are expressed as the mean $\pm$ S.E.M. The values were analyzed by one-way analysis of variance and post hoc analysis using Newman-Keuls multiple comparison, Mann-Whitney test, and *t* test (Prism, version 5.0; GraphPad Software Inc., San Diego, CA). *P* values less than 0.05 were considered statistically significant.

Results

Effects of Biphalin on Brain Edema and Infarct Formation Induced by tMCAO. The ischemic brain hemisphere showed significant swelling compared with the contralateral hemisphere, indicating significant edema formation during stroke (data not shown). Treatment with biphalin (given to animals 10 min after reperfusion at the concentration of 5.7 $\mu\text{mol}/\text{kg}$ i.p.) decreased infarct formation in tMCAO, evidenced by TTC staining (Fig. 1A). The infarct ratio was decreased by 52.2% reduction with biphalin treatment in the tMCAO model ($P < 0.05$; Fig. 1B). At the same time, the edema ratio showed a statistically significant decrease in the biphalin-treated group compared with the control group (66.6% reduction; $P < 0.05$; Fig. 1C).

Effects of Biphalin on Brain Edema and Infarct Formation Induced by pMCAO. Treatment with biphalin (given to animals 60 min after occlusion at the concentration of 5.7 $\mu\text{mol}/\text{kg}$ i.p.) decreased infarct formation in pMCAO, evidenced by TTC staining (Fig. 2A). The infarct ratio was significantly decreased with biphalin treatment (56.4% reduction; $P < 0.05$; Fig. 2B). At the same time, the edema ratio showed a statistically significant decrease in the biphalin-treated group compared with the control group (58.3% reduction; $P < 0.05$; Fig. 2C).

Effects of Biphalin on Neurological Damage after tMCAO Model. Neurological evaluation was carried out in tMCAO groups. Biphalin administration 10 min after reperfusion significantly improved the neurological behavior of animals compared with the vehicle-treated control group (31.7% improvement in tMCAO groups; $P < 0.05$; Fig. 3).

Histological Staining. Biphalin treatment decreased FJC-positive neuronal cells compared with the vehicle-treated control group in different regions of the brain: hippocampus, cortex, and striatum in tMCAO (Fig. 4).

Similar results were obtained in DHE staining experiments. Biphalin treatment reduced DHE fluorescence signals in the hippocampus, cortex, and striatum compared with vehicle-treated groups.

Locomotor Activity Measurement. A set of locomotor activity parameters were monitored by a VersaMax Monitoring System, including horizontal activity, vertical activity, total distance, number of movements, movement time, stereotypy time, stereotypy counts, stereotypy time, and center distance. All eight parameters were significantly decreased with vehicle-treated animals subjected to the tMCAO model compared with the sham animal group ($P < 0.05$). With biphalin treatment 10 min after reperfusion, all locomotor parameters were significantly improved, except stereotypy time compared with the MCAO group ($P < 0.05$). (Table 1).

Effects of Biphalin in Decreasing Translocation of Conventional Isoforms of PKC. Because PKC membrane translocation is a prerequisite for its activation, we measured particulate and cytosolic PKC isoform immunoreactivities in the pMCAO model. With biphalin treatment, the amount of particulate (membrane)-associated conventional isoforms of PKC decreased in the penumbra area concomitant with the increase in the cytosolic fraction compared with the vehicle-treated group. Biphalin significantly decreased the translocation of PKC from the cytoplasm to membrane in conventional isoforms βI , βII , and γ (Fig. 5B). In contrast to the penumbra area, the cytosolic and particulate (membrane)

Fig. 1. Effect of intraperitoneal injection of biphalin (5.7 μ mol/kg, administered 10 min after reperfusion) or its vehicle on edema formation and infarct ratio in a transient MCAO model (60-min occlusion, 24-h reperfusion). A, representative TTC staining of vehicle- and biphalin-treated animal brains. B, infarct ratio in vehicle- and biphalin-treated animal brains. C, extent of edema formation in vehicle- and biphalin-treated animal brains. *, $P < 0.05$; $n = 6-8$ per group.

Fig. 2. Effect of intraperitoneal injection of biphalin (5.7 μ mol/kg, administered 60 min after the occlusion) or its vehicle on edema formation and infarct ratio in the 6-h permanent MCAO model. A, representative TTC staining of vehicle- and biphalin-treated animal brains. B, infarct ratio in vehicle- and biphalin-treated animal brains. C, extent of edema formation in vehicle- and biphalin-treated animal brains. *, $P < 0.05$; $n = 6-8$ per group.

protein expression of the conventional PKC isoforms did not significantly increase or decrease in the ischemic core area.

Effects of Biphalin in Decreasing the Expression of NKCC. In the vehicle-treated group subjected to pMCAO, the particulate (membrane) fraction expression of NKCC was significantly increased in the penumbral brain area compared with the sham animal group ($P < 0.01$; Fig. 6). With biphalin treatment, NKCC expression was significantly decreased compared with the vehicle-treated group. We did not observe significant changes in the expression of NKCC in the

core brain areas with the vehicle or biphalin treatment groups.

Effects of Biphalin in Decreasing the Cell Volume in Primary Neuronal Cells by Inhibiting PKC. The PKC activator PMA induced significant cell volume increases within 15 min to 2 h, and this effect was decreased in 3-h incubation (Fig. 7A). The nonselective OR agonist biphalin (0.01 nM) significantly reduced the level of urea uptake with the induction of PMA ($P < 0.05$; Fig. 7B). In addition, the nonselective opioid antagonist naloxone reversed the ob-

Fig. 3. Neurological score evaluation of intraperitoneal injection of biphalin (concentration was 5.7 $\mu\text{mol/kg}$, administered 10 min after the reperfusion) or its vehicle treated in the transient MCAO model (60-min occlusion, 24-h reperfusion). *, $P < 0.05$; $n = 6$ –8 per group.

served effects of biphalin (0.01 nM) with the induction with PMA ($P < 0.05$; Fig. 7B). The NKCC1 inhibitor bumetanide decreased PMA-induced increase of urea uptake (Fig. 7B). OGD-exposed primary neuronal cells showed a significant increase in urea uptake ($P < 0.01$; Fig. 7C) compared with normoxic controls. The nonselective OR agonist biphalin decreased urea uptake during the OGD condition. Furthermore, the NKCC1 inhibitor bumetanide also decreased urea uptake in OGD conditions (Fig. 7C).

Discussion

In this study, we confirmed that OR activation with a nonselective OR agonist, biphalin, exerts antiedematous and neuroprotective effects during stroke by using both in vivo pMCAO and tMCAO models as well as in vitro primary neurons exposed to OGD.

During ischemic stroke, reduction of cerebral flow triggers a complex cascade of events, eventually causing cell apoptosis and necrosis. Immediate injury begins with decreased nutrient supply, which causes energy-dependent Na^+/K^+ -ATPase and $\text{Ca}^{2+}/\text{H}^+$ -ATPase shutdown, leading to cellular ionic imbalances. The imbalance of Na^+ , Ca^{2+} , and Cl^- causes cytotoxic edema (Durukan and Tatlisumak, 2007). Intracellular calcium overload upon onset of injury via calcium channels and intracellular release from the endoplasmic reticulum, mitochondria, synaptic vesicles, etc., causes enhanced activation of PKC, phospholipase A2, and phospholipase C and in turn activates various proteases and endonucleases, further leading to blood-brain barrier disruption, membrane phospholipid degradation, and generation of reactive oxygen species, eventually causing apoptosis and necrosis (Durukan and Tatlisumak, 2007). Furthermore, excitatory amino acids, especially glutamate, are released to further increase the intracellular Ca^{2+} , Na^+ , and Cl^- levels that amplify the ischemic damage.

Classically, OR activation has been shown to result in the inhibition of calcium entry via reducing voltage-gated L-type calcium channels (Moises et al., 1994) and N-type and P-type calcium channels (Gross and Macdonald, 1987). Meanwhile,

this activation exhibits the inhibition of glutamate release and response in cultured neuronal cells (Zhu and Pan, 2005). In the present study, we administrated biphalin (5.7 $\mu\text{mol/kg}$ i.p.) in pMCAO and tMCAO animals 60 min after occlusion and 10 min after reperfusion, respectively. It is important that we tested the neuroprotective effect of biphalin at a clinically relevant time window (after ischemia). To detect the ischemic core tissue, we used TTC staining as a marker of tissue dehydrogenase and mitochondrial dysfunction. We also used FJC staining to detect the progression of neurodegeneration and DHE staining to assess free radical damage in penumbral tissue. As expected, biphalin not only decreased neurodegeneration but also reduced free radical damage in penumbra areas in the cortex, hippocampus, and striatum, as shown by FJC and DHE staining in tMCAO (Fig. 4). The free radical scavenging effect of OR activation could be supported by the previous reports showing decreased nitric-oxide production via reducing glutamate release (Goyagi et al., 2003) or increased activity of antioxidant enzyme (superoxide dismutase and glutathione-Px) (Yang et al., 2009) after OR activation.

Because the behavioral changes after stroke have high relevance with respect to patient recovery, we assessed the neurological functional improvement in biphalin-treated stroke animals by classic neurological score method and the VersaMax animal monitoring system. The qualitative assessment of animals using the classic neurological deficit score was further confirmed with various quantitative assessments of locomotor functions, which might not be evident from merely assigning scores by the classic method. Biphalin treatment significantly improved not only neurological function but also locomotor parameters, including horizontal activity, total distance, number of movements, movement time, stereotype counts, and center distance, etc., which further supports our observation of the neuroprotective effect of biphalin treatment.

Many investigative compounds belonging to different pharmacological classes have been shown to exhibit neuroprotective effects, using preclinical MCAO models. However, most studies dealt with infarct volume rather than brain edema. Nevertheless, enclosed brain structures and the paucity of lymphatic drainage within the cranium make the brain particularly sensitive to small changes in brain water. Brain edema is the predominant cause of neurologic deterioration among the mass effects (Bounds et al., 1981). Prolonged edema from brain ischemia can cause life-threatening brain herniation of the vital structures. The clinical administration of mannitol and glycerol is restricted to the early stage of stroke, before blood-brain barrier breakdown, and exhibits the major drawbacks, such as rebound intracranial pressure or increase in cerebral volume with the secondary edema (Goluboff et al., 1964; Larsson et al., 1976). Very limited drug candidates have been evaluated targeting brain edema during stroke. In regard to opioid agonists, activation of KOR by 2-(3,4-dichlorophenyl)-*N*-methyl-*N*-[(1*R*,2*R*)-2-pyrrolidin-1-ylcyclohexyl]acetamid (U-50488) decreased brain edema after ischemia (Guéniau and Oberlander, 1997). With the need for new drug candidates, we decided to evaluate multiple benefits of the nonselective opioid agonist biphalin, in producing neuroprotective effects as well as antiedematous effects in both tMCAO and pMCAO stroke models. In our previous studies, we demonstrated superior antiedematous

Fig. 4. Representative images of evaluation of neuronal cell death (Fluoro-Jade C staining) and oxidative stress (DHE staining) in different regions (hippocampus, cortex, and striatum) of brain after transient MCAO and biphalin (5.7 $\mu\text{mol/kg}$ i.p.) or vehicle treatment ($n = 5-6$ per group).

effects of biphalin compared with selective OR agonists, including κ -selective agonist U-50488, using in vitro-hippocampal slice perfusion model (Yang et al., 2011). In addition, in that study biphalin significantly decreased cell volume in primary neuronal cells subjected to OGD, which was reversed with naloxone. Observations in this study are in good

correlation with previous results using a hippocampal slice perfusion model (Yang et al., 2011).

To understand the possible mechanisms of neuroprotection as well as the antiedematous effects of biphalin, we studied the effect of OR activation on PKC expression. Some investigators have identified that acute activation of ORs de-

creases PKC activity (Liu et al., 2008). Moreover, PKC inhibition have been shown to be neuroprotective in stroke (Bright and Mochly-Rosen, 2005). In our study, we demonstrated PKC expression in two distinct brain injury areas, core and penumbra. We observed that biphalin reduced the translocation of three conventional isoforms of PKC in penumbra brain tissue. In addition, we demonstrated that the PKC activator PMA induces increased cell volume in normoxic neurons, compared with control untreated cells. It is noteworthy that biphalin also reduces PMA-induced cell volume increase, suggesting that PKC-dependent signaling is a key mediator of neuronal cellular edema induced by OGD. We also verified that biphalin reduction of PMA-induced cellular edema was mediated by opioid receptor activation by testing the naloxone sensitivity of this pathway. The nonselective opioid receptor antagonist naloxone significantly reversed the effects of biphalin on PMA-induced cellular edema, suggesting that these edematous pathways are mediated by opioid receptor activation. The mechanism of PKC inhibition by biphalin is not well understood. Opioid receptor activation causes decreased intracellular Ca²⁺. Calcium is

one necessary factor to trigger the activation of conventional isoforms of PKC. This might be one possible mechanism contributing to the inhibition of PKC by opioid agonist. Another mechanism of opioid-induced PKC inhibition may involve activation of specific protein phosphatase isoforms, which have been shown in our previous studies to be associated with the effects of nicotine on brain endothelial cells' response to hypoxia/aglycemia and NKCC protein expression and activity (Abbruscato et al., 2004). In the future, we plan to investigate these two possible pathways.

To further decipher the antiedematous and neuroprotective effects of biphalin, we investigated changes in NKCC expression in penumbral brain areas. Brain edema formation mainly involves ion transporter dysfunction. Widely studied, NKCC has been implicated to play a key role in edema formation during ischemia (O'Donnell, 1993). This view is supported by the studies of Chen et al. (2005) demonstrating that NKCC1 knockout mice exhibited 40% reduction in infarct area and 1.1% reduction in water content of brain. Increased NKCC activity and expression were generally shown to be associated with increased phosphorylation (Kahle et al., 2009) under both physiological and pathological conditions such as cerebral ischemia. We and others have shown that NKCC phosphorylation increased activity in endothelial cells during OGD conditions (Yang et al., 2006) and in the cortex region in the rat cerebral focal MCAO model (Yan et al., 2003), as well as in astrocyte cells under 2-h OGD conditions (Lenart et al., 2004). PKC regulates various protein activities by phosphorylation of serine and threonine residues. It is postulated that phosphorylation of NKCC is the main operative mechanism, which also regulates NKCC activity (Yang et al., 2006). In our study, we found that the NKCC inhibitor bumetanide decreased PMA-induced increases in intracellular volume, indicating PKC regulation of

TABLE 1
Effect of stroke and biphalin treatment on mouse locomotor activity
Data represent the mean ± S.E.M. of six to seven independent determinations.

Parameter	Control	MCAO	Biphalin-Treated
Horizontal activity	2889 ± 747	265 ± 148*	485 ± 110#
Total distance, cm	1912 ± 572	116 ± 58*	319 ± 173#
Number of movements	148 ± 57	11 ± 5*	35 ± 11#
Movement time, s	182 ± 74	11 ± 6*	37 ± 12#
Stereotype counts	1247 ± 324	132 ± 67*	192 ± 58#
Number of stereotypy	118 ± 37	19 ± 8*	28 ± 6#
Stereotypy time, s	127 ± 28	30 ± 10*	26 ± 8
Center distance, cm	456 ± 173	12 ± 7*	105 ± 74#

*, *P* < 0.05 compared with the control group.
#, *P* < 0.05 compared with the MCAO group.

Fig. 5. Effect of biphalin on the membrane translocation of PKC in the ischemic core and penumbra regions of brain after 6-h permanent MCAO. A, representative Western blotting of PKC (α, β I, β II, and γ) in the ischemic core and penumbra regions of mouse brains treated with biphalin (5.7 μmol/kg i.p., 60 min after occlusion) or its vehicle. B, densitometric analysis of Western blotting results for conventional isoforms of PKC. The values of membrane protein are expressed as the percentage of the intensity of the band derived from the sham animal group. *, *P* < 0.05; ***, *P* < 0.001; *n* = 3.

Fig. 6. Effect of biphalin on the expression of NKCC in the ischemic core and penumbra regions of brain after 6-h permanent MCAO. A, representative Western blotting of NKCC in the ischemic core and penumbra regions of mouse brains treated with biphalin (5.7 μmol/kg i.p., 60 min after occlusion) or its vehicle. B, densitometric analysis of Western blotting results for NKCC. The values are expressed as the percentage of the intensity of the band derived from the sham animal group. *, *P* < 0.05; **, *P* < 0.01. con, control; pen, penumbra.

Fig. 7. Neuronal cell volume (urea uptake) in normoxic (Nor) or OGD conditions in the presence or absence of PMA, biphalin, or bumetanide. **A**, time course of urea uptake in normoxic primary cortical neurons treated with PMA (0.01 μ M). **B**, urea uptake in bumetanide- and biphalin-treated (0.01 nM, 1 h) cortical neurons in the presence or absence of the nonselective opioid antagonist naloxone (30-min preincubation with PMA before biphalin treatment). **C**, urea uptake in bumetanide-treated (20 μ M, 20 min) and biphalin-treated (0.01 nM, 1 h) cortical neurons in the presence of OGD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

the cell volume through NKCC (Fig. 7). Consistent with other reports, we also found increased NKCC expression in ischemic penumbral areas of brain. Biphalin treatment resulted in decreased NKCC protein expression compared with control MCAO vehicle-treated animals (Fig. 6). In addition, under physiological and pathological conditions glutamate has been shown to stimulate neuronal NKCC1 activity by activation of *N*-methyl-D-aspartate and non-*N*-methyl-D-aspartate ionotropic receptors (Sun and Murali, 1998; Su et al., 2002). Our observation of inhibition of PKC-dependent NKCC activity during ischemia is an additional mechanism by which OR activation could produce antiedematous effects and provides new insight into these possible neuroprotective pathways.

Taken together, we report here that the dimeric enkephalin OR agonist biphalin exhibits neuroprotective effects in pMCAO and tMCAO in vivo models of stroke. Biphalin not only decreases both infarct and edema ratios, but also reduces the number of dying neuronal cells in the penumbra area associated with stroke. One possible mechanism of the neuroprotective effects of biphalin is through reduced PKC expression. The antiedematous effect of biphalin can be attributed to reduced PKC-dependent NKCC stimulation, resulting in less cellular edema. However, multimodal mechanisms of OR activation producing neuroprotective and antiedematous effect cannot be ruled out, resulting from reduced glutamate release, decreased intracellular calcium, and increased antioxidant enzymes, which have been previ-

ously suggested by others. To further support our findings in this study, we have undertaken the investigation of additional neuroprotective mechanisms of OR activation by biphalin in stroke and its specific effects on glutamate release.

Authorship Contributions

Participated in research design: Yang and Abbruscato.

Conducted experiments: Yang and Wang.

Contributed new reagents or analytic tools: Yang and Karamyan.

Performed data analysis: Yang, Shah, and Abbruscato.

Wrote or contributed to the writing of the manuscript: Yang, Shah, Karamyan, and Abbruscato.

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