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ORIGINAL ARTICLE

Gastrodin ameliorates microvascular reperfusion injury–induced pyroptosis by regulating the NLRP3/caspase-1 pathway

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Abstract

Inflammation is a pivotal feature of myocardial reperfusion–induced microvascular injury and dysfunction. However, the molecular mechanisms by which myocardial reperfusion triggered inflammation remain incurable. The NLRP3 inflammasome is a key intracellular sensor that detection of cellular stress to activation of caspase-1, and consequent IL-1β maturation and pyroptotic cell death. Here, we showed that NLRP3 inflammasome played a key role in myocardial reperfusion–induced microvascular injury. We observed NLRP3 inflammasome activation and pyroptosis in both cardiac microvascular endothelial cells and myocardial I/R animal model. Gastrodin, an effective monomeric component extracted from the herb Gastrodia elata BIume, blocked cardiac microvascular endothelial cell pyroptosis via inhibiting NLRP3/caspase-1 pathway. Gastrodin also reduced interleukin-1β (IL-1β) production in vivo and in vitro. Furthermore, gastrodin treatment attenuated infarct size and inflammatory cells infiltration and increased capillary formation. Gastrodin is thus a potential therapeutic for NLRP3-associated inflammatory disease.

Keywords Ischemia/reperfusion · NLRP3 inflammasome · Pyroptosis · Gastrodin

Introduction

Acute myocardial infarction (AMI) is one of the most important causes of morbidity and mortality in cardiovascular disease [9, 25]. Following an AMI, the most effective treatment for salvaging viable myocardium, and reducing myocardial infarct size, is timely restoring of the infarct-related artery (IRA), which has contributed to an important decrease in mortality by limiting myocardial necrosis. Yet, despite successful revascularization of the infarct-related artery, perfusion of the ischemic myocardium is not or is incompletely restored due to myocardial "no-reflow" phenomenon [15], which is severe microvascular dysfunction or loss of integrity leading to microvascular obstruction (MVO). The pathophysiology of MVO is complex, whereas inflammatory responses during microvascular injury have been recognized as a key hallmark. However, the molecular mechanisms involved in inflammatory reaction still remain obscure. Hence, there is need to fully understand the mechanisms of inflammatory reaction and seek for novel therapeutic strategies.

NLRP3 (NOD-like receptor protein 3), a multimeric protein complex, engages capase-1, requiring the adapter protein apoptosis–associated speck-like protein containing a CARD (ASC) [2, 4]. Furthermore, the activation of caspase-1 can regulate the cytokines such as IL-1β and IL-18 production and secretion by cleavage of pro-IL-1β and pro-IL-18 [20, 34]. Activation of inflammasome-associated inflammatory caspases drives cleavage of the pro-pyroptotic factor gasdermin D, generating an N-terminal fragment that

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oligomerizes to form pores on the cell membrane and cause cell death known as pyroptosis [3, 24, 31]. NLRP3 inflammasome can sense some endogenous "danger signals" triggering sterile a wide variety of inflammatory diseases, including cardiovascular, renal, neurodegenerative diseases and type 2 diabetes [1, 13, 14, 30, 31]. But the medications targeting NLRP3 inflammasome are not available in clinic. Therefore, NLRP3 inflammasome has been regarded as a potential drug target for treatment of inflammatory diseases.

Gastrodian (GAS), a traditional Chinese medicine monomeric component, has been traditionally used for the treatment of cardiovascular and cerebrovascular diseases for centuries [11, 22]. Previous studies have demonstrated that GAS has possessed multiple pharmacological properties including anti-oxidant, anti-inflammatory, and hypoxiatolerance [21, 26, 33]. However, the molecular mechanisms and the target of GAS to the protection microvascular injury induced by myocardial reperfusion injury are still unknown. We hypothesize that GAS may protect microvascular injury against I/R injury through inhibition of NLRP3 inflammasome activity and cardiac microvascular endothelial cell pyroptosis.

Materials and methods

Mouse ischemia-reperfusion injury model

Adult male C57BL/6J mice (18–22 g) used in the studies were obtained from Model Animal Research Center of Kunming Medical University (Kunming, China). The C57 mice were anesthetized with 1% pentobarbital. After anesthesia, the hearts were then exposed between the fourth and fifth ribs and the left anterior descending (LAD) coronary artery was ligated 6–0 silk suture. The slipknot was released for 1 h or 6 h after occlusion for 45 min. In Sham-operated animals, a silk suture was passed under LAD without ligation.

Drug administration protocol

GAS was dissolved in saline and intraperitoneally administrated (once a day, 100 mg/kg) for 3 days before ligation of the left anterior descending (LAD); 15 min before reperfusion, an additional dose of 100 mg/kg gastrodin was immediately injected into the peritoneal in a randomized. MCC950 (Selleck, CA, USA), a potent, selective, small-molecule inhibitor of NLRP3, was dissolved in sterile saline; intraperitoneal injection (15 mg/kg) was repeated daily on day 1 to day 3 before ligation of the left anterior descending (LAD); 15 min before reperfusion, an additional dose of 15 mg/kg MCC950 was immediately administrated in the peritoneal in a randomized. Thereafter, mice were randomly divided into the following groups: Sham group, I/R group, I/R+GAS group, MCC950+I/R group.

Assessment of myocardial infarct size

The region of ischemia and the infarct size was measured by double staining with TTC and Evan's blue dye. At the end of the 6-h reperfusion period, the slipknot of the LAD was released and 2% Evan's blue dye was injected into left ventricular cavity. The heart was quickly excised and left ventricles were sliced transversely into approximately 1.5–2.0 mm in thickness and incubated for 45 min in a 2% solution of buffered TTC at 37 °C. Slices were photographed with a digital camera in order to identify normal (stained using Evan's blue dye), infarcted (unstained by TTC and Evan's blue dye), and the non-ischemic (stained brick red by TTC) myocardium. The area at risk (AAR) portion of the LV was stained red and white, whereas the infarct size (IS) was stained white and normal myocardium stained dark blue. The heart slices were photographed digitally. After this procedure, the images were analyzed using Image J; the IS and AAR were calculated as a percentage of the LV.

Immunohistochemistry

Briefly, immediately after heart excised, infarcts, adjacent to infarcts, and remote region tissues with a distance of 0.5 cm from the margin of the heart were fixed in 10% buffered formalin. Afterwards, the specimens were dehydrated with a graded ethanol series and paraffin-embedded. The sections were subsequently incubated with 0.3% hydrogen peroxide in PBS to block endogenous peroxidase activity and treated with 10 mM citrate buffer (pH 6.0) to retrieve the antigen, followed by rinsing in phosphate-buffered saline (PBS). Subsequently, the sections were blocked with 5% goat serum in PBS for 2 h and incubated overnight at 4 °C with the following primary antibodies: rabbit polyclonal anti-F4/80 antibody (1:100, Cell Signaling Biotechnology). Immunoreactivities were performed with the labeled streptavidin-biotin peroxidase and visualized using diaminobenzidine tetrahydrochloride (DAB) staining. Captured images were further analyzed by software Image J.

Cells culture and hypoxia/reoxygenation

Human cardiac microvascular endothelial cells line (HCMECs) were obtained from the Bei Na Chuanglian Biotechnology (BNCC, Wuhan, China). The passage number we used was the second or third. Cells were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM: Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (BI: Israel, Middle East) and 100 IU/ml penicillin and 100 IU/ml streptomycin (Hyclone, Logan, UT, USA). The cells were cultured in a humidified environment in consisting of 95% air and 5% CO_2 at 37 °C. Briefly, oxygen-serum deprivation injury occurred by placing cells in a hypoxic atmosphere (1% O_2, 5% CO_2, 94% N_2) in the absence of serum

medium for 2 h. After 2 h, the medium was exchanged for oxygenated and was in the presence of serum medium, and the culture was incubated at 37 °C for 2 h.

Western blot analysis

Expression of NLRP3 inflammasome was analyzed by western blotting. Cells were washed with ice-cold PBS and then lysed in RIPA buffer and fresh 0.1 mM phenylmethyl sulfonyl fluoride (PMSF). The cell lysates were prepared by scraping and centrifugation. Cellular protein concentrations were determined by the BSA protein assay (Thermo Fisher, USA). Cell lysates were subjected to SDS-PAGE under reducing conditions, and the protein bands then transferred to a PVDF membrane. The membrane was blocked for 2 h at normal temperature with 5% skim milk in Tris-buffered saline (TBS) and then incubated overnight at 4 °C with the primary antibodies as follows: the primary antibodies against NLRP3 (1:1000, Abclonal), caspase-1 (1:1000, AdipoGen), IL-1β (1:1000, Santa Cruz biotechnology), and β-actin (1:2000, Santa Cruz biotechnology) were used. After washing, membranes were incubated with a horseradish peroxidase (HRP)-conjugated IgG secondary antibody in accordance with the origin of the primary antibody for 2 h at room temperature. Antibody-reactive bands were visualized by an enhanced chemiluminescence (ECL) system (Amersham Imager 600). The expression levels of β-actin served as an internal control for protein loading.

Immunofluorescence

For cytofluorescense staining, cells grown on glass coverslips were fixed in 4% paraformaldehyde for 20 min and permeabilized in 0.1% Triton X-100 for 15 min. Cells on coverslips were incubated with normal goat serum for 2 h. After extensive washing in PBS, cells were incubated with primary rabbit anti-NLRP3 (1:300, Abclonal) and mouse anti-caspase-1 (1:200, AdipoGen) antibody overnight at 4 °C. After three washes with PBS, cells were incubated with secondary FITC-conjugated anti-ribbit IgG or FITC anti-rabbit IgG antibody for an additional 2 h at room temperature. Stained cells were photographed under a fluorescence microscope (Olympus, Tokyo, Japan) with the B-2A (EX: 450-490, DM: 505, BA: 520) and G-2A (EX: 510-560, DM: 575, BA: 590) filters. The fluorescence of cells was quantified using ImageJ software (version 1.37; National Institutes of Health, Bethesda, MD, USA).

Caspase-1 activity and myocardial enzymatic assay

CMECs were stained for 1 h using caspase-1assay (Ac-YVAD-*p*NA) kit according to the manufacturer's instructions. Active caspase-1 was measured at 405 nm by a microplate reader following the manufacturer's instructions. The serum levels of creatine kinase (CK), creatine kinase isoenzyme myocardial band (CK-MB), lactate dehydrogenase (LDH), and troponin I (TnI) were measured using automatic biochemical analyzer (cobas c 311, Mannheim, Germany).

TUNEL assay

Myocardial apoptosis was detected using a commercial kit (Beyotime, China), according to the Manufacturers' instructions. Nucleus were stained with DAPI. Fluorescent pictures were photographed under a fluorescence microscope (Olympus, Tokyo, Japan).

Statistical analysis

Data are reported as mean ± SEM and were analyzed using GraphPad Prism 5. Differences between groups were assessed by *t* tests (one measured variable) or by a two-way ANOVA with Bonferroni post hoc testing. $P < 0.05$ was considered statistically significant.

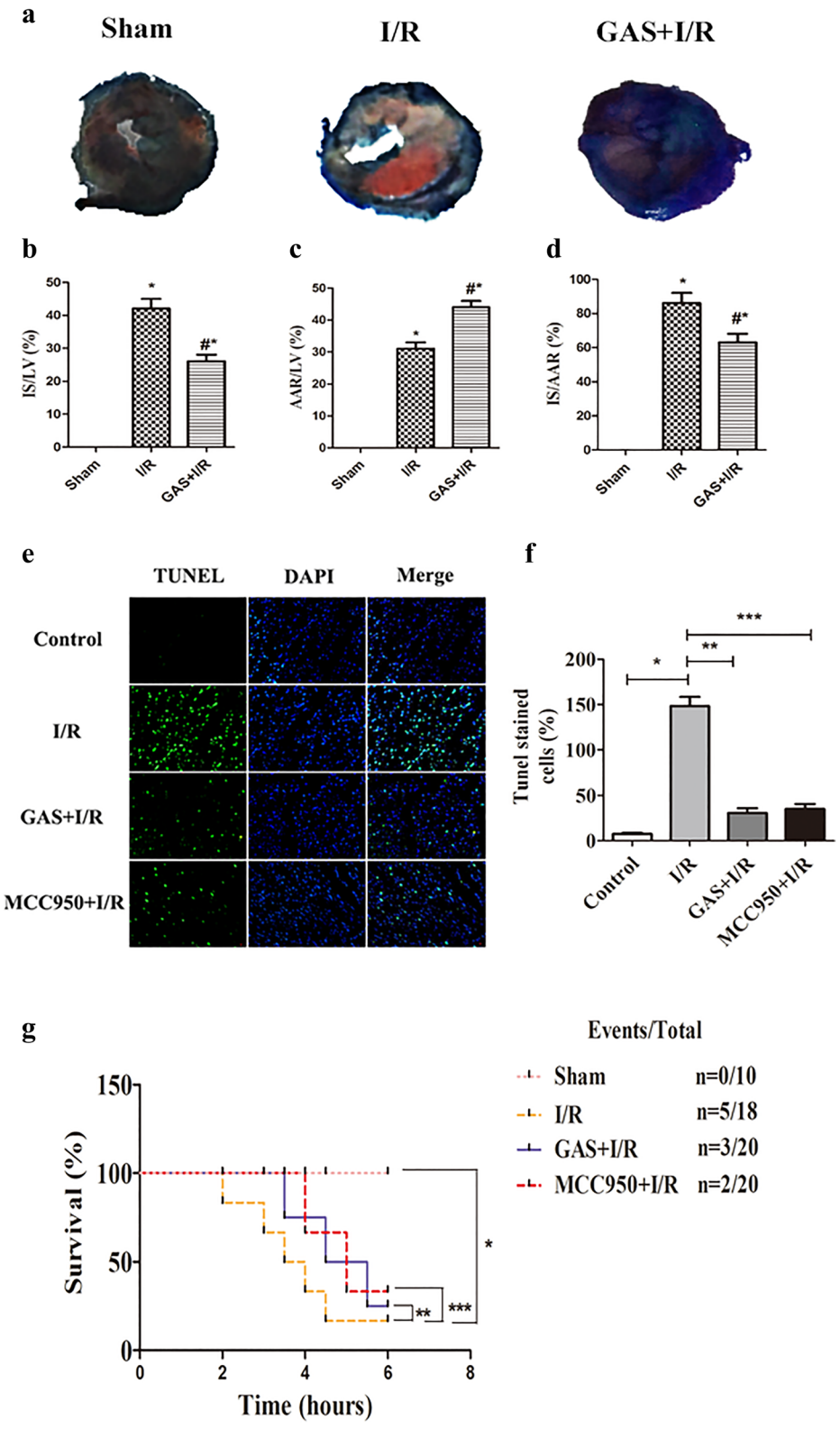
Results

Assessment of animals survival

A total of 68 mice were used in the survival study. All of the animals survived in the Sham group. Mortality within 6 h following reperfusion after 45 min of ischemia was 14.7% (10 mouse out of 68 died). However, the mortality of administration gastrodin and MCC950 groups was respectively15% (3 dead out of 20), 10% (2 dead out of 20) compared with mortality in model group of 27% (5 dead out of 18) (Fig. 1g, $P < 0.05$).

Gastrodin reduced the infarct size and cardiomyocyte apoptosis after myocardial I/R injury

Myocardial infarct size was assessed at 6 h after reperfusion. Figure 1a shows the effect of GAS pretreatment on the infarct size. The IS as percentage of the LV (IS/LV) of the I/R group was higher compared with that of the GAS+I/R group (Fig. 1b, $P < 0.05$). The AAR as the percentage of the LV (AAR/LV) of the I/R group was lower compared with that of the GAS+I/R group (Fig. 1c, $P < 0.05$). Compared to the Sham group, the ratio of infarct size to ARR in I/R markedly increased (Fig. 1d, $P < 0.05$). However, GAS pretreatment significantly decreased the ratio of infarct size to ARR compared to that of the untreated I/R group (Fig. 1d, $P < 0.05$). Our results also indicated that cardiomyocyte apoptosis was increased in the I/R group compared with the Sham group; GAS and MCC950 decreased the percentage of apoptotic cardiomyocytes during the I/R (Fig. 1e, f).


a
Sham
I/R
GAS+I/R
b
IS/LV (%)
c
AAR/LV (%)
d
IS/AAR (%)
Sham
I/R
GAS+I/R
e
TUNEL
DAPI
Merge
Control
I/R
GAS+I/R
MCC950+I/R
f
Tunel stained cells (%)
Control
I/R
GAS+I/R
MCC950+I/R
g
Survival (%)
Time (hours)
Events/Total
Sham n=0/10
I/R n=5/18
GAS+I/R n=3/20
MCC950+I/R n=2/20

◀ **Fig. 1** Effect of gastrodin on myocardial infarct size. **a** Representative TTC-Evan's blue stained sections of hearts from each group. Brick red stained area represents the area at risk (AAR), whereas the white area shows the infarcted size (IS). **b–d** Ratio of IS/LV, AAR/LV, and IS/AAR. Data are expressed as mean ± SEM ($n = 5$ for each). *$P < 0.01$ vs. Sham group, #*$P < 0.05$ vs. I/R group. **e** TUNEL staining in I/R injury tissue. Green fluorescence represents TUNEL-positive apoptotic nucleus; blue fluorescence represents total cardiomyocytes nucleus. **f** Apoptosis positive cells of each group. Data are expressed as mean ± SEM ($n = 3$ for each). *$P < 0.05$ vs. control group, **$P < 0.05$ vs. GAS+I/R group, ***$P < 0.05$ vs. MCC950+I/R group. **g** Survival analysis for mice subjected to ischemia and following 6 h perfusion (*$P < 0.05$ vs. Sham group, **$P < 0.05$ vs. GAS+I/R group, ***$P < 0.05$ vs. MCC950+I/R group, Kaplan-Meier survival analysis)

Gastrodin regulates inflammatory cell infiltration and capillary formation after myocardial I/R injury

Inflammatory cell infiltration is key contributor for microvascular injury (Fig. 2). To explore the underlying protective effects of GAS pretreatment on microvascular injury during myocardial I/R, myocardial inflammatory cell infiltration was examined by immunohistochemistry. We then compared the infiltration of macrophages and neutrophils in the Sham and I/R groups (Figs. 3a and 4a). The numbers of infiltrated macrophages and neutrophils were increased in the ischemic area of myocardium in the I/R group compared with the Sham group (Figs. 3b and 4b, $P < 0.05$). Yet, early in the perfusion (at 1 h of reperfusion), GAS pretreatment increased macrophage infiltration in the ischemic and border areas in comparison to that of the untreated I/R group (Fig. 2c–f, $P < 0.01$); however, GAS or MCC950 pretreatment decreased macrophages infiltration compared to that of untreated I/R group at 6-h post-myocardial I/R injury ($P < 0.01$). GAS or MCC950 administration also attenuated infiltration of CD11b neutrophils compared to that of the untreated I/R group (Fig. 4c–f).

Capillary density was further assessed using immunofluorescence analysis of endothelial marker CD31; compared with the I/R group, GAS pretreatment contributed to capillary formation in the border area (Fig. 2d). To evaluate GAS whether could contribute to vascular endothelial cell proliferation, myocardial microvascular endothelial cells (CMECs) were cultured and hypoxia reoxygenation model was established. Compared with hypoxia/reoxygenation (H/R) group, the expression of VEGF markedly increased in the GAS+I/R group. In vivo, consistent with in vitro, GAS treatment upregulated the expression of VEGF (Fig. 2a–c, $P < 0.05$).

I/R injury activates the NLRP3 inflammasome to induce IL-1β secretion

Previous study showed that IL-1β expression in heart induced by I/R, and inhibition of IL-1β suppressed myocardial injury post-myocardial reperfusion [16]. However, it is uncertain whether IL-1β is involved in microvascular injury after I/R. In this work, the expression of IL-1β in CMECs was analyzed at different reperfusion time points using western blot. We found that IL-1β was significantly increased at 2 h after hypoxia reoxygenation (Fig. 5a, c). Recent evidence demonstrated that a sterile inflammatory response triggered by cell and tissue damage was mediated through a multiple-protein complex called NLRP3 inflammasome. The activation of NLRP3 inflammasome leads to IL-1β production. Therefore, we hypothesize NLRP3 inflammasome that is a key factor in microvascular dysfunction after myocardial reperfusion. We evaluated the expression of NLRP3 in CMECs at different reperfusion time points; western blot analysis revelated that NLRP3 level was higher in hypoxia reoxygenation compared with the control group and peaked at 2 h after hypoxia reoxygenation (Fig. 5a, b, $P < 0.01$). Immunofluorescence analysis also showed that elevated expression level of NLRP3 in hypoxia reoxygenation compared to the control group (Fig. 5d).

We further investigated the inflammasome activation in I/R injury, C57BL/6J mice were subjected to 45 min ischemia and 1 or 6 h reperfusion, and then NLRP3, caspase-1 levels were evaluated using immunofluorescence analysis, which demonstrated that I/R injury remarkably increased NLRP3 and caspase-1 expression in the ischemic and border area compared to the Sham group (Fig. 9a, b).

Effects of MCC950 on NLRP3 inflammasome activation in response to I/R injury

The effect of MCC950 on NLRP3 inflammasome activation was tested in CMECs and mouse heart. CMECs were subject to hypoxia reoxygenation, before reoxygenation pretreated with different concentrations MCC950 (1, 10, 100 μM), especially at 1 μM, remarkably decreased the expression of NLRP3, caspase-1, and GSDMD (Fig. 6b–d). Correspondingly, the expression of caspase-1 and GSDMD was inhibited by MCC950 (Fig. 6a). A high dose of MCC950 did not block NLRP3 expression and did not consistently affect the expression of caspase-1 and GSDMD in CMECs (Fig. 6a). The inhibitory effect of MCC950 (15 mg/kg) treated animals on NLRP3 and caspase-1 activation was confirmed using immunofluorescence analysis (Fig. 10a, b); the result showed that NLRP3 and caspase-1 activation were significantly decreased in the MCC950 group in comparison to the I/R group.

Gastrodin attenuates the NLRP3 inflammasome activation in vitro and in vivo

We further examined the effect of GAS on NLRP3/caspase-1 activation and the production of mature IL-1β. To determine the optimal concentration of GAS, the expression of NLRP3 inflammasome after pretreatment with different doses of GAS was analyzed using western blotting. As shown in Fig. 7a, GAS led to decrease the expression of NLRP3 and IL-1β secretion

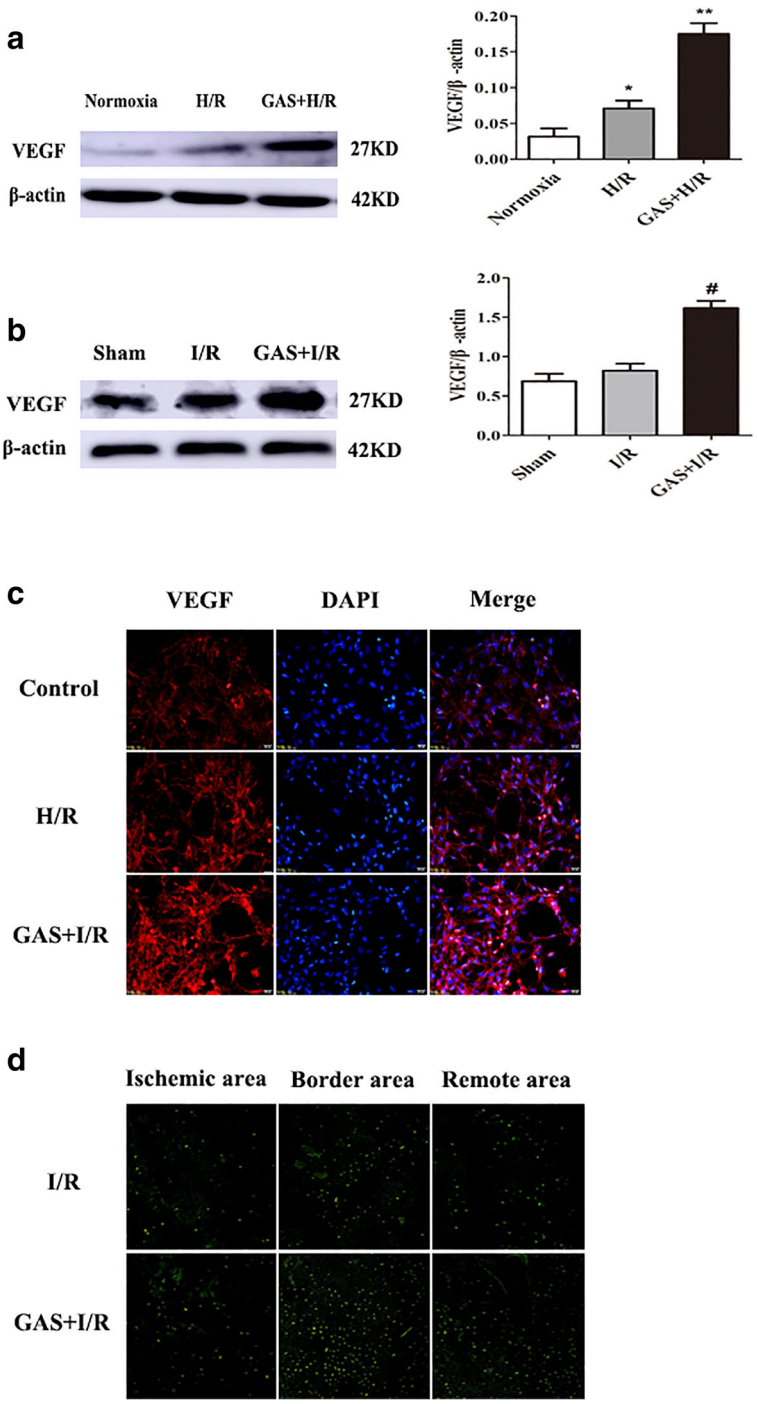


Fig. 2 Gastrodin promoted angiogenesis in myocardial I/R injury. **a** Representative western blotting bands and densitometric quantification of VEGF in vitro. **b** The expression of VEGF and in the Sham, I/R, and GAS+I/R groups at 6 h after myocardial I/R injury. **c** Immunofluorescence staining for VEGF in CMECs. **e** The sections of the heart were analyzed by immunofluorescence staining with antibody against CD31 (endothelial cells). Data are expressed as mean ± SEM ($n = 3$ for each). *$P < 0.05$ vs. control group, **$P < 0.05$ vs. H/R group. #$P < 0.05$ vs. I/R group

medicated by NLPR3/caspase-1 pathway in comparison to the hypoxia reoxygenation group (Fig. 7b, c, $P < 0.01$). Pretreatment with GAS (20, 40, 80, and 100 μM), especially at 40 μM, remarkably decreased the expression of NLRP3 inflammasome (Fig. 7b, $P < 0.01$). Yet, 40 μM GAS was used in subsequent experiments. Consistent with the western blotting results, immunofluorescence analysis also revealed that GAS pretreatment markedly decreased the expression of NLRP3 in comparison to the hypoxia reoxygenation group (Fig. 7d).

To further verify the role of the GAS in I/R injury, GAS was administered in C57BL/6J mice for 3 consecutive days (100 mg/kg) via intraperitoneal injection before ischemia; 15 mins before reperfusion, an additional dose of 100 mg/kg gastrodin was immediately injected into the peritoneal (Figs. 8 and 9). NLRP3 expression was measured in mice after 45 min ischemia and 1 or 6 h reperfusion using immunofluorescence. The results indicated that the level of NLRP3 was weakly expressed in the GAS-treated group in comparison to the

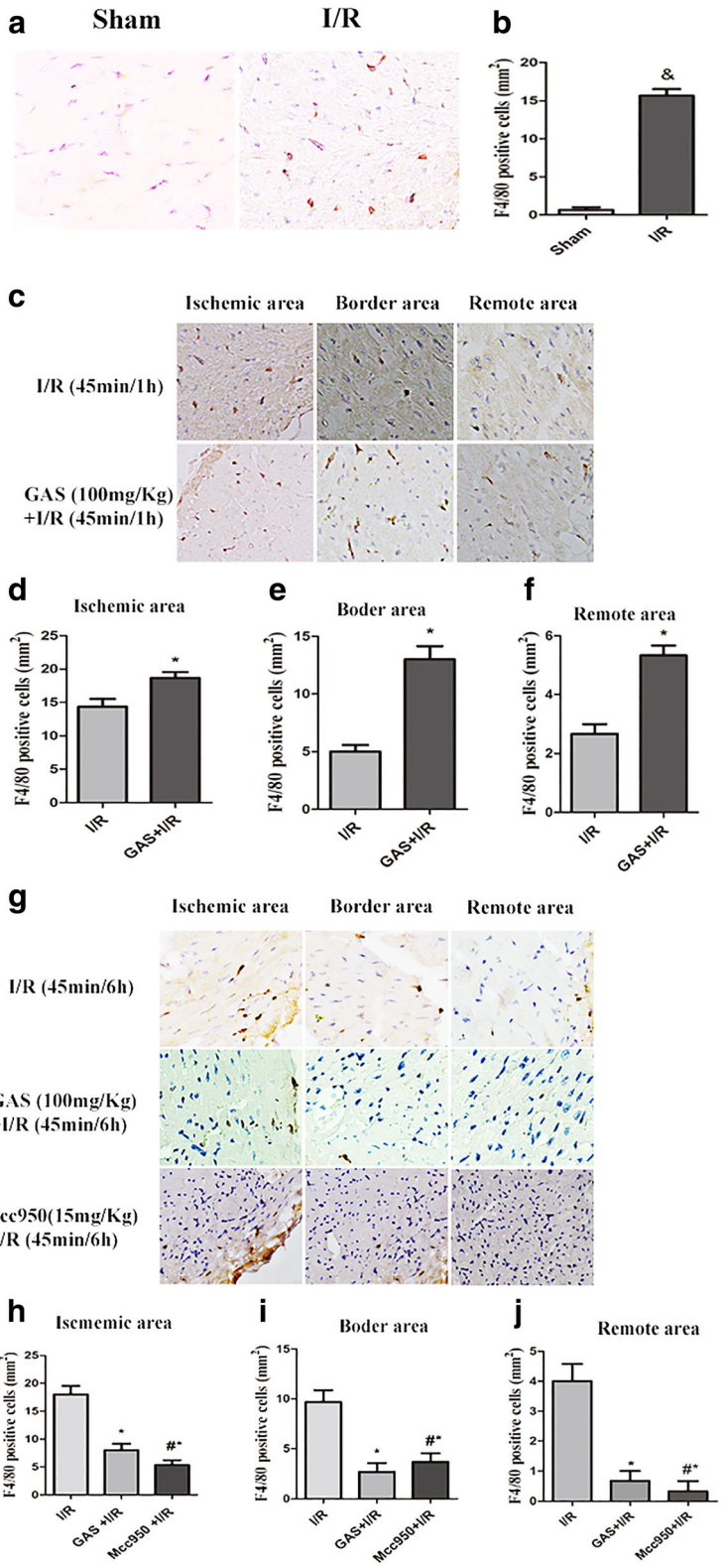


Fig. 3 Effect of gastrodin or MCC950 on macrophage infiltration was immunohistologically analyzed by staining with antibody against F4/80. **a** F4/80$^+$ cells were upregulated in heart at 1 h after myocardial I/R. **b** The number of F4/80$^+$ cells was quantified. **c** Gastrodin enhances ischemic area macrophage infiltration at 1 h after I/R injury. **d**–**f** The percentage of F4/80$^+$ cells was counted in ischemic, border, and remote area. **g** Gastrodin or MCC950 suppressed ischemic area macrophage infiltration at 6 h after I/R injury. **h**–**j** The proportion of F4/80$^+$ cells was counted in ischemic, border, and remote area. Data are expressed as mean ± SEM ($n = 5$ for each). Scar bar = 20 μm. $^{\&}P < 0.05$ vs. Sham group, $^{*}P < 0.01$ vs. GAS+I/R group, $^{\#*}P < 0.01$ vs. MCC950+I/R group

vehicle-treated group (Fig. 10a). Similar trend was observed in the expression of caspase-1 (Fig. 10b). The level of CK, CK-MB, LDH, and TnI was significantly increased in the I/R group as compared with the Sham group. Administration with gastrodin inhibited the increase in CK, CK-MB, LDH, and TnI levels (Fig. 10c–f, $P < 0.05$). Circulation markers of CK and TnI were lower in mice treated with MCC950 (Fig. 10g–h, $P < 0.05$).

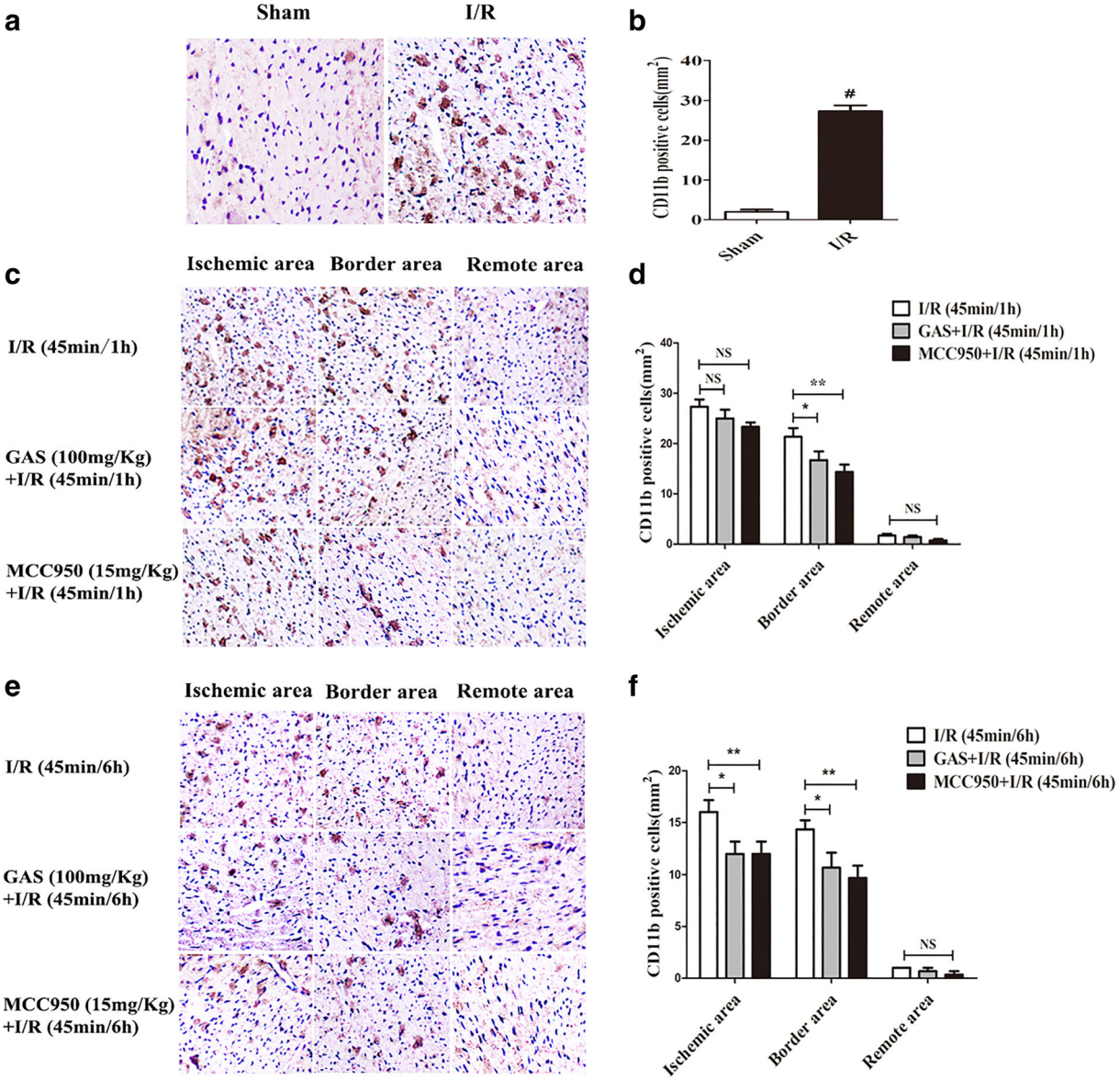


Fig. 4 Effect of gastrodin or MCC950 on neutrophil infiltration was immunohistologically analyzed by staining with antibody against CD11b. **a** CD11b cells were increased in ischemic and border areas after I/R. **b** The number of CD11b cells was quantified. **c** Heart sections were immunohistologically analyzed by staining with antibody against CD11b. **d** The percentage of CD11b cells was counted in ischemic, border, and remote area. **e** Gastrodin or MCC950 attenuated ischemic area neutrophil infiltration after I/R injury. **f** The proportion of CD11b cells was counted in ischemic, border, and remote area. Data are expressed as mean ± SEM ($n = 5$ for each). Scar bar = 20 μm. $^{\#}P < 0.01$ vs. Sham group, $^{*}P < 0.05$ vs. GAS+I/R group, $^{\#*}P < 0.05$ vs. MCC950+I/R group

Gastrodin attenuates the NLRP3/caspase-1-mediated pyroptosis in vitro and in vivo

The pore-forming protein gasdermin D (GSDMD) was recently identified as a type of proinflammatory programmed cell death induced by inflammasome called pyroptosis. We hypothesized that pyroptosis played an important role in the pathophysiology of myocardial I/R injury. We explored the role of NLRP3/caspase-1-mediated pyroptosis in CMECs and C57BL/6J mice after myocardial I/R injury. The expression of NLRP3, caspase-1, IL-1β, and GSDMD was higher in hypoxia reoxygenation group compared with the control group (Fig. 8b–e, $P < 0.05$) measured by western blotting and immunofluorescence (Fig. 8a, g, h). To verify if GSDMD has higher expression in the myocardial tissue induced by myocardial I/R injury, immunohistochemical staining indicated that, consistent with in vitro result, the number of positive cells in the I/R group was higher than that in the Sham group (Fig. 11a, b, $P < 0.05$). Administration of GAS could reverse this increase (Fig. 11c–e), implying that pyroptosis mediated by NLRP3/caspase-1 pathway may be involved in the pathophysiology of myocardial I/R.

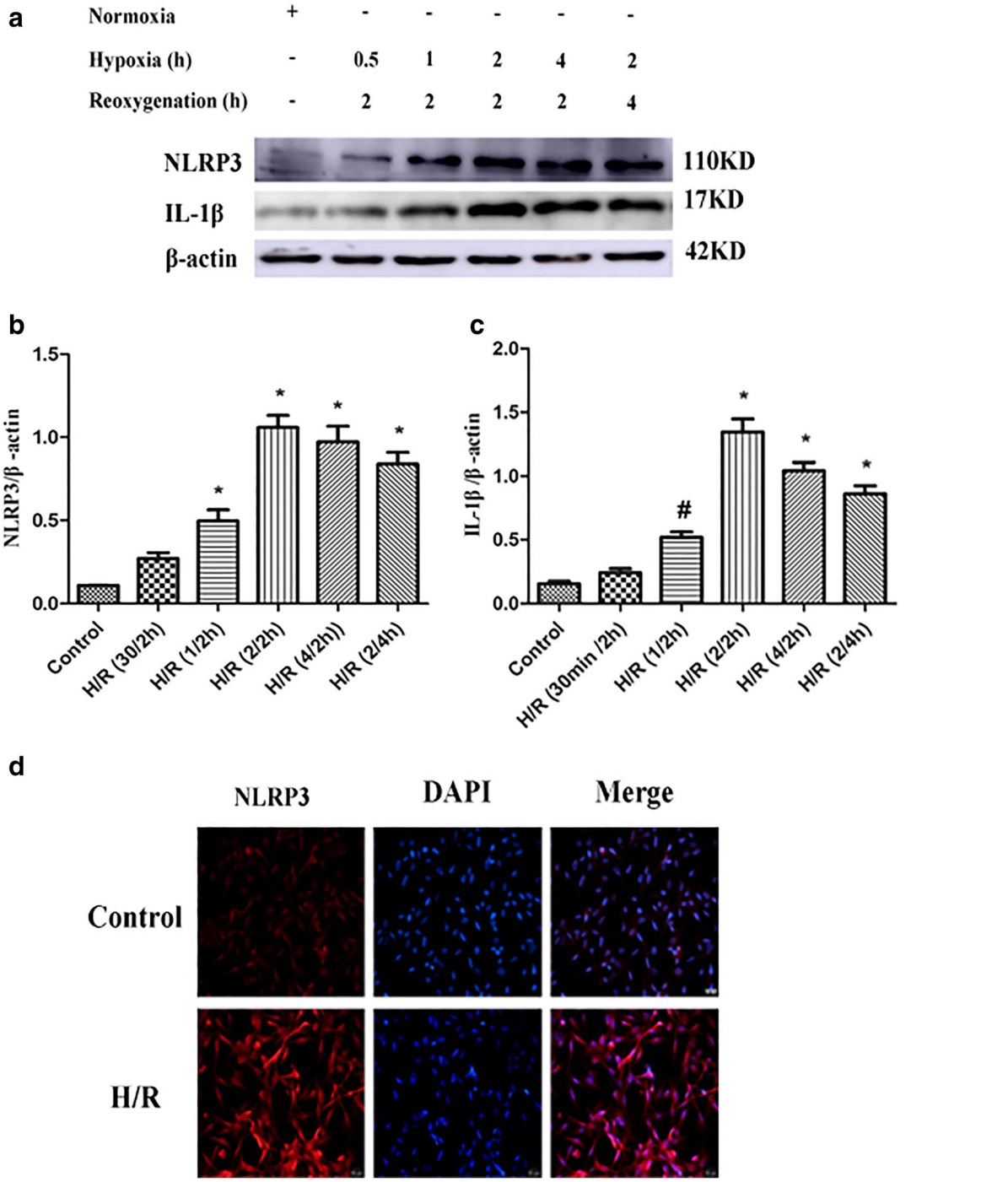


Fig. 5 H/R activates the NLRP3 inflammasome to induce IL-1β secretion in CMECs. **a**–**c** Representative western blotting bands and densitometric quantification of NLRP3 and IL-1β in the CMECs at different hypoxia/reoxygenation (H/R) times. **d** Immunofluorescence staining for NLRP3 in CMECs at 2 h of hypoxia/2 h of reoxygenation. Data are presented as the mean ± SEM. $^{\#}P < 0.05$, $^{*}P < 0.01$ vs. control group

Discussion

Here, we have characterized the role of gastrodin to pyroptosis for the first time. The point to the pyroptosis in the process of myocardial reperfusion injury will serve as a potential therapeutic target. This is supported by the following findings: IL-1β secretion mediated by NLRP3/caspase-1 was high expression in the pathophysiology of microvascular and myocardial I/R injury; pyroptosis was induced by hypoxia reoxygenation in CMECs and myocardial I/R injury mice; GAS played a key role in protecting against CMECs and myocardial tissue damage by inhibiting pyroptosis mediated by NLRP3/caspase-1

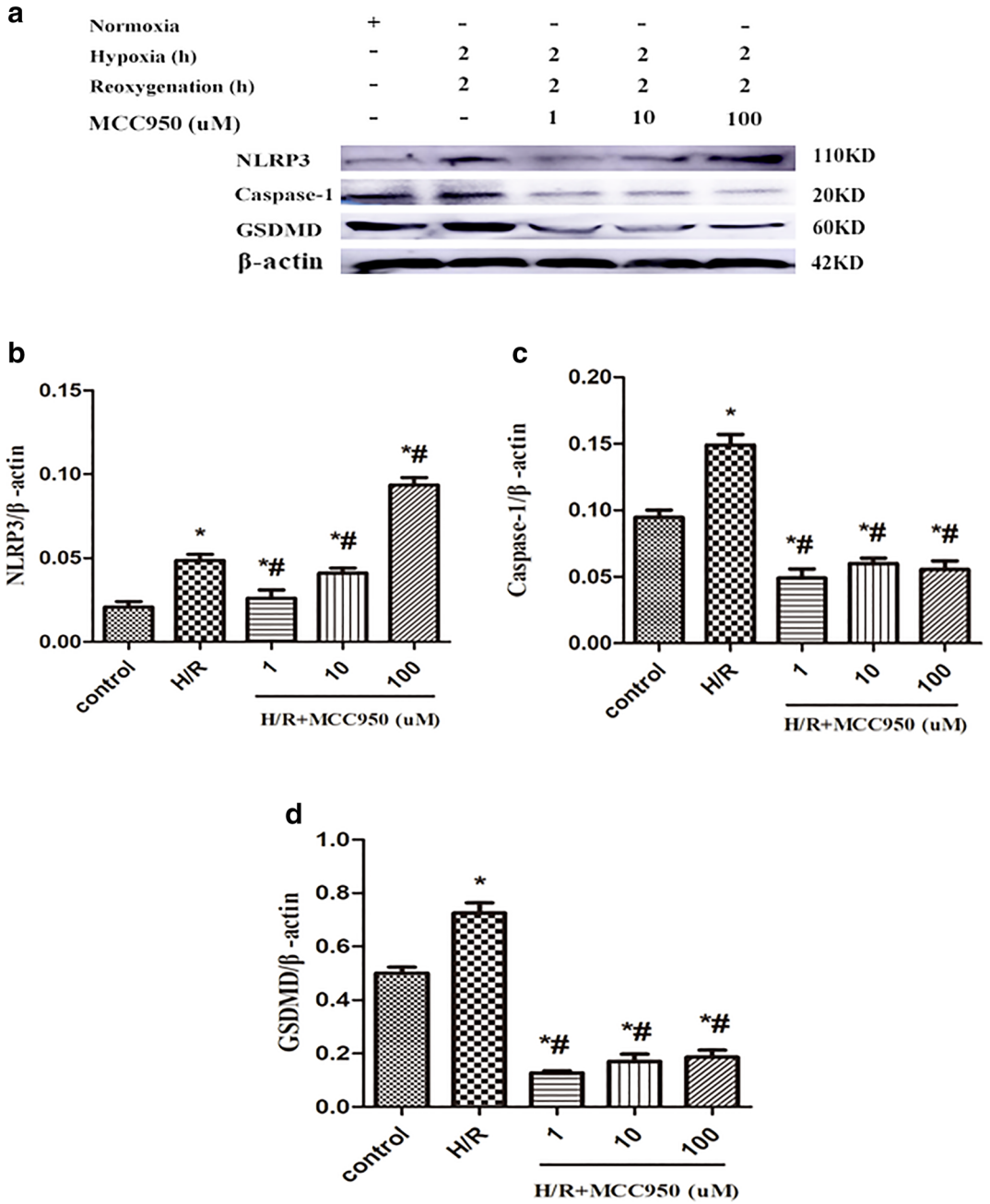


Fig. 6 Effects of different MCC950 concentrations (1, 10, 100 μM) on NLRP3 inflammasome activation in response to I/R injury. **a**–**d** Representative western blotting bands and densitometric quantification of NLRP3, caspase-1, and GSDMD in the CMECs after H/R. Data are presented as the mean ± SEM ($n = 3$ for each). $^{*}P < 0.05$ vs. control group, $^{\#*}P < 0.05$ vs. H/R group

pathway; GAS reduced inflammatory cytokine expression and subsequent injury such as infarct size development in myocardial I/R injury. These findings clarified the molecular events in the pathophysiology of microvascular and myocardial I/R injury. GAS could suppress CMECs and myocardial tissue pyroptosis by inhibiting NLRP3/caspase-1 activation and might provide a novel therapy for myocardial I/R injury.

Acute myocardial infarction is the most severe clinical manifestation of coronary artery disease, which remains a leading cause of mortality and morbidity in the world [28]. Although reperfusion therapy is successful in preventing heart damage, which is then further exacerbated by an intense and highly specific inflammatory response. However, the inflammatory response to myocardial reperfusion has proven to be more complex than previously [30, 31]. The process of inflammation plays an important role not only in removing necrotic cell debris and repairing injured tissue, but that it also contributes to further injury. Therefore, it is important to balance the inflammatory response. Increasing evidence demonstrated that IL-1β was a key mediator of inflammation in I/R

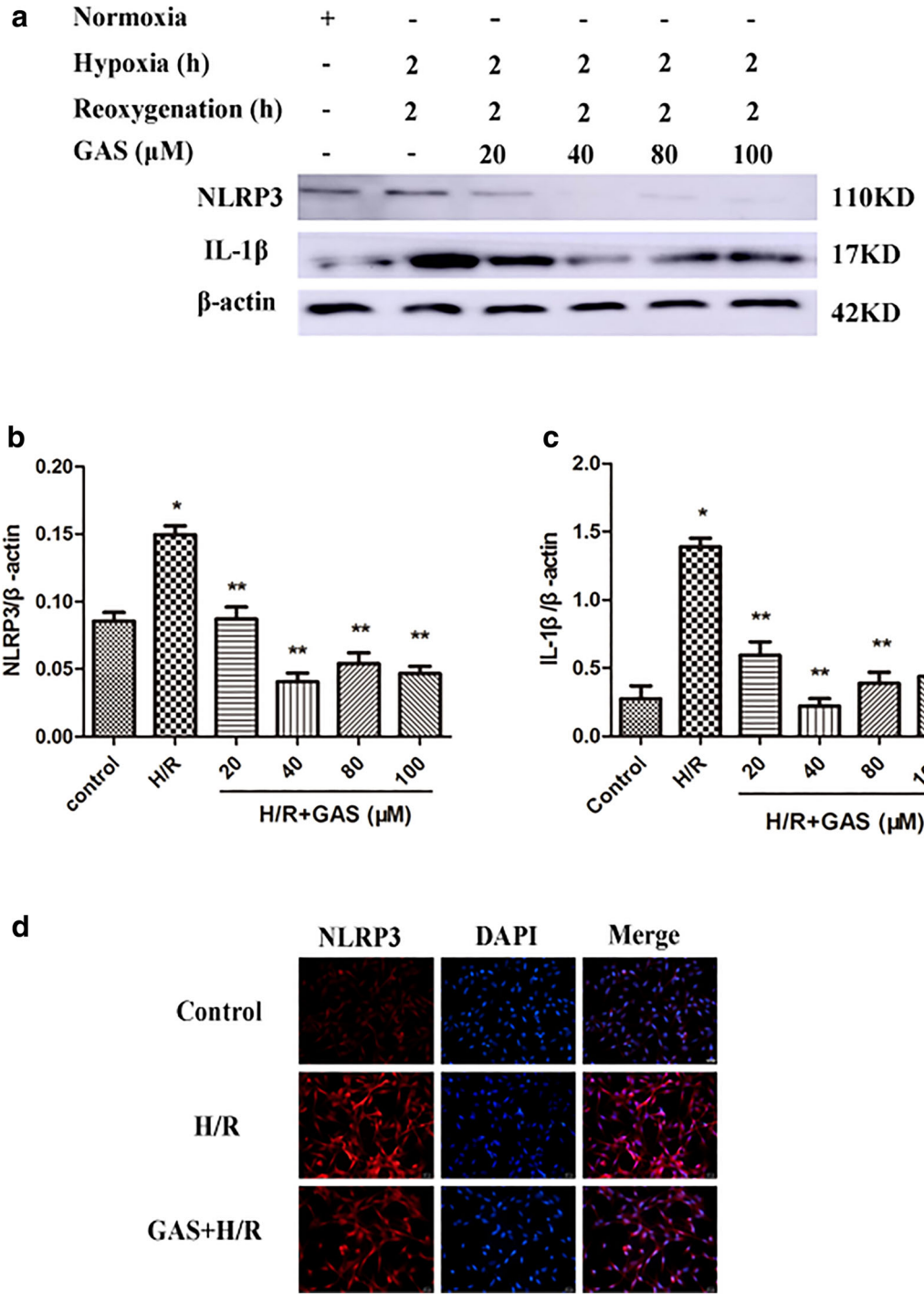


Fig. 7 Gastrodin inhibits the activation of the NLRP3 inflammasome to induce IL-1β secretion in CMECs. **a**–**c** Western blot analysis and densitometric quantification of NLRP3, IL-1β in CMECs pretreated with different concentrations of GAS (20, 40, 80, 100 μM) before 2 h of reoxygenation. **d** Immunofluorescence staining for NLRP3 in CMECs pretreated with GAS (40 μM) before 2 h of reoxygenation. Data are presented as the mean ± SEM. $^{*}P < 0.01$ vs. control group, $^{**}P < 0.01$ vs. H/R group

injury [17]. It is uncertain whether IL-1β is involved in microvascular damage; our result indicated that IL-1β was activated in CMECs induced by H/R, suggesting that IL-1β may play an important role in microvascular injury. However, recent study suggested that a sterile inflammatory response triggered by cell or tissue damage and endogenous danger signals was mediated by NLRP3 inflammasome, a novel regulator of inflammatory and cell death [27]. Hence, we hypothesized that the production of IL-1β was dependent NLRP3 inflammasome activation. Our research manifested that H/R remarkably increased NLRP3 expression in CMECs in a time-dependent manner. NLRP3 activation was also activated in I/

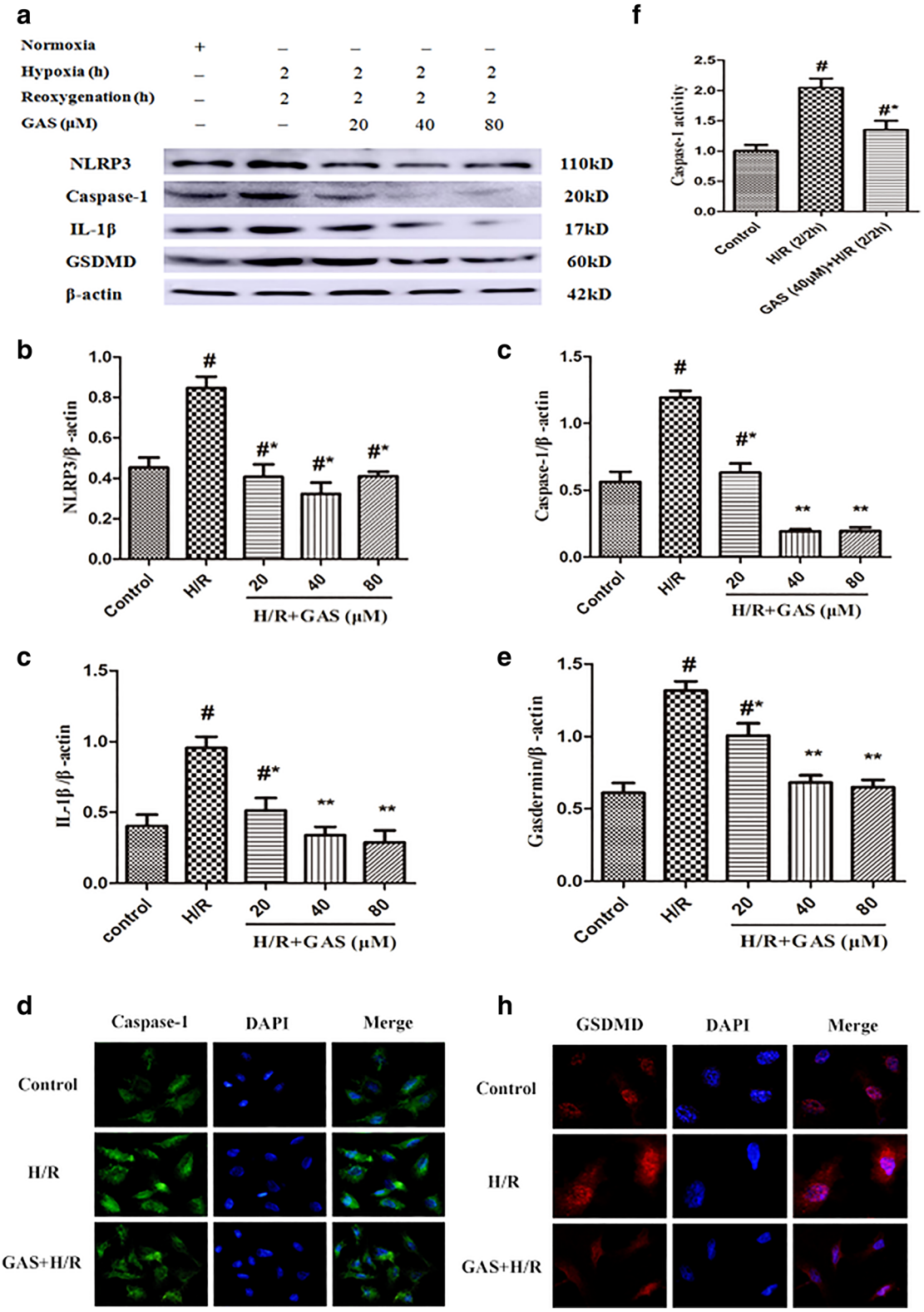


Fig. 8 Gastrodin inhibits the activation of NLRP3/caspase-1 pathway to suppress CMECs pyroptosis. **a**–**e** Western blot analysis and densitometric quantification of NLRP3, caspase-1, GSDMD, and IL-1β in CMECs pretreated with different concentrations of GAS (20, 40, 80 μM) before 2 h of reoxygenation. **f** Caspase-1 activity was determined by an enzyme activity assay kit in CMECs. **g**–**h** CMECs were analyzed by immunofluorescence staining with antibodies with caspase-1 and GSDMD. Data are expressed as mean ± SEM ($n = 3$ for each). $^{\#}P < 0.05$ vs. control group, $^{\#*}P < 0.05$ vs. H/R group, $^{**}P < 0.01$ vs. H/R group

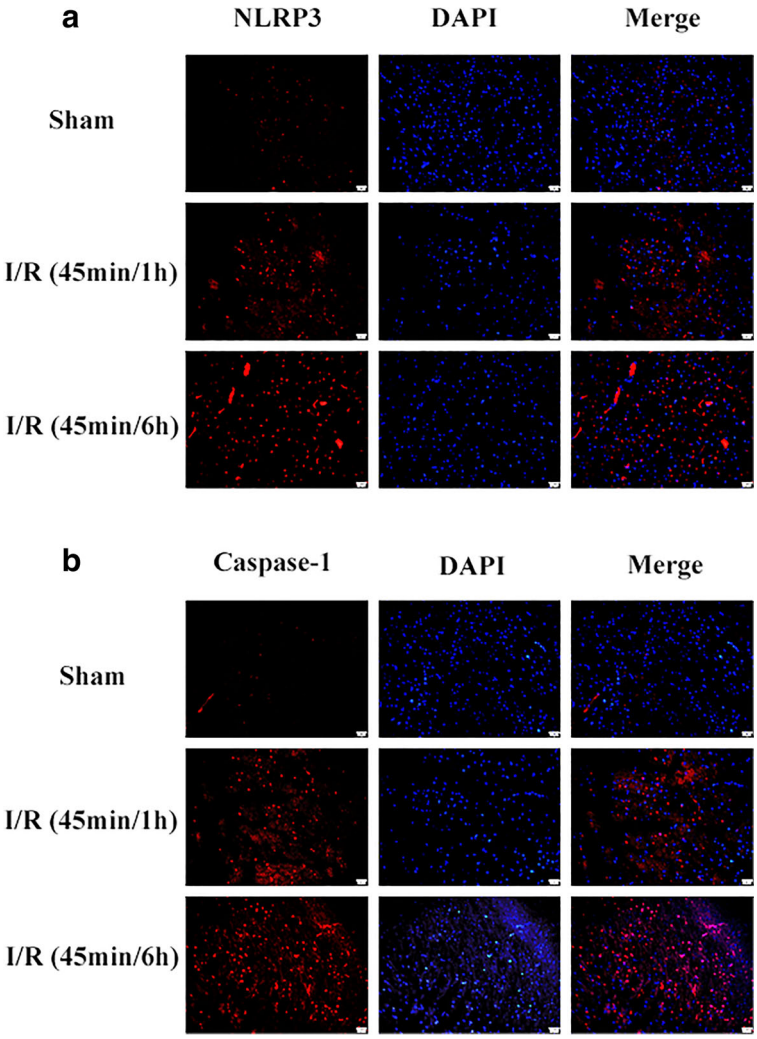


Fig. 9 NLRP3 and caspase-1 experssion in heart after I/R injury. **a**, **b** The sections of the ischemic area were analyzed by immunofluorescence staining with antibodies against NLRP3 and caspase-1 at 1 h and 6 h after myocardial I/R injury

R model, which promoted macrophage (F4/80) and neutrophil (CD11b) infiltration to ischemic area and increased infarct size. These findings manifested that NLRP3 inflammasome played a critical role in the pathophysiology of microvascular injury.

The dysregulation of inflammasomes can cause uncontrolled inflammation and is involved in many human diseases [7, 8, 23, 32]. Yet, the most widely studied inflammasome receptor in the heart is NACHT, LRR, and PYD domain-containing protein 3 (NOD-like receptor protein 3, NLRP3), which is activated in response to danger-associated molecular patterns (DAMPs) such as mitochondrial damage during myocardial reperfusion injury [29]. Activation of the NLRP3 inflammasome triggers further myocardial injury through the secretion of IL-1β and promotion of pyroptotic cell death in the wave-front of reperfusion injury. Previous studies indicated that strategies blocking the activation of the NLRP3 inflammasome in the early reperfusion period after acute myocardial infarction attenuated the overall size of the infarct [5]. In addition, detect danger-associated molecular patterns (DAMPs) to induce inflammatory innate immune responses against cardiomyocyte or endothelial cells death [30]. Hence, NLRP3 inflammasome has been regarded as a potential drug target for inhibition inflammatory response.

Pyroptosis is a form of necrotic and inflammatory programmed cell death induced by inflammatory caspases [16]. Pyroptosis requires cleavage and activation of the pore-forming effector protein gasdermin D driven by caspases [10, 24]. Recent advances indicated that pyroptosis played a regulatory role in a variety of infectious and noninfectious diseases, which was prevented by treatment with inhibitor MCC950 [6, 12], a potent, selective, small-molecule that can inhibit NLRP3 inflammasome activation both in vitro and in vivo [5]. However, little is known the role of pyroptosis in myocardial reperfusion induced microvascular dysfunction or injury. In this regard, we hypothesize that pyroptosis plays a critical role in the mechanism of myocardial I/R injury. In our study, the morphological and molecular evidence of pyroptosis was observed in both I/R heart tissue and CMECs, suggesting pyroptosis played an important role in

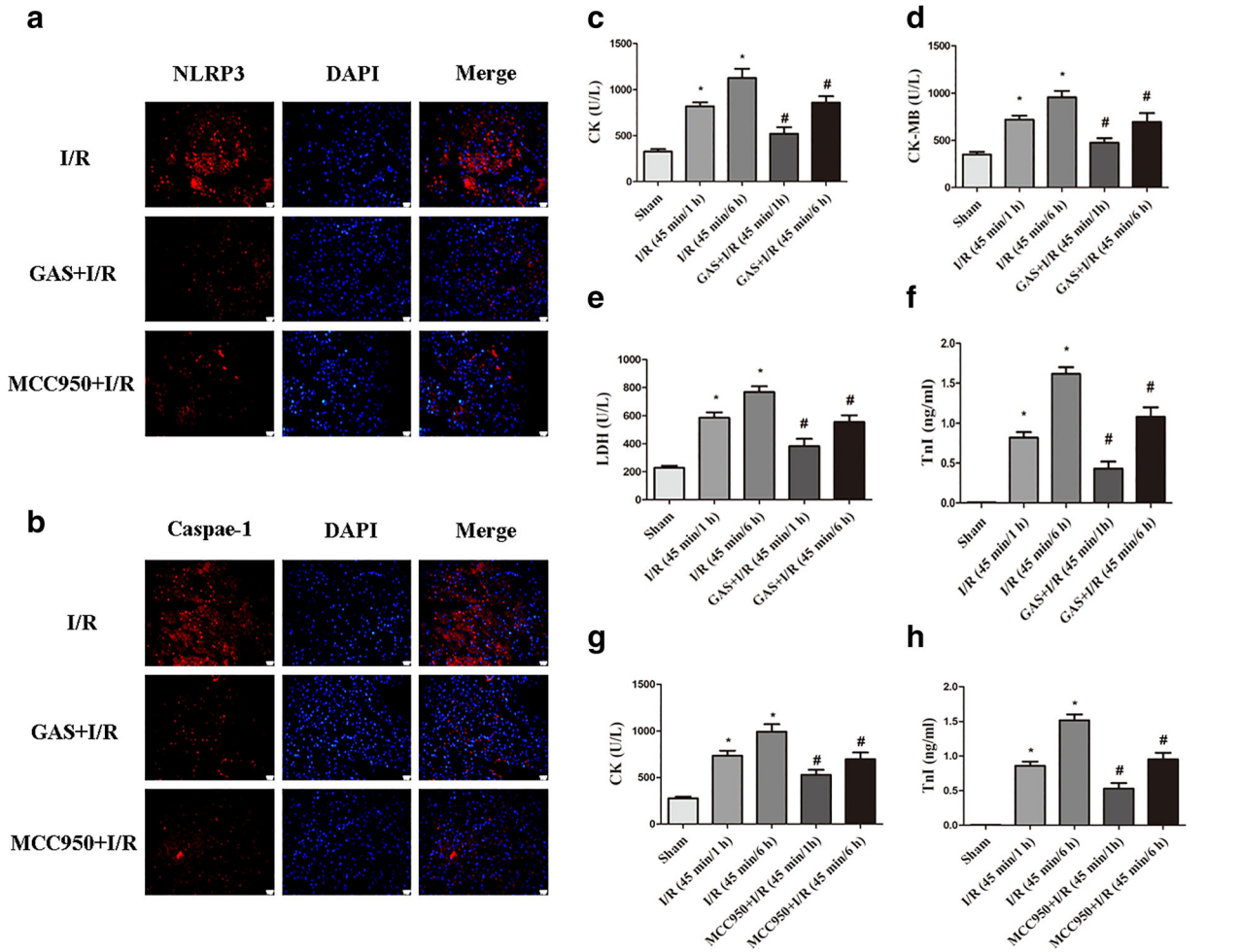


Fig. 10 Effect of gastrodin or MCC950 on immunofluorescence analysis of the markers of NLRP3, caspase-1, and myocardial enzyme activity after I/R injury. **a**, **b** Mice treated with gastrodin (100 mg/kg) or MCC950 (15 mg/kg) remarkably reduce NLRP3 and caspase-1 activation after myocardial I/R injury. CK (**c**), CK-MB (**d**), LDH (**e**), and TnI (**f**) activity in serum from Sham, I/R, and gastrodin+I/R groups. CK (**g**), TnI (**h**) activity in serum from the Sham, I/R, and MCC950+I/R groups. Data are presented as the mean ± SEM ($n = 3$ for each). $^{*}P < 0.05$ vs. Sham group, $^{\#}P < 0.05$ vs. I/R group

determining cardiac infarct size. Thus, targeting pyroptosis or upstream proinflammatory caspases therapeutically might exert cardioprotective effects.

Gastrodin is an active compound isolated from the roots of a plant used in ancient Chinese traditional medicine that has been traditionally used for the treatment cardiovascular and cerebrovascular diseases [22, 26]. Previous studies demonstrated that GAS has anti-inflammatory, anti-oxidant effect, and improved microvascular flow and circulatory functions [18, 19]. It has been reported that GAS pretreatment could ameliorate cerebral ischemic injury and arrhythmia during myocardial I/R. In our previous study, it indicated that GAS could also prevent cardiomyocytes apoptosis. However, whether GAS pretreatment can inhibit pyroptosis is unclear. Our studies showed that pretreatment with GAS blocked NLRP3-dependent pyroptotic cell death during I/R in vitro and in vivo, suggesting that GAS played a pivotal role in protecting against CMECs and heart tissue by inhibiting pyroptosis.

Recent studies indicated that pyoptosis was mediated by the NLRP3/caspase-1 pathway. Thus, we hypothesized that GAS could inhibit the NLRP3 inflammasome activation to prevent further pyroptosis. Our result implied that myocardial I/R triggered canonical inflammasome depended on the NLRP3/caspase-1 pathway for production of proinflammatory cytokine IL-1β and activated GSDMD for induction of pyroptosis. GAS inhibiting NLRP3/caspase-1 pathway ameliorated pyroptosis in experimental model of I/R, reducing myocardial infarct size and inflammatory cell infiltration.

In summary, the present study demonstrated that the NLRP3/caspase-1 pathway might play a key role in CMECs and heart tissue pyroptosis and increased infarct size during myocardial I/R injury. GAS, at least partly, reverses CMECs

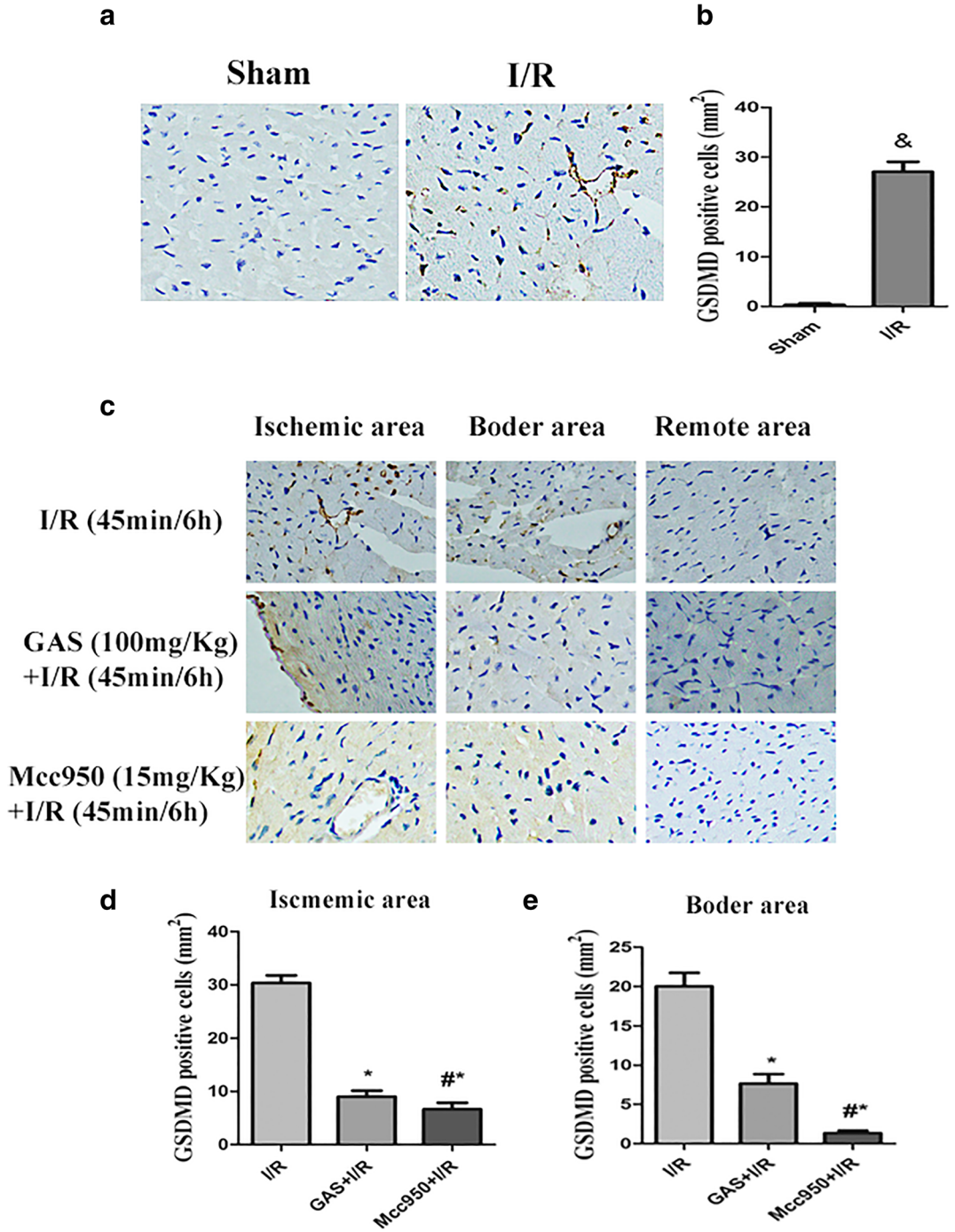


Fig. 11 Effect of gastrodin or MCC950 on the number of GSDMD positive cells in heart after myocardial I/R. **a** Pyroptosis-associated protein was upregulated in heart at 6 h after myocardial I/R injury. **b** The percentage of GSDMD$^+$ cells was counted. **c** The sections were obtained from the ischemic, border, and remote areas of WT mice at 6 h after myocardial I/R injury, mice treated with gastrodin (100 mg/kg) or MCC950 (15 mg/kg) remarkably inhibit pyroptosis after myocardial I/R injury. **d**–**e** The number of GSDMD$^+$ cells was quantified. Data are expressed as mean ± SEM ($n = 3$ for each). Scar bar = 20 μm. $^{\&}P < 0.01$ vs. $^{*}P < 0.01$ vs. GAS+I/R group, $^{\#*}P < 0.01$ vs. MCC950+I/R group

and heart tissue pyroptosis and decreases myocardial infarct size and inflammatory cell infiltration through inhibiting NLRP3/caspase-1 signaling pathway. Furthermore, the deep molecular mechanism of gastrodin modulating pyroptosis will be further explored in our later research.

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Compliance with ethical standards

All animal experimental protocols were reviewed and approved by the Ethics Committee of Kunming Medical University for the use of laboratory animals.

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

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