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ORIGINAL PAPER

Ginsenoside Rd Attenuates Tau Protein Phosphorylation Via the PI3K/AKT/GSK-3β Pathway After Transient Forebrain Ischemia

Xiao Zhang · Ming Shi · Ruidong Ye · Wei Wang · Xuedong Liu · Guangyun Zhang · Junliang Han · Yunxia Zhang · Bing Wang · Jun Zhao · Juan Hui · Lize Xiong · Gang Zhao

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Abstract Phosphorylated tau was found to be regulated after cerebral ischemia and linked to high risk for the development of post-stroke dementia. Our previous study showed that ginsenoside Rd (Rd), one of the main active ingredients in Panax ginseng, decreased tau phosphorylation in Alzheimer model. As an extending study, here we investigated whether Rd could reduce tau phosphorylation and sequential cognition impairment after ischemic stroke. Sprague–Dawley rats were subjected to focal cerebral ischemia. The tau phosphorylation of rat brains were analyzed following ischemia by Western blot and animal cognitive functions were examined by Morris water maze and Novel object recognition task. Ischemic insults increased the levels of phosphorylated tau protein at Ser199/202 and PHF-1 sites and caused animal memory deficits. Rd treatment attenuated ischemia-induced enhancement of tau phosphorylation and ameliorated behavior impairment. Furthermore, we revealed that Rd inhibited the activity of Glycogen synthase kinase-3β (GSK-3β), the most important kinase involving tau phosphorylation, but enhanced the activity of protein kinase B (PKB/AKT), a key kinase suppressing GSK-3β activity. Moreover, we found that LY294002, an antagonist for phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway, abolished the inhibitory effect of Rd on GSK-3β activity and tau phosphorylation. Taken together, our findings provide the first evidence that Rd may reduce cerebral ischemia-induced tau phosphorylation via the PI3K/AKT/GSK-3β pathway.

Keywords Ginsenoside Rd · PI3K/AKT · GSK-3β · Tau phosphorylation · Ischemic stroke

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X. Zhang · M. Shi (✉) · X. Liu · G. Zhang · J. Han · Y. Zhang · B. Wang · J. Zhao · J. Hui · G. Zhao (✉)
Department of Neurology, Xijing Hospital, Forth Military Medical University, Xi'an, People's Republic of China
e-mail: biomidas@fmmu.edu.cn

G. Zhao
e-mail: zhaogang@fmmu.edu.cn

R. Ye
Department of Neurology, Jinling Hospital, Nanjing University School of Medicine, Nanjing, People's Republic of China

W. Wang
Department of Microbiology, Institute of Clinical Medicine, Oslo University Hospital, Oslo, Norway

L. Xiong
Department of Anesthesiology, Xijing Hospital, Forth Military Medical University, Xi'an, People's Republic of China

Introduction

Ischemic stroke is a major cause of mortality and disability in human and the second leading cause of death in developed countries [1]. Apart from physical disability, post-stroke dementia (PSD) is the second most common consequence caused by stroke [2]. Epidemiological studies have shown that the incidence of PSD is about 32 % 1 year after stroke and 12 times higher than in normal population 4 years after stroke [3, 4]. PSD is associated with a high mortality rate in stroke patients and seriously affects the quality of their lives [3, 4]. Growing evidence showed that many of these dementias developed progressively and the primary damage was not the direct cause of the PSD in most of the cases [5].

It is reported that PSD shares some common risk factors and neuropathology features with a well-known Alzheimer's disease (AD), e.g. formation of neurofibrillary tangles (NFTs) mainly composed of hyperphosphorylated tau protein [6–9]. Tau protein is microtubule-associated proteins that is expressed abundantly in central nervous system (CNS) [10]. It induces microtubule assembly and stabilizes microtubules by its phosphorylation states which are mainly regulated by phosphatidylinositol 3-kinase/protein kinase B/Glycogen synthase kinase-3β (PI3K/AKT/GSK-3β) pathway [11–16]. However, hyperphosphorylation of tau forms NFTs and leads to dysfunction of synapses, degeneration of neurons, and cognitive impairment [10, 17]. Therefore, inhibition of tau phosphorylation may be a potential therapeutic strategy for preventing such neurodegenerative diseases [18].

Ginsenosides are the most active ingredients in *ginseng* which has been widely used as a traditional herbal medicine for thousands of years in eastern Asia [19]. Our preclinical and clinical studies showed that ginsenoside Rd (Rd), a monomer compound extracted from *ginseng*, was effective and safe for the treatment of acute ischemic stroke [20–27], suggesting that Rd may be a promising neuroprotectant. Moreover, our latest studies showed that Rd inhibited tau phosphorylation and ameliorated cognitive function in rat model of AD [28–30]. Thus, as an extending study, here we investigated whether Rd could affect experimental cerebral ischemia-induced tau phosphorylation and sequential cognition dysfunction and further explored the underlying mechanisms in this process.

Materials and Methods

Materials

Rd with a purity of 98 % was obtained from Tai–He Biopharmaceutical Co. Ltd. (Guangzhou, China). The stock solutions were prepared in saline containing 10 % 1,3-propanediol (v/v). cresyl violet and 2,3,5-triphenyltetrazolium chloride (TTC) were purchased from Sigma-Aldrich Inc. (St. Louis, Mo, USA). PHF-1 antibody was obtained from Abcam (Cambridge, UK) and other antibodies were purchased from Cell Signaling (Carlsbad, CA, USA). All other reagents were from commercial suppliers and of standard biochemical quality.

Focal Cerebral Ischemia

Male Sprague–Dawley rats weighing 270–320 g, provided by the Animal Breeding Center, affiliated to the Fourth Military Medical University, China, were used in this study. Animal protocols were approved by the Ethics Committee for Animal Experimentation of the Fourth Military Medical University. The focal cerebral ischemia was induced by 2 h of middle cerebral artery occlusion (MCAO) as described previously with modifications [24]. In brief, animals were anesthetized with a mixture of isoflurane (1.5–2 %), oxygen and nitrogen. Body temperature in the rectum was maintained at 37.0 °C using a thermostatically controlled heating blanket. A 4–0 monofilament coated with poly-L-lysine was introduced through the internal carotid artery to occlude the origin of the middle cerebral artery (MCA). The induction of focal cerebral ischemia was verified with laser Doppler flowmetry (PeriFlux 5000; Perimed AB, Sweden). A drop in regional cerebral blood flow (CBF) below 30 % from baseline after the insertion of the filament was considered to be sufficient for induction of focal cerebral ischemia. Control animals were subjected to the same surgical procedures except that the suture was not advanced into the MCA. Rd with a concentration of 30 mg/kg or vehicle was applied intraperitoneally 1 h before MCAO and 10 mg/kg per day until animals were sacrificed.

Nissl Staining

Nissl staining was performed as we previously described with some modifications [30]. Briefly, the sections of brains were placed in xylene twice (15 min/every time), then placed in graded alcohols (100, 90, 80, 70, 50 %, 5 min/every time), immersed in 0.1 % cresyl violet 5 min, dehydrated through graded alcohols (95, 100 %, 2 min/every time), placed in xylene twice again (15 min/every time) and mounted by neutral gum, the numbers of remaining neurons in ipsilateral Hippocampal CA1 region were calculated for each experimental group.

TTC Staining

TTC stain was performed as we previously described [26]. Briefly, rats in each group were anesthetized and decapitated. Each brain was sliced into 2 mm sections. Serial coronal sections were soaked in 2 % TTC phosphate buffer at 37 °C for 10 min in the dark. Normal brain tissues were stained red, while infarct tissues were not stained (white). The sections were soaked in 4 % paraformaldehyde phosphate buffer for 30 min, arranged in order and scanned. The ratios percentages of infarct areas to the total brain areas were calculated.

Novel Object Recognition (NOR)

The NOR takes advantage of the natural tendency of rats to explore a novel object more than a familiar object and was used to evaluate the working memory from post-occlusion

day (POD) 26 to POD 32 as previously described [31]. Briefly, rats were trained in the experimental chamber with two identical objects. They were allowed to explore the objects for 10 min. Memory retention was assessed either 1 h (for short-term retention) or 24 h (for long-term retention) after training. The animals were allowed to explore two different objects for 5 min. One of the objects was identical to those explored during the training session (familiar object) and the other was a different object (novel object). The animals were regarded to be exploring when they were facing, sniffing, or biting the object. The time spent exploring object was recorded and analyzed using ANY-maze software (Stoeling Co, Wood Dale, IL, USA). The relative time of novel object exploration was calculated as the index of discrimination calculated as follows: (Tnovel − Tfamiliar)/(Tnovel + Tfamiliar). The total times of exploration for each group in training and testing sessions were compared to verify that there were no differences between groups in this parameter. Animals showing low exploration times were excluded from the experiments.

Morris Water Maze

To assess the spatial memory deficit of rats after MCAO, Morris water maze (MWM), including 5 days of spatial acquisition and 1 day of probe trial (POD 26–32), was performed as we previously described [32]. Rats were individually trained in a circular pool (150 cm diameter, 60 cm height) filled with water (22 °C). The platform was submerged about 1.5 cm below the surface of the water infiltrated with 80 ml Chinese ink. On each day, the animal was subjected to three trials and each trial lasted either until the rat found the platform or for 120 s. Time to reach the platform (latency), length of swim path, and swim speed were recorded semi-automatically by a video tracking system. 24 h after the last learning session, the platform was removed from its previous location and the animals were given a probe trial in which they had 60 s to search for the platform. Time spent in the target quadrant, number of times animals crossed above the former target site where the platform had been located (crossovers) were recorded during the probe trials. To control for possible differences in visual acuity or sensorimotor function between groups, a cued task were performed using a visible platform raised 0.5 cm above the surface of the water.

Primary Culture of Neurons

Neurons were cultured from Sprague–Dawley rat embryos as previously described with some modifications [23]. Animal procedures were in accordance with NIH Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996). All efforts were made to minimize animal number and their suffering. After trituration and trypsinization, the single-cell suspension from embryos Hippocampus was seeded in 6-well plates coated with poly-L-lysine (50 μg/ml) at the density of (2–2.5) × 10^5/cm^2. Cells were maintained in Neurobasal medium supplemented with 2 % B-27, 0.5 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin in a humidified atmosphere of 5 % CO_2 at 37 °C.

Oxygen–Glucose Deprivation (OGD)

OGD was carried out as described by Li et al. [22]. Briefly, the culture medium was replaced with pre-warmed Dulbecco's modified eagle medium (DMEM) without glucose and serum. The cell cultures were then transferred into an anaerobic chamber equilibrated with 95 % N_2 and 5 % CO_2. The chamber was kept in a 37 °C incubator. Control cultures were maintained in a normal oxygenated DMEM containing 25 mM glucose. After 2 h, cultures were placed back to the normoxic incubator with normal culture medium. LY294002 (5 μM) was pretreated 12 h before OGD. Rd (10 μM) was added in the culture medium during OGD and reoxygenation.

Western Blot

Ipsilateral hippocampus after MCAO was homogenized on ice in the RIPA lysis buffer (Beyotime, China) containing 0.5 mM PMSF. Western blot was performed according to Ye R et al. [24]. Briefly, protein samples were electrophoresed on a 10 % SDS-PAGE and subsequently transferred to PVDF membrane (Millipore, USA). The membrane was incubated in blocking buffer containing 5 % non-fat dried milk at room temperature for 1 h and then probed with the primary antibody (PHF-1 at 1:500; p-AKT at 1:500; tau-5 at 1:1,500; AKT at 1:2,000; S199/202 at 1:500 GSK-3β at 1:1,000; p-GSK-3β at 1:500 and GAPDH at 1:3,000) in blocking buffer at 4 °C overnight. The membrane was washed three times with TBST (TBS and 0.1 % Tween 20) and then incubated with HRP-conjugated secondary antibody at room temperature for another 1 h. Specific signals of proteins were visualized by chemiluminescence using the ECL western blotting detection system (GE Healthcare UK). For quantitative analysis, the ratio of the specific signals of protein (relative intensity of the signal) to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein was calculated.

Statistical Analysis

Results were analyzed with one-way ANOVA followed by Tukey post test for multiple comparisons. Origin-pro 8

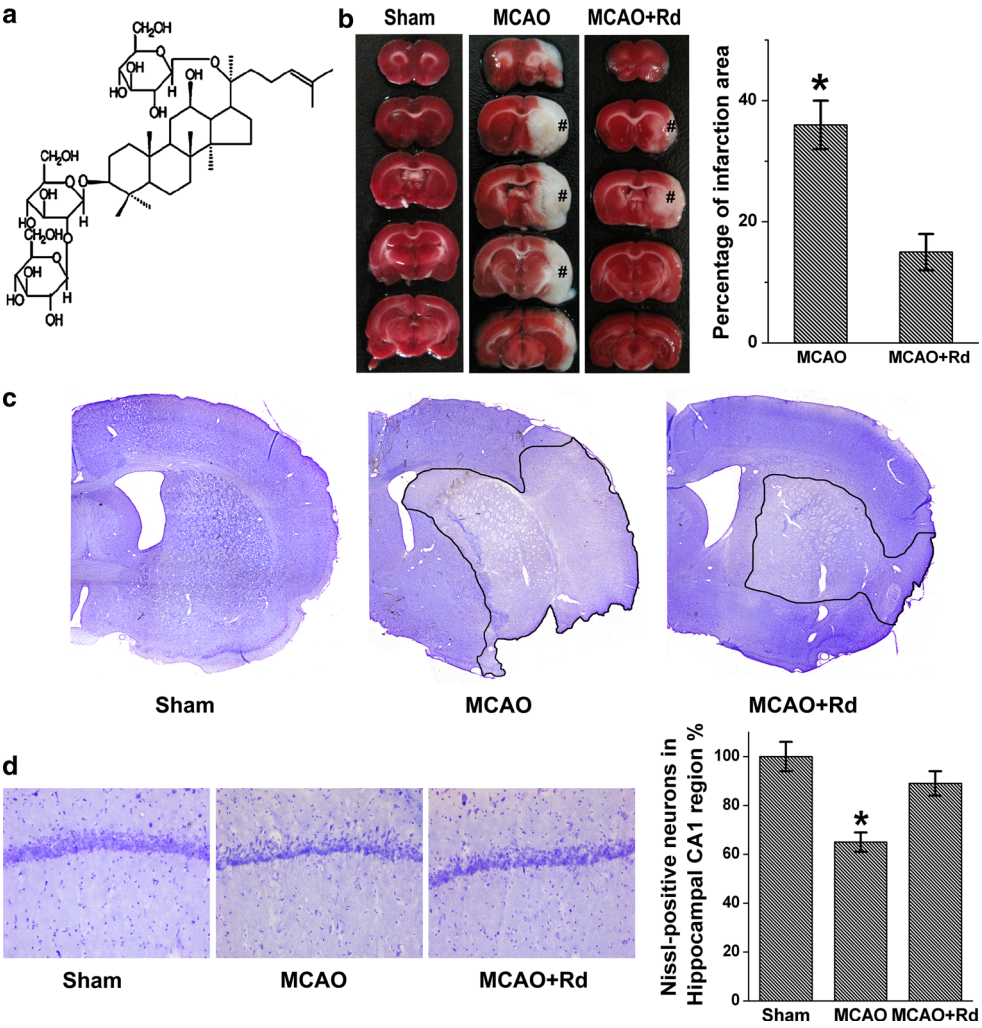

Fig. 1 The neuroprotective effect of Rd after rat MCAO. Infarction sizes were evaluated by TTC (**b**) and Nissl staining (**c**) 1 day after MCAO. **a** The structural formulae of Rd. **b** TTC staining of infarcts in brain sections (*white areas* marked by *number sign*). **c** Nissl staining of infarcts in brain sections (boxed by *black lines*). (**d**) Nissl staining of cell loss in ipsilateral hippocampal CA1 region 28 days after MCAO. Data are expressed as mean ± SEM (n = 9). Mean values in Sham groups were scaled to 100 % (Nissl staining). * $p < 0.05$ versus MCAO + Rd group

software was used for statistical tests. Statistical significance was established at $p < 0.05$.

Results

Rd Reduces Infarction Volume and Hippocampal Cell Loss After MCAO

Ginsenoside Rd (Dammar-24[25]-ene-3,12,20[S]-triol-]20-O-β-d-gluco-pyranosyl]-3-O-β-d-glucopyranosyl-(1 → 2)-β-dglucopyranoside[Rd]; Fig. 1a) is one of the major ginsenosides in the ginseng root [27]. Rd has exhibited an encouraging neuroprotective efficacy against cerebral ischemic stroke in both laboratory and clinical studies [20–27]. Consistently, in this study we showed that Rd administration could reduce infarction volume POD 1 after MCAO, as revealed by TTC and nissl staining (Fig. 1b, c), we also found that Rd could decrease cell loss in ipsilateral hippocampal CA1 region POD 28 after MCAO (Fig. 1d), these results suggested that Rd was effective in the treatment of ischemic stroke.

Rd Improves Cognitive Function After MCAO

NOR and MWM were respectively performed to assess the effects of Rd on working and spatial memory after ischemic brain injury. For working memory, the rats subjected to MCAO injury spent less time exploring the novel objects, compared with the sham group. Relative to the MCAO group, the rats in Rd-treated group spent a greater proportion of time exploring the novel objects in either 1 h or 24 h tests (Fig. 2a, b). For spatial learning and memory, mean latency of finding the platform and the path length to reach the platform declined progressively during the training period in sham rats. By contrast, the rats in MCAO group had more time of latency and longer distance of path length. Less time in target quadrant and platform

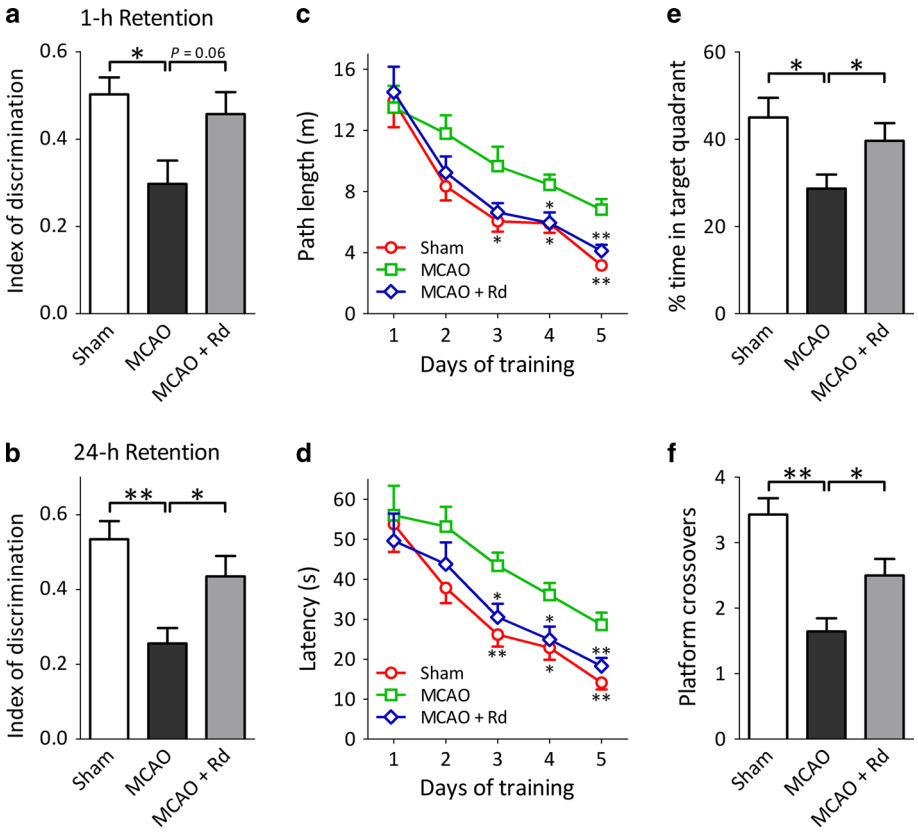

Fig. 2 Effects of Rd on cognitive function after rat MCAO. Learning and memory performance were evaluated in novel object recognition (NOR) (**a**, **b**) and Morris water maze (MWM) (**c**–**f**) task 28 days after MCAO. Performance on object recognition was tested 1 h (**a**) and 24 h (**b**) after training with two identical objects. In MWM task, spatial acquisition trial [path length (**c**), escape latency (**d**)] and probe trial [time in garget quadrant (**e**), platform crossovers (**f**)] were performed in order to detect spatial learning and memory ability. Data are expressed as mean ± SEM (n = 14). * $p < 0.05$, ** $p < 0.01$ versus MCAO group

crossovers was also found in MCAO group. However, these changes were markedly attenuated by Rd administration (Fig. 2c–f). In addition, all the rats could swim easily in all three groups and swimming speed of three groups did not differ during pre-training (data not shown). These results above indicated that Rd could improve working memory and spatial learning function after ischemic brain injury.

Rd Attenuates Tau Protein Phosphorylation After MCAO

Hyperphosphorylated tau is highly associated with cognitive impairment and has been found in the ischemic brain [6–11, 17, 33, 34]. To determine the effects of Rd on the level of phosphorylated tau (p-tau) in vivo, we investigated the expression of total tau (tau-5) and p-tau (PHF-1 and S199/202) 24 h/7 days/14 days and 28 days after MCAO (Fig. 3, Fig. S1). Western blotting results showed that PHF-1 and S199/202 levels increased in all of these time points, but tau-5 was not affected after MCAO, consistent with previous reports [33, 34]. Rd treatment significantly reduced MCAO-induced increase of p-tau levels at these time points (Fig. 3a–d, Fig. S1a-d) but did not affect tau-5 expression (Fig. 3e, f, Fig. S1e-f). These results indicated that Rd could attenuate tau protein phosphorylation after ischemic injury.

Rd Enhances GSK-3β and AKT Phosphorylation After MCAO

GSK-3β is one of the most important kinases involving in tau phosphorylation in the brain [12–16] and can be inhibited by AKT, a kinase involving in survival signaling pathways [35–37]. We then explored whether Rd could affect PI3K/AKT/GSK-3β pathway in MCAO rats (Fig. 4). The activities of GSK-3β and AKT kinases are dependent on their phosphorylation states. To be phosphorylated inhibits GSK-3β (at Ser9 site) but activates AKT. Western blotting results showed that the levels of p-GSK-3β and p-AKT were reduced 24 h after rat MCAO (Fig. 4a, c) and returned to normal levels 28 days after surgery (Fig. 4b, d), MCAO also changed p-GSK-3β and p-AKT levels at POD 7 and POD 14 (Fig. S2), these results were consistent with previous reports [38]. With the treatment of Rd, the levels of p-AKT and p-GSK-3β were markedly increased in these time points, indicating that Rd may suppress GSK-3β activity but enhance AKT activity.

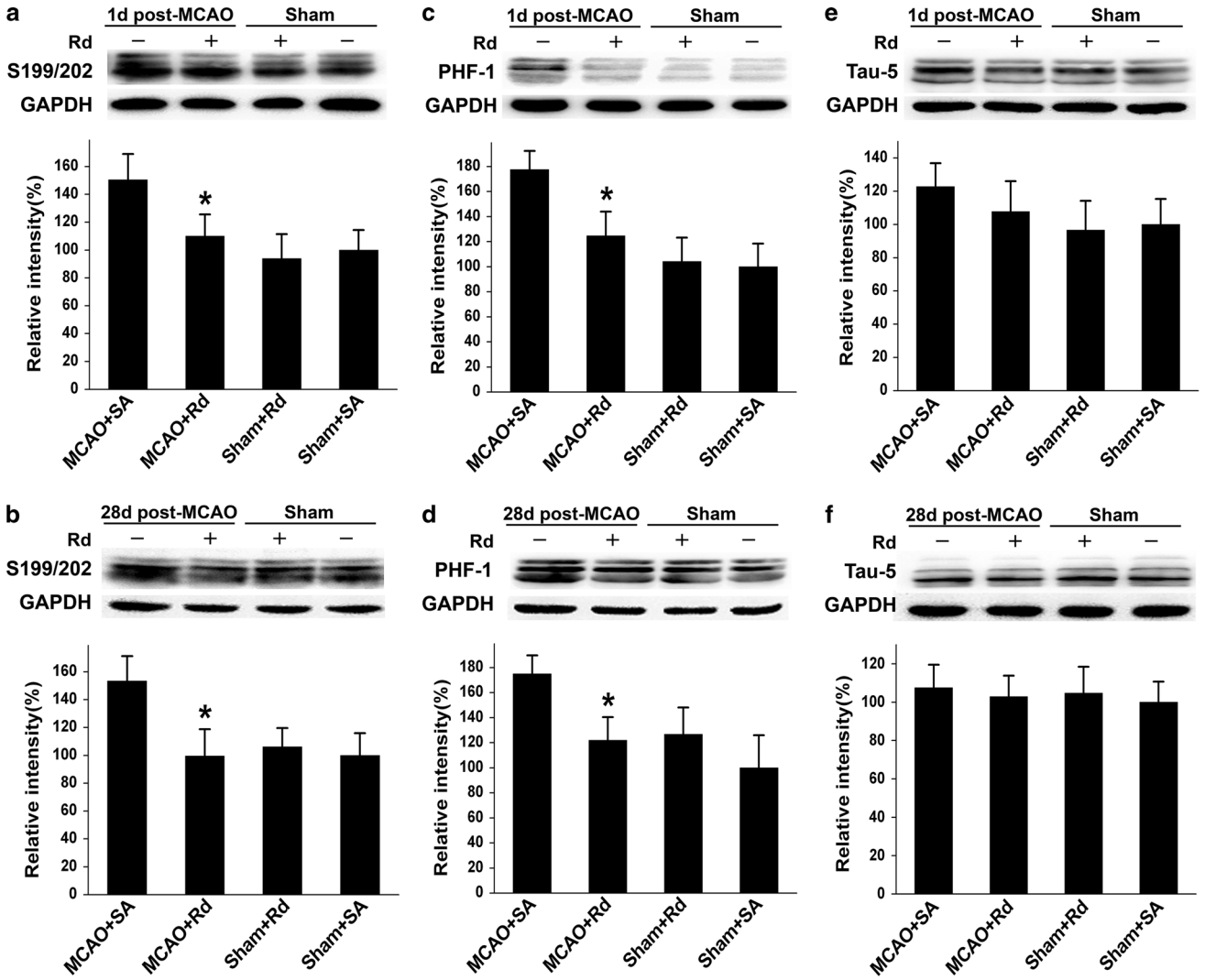

Fig. 3 Effects of Rd on p-tau expression in ischemic rat brain 24 h and 28 days after MCAO. Western blotting analysis was performed using antibodies against S199/202 (**a** 24 h and **b** 28 days) or PHF-1 (**c** 24 h and **d** 28 days) or tau-5 (**e** 24 h and **f** 28 days) as described in Methods. Data are expressed as mean ± SEM (n = 6). GAPDH was used as internal control. Mean values in vehicle-treated (Sham) groups were scaled to 100 %. * $p < 0.05$ versus MCAO group, *SA* saline

Rd Affects Tau Phosphorylation Via PI3K/AKT/GSK-3β Pathway

Since AKT and GSK-3β are key participants in the PI3K/AKT/GSK-3β signaling pathway, we then investigated whether Rd affected tau phosphorylation via this pathway by using a PI3K/AKT pathway inhibitor LY294002 on MCAO rats and primary cultured neurons exposed to OGD injury. Western blotting results showed that compared with the sham group, MCAO increased p-tau but decreased p-GSK-3β levels, which was attenuated by Rd administration (Fig. 5a, b, d), consistent with our previous results (Fig. 4). With the treatment of LY294002, the inhibitory effects of Rd on tau phosphorylation and GSK-3β activity were significantly blocked (Fig. 5a, b, d). Our in vitro study also drew a similar conclusion. That is, Rd could reduce OGD induced tau phosphorylation and GSK-3β activation, but these effects were abolished by LY294002 administration (Fig. S3). It was noted that tau-5 expression was not affected with the treatment of Rd or LY294002 (Fig. 5c).

Discussion

In the present study, we investigated the effects of Rd on tau protein phosphorylation as well as cognitive function in MCAO rats and showed that Rd attenuated cognitive impairment and decreased the levels of p-tau protein after ischemic injury. Moreover, Rd could enhance AKT and GSK-3β phosphorylation, which was blocked by PI3K/

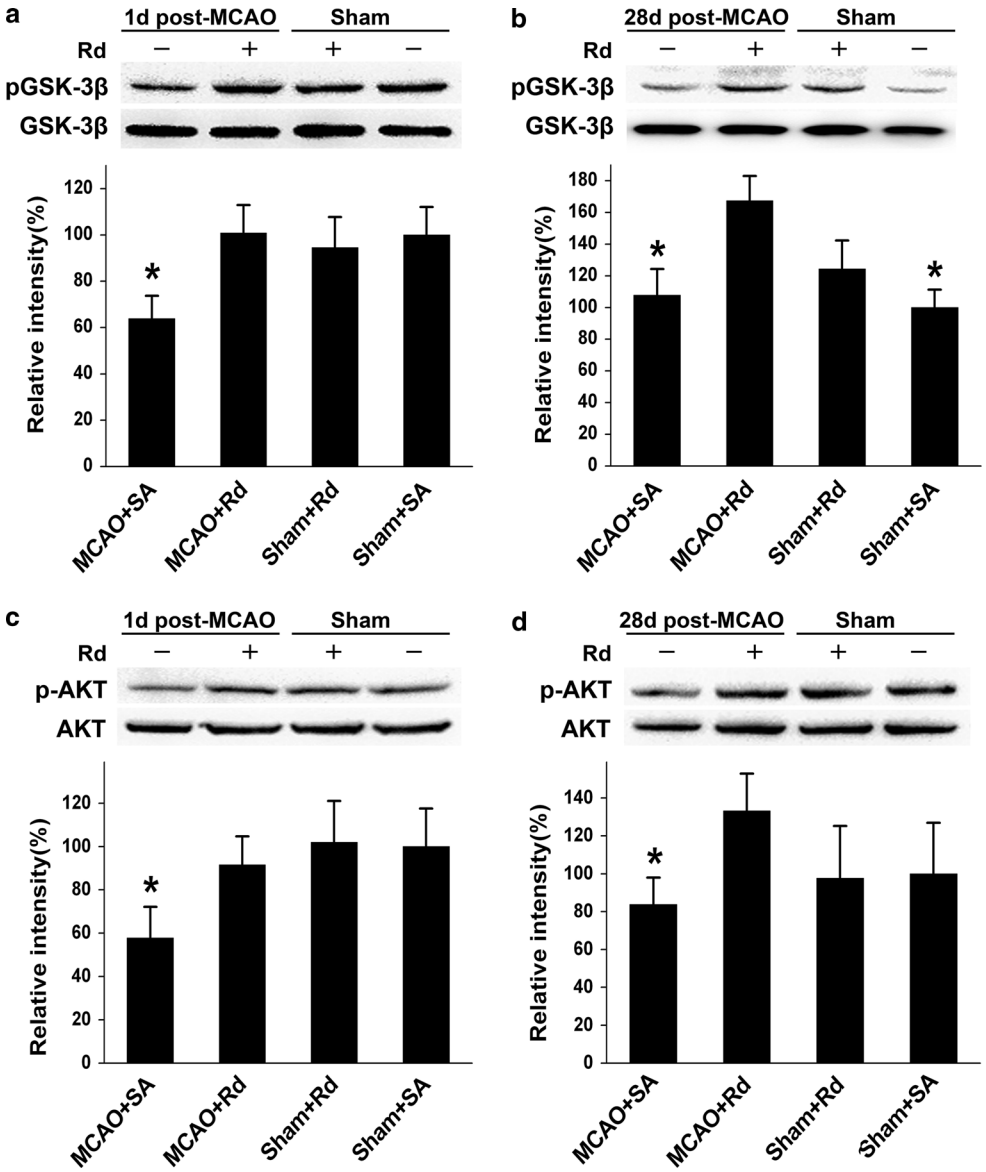

Fig. 4 Effects of Rd on AKT and GSK-3β phosphorylation in ischemic rat brain after MCAO. Western blotting analysis was performed using antibodies against p-GSK-3β/GSK-3β (**a** 24 h and **b** 28 days) or p-AKT/AKT (**c** 24 h and **d** 28 days) as described in Methods. Data are expressed as mean ± SEM (n = 6). Mean values in vehicle-treated (Sham) groups were scaled to 100 %. * $p < 0.05$ versus MCAO + Rd group, *SA* saline

AKT inhibitor LY294002, suggesting that PI3K/AKT/GSK-3β signaling pathway was involved in the inhibitory effects of Rd on tau protein phosphorylation, which may account for cognitive function improvement after Rd administration.

Ischemic stroke not only cause physical disability, but also PSD [2]. In community-based studies, the prevalence of dementia in people with a history of stroke was about 5.8-times higher than in those who have not had stroke [39, 40]. In hospital-based studies, the incidence of PSD ranges from 5.9 to 32 % [3]. Ischemic stroke-induced progressive cognitive impairment was also confirmed in animal model of stroke [41–43]. The mechanism underlying PSD is still unclear. Tatemichi et al. [44] reported that more than 60 % of PSD patients developed AD pathology. Many pre-clinical studies also found that focal cerebral ischemia induced AD-like pathological changes in rodent model [8, 33, 34, 45]. Consistently, the present study showed MCAO injury caused cognitive deficits and tau protein phosphorylation (Figs. 2, 3).

Preventing tau hyperphosphorylation was proposed to be a potential therapeutic strategy for AD-like diseases [18].

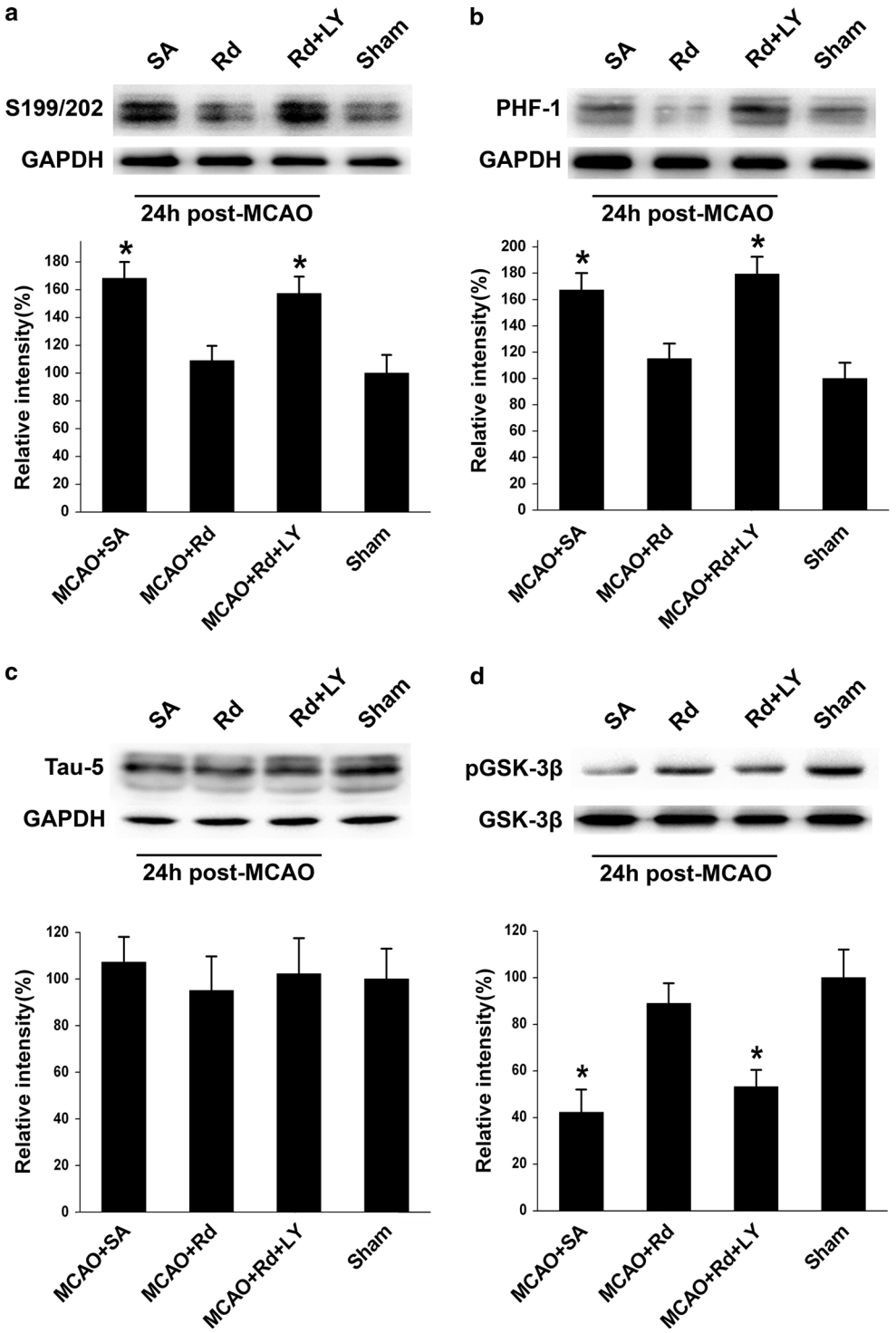

Fig. 5 PI3K/AKT/GSK-3β pathway is involved in the effect of Rd on tau phosphorylation in vivo. Western blotting analysis of proteins extracted from ischemic rat brain 1 day after MCAO was performed using antibodies against S199/202 (**a**) or PHF-1 (**b**) or tau-5 (**c**) or p-GSK-3β (**d**) as described in Methods. LY294002 (LY, 5 g/l, 5 μl, i.c.v.) was pretreated 1 h before MCAO. Data are expressed as mean ± SEM (n = 6). GAPDH was used as internal control. Mean values in sham-treated groups were scaled to 100 %. * $p < 0.05$ versus MCAO + Rd group, *SA* saline

Our previous study showed that Rd could inhibited tau phosphorylation in rat model of AD [28–30]. As an extending study, the present study showed that daily administration of Rd could also inhibit tau phosphorylation and ameliorate cognitive function after experimental cerebral ischemia in rats (Figs. 2, 3).

The PI3K/AKT/GSK-3β pathway is pivotal to maintenance of the neuronal network, cell survival, and longevity. Dysregulation of this signaling pathway is the major mechanism underlying the pathology of neurodegenerative diseases, such as AD [46]. GSK-3β is a major tau kinase playing a crucial role in AD-like tau hyperphosphorylation [12–16] and its activity can be inhibited by AKT at Ser^9-phosphorylation site [46]. Our present study showed that AKT was inactivated but GSK-3β was activated after MCAO (Fig. 4), consistent with a previous study [38], and these results may partly explain why p-tau levels increased after stroke. Furthermore we showed that Rd inhibited GSK-3β but enhanced AKT activities whereas PI3K/AKT inhibitor LY294002 could block this effect of Rd (Fig. 5), suggesting that Rd may affect tau phosphorylation via PI3K/AKT/GSK-3β pathway.

Estrogen receptor (ER)-mediated PI3K/AKT activation is well-documented in protection against cell death. Ginsenosides were structurally and functionally similar to 17β-estradiol and activated AKT pathways [47, 48]. Thus, we proposed that acting on ER may be responsible for Rd-induced AKT activation and sequential p-Tau down-regulation. Yet, in our previous studies Rd was showed to prevent from glutamate/oxygen–glucose deprivation (OGD)-induced apoptosis in cultured neurons and reduce infarction volume after transient focal ischemia in rats [20–27], Rd could also increase Glutamate transporter-1 (GLT-1) expression in astrocytes for glutamate clearance [49]. Thus, apart from reducing p-Tau levels, other mechanisms may involve in the ameliorate effects of Rd on cognitive function after cerebral ischemic injury as well. Further investigation is still needed.

In conclusion, in the present study we provide the first evidence that Rd could improve cognitive function and reduce tau protein phosphorylation via the PI3K/AKT/GSK-3β pathway after experimental cerebral ischemia, indicating that Rd may serve as a promising drug for the treatment of PSD.

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Conflict of interest The authors declare that they have no conflict of interest.

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