

Original Article

Ginsenoside Rg1 suppressed inflammation and neuron apoptosis by activating PPAR γ /HO-1 in hippocampus in rat model of cerebral ischemia-reperfusion injury

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Abstract: Generally accepted, inflammation and neuron apoptosis are two characterized pathological features of cerebral ischemia-reperfusion (IR) injury. Ginsenoside Rg1 was reported showing distinct neuroprotective effect in cerebral IR injury but the underlying mechanisms are still unclear. PPAR γ /Heme oxygenase-1 (HO-1) signaling was proved effective in suppressing both apoptosis and inflammation. This study was aimed to investigate whether PPAR γ /HO-1 signaling was involved in cerebral IR injury and ginsenoside Rg1's neuroprotective effect in cerebral IR injury. Cerebral IR injury was induced by middle cerebral artery occlusion in rats. The PPAR γ agonist rosiglitazone (ROZ) and the HO-1 inhibitor zinc protoporphyrin-IX (ZnPP) and ginsenoside Rg1 at various concentrations were used to treat the modeled rats. Neurological deficits, apoptosis and inflammation in hippocampus were evaluated. Furthermore, HO-1 enzymatic activity, expression levels of apoptosis-related and inflammation-related proteins, concentrations of inflammatory cytokines were also determined. The results showed that PPAR γ activation by ROZ significantly attenuated neurological deficits, apoptosis and inflammation in hippocampus in cerebral IR rats. However, the neuroprotective effect of ROZ was then impaired by HO-1 inhibitor ZnPP. This effect was evidenced by changes of expression levels of PPAR γ , bcl-2, cleaved caspase-3, cleaved caspase-9, IL-1 β , TNF- α , HMGB1, and RAGE in hippocampus of modeled animals. Ginsenoside Rg1 showed similar effect to ROZ in activating PPAR γ /HO-1 in protecting against apoptosis and inflammation but also impaired by ZnPP administration. In conclusion, PPAR γ /HO-1 signaling was critical in mediating apoptosis and inflammation, which was also the therapeutic target of ginsenoside Rg1 in cerebral IR injury.

Keywords: Cerebral ischemia-reperfusion injury, PPAR γ , apoptosis, inflammation

Introduction

Resulted from either permanent or transient cerebral blood flow reduction or pause, ischemic stroke becomes one of the leading causes of morbidity and mortality globally [1]. It was believed that ischemia-reperfusion (IR) injury was the underlying pathogenesis for neurological damage in stroke [2]. Generally accepted and frequently reported, cell apoptosis is the associated molecular mechanisms of IR injury. In the brain, pyramidal neurons in hippocampal CA1 region were considered the most vulnerable neurocytes to IR injury-induced apoptosis according to previous studies [3]. Moreover, loss and dysfunction of CA1 neurons was considered to lead to secondary neurological injuries such as long-term learning deficits [4].

Inflammatory responses right after occurrence of stroke were also believed to take responsibility in brain IR injury. The immune cells such as neutrophils were activated to participate in inflammatory response [5]. Several previous studies reported that inflammatory cytokines including interleukin (IL)-1 beta, IL-10, tumor necrosis factor (TNF)-alpha mediated inflammation reactions in cerebral IR injury by inducing microcirculation dysfunction, aggravating cell apoptosis and recruiting inflammatory cell accumulation [6]. Application of anti-inflammation therapies showed significant attenuating effects on the neurological injuries in experimental stroke animal models [7]. Thus, drugs targeting inflammation and neuron apoptosis are potential ideal candidates in neuroprotective treatment of ischemic stroke.

Ginsenoside Rg1 prevent cerebral ischemia-reperfusion injury

As a typical and representative drug in traditional medicine in Eastern Asian region, ginseng has been used to treat many kinds of organic and psychosomatic diseases from ancient times. Ginsenoside Rg1 (GRg1) is one of the bioactive components extracted from ginseng, which exerts anti-inflammation, antioxidant, anti-proliferative and anti-apoptosis pharmacological activities [8, 9]. In several IR-injury related diseases, such as myocardial infarction and liver ischemia, GRg1 showed abundant protective and curative effects [10, 11]. However, mechanisms of effects of GRg1's protective role in cerebral IR injury are rare are still unclear.

Peroxisome proliferator-activated receptor γ (PPAR γ) is a member of nuclear hormone receptor superfamily, which was believed mediating many signaling pathways in various pathological conditions [12]. Activation of PPAR γ was considered protective against both apoptosis and inflammation [13, 14]. After activated by specific ligands, PPAR γ was translocated to further activate PPAR response elements (PPREs) to initiate target gene transcription. Heme oxygenase-1 (HO-1) is one of the down-stream effector of PPAR γ [15]. Notably, HO-1 was shown to suppress apoptosis and inflammation [16, 17]. It was reported in a recent study that GRg1 attenuated neurological damage in hippocampus in rat model of Alzheimer's disease by up-regulating PPAR γ [18].

Thus, we speculated that whether PPAR γ /HO-1 activation was involved in GRg1's neuroprotective effects against cerebral ischemia-reperfusion injury. To testify our hypothesis, the neurological deficits were evaluated in rat model of cerebral IR injury treated by GRg1, PPAR γ agonist and HO-1 inhibitor. Moreover, PPAR γ expression, HO-1 activity, inflammatory cytokines and apoptosis in hippocampus were also assessed. We believe that the results in this study would not only enrich our knowledge concerning the pathogenesis, but also provide solid supporting evidence for further application of GRg1 in treatment of ischemic stroke.

Material and methods

Animals, cerebral IR injury modeling and treatment

108 male Sprague-Dawley (SD) rats were randomly and evenly divided into 8 groups: namely

Sham group (Sham), ischemia-reperfusion group (IR), rosiglitazone treated ischemia-reperfusion group (ROZ + IR), rosiglitazone and zinc protoporphyrin-IX treated ischemia-reperfusion group (ROZ + ZnPP + IR), high-dose GRg1 treated ischemia-reperfusion group (HGRg1 + IR), medium-dose GRg1 treated ischemia-reperfusion group (MGRg1 + IR), low-dose GRg1 treated ischemia-reperfusion group (LGRg1 + IR), GRg1 and ZnPP treated ischemia-reperfusion group (ZnPP + GRg1 + IR). This study was approved by the Institutional Research Committee of Xi'an Jiaotong University and performed at the Surgical Dream Works Laboratory. All animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

The IR injury was modeled by middle cerebral artery occlusion (MCAO) according to protocol described previously [6]. The internal and external carotid artery were exposed and isolated. Then the distal part of external carotid artery was dissected. Monofilament nylon (4-0) was introduced into internal carotid artery through external carotid artery to perform MCAO. After 2 hours, MCAO was withdrawn to introduce reperfusion.

Rosiglitazone (GSK, 5 mg/kg) was administrated orally 6 hours prior to MCAO; ZnPP (Sigma-Aldrich, 10 mg/kg) was administrated by intraperitoneal injections with every rosiglitazone treatment; high-dose (60 mg/kg) GRg1 (Sigma-Aldrich), medium-dose (40 mg/kg) GRg1 or low-dose GRg1 (20 mg/kg) which was showed effective neuroprotective effect according to previous studies was dissolved in physiological saline and administrated by intravenous injection 1 hour before MCAO.

Neurological deficits determination

As described in previous studies [19], a five-point scale was employed to evaluate the neurological defects 24 hours after reperfusion. All investigators were blinded to animal groups. The scale was divided into five grades from 0 to 4. Grade 0 referred to no neurologic deficit (normal), grade 1 referred to flexion of forelimbs and contralateral torso when lifted by tail (mild); grade 2 referred to circling to contralateral side but maintaining normal posture at rest (moderate); grade 3 referred to leaning to the contralateral side at rest (severe); grade 4 referred to no spontaneous motor activities (very severe).

Ginsenoside RG1 prevent cerebral ischemia-reperfusion injury

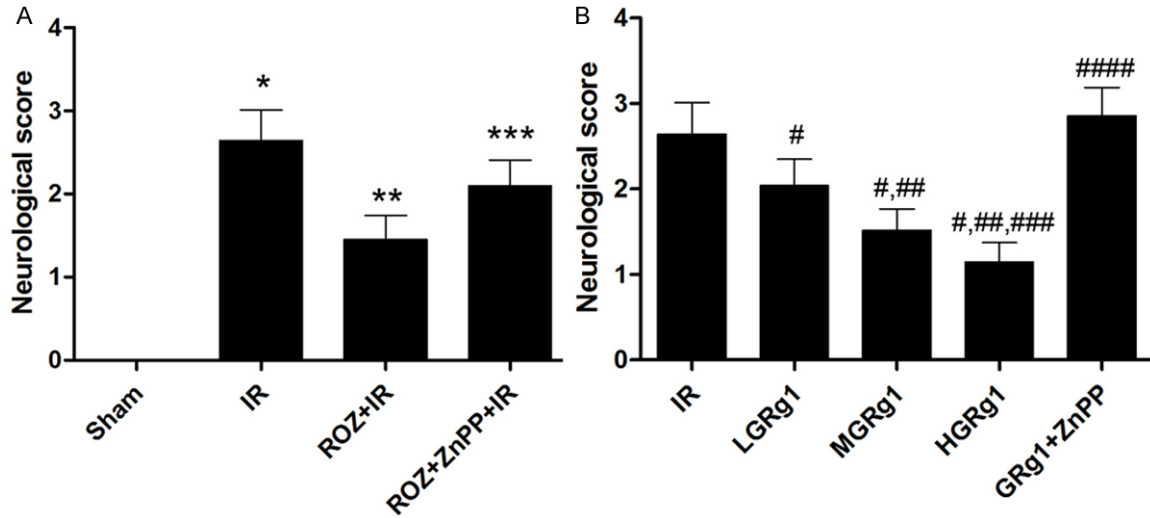


Figure 1. Effects of rosiglitazone, ZnPP and RG1 treatments on neurological impairments on IR rats. Columns on this figure indicated the neurological score in IR rats received different treatments. The neurological score was determined by a 5-point scale. A. Neurological deficits score in sham, IR, ROZ + IR and ROZ + ZnPP + IR respectively. Differences were significant when compared with *Sham, **IR, ***ROZ + IR. B. Neurological deficits score in IR, LGRg1, MGRg1, HGRg1 and GRg1 + ZnPP respectively. Differences were significant when compared with #IR, ##LGRg1, ###MGRg1, ####HGRg1.

In situ apoptosis assay

The cell apoptosis in hippocampus was assessed by TUNEL fluorescent staining according to previous descriptions. Briefly, the rats were scarified by over-dose anesthesia by chloral hydrate (10%) intraperitoneal injection. The brain tissue was harvested and further embedded by OCT (Sakura) and then cut to frozen sections of 10 μ m at -20°C. 0.1% Triton X-100 buffer was used to treat the sections. After washing by cold PBS buffer, TUNEL stain mixture (Roche) was added to the tissue sections to incubate at 37°C for 1 hour in a dark chamber. Excitation at 450 nm and observed at 550 nm, a fluorescence microscopy (Nikon) was employed to observe and quantify the apoptotic cells.

Enzyme-linked immunosorbent (ELISA) assay

The modeled rats were scarified by over-dose anesthesia by chloral hydrate (10%) intraperitoneal injection. The brain tissue was harvested and the hippocampus was isolated from ischemic cortex. Homogenates from the fresh hippocampus tissue were used for ELISA assay. Rat ELISA kits (R & D) were used to detect the concentrations of IL-1 β , TNF α and high-mobility group box-1 (HMGB1) in hippocampus homogenates. All experimental protocols were in

accordance with the instructions from the manufacturer.

HO-1 enzymatic activity evaluation

The testing sample was acquired as the supernatant which was resulted from centrifugation of hippocampus tissue homogenates at 10000 g for 15 minute at 4°C. A HO-1 enzymatic activity assay kit (GenMed) was used to evaluate the HO-1 enzymatic activity per manufacturer's instructions.

Western blotting

Western blotting assay was implemented according to protocol described previously. Harvested hippocampus tissue were homogenized by RIPA lysis buffer system (Santa Cruz) supplemented with protease inhibitor cocktail (Santa Cruz) on ice. The total protein was collected after the homogenates were centrifuged at 14000 g for 20 minutes at 4°C. The protein concentration was detected by BCA method with a BCA Protein Assay kit (Thermo Scientific). 30 μ g protein from each sample was separated by electrophoresis on SDS-PAGE gel and then transferred to PVDF membranes electronically. 5% defatted milk was used to block the possible unspecific binding. Specific antibodies against PPAR γ (Cell Signaling Tech), Bcl-2

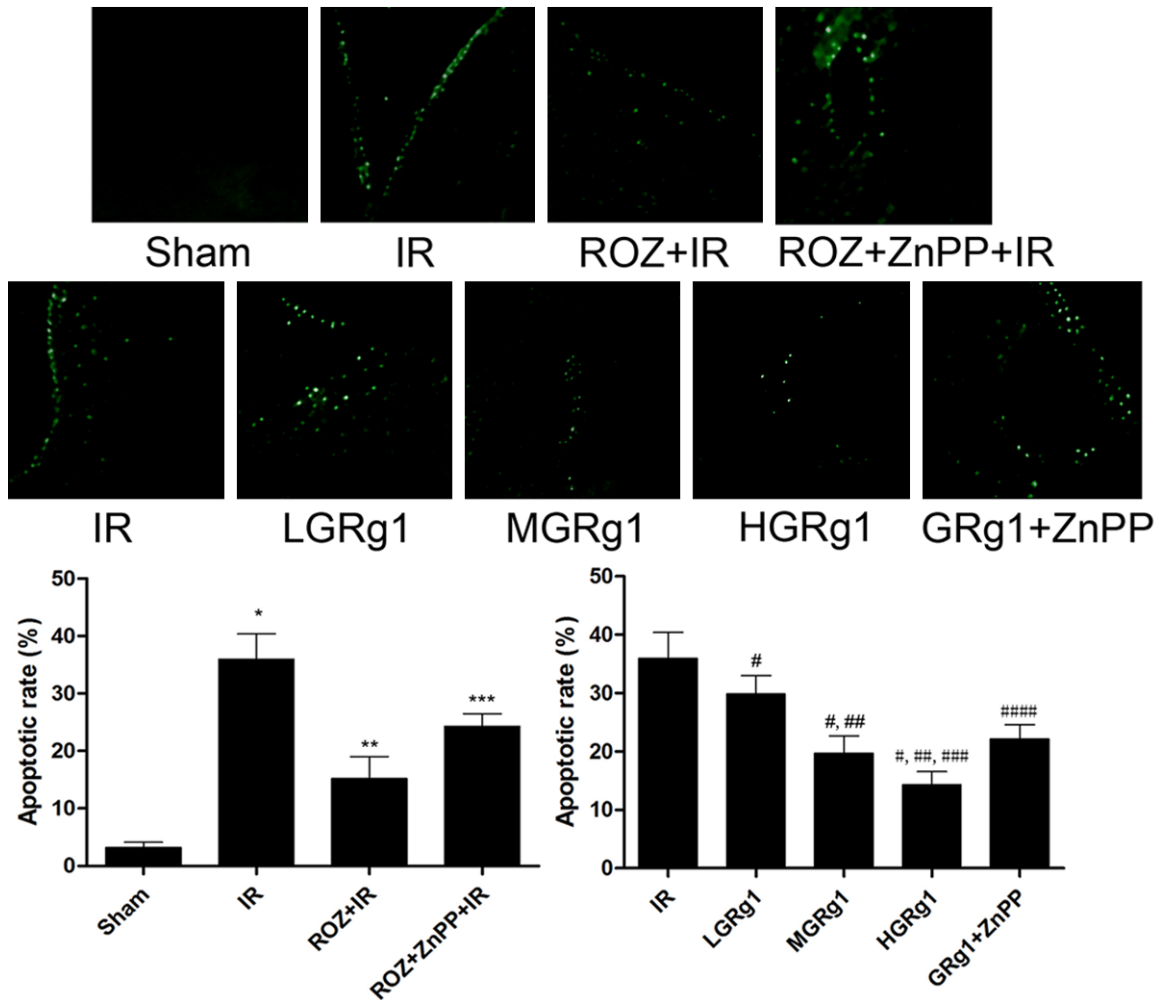


Figure 2. Effects of rosiglitazone, ZnPP and RGg1 treatments on apoptosis in hippocampus in IR rats. The upper panel in this figure showed the captured images of TUNEL fluorescent stain in hippocampus in different groups. The positive staining was shown as green fluorescence in these images. The lower panel demonstrated the calculated apoptotic rate in different groups. Differences were significant when compared with *Sham, **IR, ***ROZ + IR; Differences were significant when compared with #IR, ##LGRg1, ###MGRg1, ####HGRg1.

(Abcam), cleaved caspase-3 (Cell Signalling Tech), cleaved caspase-9 (Cell Signalling Tech), receptor for advanced glycation end product (RAGE) (Abcam) and GAPDH (Santa Cruz) were used to incubate the membranes at 4°C for 12 hours. After the horseradish peroxidase-conjugated secondary antibodies incubation, the immunoblots were detected by using Enzymatic Chemiluminescence (ECL) kit (Bio-Rad). The intensities of the immunoblots were analyzed by software ImageJ2x.

Statistical considerations

Data acquired in this study were expressed in a (mean \pm SD) manner. One-way analysis of variance (ANOVA) was used to analyze the differ-

ences between groups. A LSD analysis was followed. $P < 0.05$ was considered statistically significant.

Results

Effects of rosiglitazone, ZnPP and RGg1 treatments on neurological impairments in IR rats

We first evaluated the effects of rosiglitazone and ZnPP on neurological deficits. 24 hours after reperfusion, neurologic deficits were scored in rats. As shown in **Figure 1A**, no rats showed neurological deficit in Sham; neurologic score reduced significantly in ROZ + IR compared with IR. However, neurologic score increased in ROZ + ZnPP + IR compared with

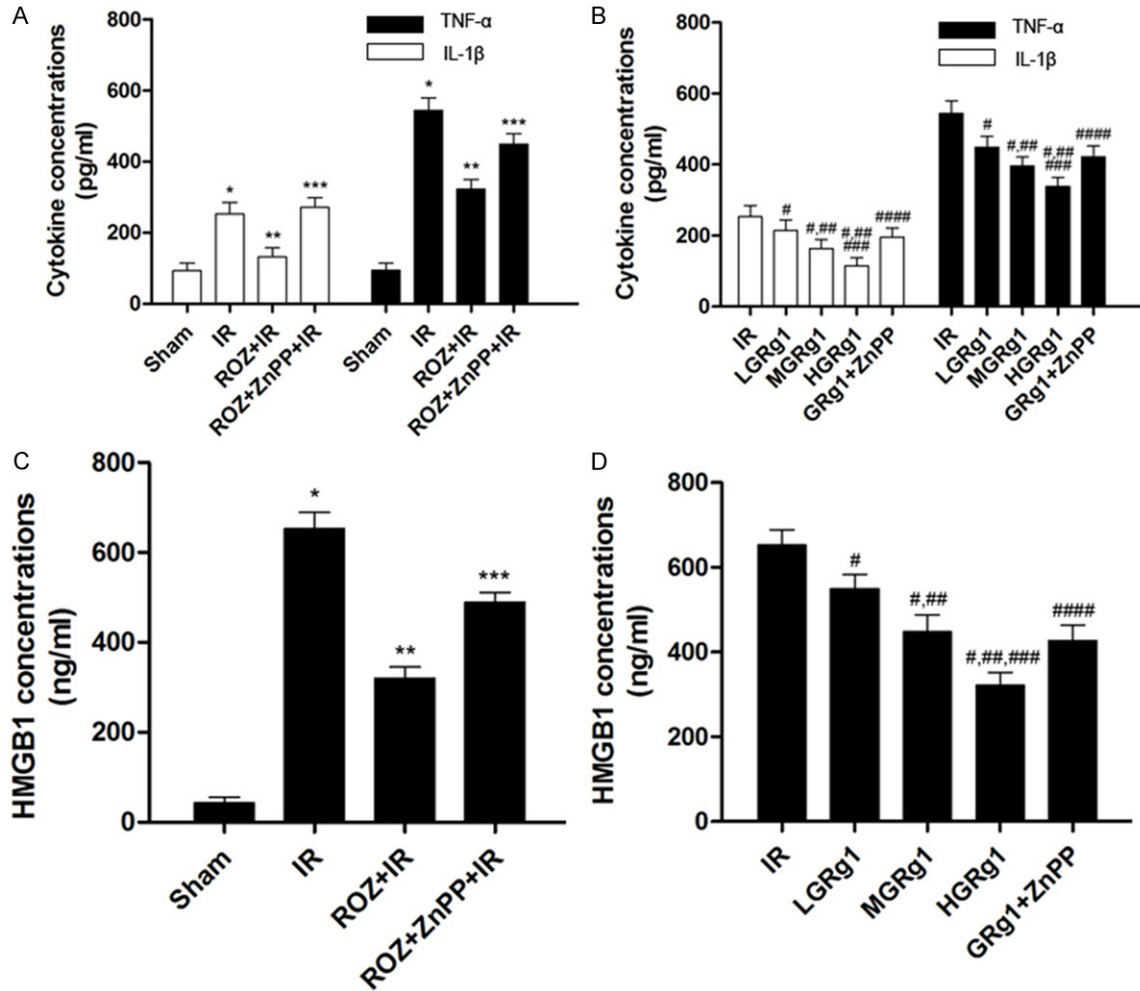


Figure 3. Effects of rosiglitazone, ZnPP and RGg1 treatments on inflammation in hippocampus in IR rats. The concentrations of inflammatory cytokines were determined by ELISA in this study. A and C showed the concentrations of IL-1 β , TNF- α and HMGB1 in sham, IR, ROZ + IR and ROZ + ZnPP + IR respectively. B and D demonstrated the concentrations of IL-1 β , TNF- α and HMGB1 in IR, LGRg1, MGRg1, HGRg1 and GRg1 + ZnPP respectively. Differences were significant when compared with *Sham, **IR, ***ROZ + IR; Differences were significant when compared with #IR, ##LGRg1, ###MGRg1, ####HGRg1.

ROZ + IR significantly. Whether RGg1 attenuated neurological impairment in IR rats? As shown in **Figure 1B**, neurological score decreased significantly in GRg1 treated rats than IR in a concentration-dependent manner. However, this effect was reversed by ZnPP treatment in ZnPP + GRg1 + IR.

Effects of rosiglitazone, ZnPP and RGg1 treatments on apoptosis in hippocampus in IR rats

TUNEL fluorescent staining was employed to detect the cell apoptosis in hippocampus region. As demonstrated in **Figure 2**, apoptotic rate in hippocampus region elevated significantly in IR compared with Sham. The apoptotic

rate decreased significantly by rosiglitazone administration in ROZ + IR than IR. Nevertheless, ZnPP reversed rosiglitazone's anti-apoptotic effect in ROZ + ZnPP + IR. In addition, GRg1 administration dramatically alleviated apoptosis in hippocampus in a concentration-dependent manner (**Figure 2**). However, apoptotic rate in ZnPP + GRg1 + IR was significantly higher than IR rats treated with GRg1 (**Figure 2**).

Rosiglitazone and RGg1 exerted anti-inflammation effect which was impaired by ZnPP in hippocampus

Figure 3 demonstrated the expression levels of several inflammatory cytokines including IL-1 β ,

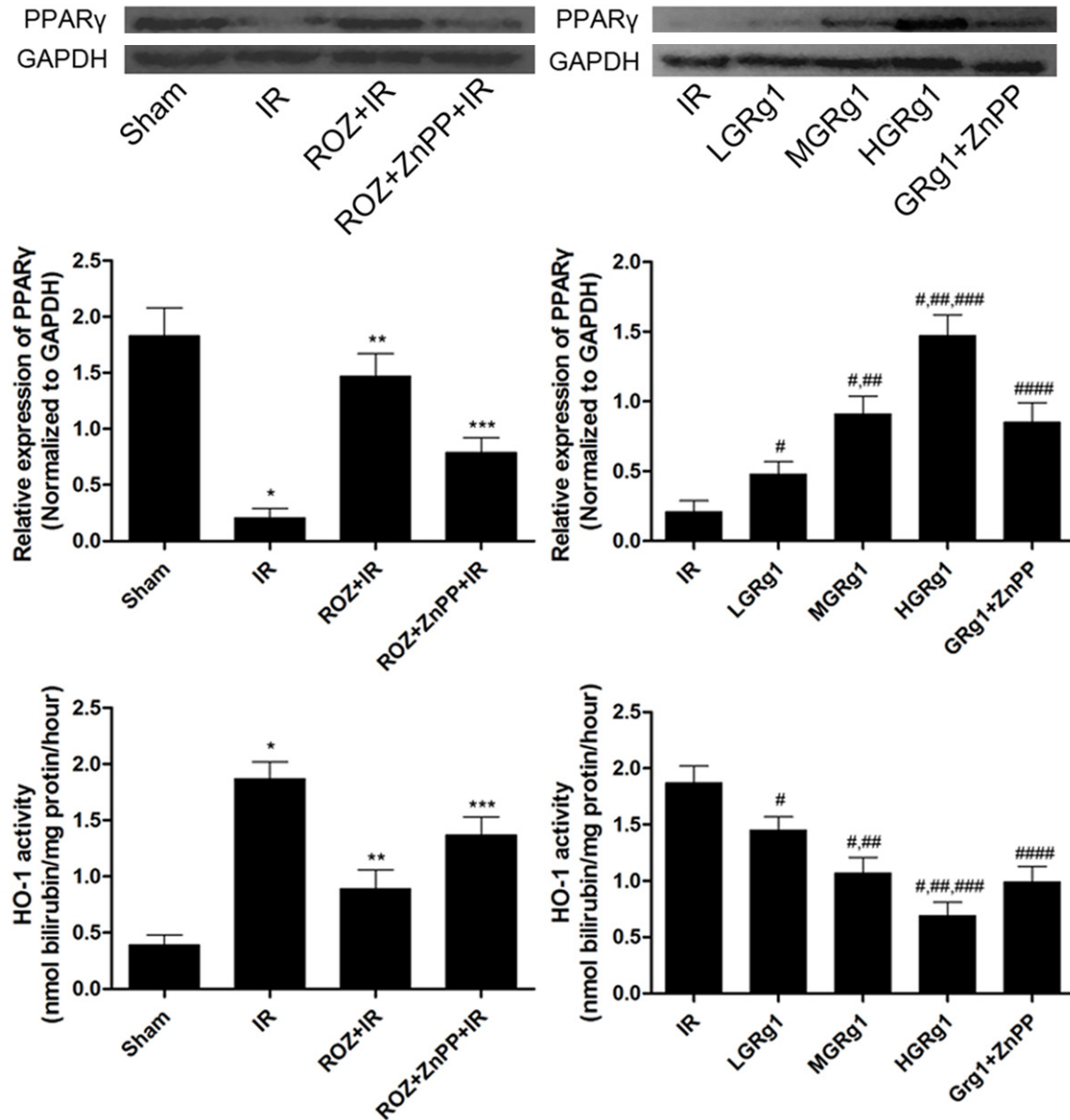


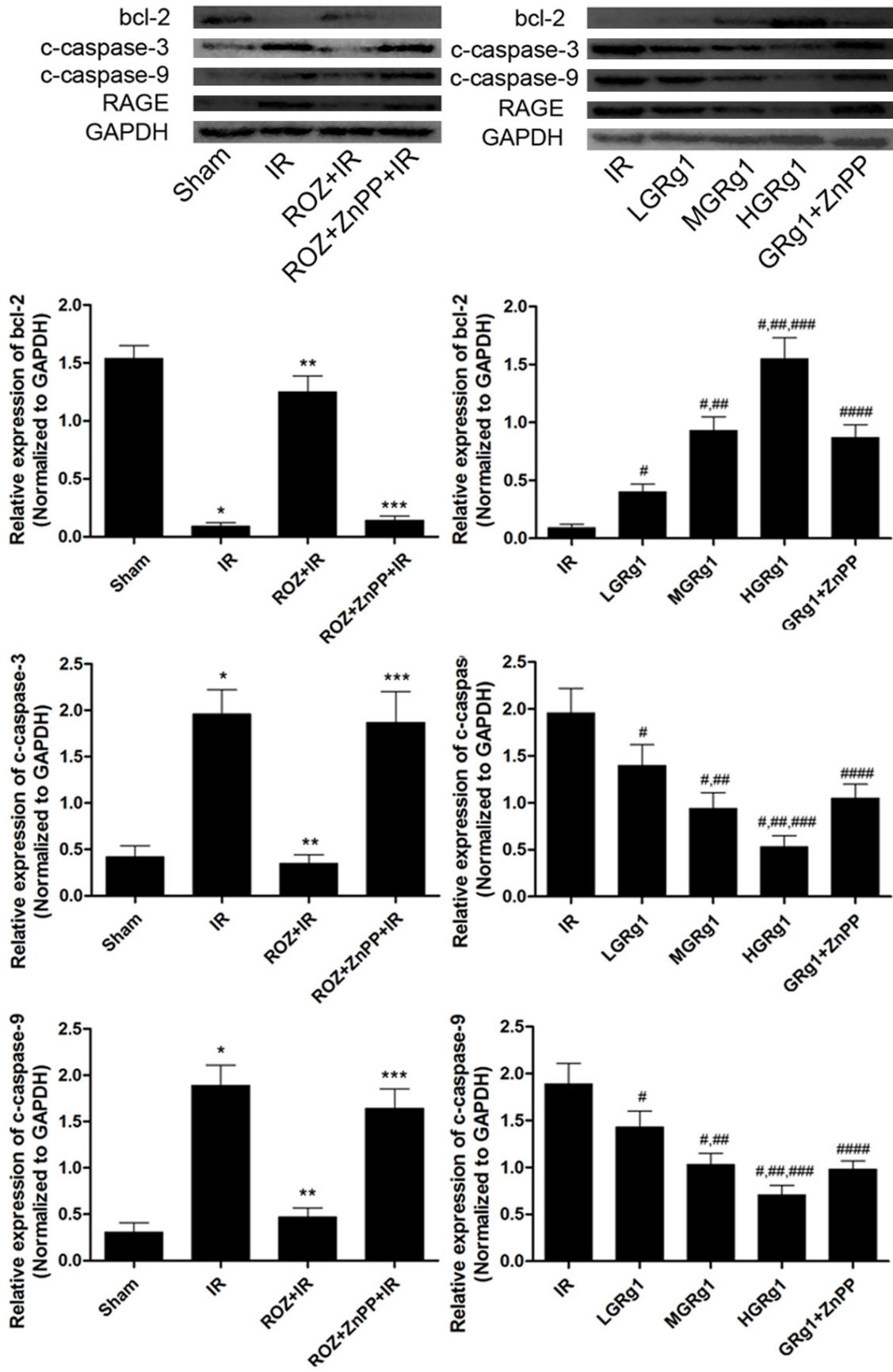
Figure 4. Effects of rosiglitazone, ZnPP and GRg1 treatments on PPAR γ /HO-1 activation in IR rats. The upper panel demonstrated the immunoblots of PPAR γ and GAPDH in different groups. The columns in the middle panel indicated the relative protein expression of PPAR γ (normalized to GAPDH) in different groups. The columns in at the bottom of this figure indicated the HO-1 activity in different groups. Differences were significant when compared with *Sham, **IR, ***ROZ + IR; Differences were significant when compared with #IR, ##LGRg1, ###MGRg1, ####HGRg1.

TNF α and HMGB1 in hippocampus homogenates from IR rats. We found that the expression levels of IL-1 β , TNF α and HMGB1 in IR were increased significantly but inhibited in ROZ + IR. We also found that ZnPP treatment in ZnPP + ROZ + IR elevated the expression level of these cytokines compared with ROZ + IR. Moreover, After RGg1 treatment, the IL-1 β , TNF α and HMGB1 concentrations decreased in IR rats. However, ZnPP treatment significantly impaired RGg1's anti-inflammatory effect (Figure 3).

Effects of rosiglitazone, ZnPP and GRg1 on PPAR γ expression and HO-1 activity in hippocampus from IR rats

Firstly, we found that PPAR γ expression and HO-1 activity was suppressed in IR rats compared with Sham. Rosiglitazone administration elevated PPAR γ expression and HO-1 activity but HO-1 activity was suppressed by ZnPP treatment. Secondly, GRg1 treatment showed similar effect on PPAR γ expression and HO-1

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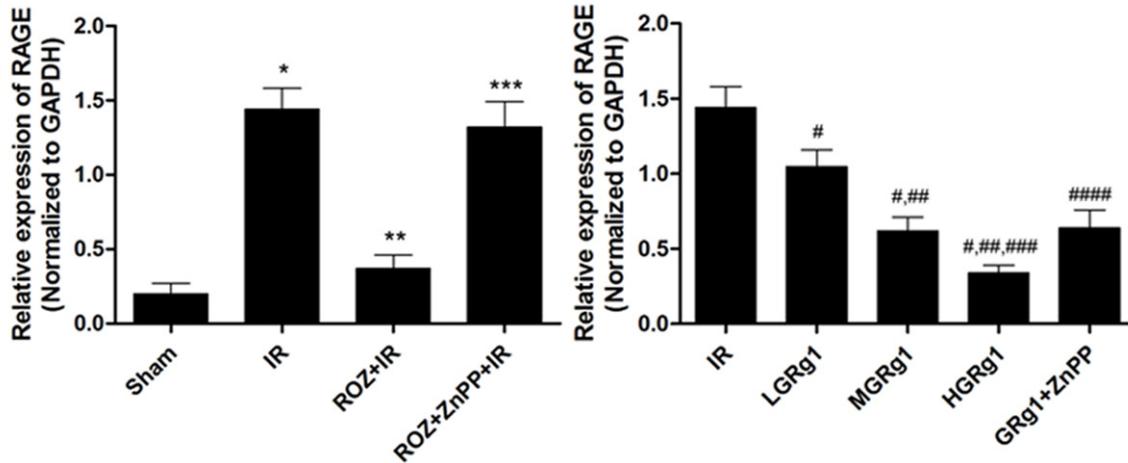


Figure 5. Effects of rosiglitazone ZnPP and GRg1 treatments on expression levels of bcl-2, cleaved caspase-3, cleaved caspase-9 and RAGE in IR rats. The upper panels showed the immunoblots of bcl-2, cleaved caspase-3 (c-caspase-3), cleaved caspase-9 (c-caspase-9), RAGE and GAPDH in different groups. The columns on the lower panels demonstrated the relative expression levels of bcl-2, c-caspase-3, c-caspase-9 and RAGE in different groups. Differences were significant when compared with *Sham, **IR, ***ROZ + IR; Differences were significant when compared with #IR, ##LGRg1, ###MGRg1, ####HGRg1.

activity as rosiglitazone but HO-1 activity elevation was also reversed by ZnPP (Figure 4).

Effects of rosiglitazone, ZnPP and GRg1 on inflammation-and apoptosis-related proteins

Bcl-2 expression was down-regulated, while expressions of cleaved caspase-3, cleaved caspase-9 and RAGE were up-regulated in IR than Sham. In ROZ + IR, Bcl-2 expression was up-regulated while expressions of cleaved caspase-3, cleaved caspase-9 and RAGE were suppressed but then reversed by ZnPP. Similar to the effect of rosiglitazone, GRg1 also up-regulated Bcl-2 expression but down-regulated expressions of cleaved caspase-3, cleaved caspase-9 and RAGE in IR rats. However, this effect was attenuated by ZnPP administration (Figure 5).

Discussion

In this present study, we firstly investigated the role of PPAR γ in cerebral IR injury in hippocampus. The cerebral IR model was introduced by MCAO, which resulted in neurological deficits deterioration. The administration of rosiglitazone, the PPAR γ agonist, significantly reduced the apoptosis and inflammation in hippocampus and thus neurological deficits and thus attenuated neurological deficits in IR rats. However, the HO-1 inhibitor ZnPP reversed PPAR γ activation's neuroprotective effect.

Secondly, we also investigated the neuroprotective effect of GRg1 in cerebral IR rats. The results suggested that PPAR γ mediated signaling was involved in the anti-apoptosis and anti-inflammation effects of GRg1 in hippocampus cerebral IR injury.

IR injury refers to the tissue and cellular damage induced by oxygen supply was restored to tissue suffered a prolonged ischemia. Cell damage were obvious in both ischemia and reperfusion phases [20]. It was believed that cell apoptosis and inflammation were characterized pathological changes during cerebral IR injury, leading to neurological dysfunctions [7]. In this study, we applied MCAO to induce cerebral IR injury in rats and the neurological deficits were found deteriorated. Furthermore, in hippocampus of cerebral IR rats, the cell apoptosis rate was found increased by TUNEL staining and the inflammation was found stimulated evidenced by inflammatory cytokines level elevation.

Indeed, the notion that PPAR γ activation protected against apoptosis and inflammation was well established in many previous studies [21, 22]. Furthermore, it has been also shown that some of these protective effects of PPAR γ were introduced by one of its down-stream effectors HO-1 [23]. HO-1 which could be categorized into stress-responsive proteins degrades pro-oxidant heme into free iron, biliverdin (bilirubin)

and carbon monoxide. HO-1 exerts its various biological activities by these enzymatic reactions. Previous studies reported that lack of HO-1 resulted in significant increase in apoptotic cell rate [24]. Other studies indicated that caspase-3 cleavage reduction was involved in HO-1 mediated apoptosis inhibitory effect [25]. Besides, by activating anti-apoptotic factors such as bcl-2, HO-1 was proved to prevent apoptosis via stabilizing mitochondrial transport carriers and maintaining mitochondrial functions [26]. Elevated HO-1 activity was also found to inhibit cytochrome C release to improve cell survival [27]. In the present study, we found that the HO-1 activity was increased after administration of rosiglitazone in cerebral IR rats; as a result, the apoptosis was reduced in hippocampus. Expression changes of corresponding proteins, including bcl-2, cleaved caspase-3 and cleaved caspase-9 were also observed. ZnPP treatment reversed this anti-apoptotic effect, which further indicated the anti-apoptotic role of PPAR γ was induced via HO-1 in cerebral IR injury.

During the onset of cerebral IR injury, complex cellular biological processes were triggered. It was reported that within several hours after cerebral IR injury, the resident glial cells were activated; circulating inflammatory cells such as neutrophils were recruited and infiltrated into brain [28]. Thus, inflammatory responses were considered playing an important role in cerebral IR injury [29]. The infiltrated inflammatory cells would secrete inflammatory cytokines. These cytokines then further activate and recruit more inflammatory cells which mediate neurological damage by releasing proteases and reactive oxygen species (ROS) [30]. In some recent studies, HMGB1 which was also considered as an inflammatory take part in several inflammatory pathological conditions such as acute lung injury, asthma and acute myocardial infarction [31, 32]. After binding to its receptor, namely RAGE, many signaling cascades were activated by HMGB1-RAGE axis, such as NF- κ B and MAPK which eventually initiated the transcription of genes of various inflammatory mediators including IL-1 β and TNF- α [33, 34] we detected by ELISA in the present study. Expression levels of IL-1 β , TNF- α and HMGB1 in hippocampus from cerebral IR rats were reduced after PPAR γ was activated by rosiglitazone which was reversed by ZnPP

administration. Considering the conclusion that HO-1 induction was coupled with subsequent reduction of HMGB1-RAGE axis, the above results in our study concerning apoptosis and inflammation indicated that PPAR γ /HO-1 was located on the central position in the network regulating apoptosis and inflammation in cerebral IR injury.

GRg1 is one of the major active ingredients of ginseng, which shows a broad spectrum of biological activities. Previous studies described the neuroprotective effects of GRg1 in treating neurodegenerative diseases [35]. However, the exact mechanisms of GRg1 in cerebral IR injury treatment are still unclear. In this study, we found that GRg1 showed dramatic curative effect in cerebral IR rats which was evidenced by improvement of neurological deficits score. In a recent study in regard of Alzheimer's disease, GRg1 was proposed to attenuate learning and memory by activating PPAR γ in hippocampus in rat [18]. Similar to this finding, we detected dramatically increased PPAR γ in hippocampus in cerebral IR rats. Furthermore, both of the apoptosis and inflammation were attenuated by GRg1 treatment. Concurrently, the HO-1 activity was also found increased in GRg1 treated rats. However, GRg1's neuroprotective effect was impaired by ZnPP administration, which was proved by neurological dysfunction, increased apoptosis and inflammation in hippocampus. Mechanically, compared with GRg1-treated cerebral IR rats, the expressions of molecules in PPAR γ /HO-1 downstream pathways were also reversed by ZnPP. These results suggested that the neuroprotective effect of GRg1 was similar to the PPAR γ agonist rosiglitazone in cerebral IR injury by attenuating apoptosis and inflammation in hippocampus through PPAR γ /HO-1 governed signaling pathways.

Disclosure of conflict of interest

None.

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