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Hispidulin exhibits neuroprotective activities against cerebral ischemia reperfusion injury through suppressing NLRP3-mediated pyroptosis

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ABSTRACT

Aim: Ischemia/reperfusion (I/R) injury is the major cause of neurological deficit following stroke. Our previous study showed neuroprotective effects of hispidulin against cerebral ischemia reperfusion injury (IRI). In this study, we further examined the involvement of pyroptosis in this neuroprotective function.

Materials and methods: IRI was simulated in a rat model by middle cerebral artery occlusion (MCAO) surgery, and the animals were treated with different doses of hispidulin. The neurological function of the rats was evaluated by the neural function defect score (NFDS), balance beam test and limb placement test. The infarct volume and brain water content were measured 72 h following IRI. Neuronal cell survival and pyroptosis in the ischemic cortex were respectively detected by Nissl staining and TUNEL assay. The relative expression of pyroptosis markers was determined by qRT-PCR, Western blotting and ELISA as appropriate. IRI was simulated *in vitro* in primary cerebral astrocytes using the OGD/R procedure. AMPKα was blocked genetically or pharmacologically using siRNA and compound C respectively. CCK-8 and LDH release assays were performed using suitable kits.

Results: Hispidulin improved the neurological symptoms of the rats after IRI, in addition to decreasing the infarct size and brain edema. Mechanistically, hispidulin exerted its neuroprotective effects *in vivo* and *in vitro* by suppressing NLRP3-mediated pyroptosis by modulating the AMPK/GSK3β signaling pathway.

Conclusion: Hispidulin is a neuroprotective agent with clinical potential against IR-induced neurological injury.

1. Introduction

Stroke is ranked as one of the top three causes of death worldwide [1]. According to the World Health Organization, approximately 15 million people are affected by stroke every year [2], of which 30% do not survive [3]. In addition, 75% of the patients who survive face long-term physical and cognitive disabilities, which has significant social and economic consequences [4]. Stroke is classified as ischemic or hemorrhagic, and the former accounts for > 80% of all cases [5]. Ischemic stroke results from the occlusion of a major cerebral artery, most frequently the middle cerebral artery (MCA), which produces a hypoxic environment that subsequently triggers apoptosis, necrosis and metabolic dysfunction in neuronal cells [3]. Currently, recombinant tissue plasminogen activator is the only drug approved by the US Food and Drug Administration for the treatment of acute ischemic stroke [6]. However, it has limited efficacy due to a narrow therapeutic window and secondary side effects [6]. Therefore, novel therapeutic agents with higher efficacy and fewer side effects are needed.

Pyroptosis, a form of programmed cell death closely associated with the inflammatory response, is activated by cleavage of caspase-1, followed by the release of pro-inflammatory cytokines [7]. The activated caspase-1 then converts the inactive forms of IL-1β and IL-18 to their active forms, which lead to pyroptosis [8]. The crucial role of the NOD-like receptor protein 3 (NLRP3) inflammasome in the initiation of pyroptosis has been well documented [9]. It is activated by various cellular stresses, which triggers the caspase-1 cascade. A recent study showed the involvement of pyroptosis in cerebral ischemia reperfusion injury (IRI) [10], although it is unclear whether its suppression can be

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neuroprotective against IRI.

Hispidulin, a flavonoid present in many Chinese herbal medicines, has potent antioxidant, antifungal, antitumor, anti-osteoporotic, anti-inflammatory and antimutagenic properties [11]. In addition, it is a ligand of the benzodiazepine and GABA-A receptors, wherein it acts as a partial positive allosteric modulator [12]. The protective effects of hispidulin against anesthesia-induced neurotoxicity have also been demonstrated [13]. In a previous study, we found that pretreatment with hispidulin improved brain function and reduced infarct area and brain edema in a rat cerebral IRI model [14]. The aim of this study was to elucidate the mechanisms underlying these neuroprotective effects.

2. Materials and methods

2.1. Establishment of rat ischemia-reperfusion (IR) model and treatment regimen

Male Sprague Dawley (SD) rats (weighing 240–280 g) were housed at 22 °C in a humidity-controlled room, and given *ad libitum* access to food and water. A total of 100 animals were randomized into the following groups (25 per group): 1) sham-operated, 2) untreated IR, 3) IR + 40 mg/kg His, and 4) IR + 80 mg/kg His. Middle cerebral artery occlusion (MCAO) surgery was performed as previously described [14]. Briefly, the rats were anesthetized with chloral hydrate, and the origin of the MCA was blocked using a nylon monofilament inserted from the external carotid. The occlusion was maintained for 2 h, and the artery was re-perfused after carefully extracting the filaments. In the sham-operated rats, neck incision was performed without arterial ligation. Hispidulin was injected intraperitoneally once daily for 3 days following IR. All animal protocols were approved by the Medical Ethics Committee of our institution.

2.2. Measurement of cerebral infarct volume

The infarct volume was measured as previously described [14]. Briefly, the freshly extracted brain tissues were sliced, fixed with 4% paraformaldehyde, and stained with 2,3,5-triphenyltetrazolium chloride at 37 °C for 10 min. The relative cerebral infarct volume was calculated.

2.3. Measurement of brain water content

Brain water content was measured as previously described [14]. Briefly, whole brains were removed, weighed, and dried for 24 h at 105 °C. The dry weight was measured the following day, and the water content was calculated as: [(wet weight-dry weight) / wet weight] × 100%.

2.4. Neurological tests

Three days following MCAO, the neurological function of the rats was assessed according to the neural function defect score (NFDS) from 0 to 7 [4]. The scoring method is listed in Table 1. The balance beam test (BBT) was used to evaluate the balancing ability of rats. The animals were placed on a wooden bar measuring 300 mm × 25 mm, and scored from 0 to 7 according to Table 2 [15]. For the forelimb placement test (FPT), the animals were held gently by the torso and moved slowly toward a table top until the dorsal forepaw surface barely touched the edge. The ability to rapidly put their forelimbs on the surface was recorded between 0 (normal) and 10 (maximal impairment) as previously described [16].

2.5. Nissl and TUNEL staining of ischemia cortex

The brain tissues were fixed with 4% paraformaldehyde and then immersed in 30% sucrose for 24 h at 4 °C. Following paraffin embedding, 5 μm brain sections were cut, and subjected to Nissl or TUNEL staining as previously described [14].

Table 1
Neurological scoring scale.

Score	Behavior
0	Rats present with symmetric activity
1	When the tail is lifted, the left forelimb cannot be extended
2	With left shoulder abduction, the left forelimb is not straight down
3	Left forelimb is close to the chest wall
4	When moving freely, the rats turned left
5	Accompanied by obvious left-front paw pushing back
6	Rats can only rotate left around the origin
7	Limbs cannot support the body weight on the left side, and the rats can only lie on the left side

Table 2
BBT scoring scale.

Score	Behavior
0	Rats can maintain balance with four feet and walk across the wooden bar
1	Rats can't walk across the wooden bar, but can maintain balance with four feet
2	Rat's paws catch the side of the wooden bar or rat's body shakes on the bar
3	One limb slips from the bar
4	Two limbs slip from the bar
5	Rats try to keep their balance but slip
6	Rats fail to keep their balance, hang on the bar and fall down
7	Rats fall down directly without trying to keep their balance

2.6. Isolation and culture of primary astrocytes

Cerebral astrocytes were isolated from neonatal SD rats (postnatal day 1) as previously described [17]. Briefly, the neonates were anesthetized with 2% isoflurane, and the cortices were removed and digested with 0.125% trypsin for 30 min at 37 °C. The digested masses were centrifuged and washed once, and the single cells were cultured in Neurobasal medium (Gibco, USA) supplemented with B27 (Gibco, USA) in a humidified atmosphere with 5% CO_2 at 37 °C.

2.7. Oxygen-glucose deprivation followed by recovery (OGD/R)

OGD/R procedure was conducted as previously described [18]. Briefly, the astrocytes were cultured in an ischemia simulating solution under 5% CO_2 and 95% N_2 for 2 h. Thereafter, the solution was replaced with normal culture medium, and the cells were maintained at 5% CO_2 and 95% air for 24 h to mimic reperfusion.

2.8. Western blotting

The cerebral cortices were homogenized with a lysis buffer (Beyotime), and the protein content in the lysates was measured using BCA assay. Western blotting was performed as per standard procedures using the following primary antibodies: xx Goat anti-rabbit IgG-HRP (Beyotime, Shanghai, China) was used as the secondary antibody, and β-actin as the internal control. The positive bands were visualized using an ECL system (Thermo Fisher, Shanghai, China).

2.9. Cell viability assay

Viable cells were evaluated using the Cell Counting Kit-8 (CCK-8) (Beyotime, Shanghai). The astrocytes were plated into 96-well plates, and the CCK-8 solution was added. Following incubation at 37 °C for 2 h, absorbance was measured at 450 nm.

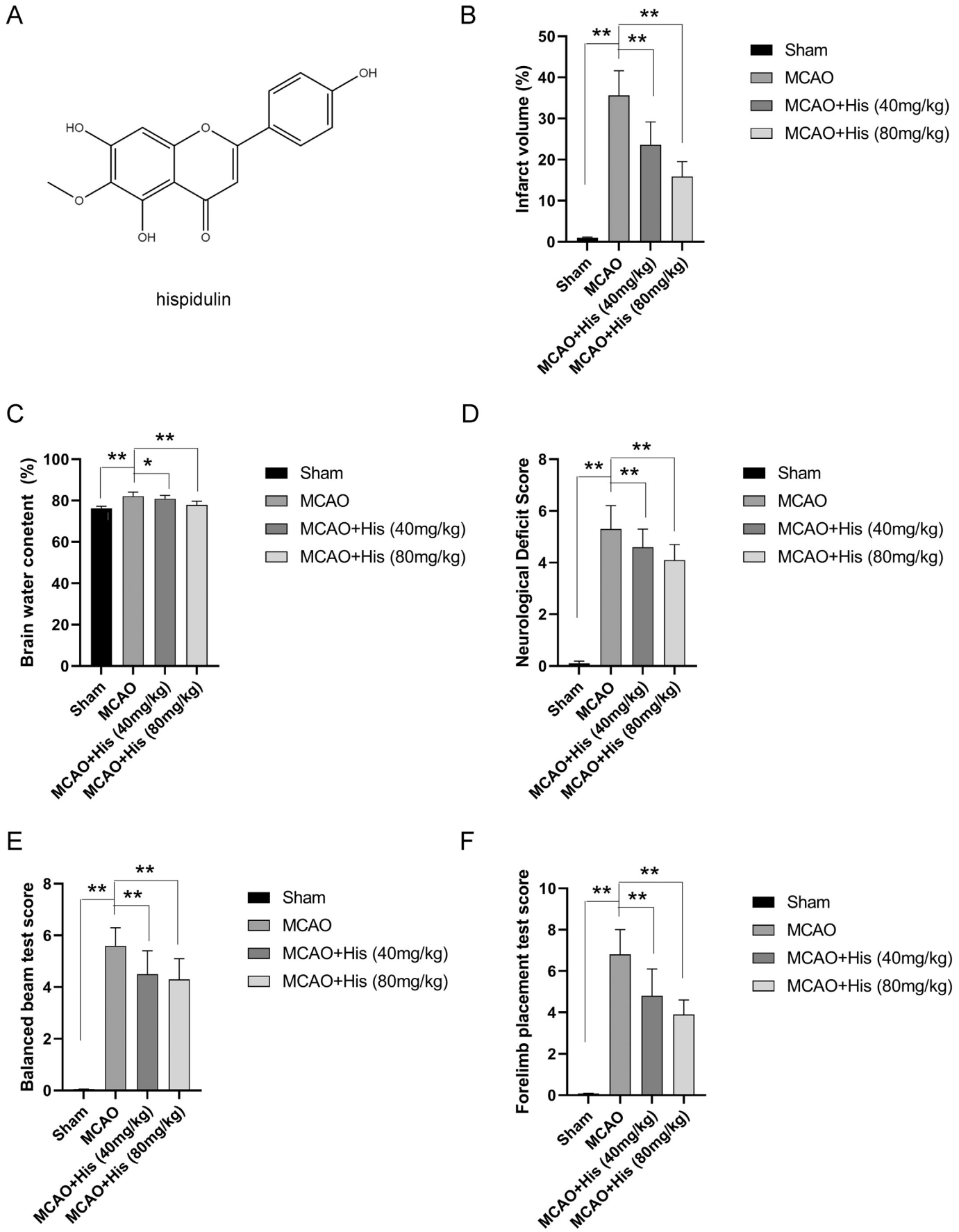

Fig. 1. Hispidulin ameliorated IR-induced neurological injury in a rat IRI model. A. Chemical structure of hispidulin. Hispidulin treatment (B) reduced the infarct volume, (C) decreased brain edema, (D) attenuated IR-induced neurological deficit, (E) restored balancing ability, and (F) attenuated IR-induced deficit in sensorimotor integration in the rat model. $N = 5$. $^{**}p < 0.01$.

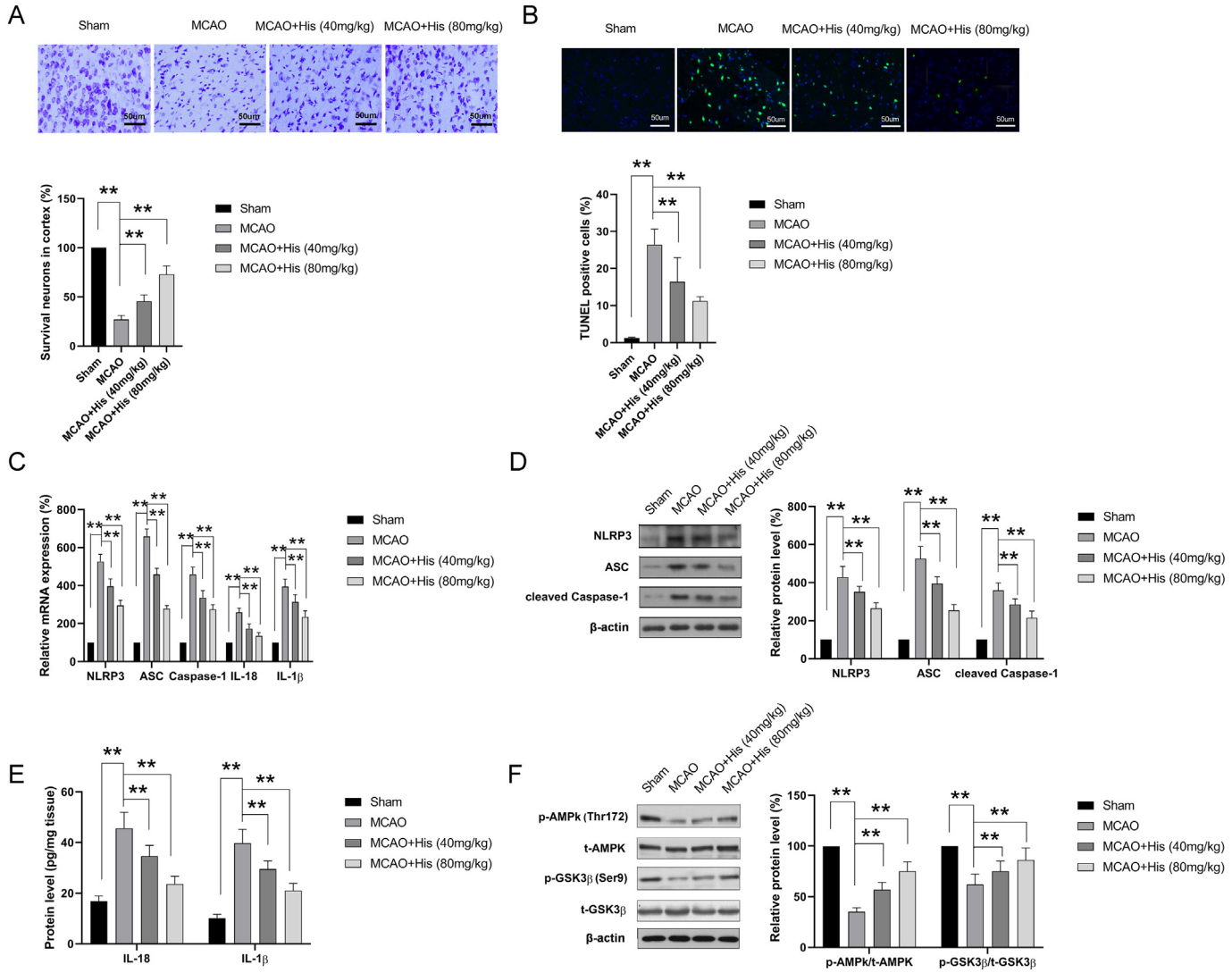

Fig. 2. Hispidulin suppressed IR-induced pyroptosis in the ischemic cortex. Effects of hispidulin on IR-induced (A) neuronal death, (B) pyroptosis, (C) upregulation of pyroptosis marker mRNA levels, (D) increase in NLRP3, ASC and cleaved caspase-1 protein levels, (E) increase in secreted levels of IL-18 and IL-1β, and (F) AMPK/GSK3β activation status. N = 5. **p < 0.01.

2.10. Measurement of LDH release and IL-1β and IL-18 production

LDH release was measured using an LDH Cytotoxicity Detection Assay Kit (TaKaRa Bio, Dalian, China) according to the manufacturer's protocols. IL-1β and IL-18 levels were measured by ELISA (Abcam, Cambridge, UK) according to the manufacturers' instructions.

2.11. Quantitative real-time reverse transcription-PCR

Total RNA was extracted from cells using TRIzol reagent and first strand cDNA was synthesized using First Strand cDNA Synthesis kit (Solarbio Bioscience & Technology Co, Shanghai, China). The primers for RT-PCR were synthesized by Sangong (Shanghai, China) using previously published sequences [19]. RT-PCR was performed using SYBR GREEN Mastermix (Solarbio, Beijing, China). The relative expression of Nrf2 to GADPH was analyzed by the $2^{-\Delta\Delta}$Ct method.

2.12. AMPKα knockdown

Cells were transfected with the AMPKα-specific siRNA (Santa Cruz Biotechnology) or scrambled siRNA as previously described [20]. AMPKα protein levels were validated by Western blotting.

2.13. Statistical analysis

All data were expressed as mean ± SD of 5 animals or 3 independent *in vitro* experiments as appropriate. Groups were compared using one-way ANOVA followed by Dunnett's *t*-test using Graphpad 8.0 software. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Hispidulin ameliorates IR-induced neurological dysfunction

The chemical structure of hispidulin is shown in Fig. 1A. As shown in Fig. 1B, MCAO significantly increased the cerebral infarct volume by 30% compared to the control rats, which was markedly decreased by hispidulin in a dose-dependent manner. Furthermore, hispidulin also alleviated the brain water content that was increased by MCAO (Fig. 1C), indicating that it can relieve cerebral edema. The IR-induced neurological deficit and the effect of hispidulin were further assessed by standardized neurological scoring and tests. As shown in Fig. 1D, IRI significantly increased the NFDS scores, indicating considerable neurological deficit, which was attenuated by hispidulin in a dose-dependent manner. In addition, IRI also impaired the balancing function and

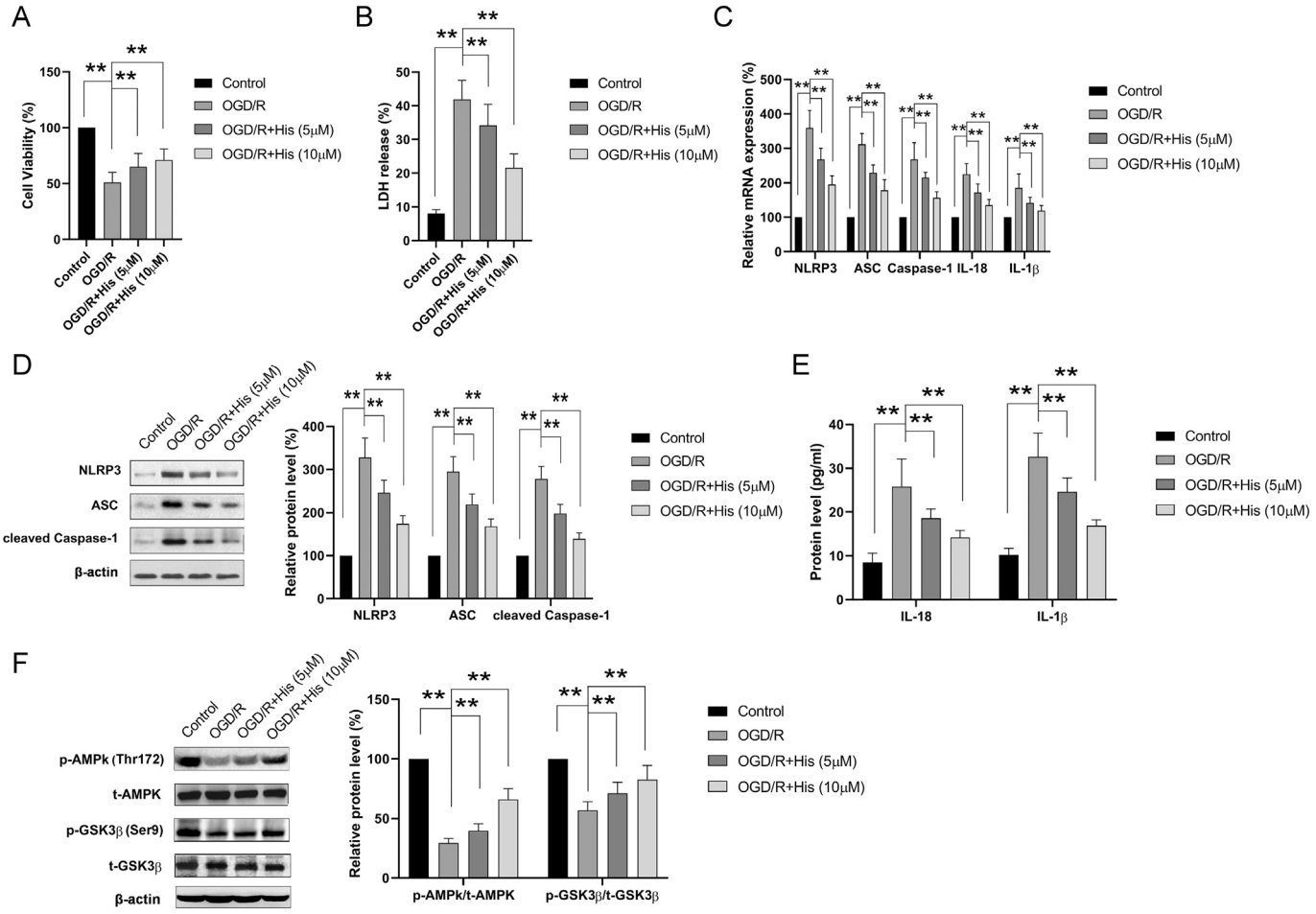

Fig. 3. Hispidulin protected OGD/R-induced neuronal injury by suppressing pyroptosis. Effects of hispidulin on OGD/R-induced (A) loss in cell viability, (B) LDH release, (C) NLRP3, ASC, caspase-1, IL-18 and IL-1β mRNA levels, (D) NLRP3, ASC and cleaved caspase-1 protein levels, (E) IL-18 and IL-1β levels, and (F) AMPK/GSK3β activation status. $^{**}p < 0.01$.

sensorimotor integration of the rats, as indicated respectively by increased BBT (Fig. 1E) and FPT (Fig. 1F) scores. Hispidulin effectively improved the balancing and sensorimotor integration abilities of rats, albeit not in a dose-dependent manner (Fig. 1E, F). Taken together, hispidulin attenuated IR-induced brain damage and neurological dysfunction.

3.2. Hispidulin attenuates IR-induced pyroptosis in the ischemic cortex

Following MCAO, the surviving neurons in the ischemic cortex decreased to < 30% compared to the sham-operated controls (Fig. 2A). Hispidulin treatment increased the proportion of surviving neurons to 40% and 70% at the doses of 40 mg/kg and 80 mg/kg respectively. Consistent with this, IRI increased the percentage of TUNEL-positive cortical neuronal cells, which was significantly decreased by hispidulin in a dose-dependent manner (Fig. 2B). To determine whether hispidulin specifically targeted IR-induced pyroptosis, we analyzed the expression levels of pyroptosis markers. As shown in Fig. 2C, NLRP3, ASC, caspase-1, IL-18 and IL-1β mRNAs were markedly increased in the ischemic cortex compared to the sham-operated group, and restored to normal levels by hispidulin treatment. The results were verified at the protein levels as well using Western blotting (Fig. 2D) and ELISA (Fig. 2E) as appropriate. Previous studies have shown that the NLRP3 inflammasome is suppressed by the inhibition of GSK3β [21], and that hispidulin can inhibit GSK3β by activating AMPK [14]. Consistent with these findings, IRI significantly decreased the level of phosphor-AMPK (Thr172) and phosphor-GSK3β (Ser9) (Fig. 2F). In addition, hispidulin-mediated inhibition of NLRP3 and pyroptosis was associated with increased phosphorylation of AMPK and GSK3β (Fig. 2F). Taken together, hispidulin can inhibit IRI-induced pyroptosis in the ischemic cortex *via* AMPK activation and GSK3β inhibition.

3.3. Hispidulin inhibits IR-induced pyroptosis by modulating AMPK/GSK3β signaling

To further examine the mechanisms underlying the neuroprotective role of hispidulin, an *in vitro* OGD/R model simulating IRI was established. As shown in Fig. 3A, OGD/R decreased viability of the astrocytes to 50%, which was partially restored to 60% and 70% by 5 μM and 10 μM hispidulin respectively. In addition, LDH release increased markedly following OGD/R due to leakage from dying cells, which was significantly attenuated by hispidulin (Fig. 3B). The OGD/R-induced cell death involved pyroptosis, as indicated by elevated levels of NLRP3, ASC, caspase-1, IL-18 and IL-1β (Fig. 3C), which were significantly attenuated by hispidulin (Fig. 3D, E) similar to that seen with the *in vivo* model of IRI. In addition, OGD/R also led to AMPK inhibition and GSK3β activation, and hispidulin treatment reversed these changes.

To validate our hypothesis that hispidulin suppressed pyroptosis *via* the AMPK/GSK3β signaling pathway, we silenced AMPKα expression in the primary astrocytes (Fig. 4A). AMPKα knockdown not only negated the effects of hispidulin on AMPK and GSK3β levels (Fig. 4B), but also abrogated its ability to rescue neuronal cells from OGD/R-induced cell death (Fig. 4C) and LDH release (Fig. 4D). Furthermore, hispidulin also failed to restore the levels of pyroptosis markers in the absence of

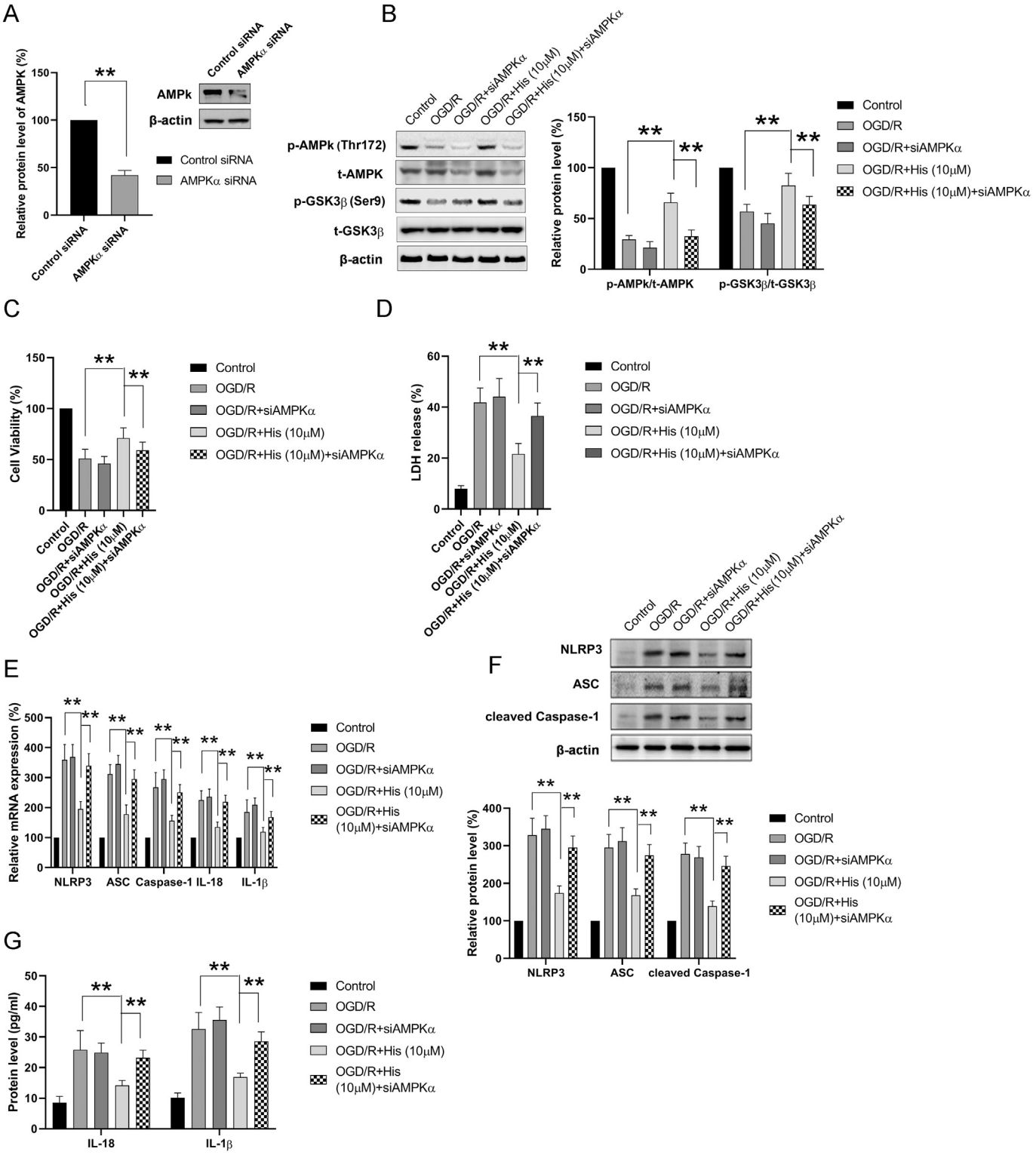

Fig. 4. AMPKα knockdown abolished the neuroprotective effects of hispidulin against OGD/R insult. A. AMPKα protein levels were reduced following gene silencing. AMPKα knockdown reversed hispidulin-induced (B) activation of AMPK and inhibition of GSK3β, (C) neuronal survival post-OGD/R, (D) reduction in LDH release, (E) downregulation of pyroptosis markers, (F) decrease in NLRP3, ASC and cleaved caspase-1 protein levels, and (G) IL-18 and IL-1β levels. **p < 0.01.

AMPK (Figs. 4E–G). We also inactivated AMPK pharmacologically using the specific inhibitor compound C, which abolished hispidulin-mediated activation of AMPK without affecting the total AMPK levels, as well as inhibition of GSK3β signaling (Fig. 5A). Furthermore, compound C also abrogated the pro-survival (Figs. 5B, C) and anti-pyroptotic (Figs. 5D–F) effects of hispidulin on neuronal cells following OGD/R. Taken together, hispidulin protected neuronal cells against OGD/R-induced injury by suppressing pyroptosis *via* the AMPK/GSK3β signaling pathway.

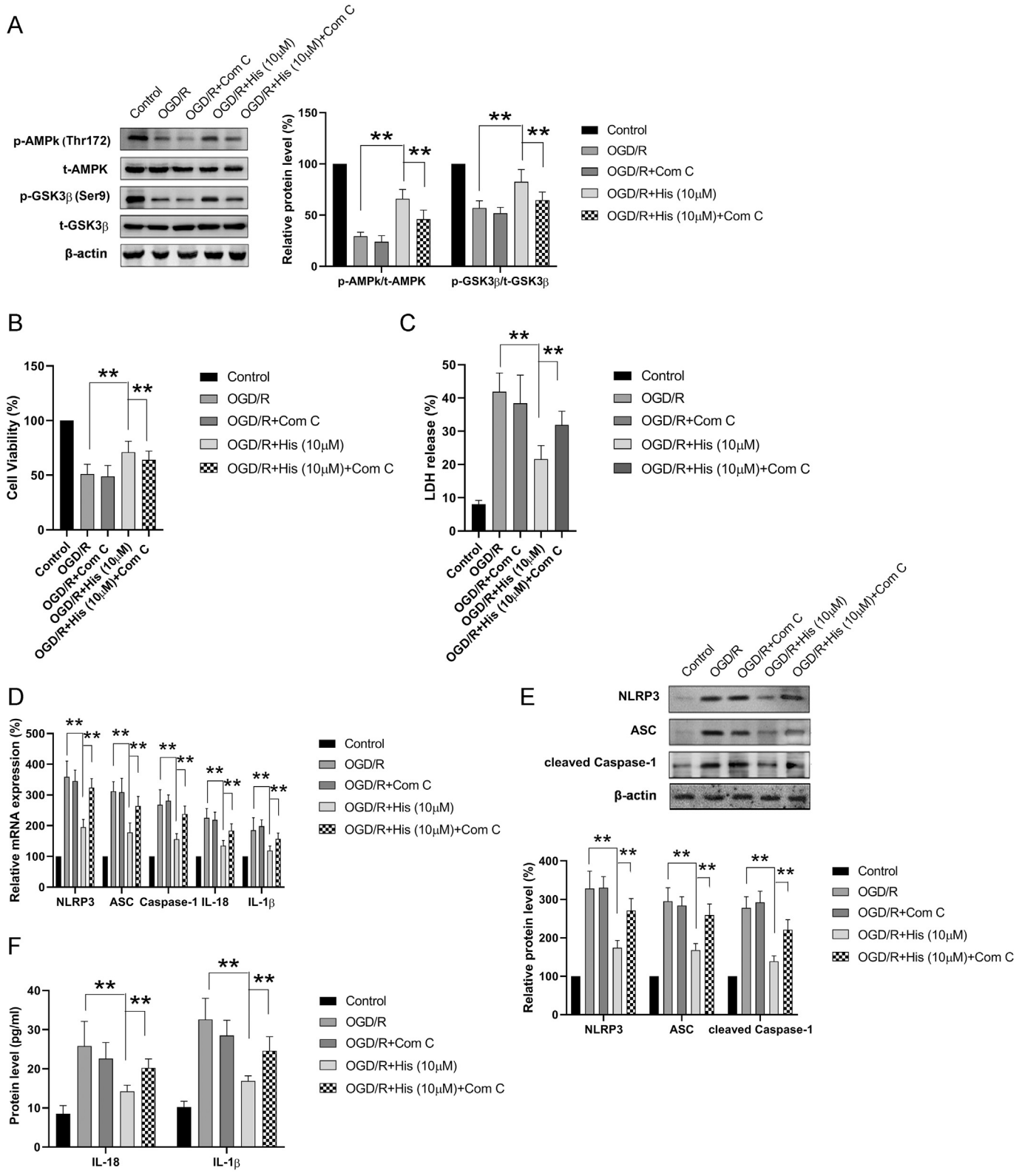

Fig. 5. Pharmacological inhibition of AMPK abrogated the neuroprotective effect of hispidulin against OGD/R insult. Compound C reversed hispidulin-mediated (A) AMPK activation and GSK3β inhibition, (B) neuronal survival post-OGD/R, (C) reduction in LDH release, (D) downregulation of pyroptosis markers, (E) decrease in NLRP3, ASC and cleaved caspase-1 protein levels, and (F) IL-18 and IL-1β levels. **p < 0.01.

4. Discussion

In addition to apoptosis and necrosis [22], cells also undergo pyroptosis [23], an inflammatory cell death mediated by inflammasome and caspase-1 activation. This cascade converts pro-IL-1β and pro-IL-18 into their active inflammatory forms [24]. Although pyroptosis was first associated with infectious diseases, it has since been shown to be involved in alcoholic hepatitis, benign prostatic hyperplasia and human

malignancies [25–27]. Yang et al. reported that renal tubule pyroptosis contributed to renal IR injury [28], and it is also the pathological basis of myocardial IR injury in diabetic rats [29]. Therefore, targeting pyroptosis is a viable therapeutic strategy to improve the clinical outcomes in patients with ischemic stroke [30]. Recent studies showed that both valproic acid and pioglitazone attenuated cerebral IR injury by suppressing pyroptosis [31,32]. In the present study also, the natural compound hispidulin improved IR-induced neurological impairment in a rat model by suppressing pyroptosis.

Inflammasomes play a key role in pyroptosis, which is initiated with caspase-1 activation [33]. They are protein complexes consisting of the cytoplasmic receptors or PRRs including NLRs (NLRP1, NLRP3 and NLRC4) and absent in melanoma 2 (AIM2), the caspase-1 precursor (pro-caspase-1) effector, and the apoptosis-associated speck-like protein containing a CARD (ASC), which acts as the adaptor protein connecting the PRRs and pro-caspase-1 [33]. IR-induced neuronal cell death and neurological impairment are strongly correlated with NLRP3 activation [34], and targeting the latter can ameliorate IR injury [35]. In the present study, NLRP3 expression was significantly elevated following IRI in both the *in vivo* and *in vitro* models, underscoring the involvement of pyroptosis in IR-induced neurological injury. Hispidulin repressed NLRP3 expression and subsequent caspase-1 activation, indicating that its neuroprotective effects are mediated by suppressing NLRP3-mediated pyroptosis.

The NLRP3 inflammasome can be influenced by various upstream elements, such as AMPK [36]. Metformin, a specific activator of AMPK, inhibited the NLRP3 inflammasome and boosted M2 macrophage polarization to accelerate wound healing [36]. The protective activities of wedelolactone on experimental murine colitis have also been attributed to its activation of AMPK signaling [37]. In addition, previous studies have shown that hispidulin can activate AMPK signaling [13,14,20,38]. Studies on an animal model of depression showed that inhibition of GSK3β blocked NLRP3 activation in the central nervous system [21,39]. The effect of hispidulin on AMPK activation has been observed in hepatocellular carcinoma and gallbladder cancer [20,38,40]. Huang et al. found that hispidulin protected aged rats against sevoflurane-induced neuronal apoptosis by activating AMPK signaling [13]. In our previous study, we showed that the neuroprotective effects of hispidulin are mediated *via* AMPKα/GSK3β signaling [14]. Therefore, we postulated that hispidulin suppressed NLRP3-mediated pyroptosis by modulating this pathway. AMPKα knockdown or pharmacological inhibition abrogated the effects of hispidulin on both AMPK and GSK3β, which in turn neutralized its pro-survival and anti-pyroptotic action. A crosstalk between autophagy and NLRP3-mediated pyroptosis has also been demonstrated [41,42], and hispidulin can trigger autophagy in the brain tissue in response to neurotoxic insults [13]. Further studies are required to determine whether autophagy induction also plays a role in hispidulin-mediated pyroptosis inhibition following IRI.

In conclusion, hispidulin improved the neurological outcomes, and decreased the infarct size and brain edema in a cerebral IRI model, by suppressing NLRP3-mediated pyroptosis *via* AMPK/GSK3β signaling.

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Declaration of conflict of interest

None.

Authors' contributions

Ming Tang designed the study and provided the funds. Ling Li designed the study. Pengpeng An, Jing Xie, Sha Qiu and Yongji Liu performed the experiments. Jianing Wang and Xiaohui Xiu analyzed the data. Pengpeng An and Jing Xie wrote the paper.

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