

# Suppression of GRASP65 phosphorylation by tetrahydrocurcumin protects against cerebral ischemia/reperfusion injury via ERK signaling

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**Abstract.** The aim of the present study was to assess the neuroprotective effects of tetrahydrocurcumin (THC) in a mouse model of cerebral ischemia/reperfusion (I/R) injury, and to investigate the involvement of Golgi reassembly and stacking protein 65 (GRASP65) and the extracellular signal-regulated kinase (ERK) signaling pathway. Cerebral I/R injury was induced using the Pulsinelli four-vessel occlusion method. After 5 min of reperfusion, mice received THC (5, 10 or 25 mg/kg) or saline by intraperitoneal injection. After 24 h of reperfusion, mice underwent neurological evaluation. Infarct volumes were determined by triphenyltetrazolium chloride staining, and levels of superoxide dismutase and malondialdehyde were measured in brain tissue homogenates. Expression of GRASP65, phosphorylated-GRASP65, ERK and phosphorylated-ERK was determined by western blotting. THC induced a dose-dependent decrease in the phosphorylation of ERK and GRASP65. Thus, THC attenuated I/R injury-induced activation of the ERK signaling

pathway and reduced the phosphorylation of GRASP65. THC exhibited a dose-dependent protective effect against cerebral I/R injury, mediated by suppression of the ERK signaling pathway and a subsequent reduction in GRASP65 phosphorylation. The current study provided new information in the research of the cerebral ischemia-reperfusion injury mechanism.

## Introduction

Cerebral ischemia/reperfusion (I/R) injury occurs as a complication of stroke following thrombolysis and a recurrence of ductal patency. The mechanisms of I/R injury are not clearly understood, and identification of effective and safe treatments for ischemic stroke is required.

The Golgi apparatus is an organelle essential for protein synthesis and maturation. In response to conditions of stress, including physiological imbalances or disruption of cell morphology, the transcription of Golgi-associated genes can be upregulated to restore homeostasis or induce apoptosis; this mechanism is termed the Golgi stress response (1,2). Research suggests the extracellular signal-regulated kinase (ERK) signaling pathway is involved in the response to oxidative stress. A previous study demonstrated that the ERK signaling pathway regulates phosphorylation of the Golgi reassembly and stacking protein 65 (GRASP65), resulting in Golgi cisternal unstacking (3).

Curcumin (Cur), a yellow dye in the crude drug 'Turmeric' (*Curcuma rhizoma*) from the rhizome of *Curcuma longa* L., is reported to have anti-inflammatory, anti-oxidative and anti-tumor effects (4-6). Tetrahydrocurcumin (THC) is an active metabolite of Cur, and has been identified in human and rat intestinal mucosa, and in hepatic cytosol (7). THC and Cur have identical  $\beta$ -diketone structures and phenolic groups, but differ in that THC lacks the double bonds of Cur (Fig. 1). Recent research suggests that THC exerts greater anti-oxidant activity than Cur in certain *in vitro* and *in vivo* systems (8-10).

The present study examined the protective effects of THC against cerebral I/R injury in mice, and reviewed the mechanisms of Golgi stress-induced cerebral I/R injury via the ERK signaling pathway.

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**Abbreviations:** THC, tetrahydrocurcumin; ERK, extracellular signal-regulated kinase; GRASP, golgi reassembly and stacking proteins; Cur, curcumin; SOD, superoxide dismutase; MDA, malondialdehyde; ROS, reactive oxygen species; TTC, tetrzolium chloride

**Key words:** Golgi apparatus stress, cerebral ischemia reperfusion injury, tetrahydrocurcumin, extracellular signal-regulated kinase, golgi reassembly and stacking proteins

## Materials and methods

**Animals.** Male specific pathogen-free ICR mice (n=100; 2 months old; 23–27 g) were provided by the Experimental Animal Center of Wenzhou Medical University (Wenzhou, China). Mice had *ad libitum* access to food and water and were housed in temperature- and humidity-controlled conditions (temperature;  $22\pm1^{\circ}\text{C}$ ; humidity  $56\pm5\%$ ) with a 12-h light/dark cycle. All mice were treated humanely, and the study was approved by the Experimental Animal Ethics Committee of Wenzhou Medical University.

**Chemicals.** THC was purchased from Dalian Meilun Biotech Co., Ltd. (Dalian, China). Sodium chloride injections (0.9%) were purchased from Hangzhou Minsheng Pharmaceutical Group Co., Ltd. (Hangzhou, China). Dimethyl sulfoxide (DMSO), chloral hydrate (10%; Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) and 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) were purchased from Sigma-Aldrich (Merck Millipore, Darmstadt, Germany). Paraformaldehyde (4%) was provided by the Experimental Neural Organisms Institution of Wenzhou Medical University (Wenzhou, China). Total superoxide dismutase (T-SOD) kits, lipid peroxidation [malondialdehyde (MDA)] assay kits, total protein kits and bicinchoninic acid (BCA) protein assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Anti-GRASP65 (sc-398363) and anti-phospho (p)-GRASP65 (sc-389542) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA), and anti-ERK (4696S) and anti-pERK (9106L) antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

**Animal surgery and THC administration.** The mice were randomly divided into 5 groups (n=20 per group) as follows: Sham-operated controls (group A), I/R group (group B), low-dose THC (5 mg/kg; group C1), moderate-dose THC (10 mg/kg; group C2) and high-dose THC (25 mg/kg; group C3).

Cerebral ischemia was induced by Pulsinelli's four-vessel occlusion method (11). All surgeries were performed by the same person. Under anesthesia with 10% chloral hydrate (3 mg/kg), a 1.5 cm incision was made in the middle of the neck and the jugular muscles were separated. The bilateral common vertebral arteries were identified and occluded by electrocoagulation. The incision was sutured and the animals were allowed to recover. The following day, the procedure was repeated and the common carotid arteries were occluded for 5 min using vitreous needles and small bulldog clamps. Sham-operated animals underwent the same surgical procedure without occlusion. Treatment injections were administered after 5 min of reperfusion. Groups A and B received intraperitoneal (i.p.) injections of equal volumes of normal saline. A 2% THC solution was prepared in DMSO; groups C1, C2 and C3 received i.p. injections of THC at a low (5 mg/kg), moderate (10 mg/kg), or high (25 mg/kg) dose, respectively.

**Neuroethological assessment.** After 24 h of reperfusion, mice were evaluated for exponents of stroke (Table I) and neurological symptoms (Table II) (12).

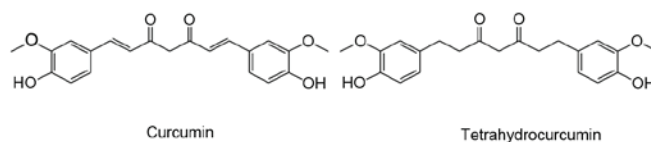


Figure 1. Chemical structures of curcumin and tetrahydrocurcumin.

**Specimen collection and preparation of pathological sections.** After 24 h of reperfusion, mice were anesthetized with 10% chloral hydrate prior to decapitation, then were transcardially perfused with normal saline. The brain was dissected via craniotomy and fixed in a 4% paraformaldehyde solution for 24 h prior to conventional paraffin embedding. The specimens were sectioned coronally (behind the optic chiasm) at a thickness of  $5\text{ }\mu\text{m}$ . Sections were stained with hematoxylin and eosin (HE), as previously described (13).

**TTC staining.** Mice were decapitated after 24 h of reperfusion. The brain was removed and cooled in normal saline at  $4^{\circ}\text{C}$  for 10 min. The specimens were sectioned coronally into four 2-mm-thick slices using a brain matrix. Slices were incubated with a 2% aqueous solution of TTC in the dark for 30 min at  $37^{\circ}\text{C}$  in a water bath, and then images using a digital camera (Canon 600D; Canon, Inc., Tokyo, Japan). Unstained areas were defined as infarcted and measured using image analysis software (Image-Pro Plus, version 6.0; Media Cybernetics, Inc., Rockville, MD, USA). Total infarct volume in the brain was calculated as the sum of the infarct volumes of the four brain slices (14).

**Assessment of cerebral SOD and MDA.** Cerebral SOD and MDA levels were measured in brain tissue homogenates prepared with a 0.9% saline solution and centrifuged at  $1,006\times g$  for 10 min at  $4^{\circ}\text{C}$ . Supernatants were collected and analyzed using the SOD and MDA kits. Concentrations of SOD and MDA were calculated based on the optical density readings obtained at 550 and 552 nm, respectively, using the Multiskan Spectrum microplate reader (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

**Western blot analysis of ERK, pERK, GRASP65 and pGRASP65.** ERK, pERK, GRASP65 and pGRASP65 levels in brain tissue homogenates were determined by western blotting. Protein extraction kits from were purchased from Jiancheng Bioengineering Institute (Nanjing, China). Total protein levels in the homogenate samples were determined using the BCA protein assay kit. Equal amounts of protein ( $20\text{ }\mu\text{g}$  sample/lane) from each sample were then subjected to 10% SDS-PAGE. The gel was transferred to a polyvinylidene difluoride membrane, and proteins were detected with antibodies against ERK (1:1,000), pERK (1:1,000), GRASP65 (1:200), and pGRASP65 (1:500).  $\beta$ -actin (1:4,000; Santa Cruz Biotechnology, Inc.) was used as a loading control. Subsequently, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit (KS001) or anti-goat (KS003) secondary antibodies (dilution, 1:3,000) for 2 h at  $24^{\circ}\text{C}$ , and were visualized using Western Blotting Chemiluminescence Reagent, followed by exposure to X-ray films. Blots were quantified using BandScan software (version 5.0; Glyko, Inc., Novato, CA, USA).

Table I. Exponents of stroke.

Symptom	Score
Messy fur, tremor	1
Decreasing exercise or bovine movement	1
Bovine feeling of ears	3
Turned up head	3
Eyes opened	3
Posterior limb shaped like Chinese character eight (/\)	3
Upper eyelid drooping	1
Circling	3
Eclampsia or paroxysmal movement	3
Exceedingly weak	6

The total score is 25 and divided into three categories: Symptom (score, 0-3), possible injury (score, 3-9), obvious injury (score,  $\geq 10$ ).

Table II. Neurological symptom assessment.

Symptom	Score
Idiopathic excavation	0
Walking while being stimulated	1
Cannot move	2
Normal gait	0
Ataxia	1
Crawling	2
No gait	3
Able to feed	0
Cannot feed	1
Able to drink	0
Cannot drink	1
Removable to pains	0
Only head or trunk can move	1
Limbs can retrace or no reaction	3

The total score is 15, and divided into four categories: Normal (score, 0), mild injury (score, 1-3), moderate injury (score, 4-6), severe injury (score, 7-15).

**Statistical analysis.** Statistical comparisons were made using analysis of variance (ANOVA) followed by post-hoc Bonferroni tests. Data are presented as the mean  $\pm$  standard deviation. Neuroethological and stroke assessment data were analyzed using the Friedman test. Friedman two-way ANOVA was used for multiple comparisons. Statistical analyses were performed using SPSS software (version 19.0; IBM SPSS, Armonk, NY, USA).  $P < 0.05$  were considered to indicate a statistically significant difference.

## Results

**Neuroethological assessment following cerebral I/R.** After 24 h of reperfusion, various neurological symptoms were

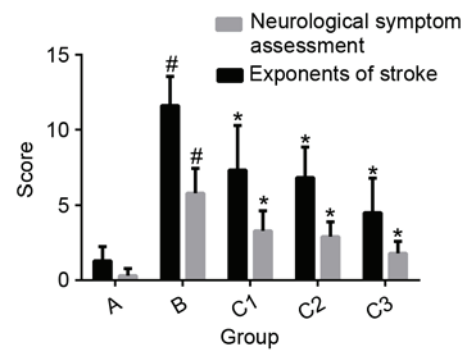


Figure 2. Neuroethological assessment of the mice in each group following I/R for 24 h.  $^{\#}P < 0.01$  vs. group A,  $^*P < 0.01$  vs. group B. I/R, ischemia/reperfusion; Group A, sham; Group B, I/R control; Group C1, low-dose tetrahydrocurcumin; Group C2, moderate-dose tetrahydrocurcumin; Group C3, high-dose tetrahydrocurcumin.

observed: Decreased exercise, lack of feeding, bovine-like reaction, ataxia (in some mice), circling with the tail lifted, posterior limbs positioned as the Chinese character for eight (/\), upper eyelid drooping or inability to open the eyes, and messy fur. Compared with group B (normal saline-injected mice), the exponents of stroke and neurological assessment scored were significantly decreased in the THC-treated groups ( $P < 0.01$ ; Fig. 2).

**Effect of THC on ischemic neuronal necrosis.** In sham-operated mice (group A), cells were closely arranged, regularly shaped and uniformly stained by HE (Fig. 3). By contrast, cells from mice with I/R injury (group B) were loosely arranged with various abnormal pathological changes, including karyopyknosis, interstitial edema, neuronal degeneration and vacuolization. Cells from mice administered the high dose of THC (25 mg/kg; group C3) were mostly normal, though karyopyknosis was evident in some cells (Fig. 3). Cells from mice administered the low dose of THC (5 mg/kg; group C1) exhibited more severe apoptosis, with higher levels of karyopyknosis, irregular arrangement, interstitial edema, neuronal degeneration and vacuolization compared with group C3. In mice administered the moderate dose of THC (10 mg/kg; group C2), the cellular effects were between those observed in groups C1 and C3 (Fig. 3).

**Effect of THC on infarct size following I/R injury.** After 24 h of reperfusion, extensive infarction was evident in group B mice. The infarct size was significantly reduced in all THC treatment groups compared with group B ( $P < 0.01$ ; Figs. 4 and 5), and the effect was dose-dependent. No infarction was observed in the sham-operated group.

**Effect of THC on SOD and MDA.** Compared with group B, mice administered THC exhibited significantly increased levels of SOD ( $P < 0.01$ ) and lower levels of MDA in brain tissue homogenates ( $P < 0.01$ ; Fig. 6). The effects of THC on SOD and MDA were dose-dependent.

**Effect of THC on the expression of ERK, pERK, GRASP65 and pGRASP65.** Western blot analysis demonstrated the protein expression of ERK in ischemic brain tissue did not



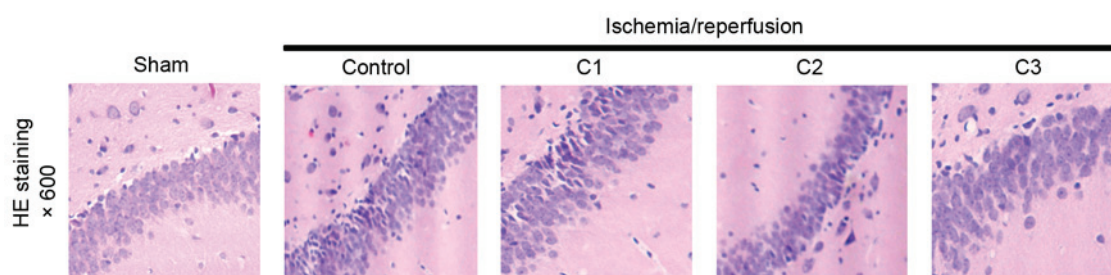


Figure 3. Changes in the neurocyte morphology of cerebral ischemia/reperfusion injury mice. Stained with HE. Magnification, x600. HE, hematoxylin and eosin; C1, low-dose tetrahydrocucumin; C2, moderate-dose tetrahydrocucumin; C3, high-dose tetrahydrocucumin.

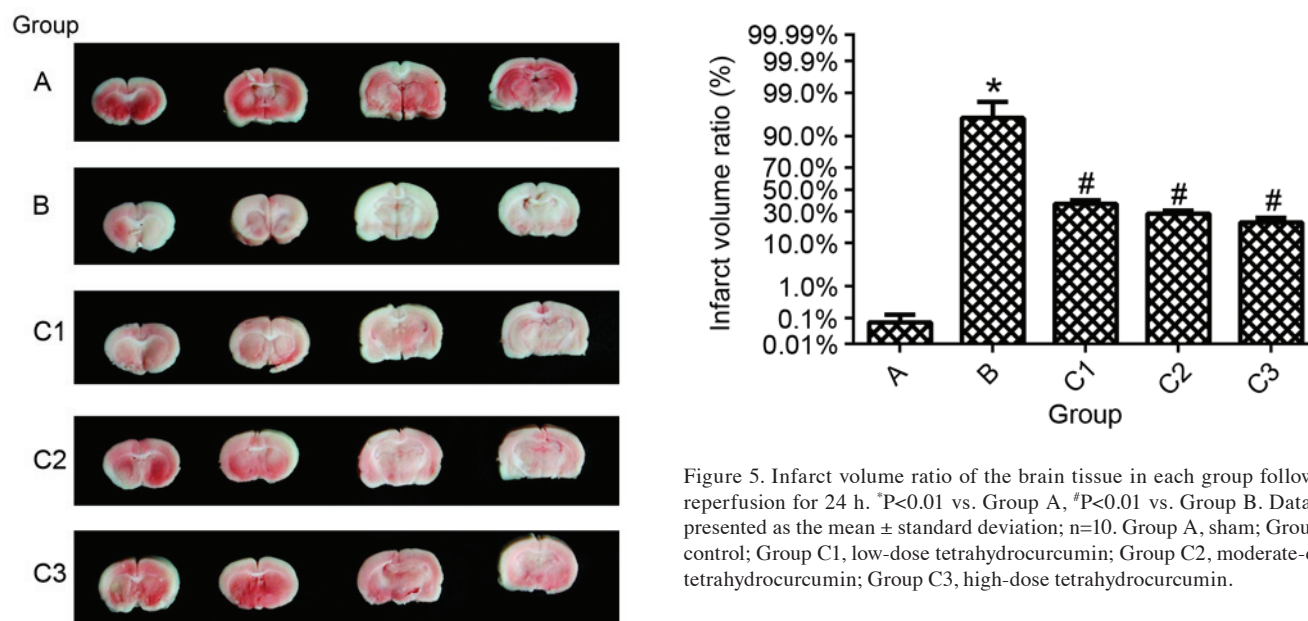


Figure 4. Infarct size of brain tissue following reperfusion for 24 h stained with triphenyltetrazolium chloride. Group A, sham; Group B, control; Group C1, low-dose tetrahydrocucumin; Group C2, moderate-dose tetrahydrocucumin; Group C3, high-dose tetrahydrocucumin.

differ the between groups ( $P>0.05$ ; Fig. 7). However, the level of pERK and the pERK/ERK ratio were significantly increased in group B compared with group A ( $P<0.01$ ; Fig. 7). The pERK/ERK ratio as significantly reduced in groups C1 ( $P<0.05$ ), C2 ( $P<0.01$ ) and C3 ( $P<0.01$ ) compared with group B (Fig. 7). This suggests that the ERK signaling pathway was suppressed by THC.

Expression of total GRASP65 did not differ significantly between groups ( $P>0.05$ ; Fig. 7). However, the level of pGRASP65 was significantly increased in group B compared with group A ( $P<0.01$ ; Fig. 7). In groups C1, C2 and C3, pGRASP65 was significantly reduced compared with group B ( $P<0.01$ ; Fig. 7).

The results of the present study suggest that the upregulated phosphorylation of GRASP65 following I/R injury may be due to, at least partially, the increased activation of the ERK signaling pathway induced by THC. Thus, THC protects against cerebral I/R injury via suppression of GRASP65 phosphorylation, and this effect may be mediated by the ERK signaling pathway.

## Discussion

The ERK signaling pathway has an important role in cerebral I/R injury. ERK is involved in growth and differentiation, mediated by growth factor receptors, and is also activated by oxidative stress, cerebral ischemia and the release of neurotransmitters under certain pathological conditions (15,16). In the current study, the level of pERK and the pERK/ERK ratio were significantly increased in ischemic mice compared with sham-operated controls, indicating that the ERK pathway was activated in response to cerebral I/R injury. In mice administered THC, pERK was significantly reduced compared with the control, suggesting that THC inhibited I/R injury-induced activation of the ERK pathway. This effect was dose-dependent, which provides further evidence of the neuroprotective properties of THC.

GRASPs are membrane proteins involved in Golgi stacking. They regulate Golgi assembly, and cell migration, division and apoptosis. Specifically, GRASP65 mediates Golgi morphological changes during pathophysiological conditions, and is involved in cell division and apoptosis (17-21).

In the present study, expression of GRASP65 and pGRASP65 were elevated following cerebral I/R injury compared with sham-operated animals ( $P<0.01$ ). Previous research has demonstrated that GRASP65 is phosphorylated

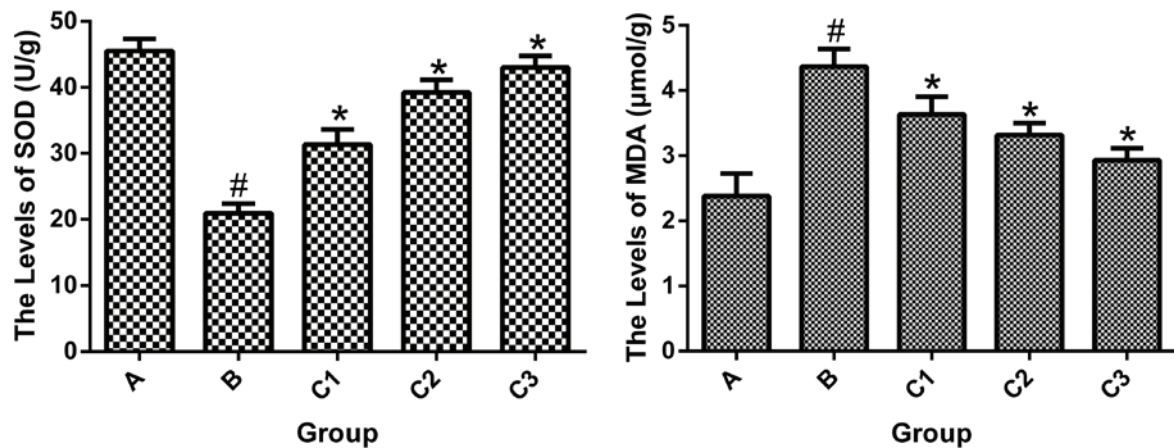


Figure 6. Changes in the levels of SOD and MDA in mice cerebral tissue homogenates. # $P < 0.01$  vs. Group A, \* $P < 0.01$  vs. Group B. Data are presented as the mean  $\pm$  standard deviation;  $n = 10$ . SOD, superoxide dismutase; MDA, malondialdehyde; Group A, sham; Group B, control; Group C1, low-dose tetrahydrocurcumin; Group C2, moderate-dose tetrahydrocurcumin; Group C3, high-dose tetrahydrocurcumin.

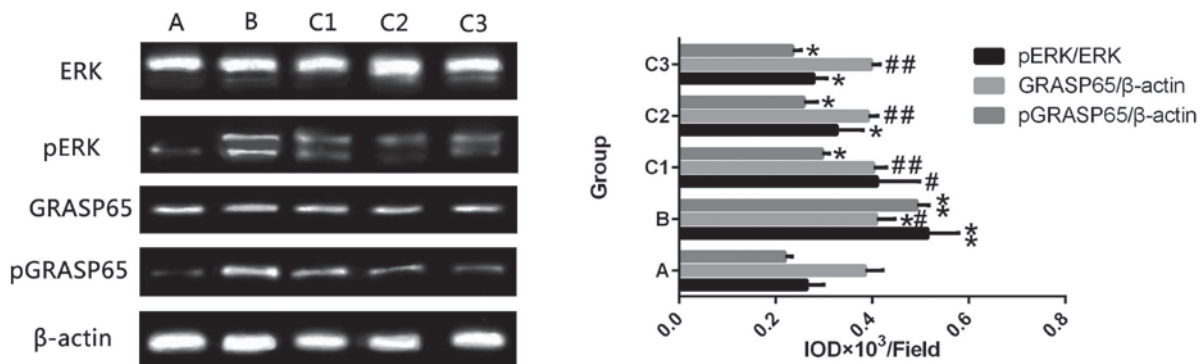


Figure 7. ERK, pERK, GRASP65 and pGRASP65 expression in mice from each group following reperfusion for 24 h. \*\* $P < 0.01$  vs. Group A, # $P > 0.05$  vs. Group A, \* $P < 0.05$  vs. Group B, \* $P < 0.01$  vs. Group B, \*\* $P > 0.05$  vs. Group B. Data are presented as the mean  $\pm$  standard deviation;  $n = 10$ . Group A, sham; Group B, control; Group C1, low-dose tetrahydrocurcumin; Group C2, moderate-dose tetrahydrocurcumin; Group C3, high-dose tetrahydrocurcumin; ERK, extracellular-signal regulated kinase; p, phosphorylated; GRASP65, Golgi reassembly and stacking protein 65.

by ERK, cyclin dependent kinase 1 and polo like kinase 1 during cell division, which leads to depolymerization and division of the Golgi (17,18,20-22).

The ERK signaling pathway may be involved in neuronal degeneration following I/R injury. In mice treated with THC, a dose-dependent decrease in the phosphorylation of GRASP65 was observed, and this effect may be mediated by ERK. The Golgi is essential for the endoplasmic reticulum and mitochondria during conditions of oxidative stress as a downstream target organelle associated with phosphorylation of GRASP65. The Golgi stress response suppresses the synthesis of required proteins, and thereby impacts the extent of I/R injury.

Reactive oxygen species (ROS) are produced as a result of incomplete reduction of oxygen within the electron transport chain. Examples of ROS include superoxide anion, singlet oxygen, hydrogen peroxide and hydroxyl radicals. These ROS typically function as signaling molecules, however during stress, they can lead to cell damage and tissue necrosis. Anti-oxidant enzymes, including glutathione reductase, catalase and SOD, are effective at neutralizing ROS. During I/R injury, SOD is involved in maintaining homeostasis and

minimizing the damaging effects of ROS (23-25). MDA is an end product of lipid peroxidation in cells, and increased levels of ROS can trigger overproduction of MDA. SOD and MDA are often used as markers of oxidative stress and anti-oxidant status, respectively. Cur is reported to have anti-oxidative properties and a neuroprotective effect against cerebral I/R injury (26). For example, Wang *et al* demonstrated that Cur attenuates oxidative stress injury induced by hypoxic-ischemic brain damage in rats. In the present study, mice treated with THC exhibited increased levels of SOD following I/R injury compared with mice given saline, and this effect was dose-dependent. Levels of MDA were increased following I/R injury, and THC attenuated this effect in a dose-dependent manner. The evaluation of neurological behavior revealed a significant dose-dependent effect of THC.

In summary, THC had a dose-dependent protective effect against cerebral I/R injury, mediated by suppression of the ERK signaling pathway and a subsequent reduction of GRASP65 phosphorylation. The current study investigated the cerebral ischemia-reperfusion injury mechanism, and suggested that THC may be a potential therapeutic agent for the prevention of ischemic brain injury.

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