Inflammation, Vol. 42, No. 5, October 2019 (© 2019)
DOI: 10.1007/s10753-019-01045-7

ORIGINAL ARTICLE

Salidroside Reduces Inflammation and Brain Injury After Permanent Middle Cerebral Artery Occlusion in Rats by Regulating PI3K/PKB/Nrf2/NFκB Signaling Rather than Complement C3 Activity

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Abstract— Salidroside, an active constituent of *Rhodiola rosea*, is neuroprotective after transient middle cerebral artery occlusion (tMCAO). However, its effects in other experimental stroke models are less understood. Here, we investigated the effect of daily intraperitoneal injections of salidroside in rats after permanent MCAO (pMCAO). Cerebral infarct volumes at 1 day after pMCAO were significantly reduced by treatment with 100 mg/kg/day salidroside, but not by 25 or 50 mg/kg/day, and this benefit of salidroside increased significantly over at least 7 days of treatment, when it was also accompanied by decreased neurological deficit scores. These observations led us to investigate the underlying mechanism of action of salidroside. 100 mg/kg salidroside for 1 day increased NeuN, Nrf2, and its downstream mediator HO-1, while it reduced nuclear NFκB p50, IL-6, and TNFα. Brusatol, a Nrf2 inhibitor, blocked the actions of salidroside on Nrf2, NFκB p50, IL-6, and TNFα. Salidroside also increased the ratio of p-PKB/PKB at 1 day after pMCAO even in the presence of brusatol. LY294002, a PI3K inhibitor, prevented all these effects of salidroside, including those on NeuN, p-PKB/PKB, Nrf2, HO-1, and pro-inflammatory mediators. In contrast, salidroside had no significant effect on the level of cerebral complement C3 after pMCAO, or on the activity of C3 as measured by the expression of cerebral Egr1. Our findings therefore suggest that salidroside reduces neuroinflammation and neural damage by regulating the PI3K/PKB/Nrf2/NFκB signaling pathway after pMCAO, and that this neuroprotective effect does not involve modulation of complement C3 activity.

KEY WORDS: Complement C3; Inflammation; Ischemic stroke; Neuroprotection; Nrf2; Salidroside.

X. Zhang and W. Lai contributed equally to this work.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10753-019-01045-7) contains supplementary material, which is available to authorized users.

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INTRODUCTION

Salidroside is a key bioactive component of the medicinal plant *Rhodiola rosea*, which is thought to be neuroprotective and to improve cognitive functions [1]. Treatment with salidroside can reduce cerebral infarct volume and neurological deficits in rats subjected to middle cerebral artery occlusion (MCAO) followed by reperfusion, whether treatment is initiated before the ischemia-reperfusion injury or up to 48 h later [2, 3]. Salidroside is known to engage a variety of neuroprotective mechanisms in transient MCAO (tMCAO), including the inhibition of inflammation through activation of the phosphoinositide 3-kinase (PI3K)/protein kinase B (PKB) pathway [4], antioxidant mechanisms associated with activation of the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway [5, 6], and the inhibition of complement activation [7]. However, the effects of salidroside in other experimental stroke models, such as after permanent middle cerebral artery occlusion (pMCAO), are less understood, particularly as the production of reactive oxygen species and the patterns of inflammation are different after MCAO if it is followed by reperfusion [8, 9].

Reactive oxygen species and inflammatory responses have both been implicated in the pathogenesis of ischemic stroke [10, 11]. Nrf2 and nuclear factor-κB (NFκB) are two key transcription factors that respectively regulate cellular responses to oxidative stress and inflammation [12–15]. Nrf2 binds to antioxidant response elements within regulatory regions of target genes and mediates transcription of antioxidant proteins, such as heme oxygenase-1 (HO-1), that are responsible for the clearance of reactive oxygen species [12, 16]. NFκB is a heterodimer, usually composed of p65 and p50 subunits, that plays a critical role in mediating pro-inflammatory signaling [15, 17]. The Nrf2 pathway and the NFκB pathway also interact through a range of molecular mechanisms [18]. However, the functional interactions between PI3K/PKB, Nrf2, and NFκB signaling are incompletely understood in ischemic brain, and their role in any actions that salidroside may have in the setting of pMCAO has not been determined.

The objectives of the present study were therefore to investigate potential neuroprotective effects of salidroside in rats after pMCAO, as well as any associated effects on PI3K/PKB, Nrf2, and NFκB signaling. Moreover, as we have previously shown that a significant component of the neuroprotective effect of salidroside in the setting of tMCAO is by inhibiting the activation of cerebral complement component C3 [7], we also set out to investigate the effects of pMCAO with and without salidroside on the levels and activity of cerebral C3.

MATERIALS AND METHODS

Animals

All animal work was performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, and approved by the Animal Care and Use Committee of Fujian University of Traditional Chinese Medicine. A total of 115 male Sprague-Dawley rats, weighing 220 ± 20 g (Slac Laboratory Animal Co. Ltd., Shanghai, China), were acclimatized for 7 days, by housing in groups of five under controlled temperature (21–23 °C) and humidity (55–75%), in a 12-h light/dark cycle with free access to standard rodent chow and water.

Experimental Models and Drug Treatments

pMCAO was performed in the rats as described previously [3, 4] except that the monofilament thread was not withdrawn after occlusion. Sham operations replicated the pMCAO procedure except that the middle cerebral artery was not occluded. After full recovery from the anesthesia, behavioral neurological deficits were scored by blinded observers according to a five-point scale, as described previously [19]. Rats with neurological deficit scores in the range of 2–4 were randomly allocated to four groups ($N = 13$–14/group) and given daily an intraperitoneal (i.p.) injections, for up to 7 days, of equal volumes of either 0.9% saline or salidroside (purity ≥ 99.7%; Lijiexun Pharmaceutical Co. Ltd., China) at a dose of 25 mg/kg/day, 50 mg/kg/day, or 100 mg/kg/day in 0.9% saline. Sham-operated rats ($N = 10$) received daily injections of an equal volume of 0.9% saline.

Infarct volumes were measured by magnetic resonance imaging (MRI) at 1 day after pMCAO (see below). Four animals from each group were then sacrificed, and their left cerebral hemispheres were dissected and frozen at − 80 °C for mRNA and protein extraction. The infarct volumes of remaining animals were measured again by

MRI at 7 days after pMCAO. All animals were then sacrificed, and their left cerebral hemispheres were dissected and stored at − 80 °C until used for mRNA and protein extraction.

To investigate the role of PI3K pathway in the early effects of salidroside, rats with neurological deficit scores in the range of 2–4 after pMCAO were assigned randomly to three groups ($N = 4$/group). Rats in the first group were given an intracerebroventricular injection of 10 μl 25% dimethyl sulfoxide (DMSO) in phosphate-buffered saline (PBS) followed by an i.p. injection of 0.9% saline. Rats in the second group received an intracerebroventricular injection of 10 μl 25% DMSO in PBS followed by an i.p. injection of salidroside (100 mg/kg) in 0.9% saline. Rats in the third group were given an intracerebroventricular injection of 10 μl of 10 μM LY294002 dissolved in 25% DMSO/PBS (Sigma-Aldrich, St. Louis, MO; [20]), followed by an i.p. injection of 100 mg/kg salidroside in 0.9% saline. The same protocol was applied to investigate the role of Nrf2 in the effects of salidroside, except that LY294002 was replaced by 10 μl of 10 μM brusatol in 25% DMSO/PBS (Tongtian Biotechnology, China; [21, 22]). Sham-operated rats ($N = 4$) received an intracerebroventricular injection of 10 μl 25% DMSO/PBS followed by an i.p. injection of 0.9% saline. The animals were sacrificed 1 day after the operation, and their left cerebral hemispheres were dissected and stored at − 80 °C for mRNA and protein extraction.

MRI and Data Analysis

MRI assessment of infarct volumes was performed as described previously, with slight modifications [23]. Briefly, each rat was anesthetized with inhaled isoflurane and immobilized in an animal holder with its head inside the imaging coil in the center of the magnet bore of a 7.0-T MRI system (Bruker BioSpin International AG, Zug, Switzerland). During scanning, body temperature was kept at 37.0 ± 1.0 °C with a heating pad placed on the torso and respiration was monitored with a pressure probe placed on the ventral side. We obtained T2-weighted images using a spin-echo sequence with coronal slice orientation and the following parameters: repetition time = 2738.308 ms, echo time = 33.00 ms, echo spacing = 11.000 ms, slice thickness = 0.8 mm, slices = 18, matrix size = 256 × 256, field of view = 30.0 × 30.0, number of excitations = 4.0.

Infarct volumes were measured in ImageJ by blinded investigators. The intact area of the ipsilateral hemisphere and the whole contralateral hemisphere area were delineated manually from individual T2-weighted MRI slice images and measured for each animal using the Region of Interest Manager software. Hemisphere volumes were calculated as the sum of areas in successive sections × section thickness. Infarct volume was expressed as a percentage of the contralateral hemispheric volume, using the following formula: infarct volume (%) = [(contralateral hemisphere volume − ipsilateral hemisphere intact volume)/contralateral hemisphere volume] × 100 [3].

Western Blot Analysis

Total protein was extracted from frozen brain tissues with ice-cold RIPA buffer (50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 0.5% NP-40, 0.1% sodium dodecyl sulfate) plus 1% protease inhibitor cocktail (Sigma-Aldrich). Nuclear protein extraction was carried out in accordance with the manufacture's instruction (Thermo Fisher Scientific, Waltham, MA). Western blot analysis was performed as described previously [3] with antibodies targeting Egr1 (1:600 dilution; #SC-110; Santa Cruz Biotechnology, Dallas, TX), C3 (1:1000; #D151005; Sangon Biotech, China), the neuronal marker NeuN (1:1000; #ab104224; Abcam, Cambridge, UK), protein kinase B (PKB; 1:1000; # 9272; Cell Signaling Technology, Danvers, MA), phosphorylated PKB (p-PKB; 1:500; # 9273; Cell Signaling Technology), NFκB p50 subunit (1:500; #SC-166588, Santa Cruz Biotechnology), Nrf2 (1:600; #ab137550; Abcam), and HO-1 (1:1000; #ab13248; Abcam). The blots were washed thoroughly and incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody (1:4000 dilution; #31460 or #31430; Thermo Fisher Scientific), and then developed with enhanced chemiluminescence AB reagent (Beyotime, Chongqing, China). The blots were stripped and re-probed with antibodies targeting β-actin (1:1000 dilution, #AF0003; Beyotime) or PCNA (1:1000 dilution; #SC-25280; Santa Cruz Biotechnology). Immunoreactive bands were visualized with a ChemiDoc XRS+ imaging system (Bio-Rad, Hercules, CA), and target proteins were quantified in ImageJ. The results are expressed as fold change relative to controls after being normalized to β-actin.

Reverse Transcription Polymerase Chain Reaction

Total RNA extraction from frozen brain tissues and reverse transcription polymerase chain reaction (RT-PCR) were performed as described previously [4]. Results were expressed as fold change relative to the mean result in the sham group, after normalization to GAPDH. All the primer sequences used for PCR are listed (Online Resource 1).

Statistical Analysis

All data are presented as means ± standard errors (SEMs) and were analyzed with one-way or two-way analyses of variance (ANOVAs) and Bonferroni *post hoc* tests in SPSS software (version 20.0) as appropriate. Neurological deficit scores were analyzed with Mann-Whitney *U* tests. A p value < 0.05 was considered statistically significant.

RESULTS

Salidroside Reduces Infarct Volumes and Improves Neurological Deficit Scores After Ischemic Brain Injury

Treatment with 100 mg/kg/day salidroside, compared with vehicle treatment, significantly reduced cerebral infarct volumes 1 day after pMCAO, and this benefit increased significantly by 7 days of treatment (Fig. 1a, b). Lower doses of 25 or 50 mg/kg/day of salidroside did not have significant benefit although there was a trend towards reduced cerebral infarct volumes (Fig. 1a, b). The increasing benefit of 100 mg/kg/day salidroside between 1 and 7 days of treatment was additionally reflected in a significant reduction in neurological deficit scores after 7 days of treatments (Fig. 1c). Sham operation did not produce any significant cerebral infarction or neurological deficit (Fig.1). We therefore went on to investigate the neuroprotective mechanism of salidroside at the dose of 100 mg/kg/day after pMCAO.

Salidroside Induces NeuN and Inhibits Pro-inflammatory Mediators Through the Nrf2 Pathway After pMCAO

We found that cerebral NeuN expression was significantly decreased, and that the nuclear NFκB p50 subunit, IL-6, and TNFα were increased, in ischemic brain at 1 day after pMCAO compared with the sham-operated rats. All these changes were reversed by salidroside (Fig. 2). Similarly, the level of nuclear Nrf2 was significantly decreased after pMCAO, and this decrease was reversed by 1 day of treatment with 100 mg/kg/day salidroside (Fig. 3a). HO-1, a downstream mediator of Nrf2 that catalyzes the first and rate-limiting step in heme degradation [24], was also significantly decreased 1 day after pMCAO, and this decrease was blocked by salidroside (Fig. 3b). The increase of nuclear Nrf2 caused by salidroside was prevented by the Nrf2 inhibitor brusatol [21, 22], as shown in Fig. 3c. Meanwhile, brusatol blocked the increase in NeuN, and the decreases of nuclear NFκB p50 subunit, IL-6, and TNFα, caused by salidroside given for 1 day after pMCAO (Fig. 4).

Activation of PI3K Mediates the Effects of Salidroside on Nrf2, NeuN, and Pro-inflammatory Cytokines

We then investigated whether the PI3K/PKB signaling was involved in the actions of salidroside after pMCAO. We found that the cerebral ratio of p-PKB/PKB was significantly decreased 1 day after pMCAO compared with the sham-operated rats. This decrease was prevented by treatment with salidroside (Fig. 5a). The effect of salidroside on the ratio of p-PKB/PKB after pMCAO was not significantly different in the presence of brusatol (Fig. 5b), but it was abolished by LY294002, a selective inhibitor of PI3K (Fig. 5c). Meanwhile, LY294002 prevented the increased Nrf2 and HO-1 caused by salidroside given for 1 day after pMCAO (Fig. 5d, e). LY294002 also reversed the increase in NeuN, and the decreases in nuclear NFκB p50 subunit, IL-6, and TNFα caused by salidroside given for 1 day after pMCAO (Fig. 6).

Complement C3 Is Not Involved in the Protective Effects of Salidroside After pMCAO

We have previously reported that salidroside inhibits activation of complement C3 after tMCAO and that this inhibition of complement activation contributes to the neuroprotective effect of salidroside after tMCAO [7]. We therefore investigated the effects of salidroside on C3 after pMCAO. Cerebral levels of C3 increased at 1 day post-pMCAO but these increases did not reach significance. However, C3 increased significantly compared with sham-operated rats by 7 days after pMCAO (Fig. 7a, b). Treatment with salidroside had no significant effect on the level of cerebral C3 at 1 day after pMCAO (Fig. 7a), a time when salidroside already had significant effects in reducing cerebral infarct volume and maintaining NeuN levels after pMCAO. Even more strikingly, salidroside had no significant effect on the level of cerebral C3 at 7 days after pMCAO (Fig. 7b), a time at which this treatment with

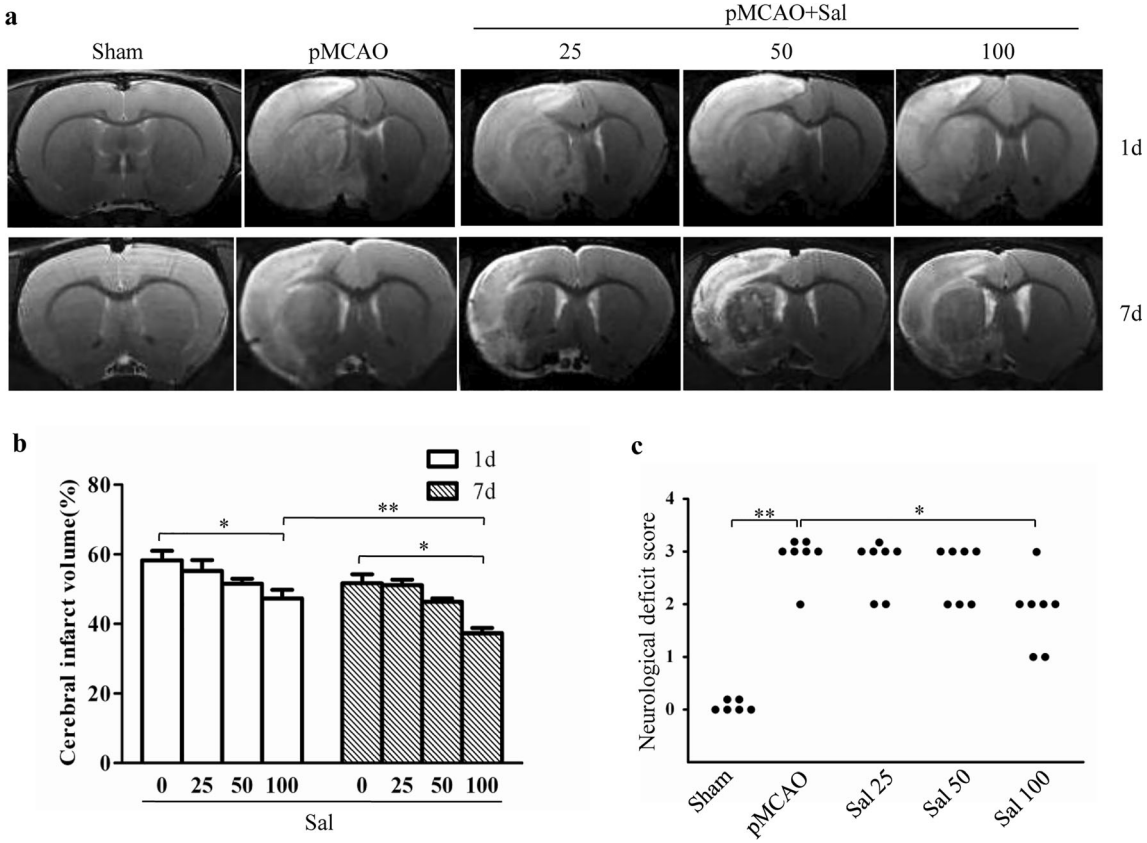

Fig. 1. Effects of different doses of salidroside on cerebral infarct volumes and neurological deficits after pMCAO. Representative T2-weighted images of brains at 1 day and 7 days after pMCAO with treatments labeled in the figure (**a**). Comparisons of means± SEMs of the ratios of infarct volumes to the volumes of the intact contralateral cerebral hemispheres (**b**). Comparisons of neurological deficit scores at 7 days after pMCAO between treatments (**c**). Salidroside (Sal) dose units are mg/kg/day. *$p < .05$ and **$p < .01$ between groups linked by brackets ($N \geq 6$/group).

salidroside produced even greater decreases in cerebral infarct volume and a significant reduction in neurological deficit scores. We have also previously reported that the inhibition of cerebral C3 activation by salidroside results in an increase of Egr1 in the sub-acute phase after tMCAO [7]. We therefore measured cerebral Egr1 levels at 7 days after pMCAO as a functionally downstream measure of the effect of salidroside on C3 after pMCAO. We found that salidroside did not significantly alter the level of cerebral Egr1 after pMCAO (Fig. 7c), consistent with a lack of effect on cerebral C3 activity in this model of cerebral ischemia.

To confirm that the lack of effect of salidroside on C3 and Egr1 after pMCAO was not due to issues with the experimental techniques, protein samples reserved from a previous study of rats subjected to tMCAO, and treated with vehicle or salidroside [3], were assayed under identical conditions to those of the pMCAO samples using the same reagents. The results confirmed that salidroside was able to suppress C3 and increase Egr1 levels after tMCAO (Online Resource 2).

DISCUSSION

Previous work has demonstrated that salidroside reduces neuroinflammation and brain injury in rats after tMCAO [2–6], but the effects of salidroside in pMCAO have not been investigated. In the present study, we show salidroside reduces infarct volumes and increases cerebral NeuN levels in pMCAO after 1 day of treatment and increasingly reduces infarct volumes with more prolonged treatment. Moreover, we show that this beneficial effect of salidroside treatment over 7 days not only reduces

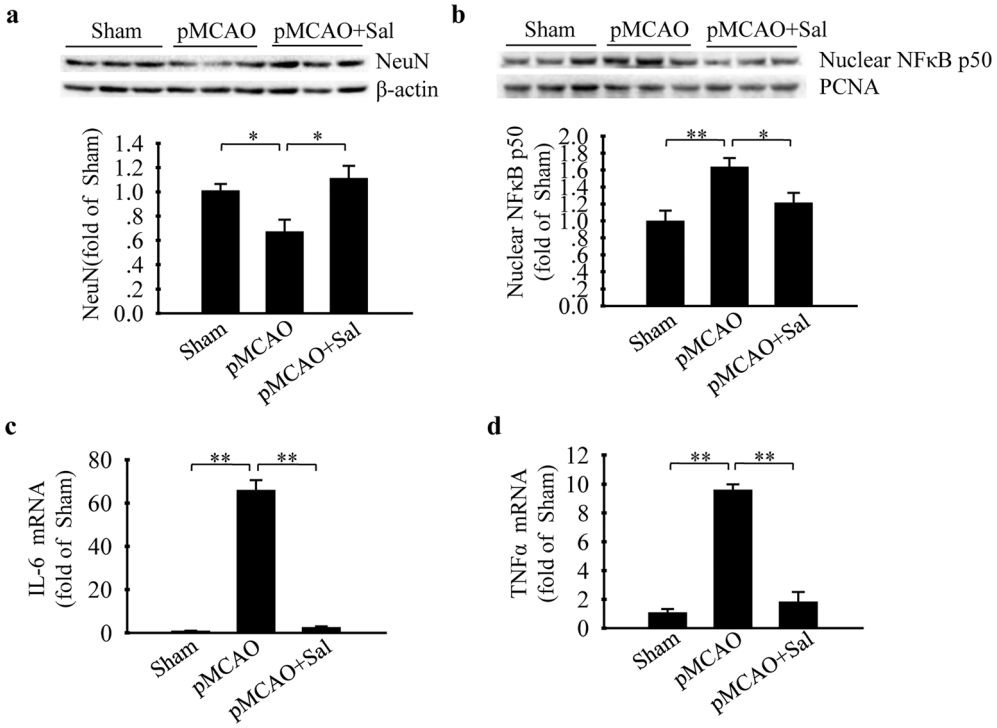

Fig. 2. Effects of salidroside on NeuN and pro-inflammatory mediators in brain at 1 day after pMCAO. Salidroside treatment induces NeuN protein (**a**) and reduces the nuclear NFκB p50 subunit (**b**). Representative Western blots probed with anti-NeuN antibody and re-probed with anti-β-actin antibody (**a**), or with anti-NFκB p50 antibody and re-probed with anti-PCNA antibody (**b**), are shown in the upper panels. NeuN levels are normalized to β-actin levels, and nuclear NFκB p50 levels are normalized to PCNA levels, and the results are shown in the bar graphs. Salidroside inhibits IL-6 (**c**) and TNFα (**d**) mRNAs. mRNA levels were measured by RT-PCR and normalized to GAPDH mRNA. All values are expressed as mean fold changes (mean ± SEM) relative to sham rats. $*p < .05$ and $**p < .01$ between groups linked by brackets.

structural injury but can significantly improve cerebral functional scores. We also demonstrate the neuroprotective ability of salidroside after pMCAO, as measured by its ability to preserve the level of cerebral NeuN, is associated with the ability of salidroside to reverse the effects of pMCAO on cerebral Nrf2 and its downstream mediator HO-1, and also to reverse the effects on cerebral nuclear NFκB p50 and the pro-inflammatory cytokines IL-6 and TNFα. We have previously reported that salidroside increases the expression of Nrf2 and HO-1 in damaged cerebral tissue 1 day after tMCAO, but the implications of these increases remain unknown [5, 6]. We found similar effects in the present study of pMCAO and also showed that brusatol, a Nrf2 inhibitor [21, 22], was able to block the actions of salidroside on Nrf2, NFκB p50, IL-6, TNFα, and NeuN, suggesting that the anti-inflammatory and neuroprotective actions of salidroside after pMCAO can at least in part be explained by the effects of salidroside to enhance the Nrf2 pathway in ischemic brain.

Interestingly, it has recently shown in neural PC12 cells that phenylethanoid glycosides, including salidroside, do not alter the expression of Keap-1, a protein which directs Nrf2 for lysosomal degradation [25], but molecular docking analysis suggests that these glycosides can bind to Keap1 and may thereby prevent its interaction with Nrf2 [26]. Consistent with this idea, the ranked docking scores of several phenylethanoid glycosides were consistent with the ranked ability of these glycosides to cause translocation of Nrf2 to the nucleus. However, of the glycosides tested, salidroside had the lowest docking scores [26] and our results with the PI3K inhibitor LY294002 strongly suggest that salidroside has other important effects on Nrf2 signaling after pMCAO. Thus, we found that LY294002 blocked the effects of salidroside on cerebral p-PKB/PKB, consistent with an inhibition of PI3K, and prevented the increase in Nrf2 caused by salidroside as well as preventing all of the downstream effects of this increase in Nrf2. This is the first time that salidroside has been shown to activate Nrf2

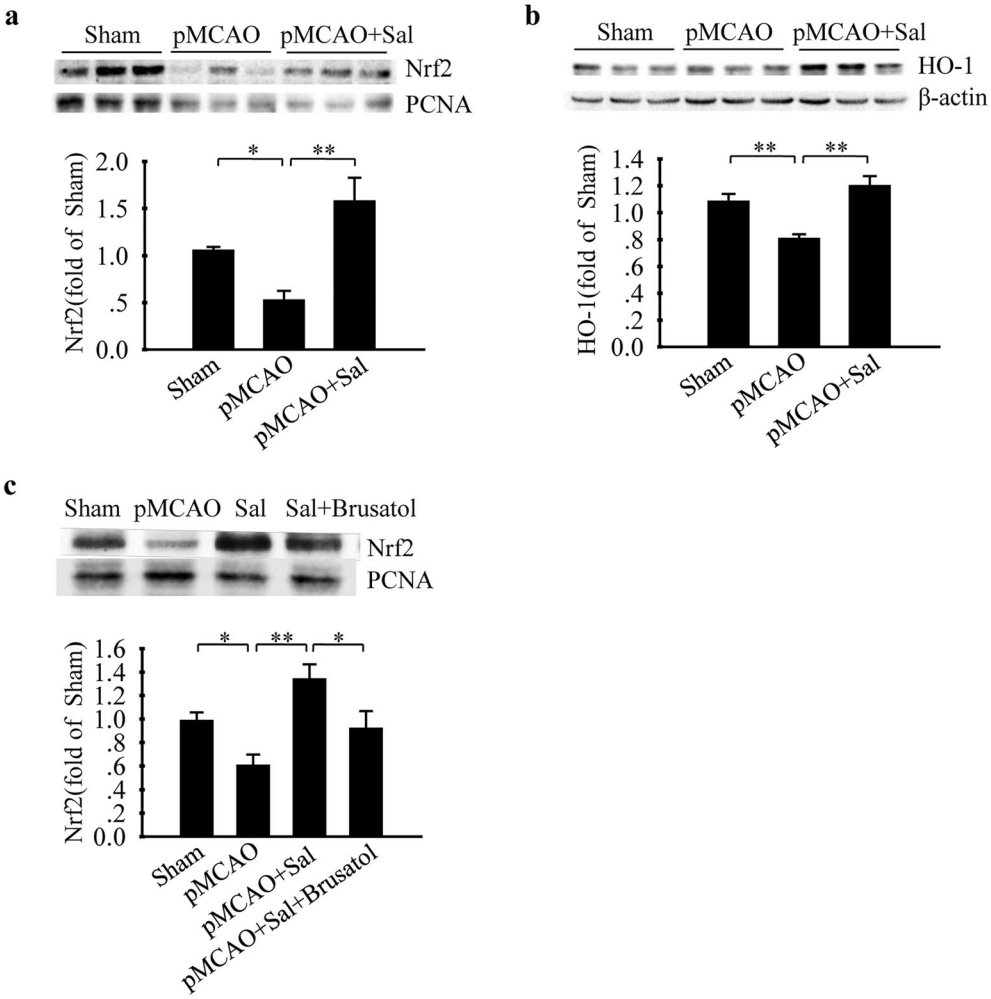

Fig. 3. Salidroside induces Nrf2 and HO-1 at 1 day after pMCAO. Salidroside induces nuclear Nrf2 (**a**) and HO-1 (**b**). Brusatol prevents the effect of salidroside to increase Nrf2 (**c**). Representative Western blots are shown probed with the antibody against Nrf2 and re-probed with anti-PCNA antibody (**a** and **c**), or probed with the antibody against HO-1 and re-probed with anti-β-actin antibody (**b**). The levels of Nrf2 normalized to PCNA (**a** and **c**), and of HO-1 normalized to β-actin level (**b**) are shown beneath the Western blots. All values are expressed as mean fold changes (means ± SEMs) relative to sham rats. $^{*}p < .05$ and $^{**}p < .01$ between groups linked by brackets.

signaling by increasing PI3K activity in neural tissues, although a similar action of salidroside has been reported in cultured cardiomyocytes [27], in the liver [28] and in immortalized mouse epithelial cells [29].

Our results with brusatol show for the first time that the Nrf2 pathway during cerebral ischemia controls the important pro-inflammatory transcription factor NFκB, and the pro-inflammatory IL-6 and TNFα. Studies in other models have demonstrated that Nrf2 and NFκB are individually involved in the cellular responses to oxidative stress and inflammation in ischemic brain [30–33]. Moreover, deficiency of Nrf2 in astrocytes induces NFκB activation and pro-inflammatory cytokine production [34], suggesting that Nrf2 can control NFκB and inflammation in these cells. We also show for the first time that both LY294002 and brusatol inhibit the ability of salidroside therapy to restore the levels of p-PKB/PKB, Nrf2, NFκB, IL-6, and TNFα towards their pre-injury levels after pMCAO. It is possible that the inhibitory effect of salidroside on these and other pro-inflammatory cytokines, driven through Nfr2, contributes to the neuroprotection afforded by salidroside in this model of cerebral ischemia without reperfusion. Interestingly, we have previously shown that salidroside exerts anti-inflammatory effects by

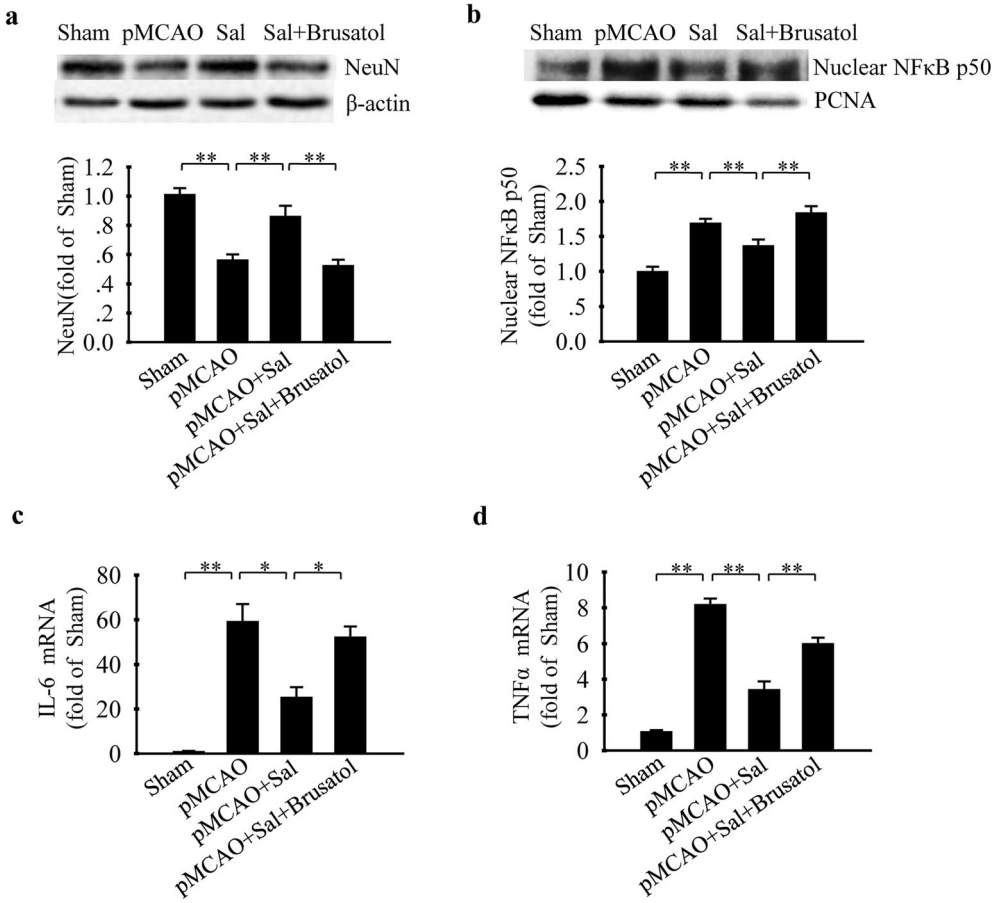

Fig. 4. Salidroside induces NeuN and inhibits pro-inflammatory mediators through activation of Nrf2 at 1 day after pMCAO. Brusatol prevents the effects of salidroside to increase NeuN (**a**) and decrease nuclear NFκB p50 (**b**). Representative Western blots are shown probed with the antibody against NeuN and re-probed with anti-β-actin antibody (**a**) or with the antibody against NFκB p50 subunit and re-probed with anti-PCNA antibody (**b**). The levels of NeuN normalized to β-actin level (**a**), and of nuclear NFκB p50 normalized to PCNA (**b**), are shown beneath the Western blots. Brusatol prevents the decreases of IL-6 (**c**) and TNFα (**d**) mRNAs caused by salidroside. mRNA levels were measured by RT-PCR and normalized to GAPDH mRNA. All values are expressed as mean fold changes (means ± SEMs) relative to sham rats. *$p < .05$ and **$p < .01$ between groups linked by brackets.

a PI3K-dependent increase in expression of hypoxia-inducible factor (HIF) in tMCAO rats [4]. Nrf2 can control the levels of HIF-1 in some non-neural cell types [35–37], but it remains to be determined whether the PI3K-dependent anti-inflammatory effects of salidroside in pMCAO and tMCAO rats depend on increased HIF expression caused by Nrf2.

In another regard, we have found that the principal neuroprotective and anti-inflammatory effects of salidroside after cerebral ischemia have significantly different underlying mechanisms depending on whether reperfusion occurs or not. This is because we have previously shown that the ability of salidroside to inhibit the activation of complement at the level of C3 is a crucial part of the neuroprotective and anti-inflammatory effects of salidroside in tMCAO, a model in which C3 is activated by the cerebral insult through both the lectin and classical pathways [7]. In contrast, we found in our present study that the dose of salidroside which provided significant neuroprotection and anti-inflammatory effects after pMCAO nevertheless failed to inhibit the raised levels of C3 seen up to 7 days after pMCAO. We have also previously shown that salidroside reverses the inhibition of Erg1 caused by increased C3 activation after tMCAO. Egr1 is a neuroprotective and neuroplasticity-associated protein that is negatively regulated by C3 [7]. In the present study,

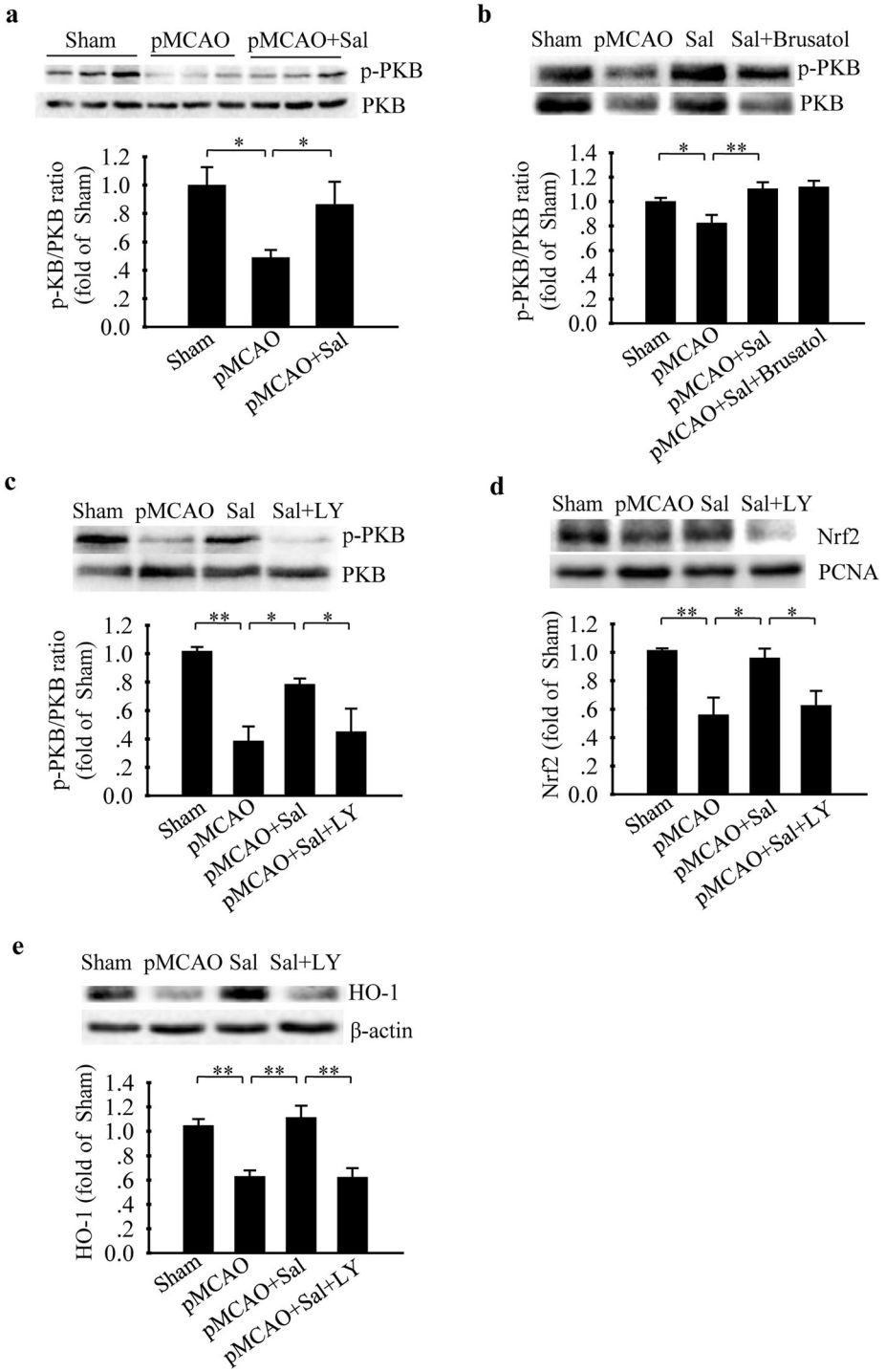

Fig. 5. Activation of PI3K/PKB signaling by salidroside drives the Nrf2 pathway at 1 day after pMCAO. Salidroside increases p-PKB/PKB ratio in the absence (**a**) or presence of brusatol (**b**). LY294002 (LY) prevents the increase of p-PKB/PKB ratio (**c**), Nrf2 (**d**), and HO-1 (**e**), caused by salidroside. Representative Western blots are shown, probed with the antibody against p-PKB and re-probed with anti-PKB antibody (**a**–**c**), or probed with anti-Nrf2 antibody and then re-probed with anti-PCNA antibody (**d**), or probed anti-HO-1 antibody and re-probed with anti-β-actin antibody (**e**). p-PKB/PKB ratios (**a**–**c**), Nrf2 levels normalized to PCNA levels (**d**) and HO-1 levels normalized to β-actin level (**e**) are shown beneath the Western blots. All values are expressed as mean fold changes (means ± SEMs) relative to sham rats. *$p < .05$ and **$p < .01$ between groups linked by brackets.

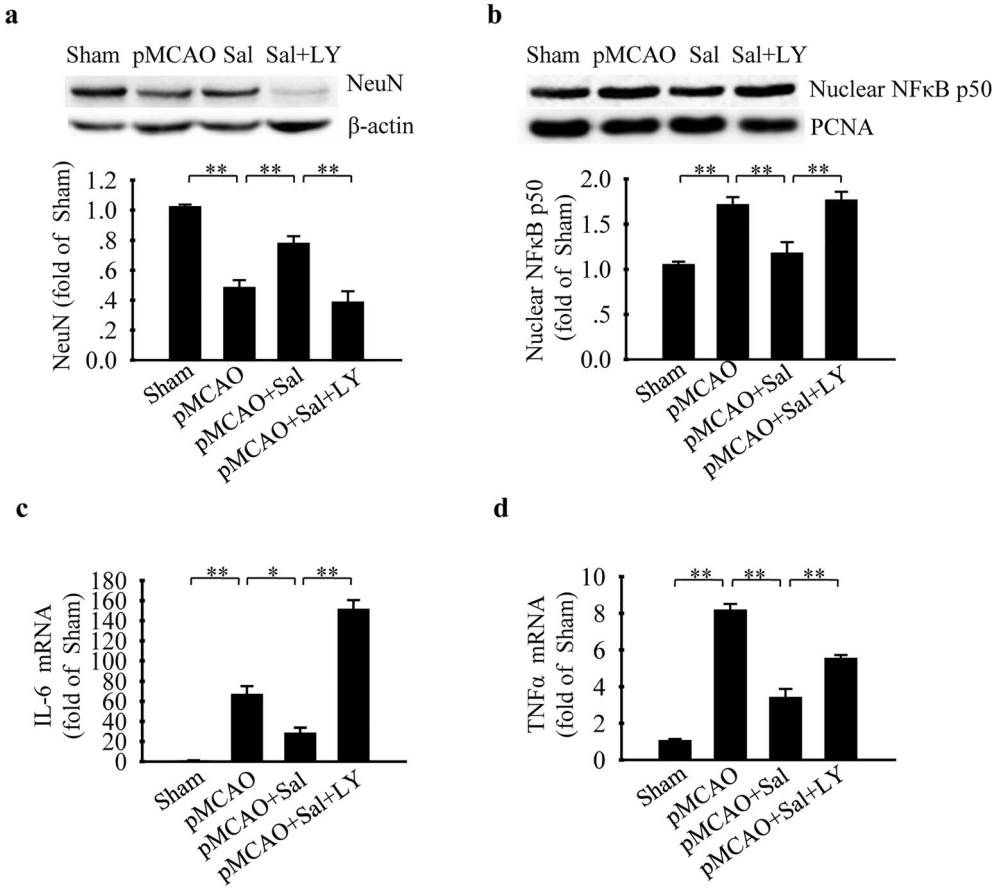

Fig. 6. Activation of PI3K/PKB signaling by salidroside drives increase of NeuN and inhibition of inflammation after pMCAO. LY294002 (LY) prevents the increase of NeuN (**a**), and the decrease of nuclear NFκB p50 (**b**), caused by salidroside. Representative Western blots are shown probed with anti-NeuN antibody and re-probed with anti-β-actin antibody (**a**), or probed with anti-NFκB p50 antibody and re-probed with anti-PCNA antibody (**b**). NeuN normalized to β-actin level (**a**), and nuclear NFκB p50 levels normalized to PCNA levels (**b**), are shown beneath the Western blots. LY294002 prevents the decreases of IL-6 (**c**) and TNFα (**d**) mRNAs, caused by salidroside. mRNA levels were measured by RT-PCR and normalized to GAPDH mRNA. All values are expressed as mean fold changes (means ± SEMs) relative to sham rats. $*p < .05$ and $**p < .01$ between groups linked by brackets.

however, we did not find an effect of salidroside on Egr1, confirming the lack of effect of salidroside on C3 activity at a time when salidroside was exerting significant neuroprotective and anti-inflammatory effects. Therefore, our evidence suggests that the neuroprotective and anti-inflammatory effects of salidroside treatment after pMCAO do not involve a salidroside-mediated suppression of cerebral C3 levels or activity, in contrast to the role of C3 inhibition in the protective effects of salidroside after tMCAO. In the case of tMCAO, we have found that salidroside quickly inhibits the lectin pathway of complement activation and reduces C3 deposition on cerebral endothelium within 1 day after tMCAO [7]. It may be significant, therefore, that we have found that salidroside only suppresses C3 expression increases in cultured endothelial cells when these are exposed to hypoxia with reoxygenation and not when they are exposed to hypoxia alone (manuscript in preparation). However, further work will be needed to establish which cerebral cell types are involved and how exactly reperfusion alters the main mechanisms of action of salidroside in protecting against cerebral ischemia.

In summary, the results of the present study in rats show that salidroside reduces brain injury and neuroinflammation after pMCAO. These protective effects depend significantly on the ability of salidroside to activate PI3K/PKB signaling, which in turn enhances the Nrf2 pathway, leading to inhibition of NFκB and the downstream

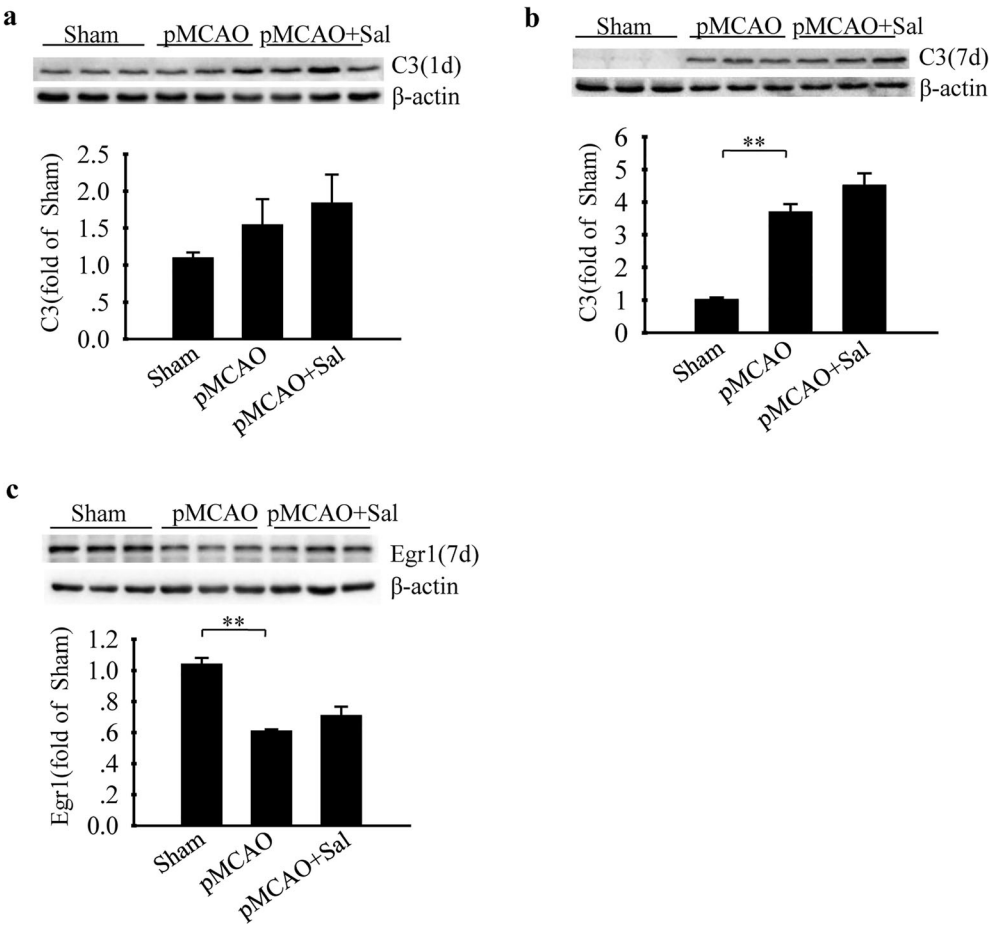

Fig. 7. Salidroside treatment has no effect on C3 and Egr1 after pMCAO. Daily treatment with 100 mg/kg does not affect the protein level of C3 1 day after pMCAO (**a**), or the protein levels of C3 (**b**) and Egr1 (**c**) 7 days after pMCAO. Representative Western blots are shown probed with anti-C3 antibody (**a** and **b**), or anti-Egr1 antibody (**c**), and re-probed with anti-β-actin antibody. C3 and Egr1 levels are normalized to β-actin and shown beneath the Western blots. All values are expressed as mean fold changes (means ± SEMs) relative to sham rats. $^{**}p < .01$ between groups linked by brackets.

production of pro-inflammatory cytokines. These protective effects of salidroside after pMCAO are not associated with inhibition of C3 activity, in contrast to our previous results that inhibition of C3 activity plays a critical role in the protective effects of salidroside when MCAO is followed by reperfusion.

ACKNOWLEDGMENTS

The authors thank the staff in the Animal Center of the Fujian University of TCM for their technical support.

FUNDING INFORMATION

This work was supported by the National Natural Science Foundation of China (projects 81473382 and 81603323), by the Collaborative Innovation Center for Rehabilitation Technology of Fujian University of TCM, and by the TCM Rehabilitation Research of SATCM (X2018002-Collaborative).

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no competing interests.

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