

A protease-activated receptor 1 antagonist protects against global cerebral ischemia/reperfusion injury after asphyxial cardiac arrest in rabbits

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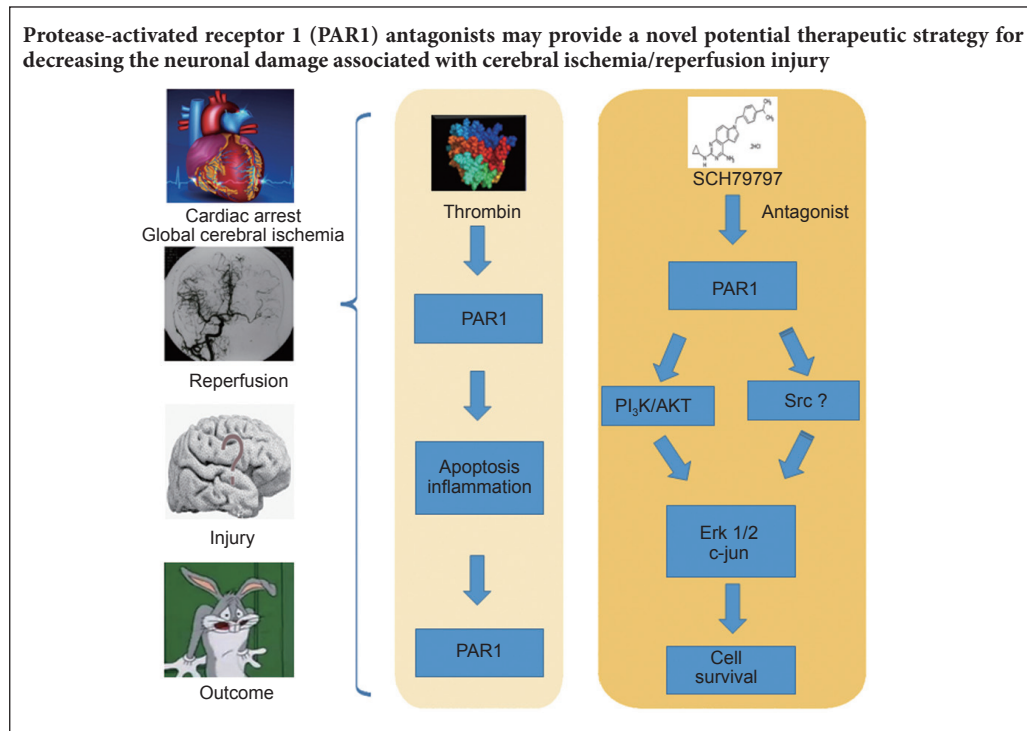
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Graphical Abstract



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Abstract

Cerebral ischemia/reperfusion injury is partially mediated by thrombin, which causes brain damage through protease-activated receptor 1 (PAR1). However, the role and mechanisms underlying the effects of PAR1 activation require further elucidation. Therefore, the present study investigated the effects of the PAR1 antagonist SCH79797 in a rabbit model of global cerebral ischemia induced by cardiac arrest. SCH79797 was intravenously administered 10 minutes after the model was established. Forty-eight hours later, compared with those administered saline, rabbits receiving SCH79797 showed markedly decreased neuronal damage as assessed by serum neuron specific enolase levels and less neurological dysfunction as determined using cerebral performance category scores. Additionally, in the hippocampus, cell apoptosis, polymorphonuclear cell infiltration, and c-Jun levels were decreased, whereas extracellular signal-regulated kinase phosphorylation levels were increased. All of these changes were inhibited by the intravenous administration of the phosphoinositide 3-kinase/Akt pathway inhibitor LY29004 (3 mg/kg) 10 minutes before the SCH79797 intervention. These findings suggest that SCH79797 mitigates brain injury via anti-inflammatory and anti-apoptotic effects, possibly by modulating the extracellular signal-regulated kinase, c-Jun N-terminal kinase/c-Jun and phosphoinositide 3-kinase/Akt pathways.

Key Words: nerve regeneration; protease-activated receptor 1; global cerebral ischemia/reperfusion; cardiac arrest; neuroprotection; SCH79797; apoptosis; inflammation; neuron specific enolase; hippocampus; neural regeneration

Introduction

Pathophysiologic responses in the brain after stroke are complicated. Brain tissue is sensitive to hypoxia, making the brain the extremely vulnerable to ischemia (Chazalviel et al., 2016; Liang et al., 2016). However, reperfusion after transient ischemia may exacerbate brain injury, a phenomenon called cerebral ischemia/reperfusion (I/R) injury (Amantea et al., 2009). In the brain, I/R injury is characterized by delayed neuronal death *via* apoptosis (Gao et al., 2015; Yan et al., 2015). The mechanisms of this delayed neuronal death are also complex and include the excessive release of excitatory amino acids (Diemer et al., 1993; Anzai et al., 2003), overload of intracellular calcium, induction of neuroinflammatory mediators, and generation of reactive oxygen species (Choi, 1996; Banasiak et al., 2000).

Protease-activated receptor 1 (PAR1), a G protein-coupled receptor, exhibits inflammatory functions and causes coagulation (Coughlin, 1999). Recent studies have shown that PAR1 activation through thrombin causes brain damage in cerebral ischemia or hemorrhage (Han et al., 2011; Chen et al., 2012; Lyden et al., 2014), and these effects are related to N-methyl-D-aspartate receptor actions. PAR1 activation increases the excitatory amino acid toxicity mediated by N-methyl-D-aspartate receptors, which leads to apoptosis (Han et al., 2011). In addition, PAR1 activation leads to brain inflammation, an important cause of brain injury (Sun et al., 2010; Alabanza and Bynoe, 2012; Tripathy et al., 2013).

Although studies have provided a causal link between PAR1 and brain I/R injury, the role of PAR1 in the damage to the damage and the mechanisms involved remain to be further elucidated. The selective PAR1 antagonist SCH79797 (Ahn et al., 2000) limits myocardial I/R injury (Strande et al., 2007) and limits renal injury through an anti-inflammatory effect (El Eter and Aldrees, 2012). In the present study, we hypothesized that a PAR1 antagonist inhibits hypoxia-induced apoptosis and attenuates cerebral I/R injury. Thus, we investigated the neuroprotective abilities of SCH79797 and the downstream effectors involved in a rabbit model of transient global cerebral I/R injury.

Materials and Methods

Animal preparation

This study was approved by the Institutional Animal Care and Use Committee of Hubei University of Medicine in Shiyang, Hubei Province, China (institutional protocol number, [2015]118). All rabbits were purchased from the Center for Animal Experiments of Hubei Province, Wuhan, China (license number, SYXK[E]-2011-0031), and received standardized care in accordance with the National Institutes of Health's guidelines for ethical animal research.

After an overnight fast with free access to water, 6–7-month-old specific-pathogen-free male Japanese white rabbits weighing 2.5–3.0 kg were used in this study. Anesthesia was induced with pentobarbital (Akorn Inc., Lake Forest, CA, USA) *via* the marginal vein of the ear (30 mg/kg, intravenously). Additional doses of 10 mg/kg were administered hourly to maintain anesthesia. Rabbits were fixed in a

supine position on an electric warming blanket to maintain a rectal temperature of $38.5 \pm 0.5^\circ\text{C}$. An inverse T-incision was made three to four cartilage rings below the thyroid cartilage, and a Y-type endotracheal tube (Tuoren Medical Appliance, Xinxiang, Henan Province, China) connected to a mechanical ventilator (HX-300S, Chengdu Taimeng Technology Co., Ltd., Chengdu, Sichuan Province, China) was inserted. Three-lead electrocardiograph electrodes placed on the chest and below the xiphoid process were used to record an electrocardiogram. The right femoral artery was exposed and cannulated, and the overlying skin was closed. A full-function heart monitor (AED Plus, Zoll Medical Corporation, Boston, MA, USA) was used to monitor cardiac vital signs, including systolic blood pressure, diastolic blood pressure, mean arterial pressure, and heart rate. The rabbits were randomly divided into the following four groups ($n = 8$ per group): sham operated (sham), saline, SCH, and LY + SCH (LY). Rabbits in the sham group received a sham operation. Rabbits in the saline group were subjected to cardiac arrest and administered saline. Rabbits in the SCH group, were subjected to cardiac arrest and treated with the PAR1 receptor antagonist SCH79797, whereas those in the LY + SCH group were subjected to cardiac arrest and treated with both SCH79797 and an inhibitor of the phosphoinositide 3-kinase (PI₃K)/Akt pathway, LY29004 (El Eter and Aldrees, 2012).

Establishment of transient global cerebral I/R injury model and drug administration

A transient global cerebral I/R injury model induced by cardiopulmonary resuscitation was established using a modified version of a previously described method (Chen et al., 2007). For the saline, SCH, and LY + SCH groups, the ventilator was turned off when the baseline hemodynamics were stable, and the trachea was clamped at the expiratory phase for approximately 5–9 minutes to induce cardiac arrest (defined as the absence of spontaneous pulse, sinus arrest as shown on the electrocardiogram, pulseless electrical activity or ventricular fibrillation, and mean arterial pressure ≤ 10 mmHg). Three minutes after cardiac arrest, chest compressions and mechanical ventilation were started. Manual chest compressions were applied at a frequency of 180–220 times/minute, with equal durations for compression and relaxation. The compression depth was one-third of the anteroposterior diameter of the chest. Simultaneously, mechanical ventilation with pure oxygen was performed at 50 times/minute, with a tidal volume of 10 mL/kg and a compression-to-ventilation ratio of 1:2, until spontaneous respiration recovered.

If resuscitation was not achieved after 2 minutes of cardiopulmonary resuscitation, advanced cardiac life support was started; adrenaline (0.25 mg/kg, intravenously) was injected *via* the marginal vein of the ear every 3 minutes, and 5% sodium bicarbonate (2 mL/kg) was injected every 30 minutes, for a maximum of three times. Chest compressions were performed intermittently at 2-minute intervals to check for restoration of spontaneous circulation. Restoration of spontaneous circulation was defined as systolic arterial pressure ≥ 60 mmHg for a minimum of 10 minutes. After 10 minutes of

extrathoracic compression and three doses of adrenaline, if restoration of spontaneous circulation did not occur, resuscitation was abandoned.

After the blood pressure and heart rate had stabilized to pre-operation levels without inotrope support, monitoring was discontinued. When spontaneous breathing had stabilized at 60 breaths/minute, artificial ventilation was discontinued, and the tracheal tubes were removed. The average time from the clipping of the trachea until the blood pressure dropped to the level of cardiac arrest was 5.05 ± 0.64 minutes. The time from cardiac arrest to the restoration of spontaneous circulation was 6.22 ± 1.20 minutes.

The PAR1 receptor antagonist SCH79797 (Tocris, Ellisville, MO, USA) dissolved in dimethyl sulfoxide (to a final dimethyl sulfoxide concentration of 0.05%) was administered to rabbits in the SCH group and the LY + SCH group at a dose of 25 $\mu\text{g}/\text{kg}$ via the marginal vein of the ear 10 minutes and again 24 hours after restoration of spontaneous circulation. To investigate the involvement of the PI₃K/Akt pathway in the effects of the PAR1 antagonist, LY29004 (Calbiochem, Cambridge, MA, USA; 3 mg/kg; dissolved in dimethyl sulfoxide to a final concentration of dimethyl sulfoxide of 0.06%) was intravenously administered to rabbits in the LY + SCH group 10 minutes before the administration of SCH79797. An equivalent volume of saline was given to the rabbits in the saline group. The tracheal tubes of rabbits in

the sham group were not clipped.

Enzyme linked immunosorbent assay (ELISA)

At 24 and 48 hours after resuscitation, rabbit blood samples were collected from the femoral artery for use in blood gas analysis and routine blood tests. A fraction of the blood was also frozen in liquid nitrogen and stored at -80°C for use in ELISAs. Serum levels of neuron specific enolase (NSE) were measured using an ELISA kit (R&D Systems Inc., Minneapolis, MN, USA) to assess neuronal damage.

Cerebral performance category score

Rabbits were evaluated 24 and 48 hours after cardiac arrest and assigned a cerebral performance category score (Noc et al., 1995; Kern et al., 1998) as an assessment of neurological dysfunction. The clinical signs included their responses to an investigator opening the cage door or lifting the rabbit, or corneal irritation, if otherwise unresponsive, as well as the ability of the rabbit to stand, move all four limbs, walk, eat, urinate, and defecate. The following scoring system was used: 1, normal; 2, slightly disabled; 3, severely disabled but conscious; 4, vegetative state; 5, died in the cage following establishment of the model.

Western blot assay

At 48 hours after establishing the model, hippocampal

Table 1 Baseline characteristics of rabbits subjected to global cerebral ischemia/reperfusion injury after cardiopulmonary resuscitation

	Sham group	Saline group	SCH group	LY + SCH group	P
Weight (kg)	2.44±0.32	2.46±0.11	2.46±0.14	2.41±0.12	> 0.05
Heart rate (beats/min)	151.30±10.18	148.76±9.10	149.20±9.35	150.14±8.46	> 0.05
Mean arterial pressure (mmHg)	67.62±3.24	68.50±2.78	67.82±2.46	68.13±2.19	> 0.05
Arterial PO ₂ (mmHg)	95.1±3.46	95.9±3.93	95.2±4.88	95.1±2.37	> 0.05
Arterial PCO ₂ (mmHg)	34.10±1.95	34.9±2.45	34.8±1.86	34.6±1.70	> 0.05
Arterial lactate (mM)	0.45±0.14	0.44±0.16	0.45±0.19	0.45±0.17	> 0.05

Sham group: Sham-operated control rabbits; saline group: rabbits subjected to cardiac arrest and administered saline; SCH group: rabbits subjected to cardiac arrest and treated with PAR1 receptor antagonist SCH79797; LY + SCH group: rabbits subjected to cardiac arrest and treated with the PI3K/Akt inhibitor LY29004 and with SCH79797. Data are expressed as the mean \pm SD. Differences among groups were analyzed by one-way analysis of variance followed by Bonferroni *post hoc* tests. PAR1: Protease-activated receptor 1; SCH: SCH79797; PO₂: partial pressure of oxygen; PCO₂: partial pressure of carbon dioxide.

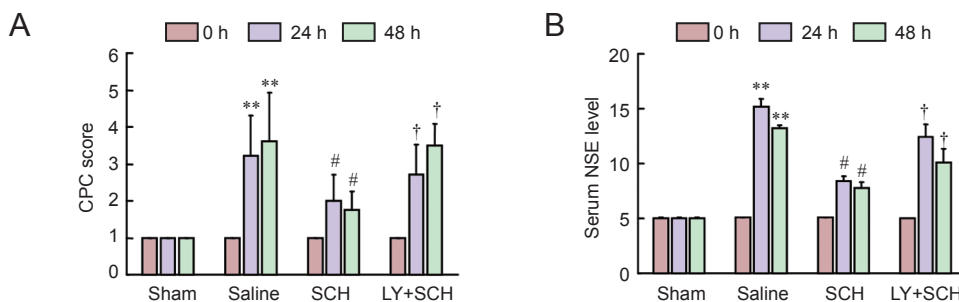


Figure 1 Effects of the protease-activated receptor 1 (PAR1) antagonist SCH79797 (SCH) on cerebral performance category (CPC) scores and serum levels of neuron specific enolase (NSE) in rabbits with transient global cerebral ischemia/reperfusion injury.

(A) CPC scores: High CPC scores indicate worse neurological function. (B) Serum NSE levels detected by ELISA. Sham group: Sham-operated control rabbits; saline group: rabbits subjected to 3 minutes of cardiac arrest and administered saline; SCH group: rabbits subjected to 3 minutes of cardiac arrest and treated with PAR1 receptor antagonist SCH79797 (25 $\mu\text{g}/\text{kg}$, intravenously) 10 minutes after cardiopulmonary resuscitation; LY + SCH group: rabbits that received the PI3K/Akt inhibitor LY29004 (3 mg/kg, intravenously) 10 minutes before SCH administration. Data are expressed as the mean \pm SD ($n = 8$). Differences among groups were analyzed by one-way analysis of variance followed by Bonferroni *post hoc* tests. ** $P < 0.01$, vs. sham group; # $P < 0.05$, vs. saline group; † $P < 0.05$, vs. SCH group.

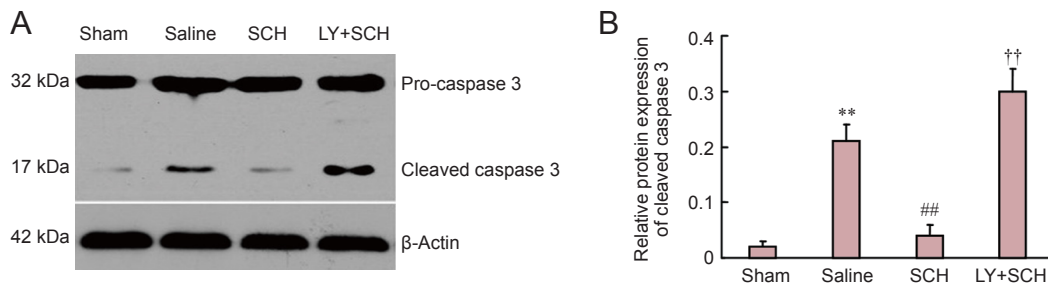


Figure 2 Effects of the PAR1 antagonist SCH79797 (SCH) and the PI3K/Akt inhibitor LY29004 (LY) on caspase 3 expression and activation after transient global cerebral ischemia/reperfusion (I/R) injury (western blot assay).

(A) Representative immunoblots for pro-caspase 3 and cleaved caspase 3 in hippocampal tissue homogenates from the studied groups 48 hours after I/R. (B) Densitometry analysis results for cleaved caspase 3 at 48 hours after I/R. The graph shows the optical density ratio of cleaved caspase 3 to β -actin using data combined from three independent experiments after normalization to the loading control. Sham group: Sham-operated control rabbits; saline group: rabbits subjected to 3 minutes of cardiac arrest and administered saline; SCH group: rabbits subjected to 3 minutes of cardiac arrest and treated with the PAR1 receptor antagonist SCH79797 (25 μ g/kg, intravenously) 10 minutes after cardiopulmonary resuscitation; LY + SCH group: rabbits that received the PI3K/Akt inhibitor LY29004 (3 mg/kg, intravenously) 10 minutes before SCH79797 administration. Data are expressed as the mean \pm SD. Differences among groups were analyzed by one-way analysis of variance followed by Bonferroni *post hoc* tests. ** $P < 0.01$, vs. sham group; ## $P < 0.01$, vs. saline group; †† $P < 0.01$, vs. SCH group. PAR1: Protease-activated receptor 1.

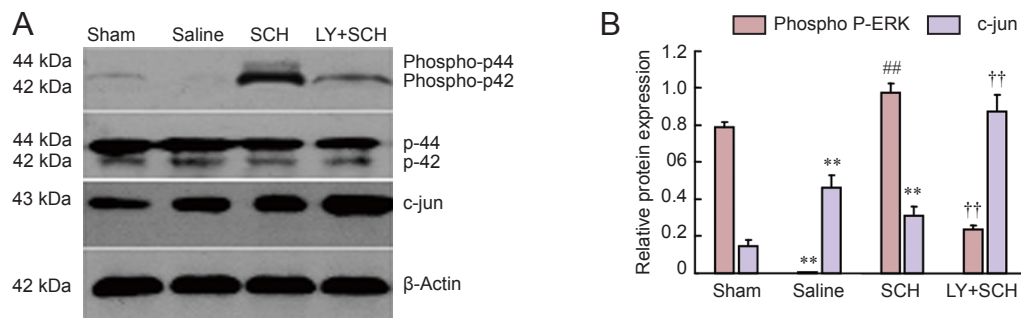


Figure 3 Effects of the PAR1 antagonist SCH79797 (SCH) and the PI₃K/Akt inhibitor LY29004 (LY) on ERK, phospho-ERK and c-Jun as assessed by western blot assay after transient global cerebral I/R injury.

(A) Representative immunoblots of ERK, phospho-ERK, and c-Jun in hippocampal tissue homogenates from the studied groups 48 hours after I/R. (B) Densitometry analysis results for ERK phosphorylation and c-Jun 48 hours after I/R. The graph shows optical density ratios of the target protein to β -actin using data combined from three independent experiments after normalization to the loading control. Sham group: Sham-operated control rabbits; saline group: rabbits subjected to 3 minutes of cardiac arrest and administered saline; SCH group: rabbits subjected to 3 minutes of cardiac arrest and treated with PAR1 receptor antagonist SCH79797 (25 μ g/kg, intravenously) 10 minutes after cardiopulmonary resuscitation; LY + SCH group: rabbits that received the PI₃K/Akt inhibitor LY29004 (3 mg/kg, intravenously) 10 minutes before SCH administration. Data are expressed as the mean \pm SD. Differences among groups were analyzed by one-way analysis of variance followed by Bonferroni *post hoc* tests. ** $P < 0.01$, vs. sham group; ## $P < 0.01$, vs. saline group; †† $P < 0.01$, vs. SCH group. p44: ERK1; p42: ERK2; PAR1: protease-activated receptor 1; I/R: ischemia/reperfusion; ERK: extracellular signal-regulated kinase; PI₃K: phosphoinositide 3-kinase; phospho: phosphorylated.

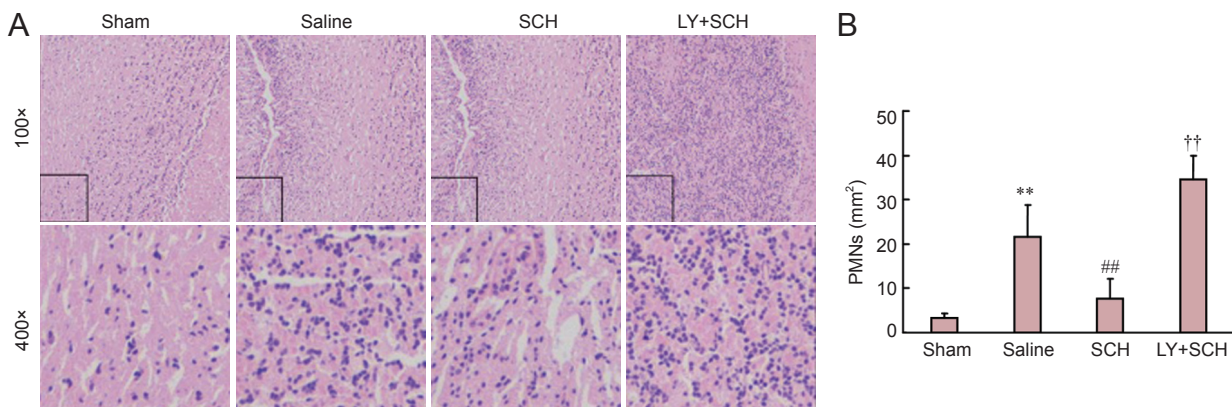


Figure 4 Effect of PAR1 antagonist SCH79797 (SCH) on the polymorphonuclear (PMN) cell infiltration caused by global transient I/R injury.

(A) Hematoxylin and eosin staining showing marked vacuolization, hemorrhage, and leukocyte infiltration in the hippocampus 48 hours after I/R. These changes are markedly attenuated by SCH79797 treatment, but are aggravated by the administration of LY29004 (LY). (B) The number of PMN cells in fields examined at $\times 400$. Data are expressed as the mean \pm SD ($n = 8$). Differences among groups were analyzed by one-way analysis of variance followed by Bonferroni *post hoc* tests. ** $P < 0.01$, vs. sham group; ## $P < 0.01$, vs. saline group; †† $P < 0.01$, vs. SCH group. Sham group: Sham-operated control rabbits; saline group: rabbits subjected to 3 minutes of cardiac arrest and administered saline; SCH group: rabbits subjected to 3 minutes of cardiac arrest and treated with PAR1 receptor antagonist SCH79797 (25 μ g/kg, intravenously) 10 minutes after cardiopulmonary resuscitation; LY + SCH group: rabbits that received the PI₃K/Akt inhibitor LY29004 (3 mg/kg, intravenously) 10 minutes before SCH79797 administration. PAR1: protease-activated receptor 1.

samples were obtained and homogenized in ice-cold buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid, 1% Triton X-100, 10 µg/mL phenylmethylsulfonyl fluoride, 12 g/mL leupeptin, and 12 g/mL aprotinin) and incubated for 10 minutes. The homogenate was then centrifuged at 4°C and 10,000 × g for 5 minutes. The supernatant was removed and stored at -80°C. Approximately 40 µg of protein per sample was loaded on a 7.5% sodium dodecyl sulfate-polyacrylamide gel. The gels were electroblotted on nitrocellulose membranes. The blots were blocked in 5% dry skim milk and then incubated at 4°C overnight with primary antibodies of mouse origin, including anti-phospho-extracellular signal-regulated kinase (ERK), anti-ERK, anti-c-Jun, and anti-β-actin (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) as well as anti-procaspase 3 and anti-cleaved caspase 3 (1:500; Sigma, St. Louis, MO, USA). After washing, the blots were incubated at 37°C with secondary antibody (horseradish peroxidase-conjugated goat anti-mouse, 1:300; Invitrogen, Carlsbad, CA, USA) for 1 hour. Washed blots were processed for band detection using enhanced chemiluminescence immunoblotting detection reagents (Amersham Life Science, Arlington Heights, IL, USA). Autoradiography was conducted using X-ray film and was followed by densitometric analysis of the bands using a Bio-Rad GS-670 imaging densitometer (Bio Rad Laboratories, Richmond, CA, USA) and Molecular Analyst Image Analysis Software (Bio Rad Laboratories).

Histological evaluation

Rabbits that survived more than 48 hours after the model was established were injected with pentobarbital (50 mg/kg, intravenously). After a deep anesthesia level was achieved, the chest was opened and the pericardium was cut before a 12G puncture needle was used to pierce the left ventricle. Normal saline was first used for left ventricular perfusion. After the outflow fluid from the venous end of catheter became clear, 4% paraformaldehyde-phosphate buffered saline was used for physiological pressure *in situ* fixation (100 mmHg). The perfusion fluid plane was 140–150 cm above the heart, and the flow lasted for 30 minutes at a rate of 2.0–2.5 mL/min before the brain and other organs were removed for *in vitro* fixation. The brain was quickly removed and was dissected using a sterile surgical blade, and the bilateral hippocampus was isolated.

Hippocampal samples were fixed in 4% paraformaldehyde for 24 hours and then embedded in paraffin; 4 µm sections were then stained with hematoxylin and eosin. Images were acquired with a Leica microscope (Leica Microsystems, Mannheim, Germany) using a high-power field (× 400). Hippocampal damage was assessed histologically based on the infiltration of neutrophils. Infiltrating neutrophils (polymorphonuclear leukocytes) were quantified by counting polymorphonuclear cells from 10 fields at 400× magnification. The results were expressed as neutrophils counted per square millimeter. Histological scoring and neutrophil infiltration assessment were performed by a specialized pathologist.

Statistical analysis

Data are expressed as the mean ± SD. Analyses were performed using the statistical software SPSS 16.0 for Windows (SPSS, Chicago, IL, USA). Differences between groups were analyzed by one-way analysis of variance followed by Bonferroni *post hoc* tests. Values of *P* less than 0.05 were considered statistically significant.

Results

Physiological, hemodynamic and arterial blood gas changes of global cerebral I/R injury after cardiopulmonary resuscitation

No significant differences in physiological and hemodynamic data or in arterial blood gas were observed at baseline among the four groups 48 hours after model establishment (*P* > 0.05; **Table 1**).

SCH79797 alleviated neurological dysfunction after transient global cerebral I/R injury

Almost all the rabbits in the saline group were unconscious at 24 and 48 hours after resuscitation, whereas the rabbits in the SCH group were conscious and showed only mild neuronal damage. The cerebral performance category scores and serum NSE levels in the SCH group were significantly decreased 24 and 48 hours after modeling as compared with those in the saline group (*P* < 0.05); however, these decreases were abolished by SCH79797 (**Figure 1**).

SCH79797 reduced apoptosis in the hippocampus after transient global cerebral I/R injury

To determine whether PAR1 was involved in the cell death of brain tissue after transient global cerebral I/R injury, an apoptosis-related protein was analyzed in the hippocampus using a western blot assay. Compared with that in the sham group, the hippocampus in rabbits subjected to cardiopulmonary resuscitation showed significant activation of caspase 3 (*P* < 0.01). SCH79797 treatment attenuated caspase 3 activation (*P* < 0.01, *vs.* saline group), whereas PI3K/Akt inhibition promoted the activation of caspase 3 (*P* < 0.01, *vs.* SCH group). However, caspase 3 activation in the SCH group was still higher than that in the sham group (*P* < 0.01; **Figure 2**).

SCH79797 increased ERK phosphorylation and decreased c-Jun levels after transient global cerebral I/R injury

To identify the downstream effectors involved in the effects of SCH79797, we first assessed ERK phosphorylation and c-Jun expression. To determine whether PI₃K, a key signaling enzyme implicated in cell survival (Cantley, 2002), is involved in the protection conferred by PAR1, the effect of the administration of LY29004, a PI₃K/Akt inhibitor, on the hippocampal cells was investigated in the presence of SCH79797.

ERK phosphorylation was increased following the administration of SCH79797, but the effects of SCH79797 were inhibited in the presence of LY29004 (**Figure 3**). The c-Jun level was decreased following the administration of

SCH79797. While in the presence of LY29004, the c-Jun level was increased. No significant changes were observed in the expression of total ERK protein in any of the groups. The levels of ERK protein in the sham, saline, SCH, and LY + SCH groups were expressed as the optical density ratios to β -actin, which were 0.62 ± 0.08 , 0.62 ± 0.09 , 0.56 ± 0.11 and 0.55 ± 0.10 , respectively.

SCH79797 effects on neutrophil infiltration after transient global cerebral I/R injury

Cerebral I/R injury induced marked neutrophil infiltration ($P < 0.01$, vs. sham group). SCH79797 treatment led to a decrease in polymorphonuclear cell infiltration ($P < 0.01$, vs. saline group). Inhibition of PI3K/Akt with LY29004 before SCH administration and the induction of cerebral I/R resulted in marked polymorphonuclear cell infiltration and inhibition of the protection conferred by SCH79797 (Figure 4).

Discussion

Previous reports of high expression levels of PAR1 in the rat and human brain suggest its potential role in regulating the function of the nervous system (Niclou et al., 1998; Striggow et al., 2001; Junge et al., 2004). The neuroprotection conferred by PAR1 deletion in various ischemic models demonstrated the toxic effects of thrombin in the central nervous system in ischemic situations (Junge et al., 2003; Olson et al., 2004), and pharmacological manipulation of PAR1 signaling has been suggested to provide a potential therapeutic target for neuroprotection in human neurological disorders (Junge et al., 2004).

The present study was designed to investigate the ability of SCH79797 to protect the rabbit brain from global cerebral I/R and the mechanisms involved. SCH79797 is a non-peptide thrombin receptor antagonist, producing effects such as antiplatelet aggregation (Ahn et al., 2000; Wu and Teng, 2006). Importantly, SCH79797 limits myocardial I/R injury (Strande et al., 2007) and limits renal I/R injury through an anti-inflammatory effect (El Eter and Aldrees, 2012). In addition, SCH79797 confers protection from secondary injury after surgically induced brain injury by decreasing both brain edema and apoptosis (Manaenko et al., 2013). These findings suggest that SCH79797 has a protective effect against I/R injury. However, the effect of blocking PAR1 signaling in the central nervous system in ischemic situations is not fully understood.

In the present study, SCH79797 was administered intravenously 10 minutes after establishing a model of transient global cerebral I/R injury model in rabbits. This model was based on the clinical treatment of cardiac arrest patients. To clarify the role of PAR1 in the present study, the effect of SCH79797 on I/R injury-induced brain damage and inflammation was investigated. Brain injury was assessed histologically and functionally. The result of each assessment showed that blocking PAR1 significantly reduced the signs of injury as measured by these parameters. Specifically, using cardiac arrest in rabbits as a model of global cerebral I/R, we found that the PAR1 antagonist SCH79797 reduced serum NSE levels, which is used as an index of neuronal damage (Gel-

derblom et al., 2013) and of improved brain function after cardiac arrest.

We also found that treatment with SCH79797 led to a decrease in polymorphonuclear cell infiltration, as shown by histological analysis. Previous studies have demonstrated that intracellular signaling mediated by the thrombin activation of PAR1 increases the expression of proinflammatory cytokines (Naldini et al., 2000; Fan et al., 2005). Therefore, we concluded that SCH79797 ameliorated inflammation in the brain, at least in part, *via* inhibition of the secretion of some cytokines.

In addition to reducing inflammation, SCH79797 ameliorated apoptosis in the central nervous system in our model of global cerebral I/R injury. Apoptosis is orchestrated by caspases, and caspase-3, which is a key executive molecule, plays an important role in the process of apoptosis. When stimulated, caspases undergo autolytic cleavage to become fully active. The cleaved caspase-3 is responsible for the majority of proteolysis during apoptosis, and detection of cleaved caspase-3 is therefore considered a reliable marker for cells apoptosis. Using a western blot assay, protein extracts from the hippocampus were analyzed for caspase 3 protein expression. Compared with those in the sham group, rabbits subjected to 3 minutes of cardiac arrest and cardiopulmonary resuscitation showed significant expression of cleaved caspase 3 protein, as demonstrated by densitometric analysis of the blotted bands. SCH79797 treatment attenuated the cleaved caspase 3 expression, whereas PI3K/Akt inhibition prevented this protection and enhanced the expression of cleaved caspase 3.

The present study also explored the mechanisms underpinning the neural cytoprotection induced by inhibition of PAR1 using SCH79797 by assessing signaling pathways downstream of PAR1. The signal transduction pathways activated by PAR1 have not been fully described; however, they are known to differ depending on the tissues involved and the responses elicited. PAR1 couples to G proteins to activate a variety of signaling cascades and cellular responses depending on the tissues in which they are present (Coughlin, 2005).

The present study focused on the signaling pathways, associated with apoptosis or survival. Previous reports have indicated that mitogen-activated protein kinase (MAPK)/ERK and c-Jun N-terminal kinase (JNK)/c-Jun signaling are associated with neuronal survival (Xia et al., 1995; Anderson and Tolkovsky, 1999; Ham et al., 2000; Dal-Cim et al., 2013). Activation of JNK/c-Jun and concurrent inhibition of ERK are critical for induction of apoptosis in neuronal cells (Xia et al., 1995). In addition, some research indicates that the PI₃K/Akt pathway contributes to neuroprotection against cerebral ischemia and reperfusion injury (Zhu et al., 2013; Qu et al., 2015; Yu et al., 2016). Thus, we examined the ERK, JNK/c-Jun, and PI₃K/Akt pathways. We found that ERK activation was decreased 48 hours after cardiac arrest, but c-Jun was increased. We demonstrated that a PAR1 antagonist significantly increased phosphorylated ERK levels and decreased c-Jun levels. Therefore, PAR1 antagonist can inhibit

neuronal apoptosis through activation of ERK and inhibition of c-Jun, which is consistent with previous studies (Xia et al., 1995; Ham et al., 2000).

A previous study has shown that SCH79797 protects rat kidney against I/R injury *via* the PI₃K/Akt pathway (El Eter and Aldrees, 2012). To strengthen our hypothesis that the ERK signaling pathway was regulated by PI₃K/Akt, we tested the effect of PI₃K/Akt inhibition on ERK phosphorylation by using LY29004. We demonstrated that PI₃K/Akt inhibition significantly decreased SCH79797-induced ERK phosphorylation in the current study. These results are consistent with the observation that ERK and MAPK are regulated by the PI₃K/Akt signaling pathway in human oral keratinocytes (Rohani et al., 2010). In addition, PI₃K/Akt inhibition increased c-Jun levels. Therefore, these results provide evidence for the involvement of the ERK, JNK/c-Jun and PI₃K/Akt signaling pathways in the protection conferred by SCH79797.

The mechanism of the neuroprotection conferred by SCH79797 may be associated with the anticoagulation effect or against the thrombin effect, because cardiac arrest and hypoxia result in activation of coagulation (Böttiger and Martin, 2001; Crosby et al., 2003). When coagulation activation occurs, PAR1 activation through thrombin causes brain damage in cerebral ischemia or hemorrhage (Böttiger and Martin, 2001; Crosby et al., 2003). Although MAPK/ERK activation is involved in cerebral ischemic injury (Wang et al., 2012), some recent reports indicate that the MAPK/ERK pathway participates in neuroprotection against transient cerebral I/R injury (Zhu et al., 2013; Yang et al., 2014; Liu et al., 2016). Additionally, the bilateral common carotid artery occlusion model is not complete global cerebral ischemia, because the vertebrobasilar artery blood supply may exist after bilateral common carotid artery occlusion. In our study, cerebral ischemia after asphyxial cardiac arrest in rabbits was complete transient global cerebral ischemia, and had a long hypoxia time, similar to actual clinical situations, for example, in cardiac arrest caused by neonatal asphyxia. Thus, our findings are distinguished from this previous report and have greater clinical application value.

Although we found that the ERK and PI₃K/Akt signaling pathways contributed to the neuroprotection conferred by SCH79797, we did not explore the specific signaling molecules upstream of these signaling pathways. PI₃K/Akt plays an important role in the control of cell growth, proliferation, survival, and migration. The activation of Akt may ameliorate I/R injury (Förster et al., 2006). Previous research has shown that PAR1, as a member of the G protein-coupled receptor family, can bind and activate c-Src, which has been linked to the activation of MAPK, PI₃K/Akt, and other proteins (Lefkowitz and Shenoy, 2005; Xiao et al., 2010). Thus, we speculate that c-Src may be a link existing between the PI₃K/Akt and ERK signaling pathways.

Other mechanisms have also been implicated in the protection conferred by SCH79797. For example, the protection may be due to an inhibition of SCH79797 on PAR1-induced nuclear factor kappa B activation (Rahman et al., 2002).

In summary, the PAR1 antagonist SCH79797 attenuated the brain damage and dysfunction observed following cardiac arrest in rabbits. SCH79797 protected against brain injury after cardiopulmonary resuscitation by decreasing brain inflammation and apoptosis. These effects were mediated by activating the ERK and PI₃K/Akt signaling pathways and inhibiting the JNK/c-Jun pathway. We also demonstrated the occurrence of cross talk between the PI₃K/Akt and MAPK/ERK signaling pathways in the brain. These data suggest a therapeutic potential for SCH79797 in acute ischemic brain injury.

Author contributions: JNY and MX were responsible for study design. JNY was in charge of writing the paper. JC was in charge of implementing the experiments, data collection and statistical analysis. All authors approved the final version of the paper.

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