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Article

P65/NLRP3 Inflammasome Mediated Endothelial Cells Pyroptosis: A Novel Mechanism of In-Stent Restenosis

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Abstract

Background: In-stent restenosis (ISR) is one of the key causes of ischemic events after coronary stent implantation, and endothelial cell death and inflammation are considered to be important mechanisms. Pyroptosis is a proinflammatory type of programmed cell death, the effects and underlying mechanisms of endothelial cell (EC) pyroptosis in ISR remains unclear. **Method**: According to our previous work, an ISR rabbit model was established. Rabbits were divided into sham operation group and stent group. Serum was collected at 0, 4, 8, and 12 weeks to detect interleukin (IL)-1β and IL-18 levels. Rabbits' vascular EC was collected to detect NLRP3, Caspase1, GSDMD and P65 expression by western blot. NLRP3 inhibitor (MCC950) and P65 inhibitor (Helenalin) were used to pretreat EC, cell viability, lactate dehydrogenase (LDH) level of supernatant and pyroptosis related protein expression were measured in different groups. **Results**: The serum levels of IL-1β and IL-18 gradually increased with time, and the levels at the site of stent implantation were higher than the peripheral level. EC viability decreased significantly in the stent group, and protein levels of NLRP3, caspase1 and GSDMD were higher than those in the sham group. MCC950 and P65 inhibitors can reverse these effects. **Conclusions**: EC pyroptosis mediated by P65/NLRP3 inflammasome axis may promote ISR, our results provide a potential intervention target for the treatment of ISR.

Keywords

in-stent restenosis; pyroptosis; rabbits; NLRP3 inflammasome

Introduction

Percutaneous coronary intervention (PCI) is the first-line treatment for most patients with acute myocardial infarction, which greatly improved the quality of life of patients and reduced mortality rates [1,2]. However, in-stent restenosis (ISR) remains a significant challenge for patients undergoing PCI [3]. It was reported that there were about 10% of patients develop ISR [4,5]. In recent years, a number of *in vitro* and *in vivo* studies have identified some potential causes of ISR, including stent gap, residual uncovered atherosclerotic plaques and polymer damage. However, there are still lacking of further researches on the mechanisms of ISR [6,7].

High inflammation response is thought to be closely related to the occurrence of ISR. Several studies have revealed that C-reactive protein (CRP) and pro-inflammatory cellular factors such as interleukin-1β (IL-1β) level can be used to predict ISR [8–10]. It was also reported that decreased levels of TIMP-1 and IL-6 in plasma associated with the risk of ISR [11]. Liu *et al*. [12] also found that methotrexate promoted endothelial healing in ISR models by inhibiting inflammation levels. Endothelial cells (ECs) secrete inflammatory factors caused by stent implantation, which is thought to be the main cause of inflammation in vessels. Finally, EC dysfunction, accompanied by lipid deposition, smooth muscle cell proliferation and macrophage cell infiltration, formed new plaque on the stent. Our previous work and other teams demonstrated that inhibition of EC death and secondary inflammatory response can increase the endometrial coverage of stents, thereby reducing the occurrence of ISR [12,13]. These evidences suggested that EC death is a key mechanism of ISR and may be a potential intervention target for preventing ISR.

Pyroptosis is a pro-inflammatory type of programmed cell death, which has been proved to play an important role in inflammatory diseases [14,15]. Pyroptosis is characterized by activation of NLRP3 inflammasome, recruitment of ASC and caspase1, resulting in cell membrane cavities and the release of intracellular inflammatory cytokines such as IL-1β and IL-18 [16]. Although there is evidence demonstrated that ECs pyroptosis contributes to early atherosclerosis [17,18], it remains unclear whether stent implantation can lead to ECs pyroptosis, secondary inflammation response, and result in ISR, ultimately.

Therefore, in this study, based on our previous ISR rabbit model, we further investigated the effects and underlying mechanisms of ECs pyroptosis in ISR.

In-stent restenosis (ISR) is one of the key causes of ischemic events after coronary stent implantation, and endothelial cell death and inflammation are considered to be important mechanisms. Pyroptosis is a proinflammatory type of programmed cell death, the effects and underlying mechanisms of endothelial cells (ECs) pyroptosis in ISR remains unclear. The main objective of this study was to confirm the role and potential mechanism of pyroptosis in ISR.

Methods

Animal Model

All animal experimental procedure protocols are approved by the institutional research ethics committee of the Harbin Medical University, and procedures were performed in accordance with the National Institutes of Health guidelines (Guide for the Care and Use of Laboratory Animals) and the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

According to previous study, nano polymer-free sirolimus-eluting stent was implanted to establish IRS rabbit model [19]. Briefly, 48 hours before procedure, adult New Zealand white rabbits (weight about 3–4 kg) received aspirin (40 mg) and clopidogrel (75 mg). The rabbits were anesthetized (intramuscular xylazine 0.15 mL/kg and 20% chloral hydrate, 0.5 mL/kg), made two incisions from the neck, two centimeters wide. A 5F puncture needle was used to puncture the hole in the proximal end, and the guide wire was inserted. The rabbits were divided into two groups, the sham group and the stent group. Sham group: two incisions were made in the neck, use 7-0 sutures to close blood vessels and wounds and wait for the animal to wake up. Stent group: the stent is inserted into the carotid artery by using a guide wire. In order to form ISR, the stent was deployed to achieve a stent-to-artery-size ratio range of 1.2:1.4. Use 7-0 sutures to close blood vessels and wounds and wait for the animal to wake up. During feeding, rabbits were fed with a high fat diet. According to the requirements of the experiment, the carotid arteries and blood were collected at different time points. Before the animals were euthanized, optical coherence tomography (OCT) was performed to detect blood vessels, and data were analyzed by two independent technicians. The experimental flow chart is shown in **Supplementary Fig. 1**.

Plasma Collection

During breeding of New Zealand white rabbits, 3 mL of blood was collected through the auricular vein using an EDTA tube (Beyotime Biotechnology, Shanghai, China). At the end of the experiment, the New Zealand white rabbits were anesthetized. A 5F puncture needle was used to collect 3 mL of blood from the carotid stent implantation site. The auricular vein was used to collect peripheral blood 3 mL. After blood collection, centrifuge the blood (3000 r/min, 4 °C, 15 min) to obtained plasm and stored at –80 °C.

EC Isolation

According to previous study [20], rabbit carotid arteries were dissected in sterile PBS and then incubated with type IV collagenase (Sigma-Aldrich Chemical, St Louis, MO, USA) at 37 °C for 3 h. The cells were cultured in the medium containing 10% fetal bovine serum. After 1 hour of adherent, the adherent non-endothelial cells were washed by PBS and added into the new medium. Western blot was used to detect the marker of smooth muscle cell and ECs.

ELISA

We collected rabbit serum and stored it in a refrigerator at –80 °C. According to the manufacturer's instructions, the serum level of IL-18 and IL-1β were detected.

Human Blood Collected

Patients had single *de novo* coronary artery lesions and underwent regular coronary angiography (CAG) at the Second Affiliated Hospital of Harbin Medical University 2019 to October 2020. 15 patients with ISR were selected, while 15 patients without ISR who had single *de novo* coronary artery lesions and similar baseline characteristics served as the control group. ISR was defined as the presence of $>$50% diameter stenosis in the stented segment [11]. The study followed the principles outlined in the Declaration of Helsinki and it was approved by the ethics committee. All patients signed informed consent forms.

Gents

The rabbits were obtained from Animal Experiment Center, the Harbin Medical University. The primary antibody (NLRP3, Caspase1, GSDMD, P65, His-H3 and β-actin) in this study was customized by Abcam (Cambridge, United Kingdom). CCK-8 and lactate dehydrogenase (LDH) assay were obtained from Beyotime Biotechnology (Shanghai, China). ELISA kit for IL-1β and IL-18 were obtained from BOSTER Biological Technology (Wuhan, China). MCC950 (5 µM) and Helenalin (10 µM) were purchased from MCE (Monmouth Junction, NJ, USA).

Western Blot

RIPA (Shanghai, China) lysates were used to extract endothelial cell proteins from different groups. Add 200 µL RIPA to a 10 cm dish. It was cracked on ice for 30 minutes and placed in a high-speed centrifuge at 12,000 rpm for 15 minutes. Collect supernatant and add 5x loading buffer

(Shanghai, China). Heat at 100 °C Celsius for 10 minutes to denature the egg whites. SDS-page gel (Shanghai, China) was used for electrophoresis and membrane transfer. Diluted primary antibody (1:1000) (Shanghai, China) for nights at 4 °C. Wash the pvdf membrane three times, the second antibody was incubated at room temperature for 1 h. Wash the pvdf membrane three times again. ECL kit (Shanghai, China) was used to observe the strips.

CCK-8 and LDH Assay

According to the manufacturer's instructions, endothelial cells were inoculated into 96-well plates: according to the appropriate number of plates laying cells (approx. 1–2 $\times$ 10^4), 100 µl cell suspension was added to each well with 10% CCK8 working solution. Culture for 1 hour: determine the absorbance at 450 nm. The supernatants from ECs were collected. The maximum LDH release control is added with 20 µL 10$\times$ lysis solution 45 min before adding the working solution. Then, 120 µL supernatants are transferred to a new 96-well plate, and 60 µL working solution is added to each well, incubated for 25 min at room temperature avoiding light. Thereafter the absorbance is measured at 490 nm.

Statistical Analysis

All the results were presented as mean $\pm$ 95% Cl, and all the data were measured by independent experiments. All statistical analyses were performed using IBM SPSS software v. 22.0 (IBM Corp., Armonk, NY, USA). In this experiment, comparisons were made between 2 groups, and there were few biological replicates in each group, so it was difficult to judge whether it was consistent with normal distribution. The Mann–Whitney U test was performed for nonparametric data between the 2 groups. The statistical significance level was set at $p < 0.05$.

Results

Serum IL-1β and IL-18 Level was Increased in ISR Model

We used scanning electron microscopy to observe the nano Polymer-free sirolimus-eluting stent, which is a stent with no overcoat and a gap on the surface of the stent for drug release (**Supplementary Fig. 2**). According to our study, an ISR rabbit model was established, Fig. 1 showed typical pathologic and OCT image. All rabbits were examined by optical coherence tomography (OCT) before euthanasia, and the results were shown in **Supplementary Table 1**. These data indicated that our ISR rabbit model has excellent stability. Furthermore, we assessed the levels of pyroptosis related factors (IL-1β and IL-18) in blood from peripherally at different time points, the results showed the IL-1β and IL-18 level in peripheral blood continued to rise from 0 to 12 weeks after stent implantation, Plasma levels of IL-1β and IL-18 in ISR group were also higher than those in sham group (Fig. 2A,B). The serum levels of IL-1β and IL-18 at the site of stent implantation were also measured, it can be observed obviously that the serum levels of IL-18 and IL-1β at the site of stent implantation were significantly higher than those in peripheral blood (Fig. 2C,D). Furthermore, we compared the levels of IL-1β and IL-18 in peripheral blood of patients with ISR and coronary heart disease, and we found that serum IL-1β and IL-18 levels were significantly increased in patients with ISR compared to patients with coronary heart disease (CHD) (Fig. 2E,F). These data demonstrated that IL-1β and IL-18 level could be the biomarker for ISR.

NLRP3 Inflammasome Related Proteins were Elevated in the Intima of the ISR Model

To study the response of ECs to stent implantation, the rabbits' ECs at stent implantation site were collected after euthanasia. Morphology and cellular marker protein detection confirmed the successful isolation of endothelial cells (**Supplementary Fig. 3**). CCK8 results showed that endothelial cell viability in ISR group was significantly lower than that in sham group (Fig. 3A). The data from LDH assay and propidium iodide (PI) staining were consistent with CCK8 results (Fig. 3B–D). Polymerase chain reaction (PCR) and western blot (WB) were used to detect the expression levels of key pyroptotic proteins. The results showed that the NLRP3 messenger RNA (mRNA) level in the model group was significantly higher than that in the control group (Fig. 4A), the expression levels of key pyroptotic proteins such as NLRP3, caspase1 and GSDMD were significantly increased in the ECs of the model group (Fig. 4B,C). These data demonstrated that NLRP3 inflammasome contributes to the ISR development.

MCC950 Reversed the Decline in ECs Viability in ISR Model

To further confirm ECs underwent pyroptosis in ISR model, MCC950, a selective NLRP3 inhibitor, was used to pre-treat isolated ECs. The results from CCK8 assay and LDH assay indicated that the cell viability was obviously increased in MCC950 treatment group (Fig. 5A,B). WB also demonstrated that the increased level of GSDMD and caspase1 level in ISR model were also attenuated in MCC950 group (Fig. 5C–E). These results illustrated that ECs undergo pyroptosis in ISR rabbit model.

P65 Mediates NLRP3 Inflammasome Activation in ISR Model

P65, as a transcription factor, is involved in a variety of inflammatory diseases. To investigate whether P65 mediates pyroptosis in an ISR model, we detected the expression level of P65 in cytoplasm and nucleus respectively,

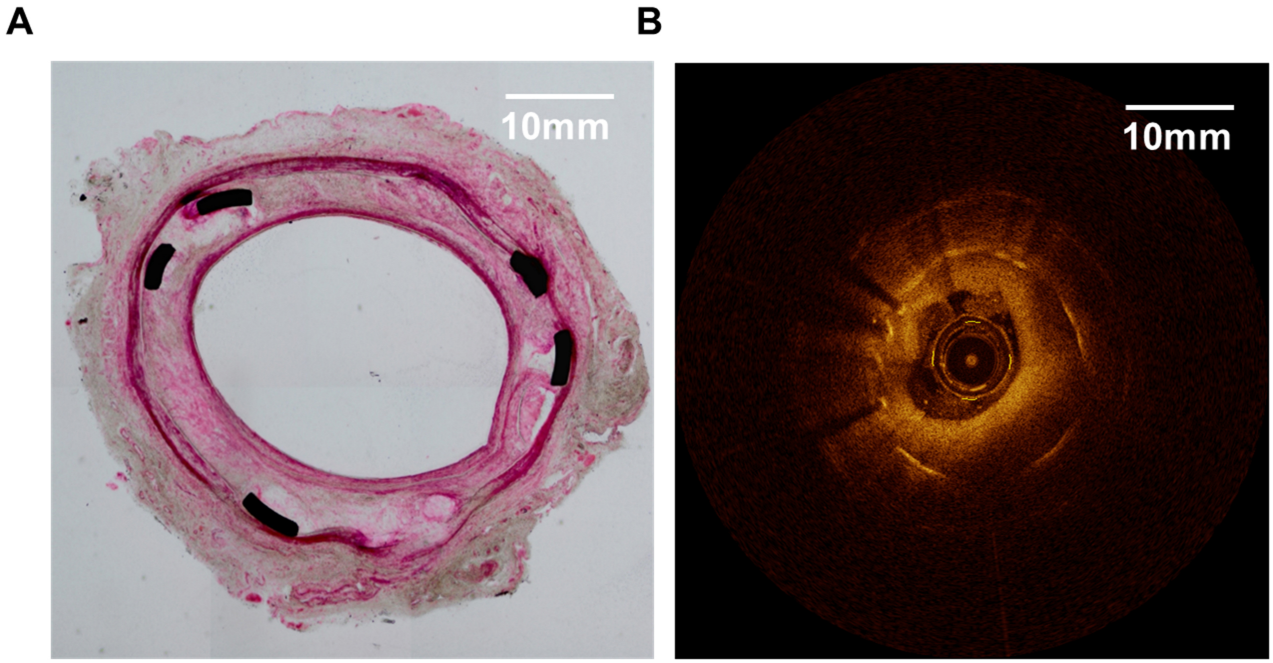

Fig. 1. Histological and optical coherence tomography optical coherence tomography (OCT) features of lesions after stent implantation in in-stent restenosis (ISR) rabbits. (A) Pathology, scale: 10 mm. (B) OCT, scale: 10 mm.

and found that in ISR model, the expression level of P65 in nucleus was increased significantly, while the expression level of P65 in cytoplasm was decreased (Fig. 6A,B). Immunofluorescence staining of P65 showed the same result (Fig. 6C). Furthermore, P65 specific inhibitors were used to treat ECs, and the results were as expected. P65 specific inhibitors significantly decreased the NLRP3, caspase1 and GSDMD level (Fig. 6E–H). These data revealed that P65 could be the potential target for ISR.

Discussion

The main findings of our manuscript are as follows: (1) In the ISR model, ECs undergo pyroptosis mediated by NLRP3 inflammasome activation. (2) P65 activation contributes to regulating ECs pyroptosis in ISR models. These results suggested that inhibition of ECs pyroptosis may be a potential intervention target for ISR.

Although PCI procedure reduces mortality and improves quality of life among acute coronary syndrome patients, ISR still occurs in 5–20% of patients [21]. ECs apoptosis and higher inflammatory response are key mechanisms of ISR. From ISR models to clinical patients, it was well established that promoting EC proliferation and anti-inflammatory therapy have positive effects on ISR [22]. However, less attention was attached to the underlying mechanism of ECs injury after stent implanted. *In vitro* experimental demonstrated that rapamycin, the main component of drug-coated stents, not only inhibits proliferation of smooth muscle cells, but also damages ECs [23]. However, up to now, the specific mechanism of ISR EC injury is still lacking *in vivo* model.

Inflammatory response is considered to be a key step in the progression of ISR. IL-6 and Lp-PLA2 levels can predict the risk for ISR in patients with acute myocardial infarction in the perioperative period [24]. Furthermore, it was also found exogenous IL-35 inhibits the progression of ISR by inhibiting macrophage polarization [25]. However, the mechanism of the inflammatory response in ISR remains unclear. Pyroptosis is a newly discovered proinflammatory programmed mode of death. It has been confirmed that pyroptosis promotes the development of chronic inflammatory diseases including atherosclerosis [15,26]. In addition to the effect of pyroptosis itself on inflammatory diseases, inflammatory factors released by pyroptotic cells can intensify the inflammatory response [27,28]. In our study, we found that the levels of IL-18 and IL-1β in peripheral blood of ISR model rabbits increased gradually with time. Additionally, the serum levels of IL-1β and IL-18 at the side of stent implantation were significantly higher than those in peripheral blood. These results suggested that stent implantation may increase the levels of IL-1β and IL-18 in ISR models. It was also found that the level of C-reactive protein (CRP) and white blood cell count were higher in patients with ISR [8]. Also, there are several studies demonstrated that some typical inflammatory markers, such as C1q/TNF-related protein and serum soluble TREM-1, were also elevated in ISR patients [29,30]. These studies only focused on levels of inflammatory markers at a single point

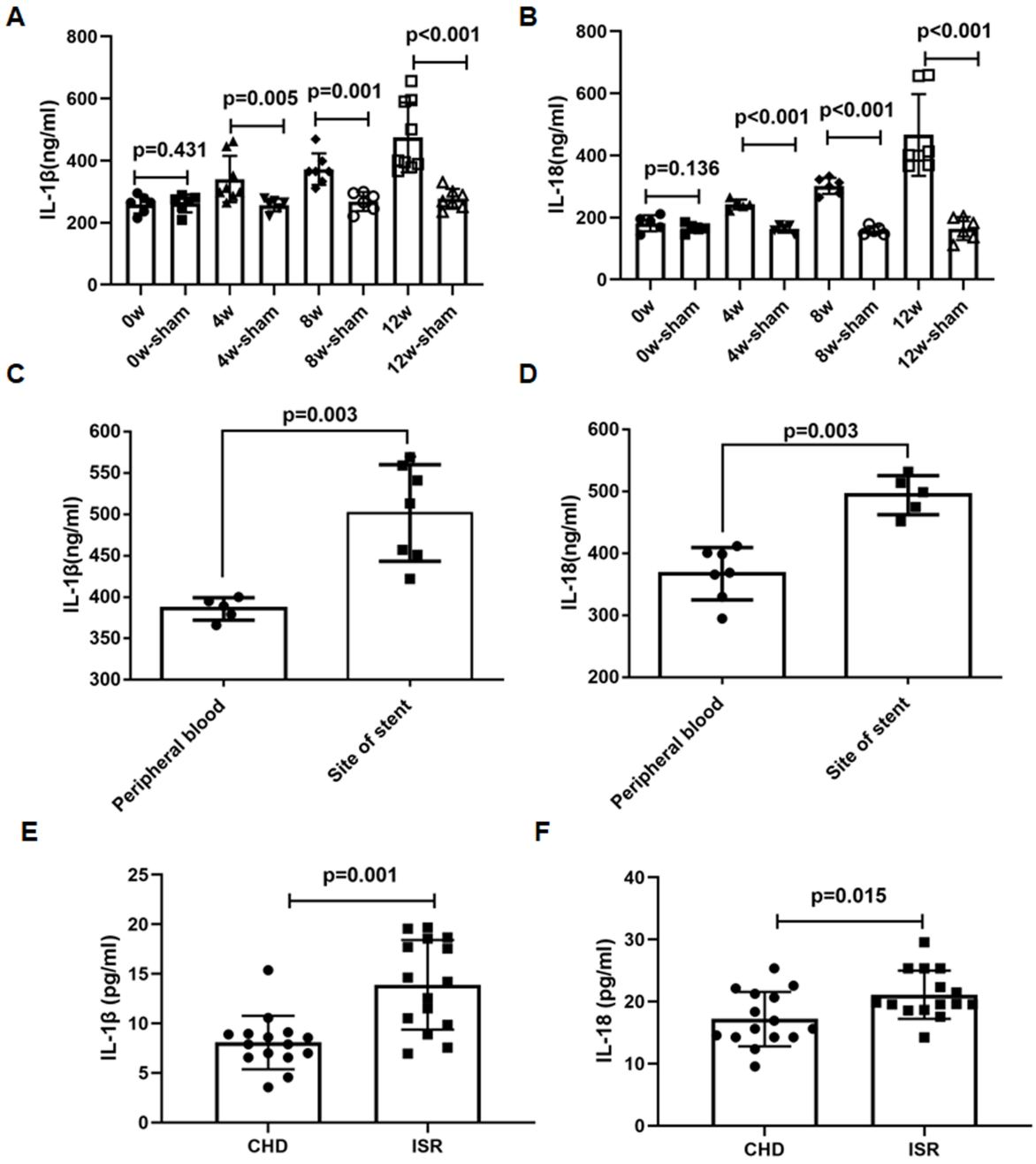

Fig. 2. The serum level of interleukin-1β (IL-1β) and IL-18 in rabbits. (A) IL-1β level in peripheral blood, Mann-Whitney U = 0. (B) IL-18 level in peripheral blood, Mann-Whitney U = 0. (C) IL-1β level in peripheral blood and blood from site of stent, Mann-Whitney U = 0. (D) IL-18 level in peripheral blood and blood from site of stent, Mann-Whitney U = 0. (E) IL-1β level in ISR and CHD patients, Mann-Whitney U = 0. (F) IL-18 level in ISR and CHD patients, Mann-Whitney U = 0.

in time, in this study, based on our previous ISR model, we identified the increased IL-18 and IL-1β levels at different time points starting from cytokines produced by pyroptotic ECs.

Moreover, we found that ECs viability isolated from the stent implanted artery were significantly less active than the control group after 48 hours of culture. It was observed that the expression of NLRP3, caspase1 and GSDMD were significantly higher in ISR model. A specific NLRP3 inhibitor, MCC950, significantly reversed the decline in ECs viability. These data suggested that there are pyroptosis occurring in ECs at the site of stent implantation in the ISR model. Furthermore, the elevated levels of IL-1β and IL-18 in ISR rabbit models may be due to ECs pyropto-

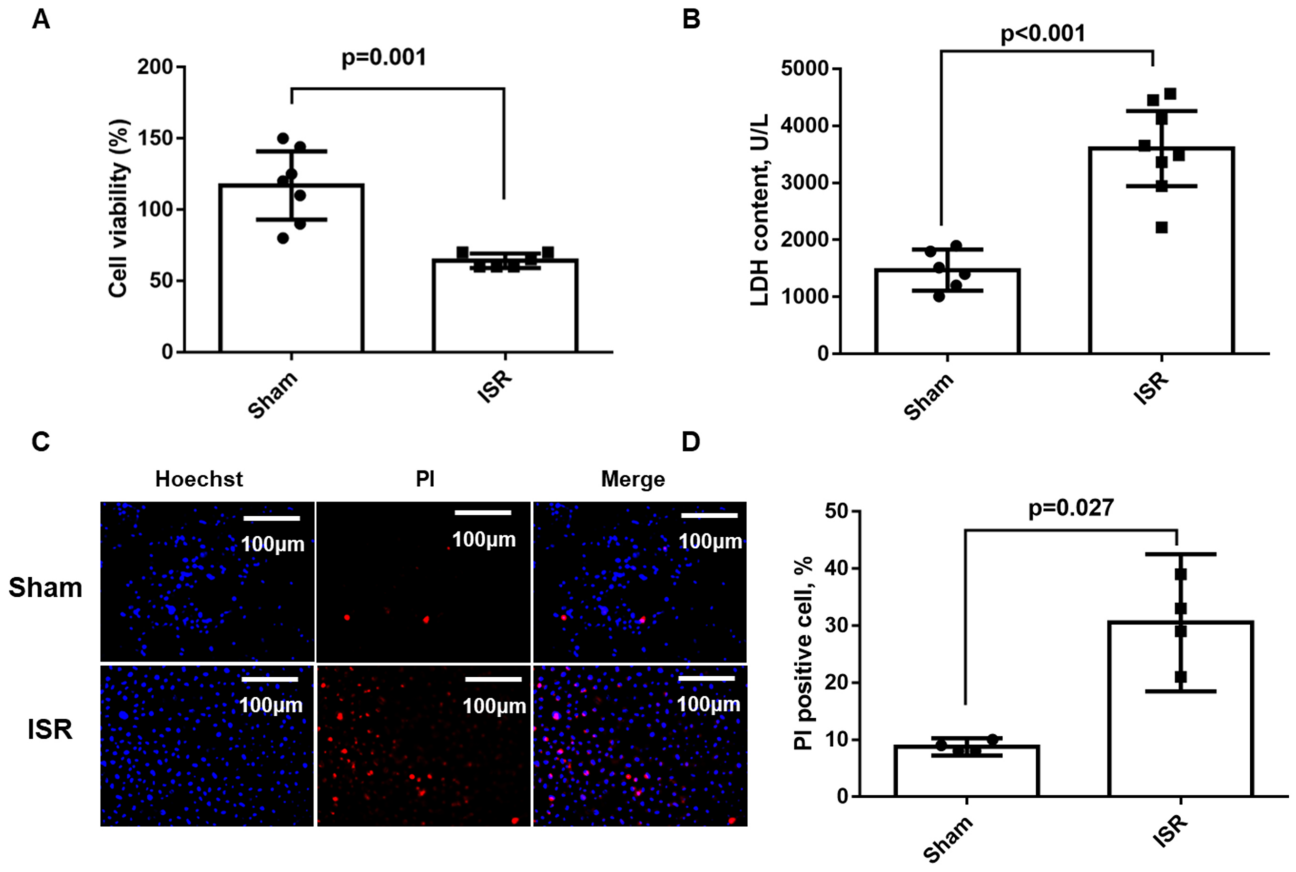

Fig. 3. The viability of endothelial cells decreased significantly in ISR group. (A) CCK8 assay, Mann-Whitney U = 0. (B) Lactate dehydrogenase (LDH) level in cell supernatant, Mann-Whitney U = 0. (C,D) PI staining, Mann-Whitney U = 0.

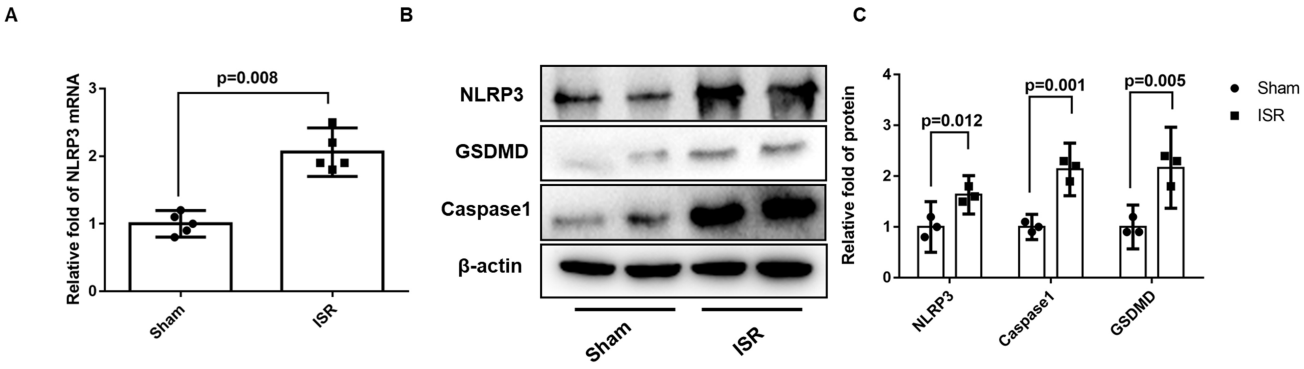

Fig. 4. The protein of NLRP3, caspase1 and GSDMD level was increased in ISR group. (A) NLRP3 mRNA level, Mann-Whitney U = 0. (B,C) The protein of NLRP3 (Mann-Whitney U = 1), caspase1 (Mann-Whitney U = 0) and GSDMD (Mann-Whitney U = 0).

sis partly. Accumulating evidence indicated that suppressing ECs apoptosis inhibits the incidence of ISR. Several monomer compounds have been shown to reduce the incidence of ISR by inhibiting ECs apoptosis [13,23]. In our study, we found there are pyroptotic ECs after stent implantation in rabbits. Moreover, with the increase of stent implantation time, the expression of pyroptosis related protein was higher. Therefore, our results suggested that ECs pyroptosis plays an important role in ISR, and its inhibition may be a potential intervention target for ISR.

P65, a classical transcription factor, regulates the transcription of numerous inflammation-related genes. NLRP3 is a key regulatory protein for cell scorching death, and studies have confirmed that P65 can bind to the promoter region of NLRP3 and promote NLRP3 expression. It was reported that oncostatin M enhance macrophage pyroptosis via P65/NLRP3 axis [31]. Moreover, quercetin suppressed NLRP3 inflammasome activation and macrophage pyroptosis by inhibiting P65 activation [15]. In our study, we found that the expression of P65 in the nucleus of ECs was

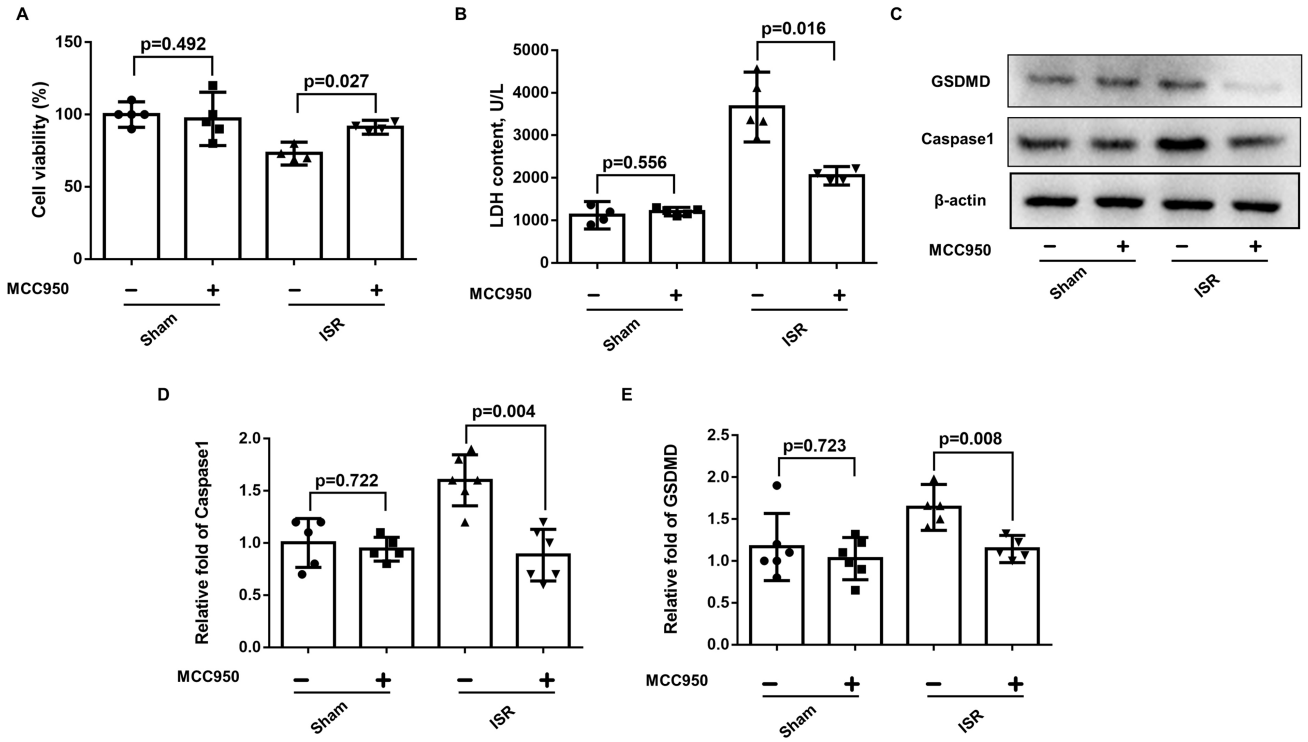

Fig. 5. MCC950 reversed endothelial cells pyroptosis in ISR model. (A) CCK8 assay, (Sham, Mann-Whitney U = 7; ISR, Mann-Whitney U = 0). (B) LDH level in cell supernatant, (Sham, Mann-Whitney U = 7; ISR, Mann-Whitney U = 0). (C–E) The protein of NLRP3, caspase1 (Sham, Mann-Whitney U = 9; ISR, Mann-Whitney U = 0) and GSDMD level (Sham, Mann-Whitney U = 10; ISR, Mann-Whitney U = 0.5).

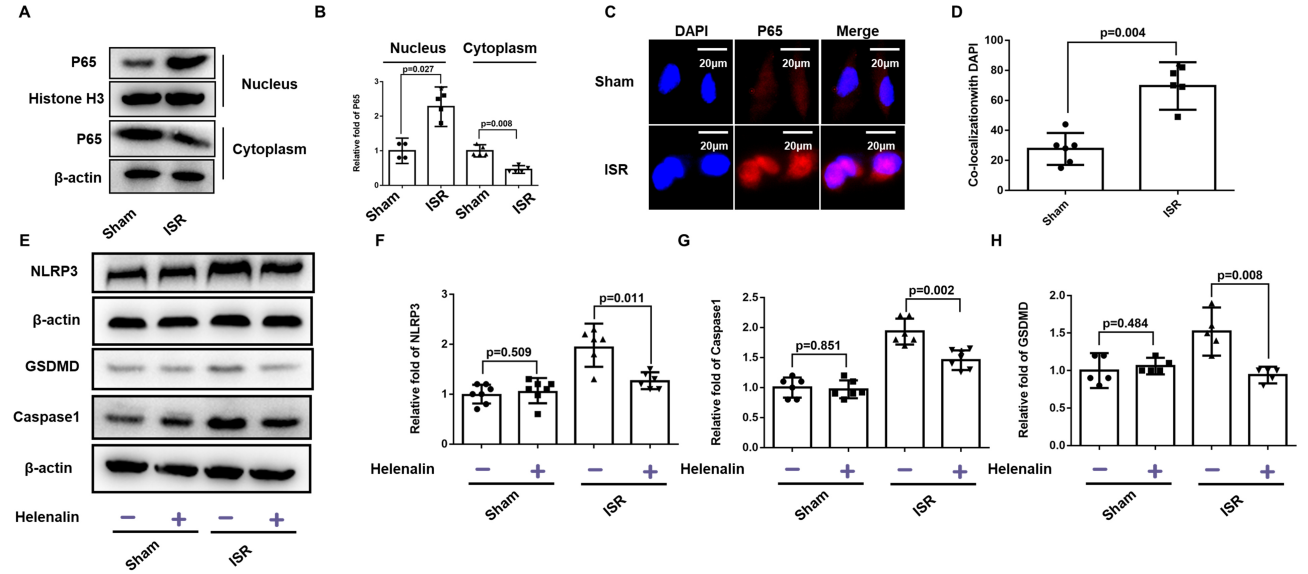

Fig. 6. P65 mediates NLRP3 inflammasome activation. (A,B) P65 level in nucleus and cytoplasm, (Nucleus, Mann-Whitney U = 0; Cytoplasm, Mann-Whitney U = 0). (C,D) P65 immunofluorescence, (Mann-Whitney U = 0). (E–H) The protein of NLRP3 (Sham, Mann-Whitney U = 19; ISR, Mann-Whitney U = 2.5), caspase1 (Sham, Mann-Whitney U = 16.5; ISR, Mann-Whitney U = 0) and GSDMD (Sham, Mann-Whitney U = 15.5; ISR, Mann-Whitney U = 0) level.

significantly increased in ISR model. Moreover, the specific inhibitors of P65 can effectively inhibit the expression of NLRP3 and other pyroptosis related proteins in ISR model. These results suggested that nuclear translocation of P65 mediates the ECs pyroptosis induced by stent implantation. Inflammatory cell infiltration is also consid-

ered to be one of the pathogenesis of ISR, and previous studies have reported that a large number of macrophages and neutrophils infiltrate in ISR models [4,8]. P65 has also been shown to promote immune cell infiltration in atherosclerosis. Furthermore, It was reported that PDE4 inhibition attenuated ISR by inhibiting immune cell adhesion via P65/VCAM1 pathway [32]. Previous study also reported that inhibition of NRF2 activity and excessive production of reactive oxygen species are also mechanisms of pyroptosis [33,34]. Whether these pathways participate in pyroptosis in ISR requires further investigation.

These results suggest that P65 is involved in ISR progression through a variety of mechanisms. Inhibition of P65 may be a potential target for improving ISR. Current drugs, such as aspirin and statins, have been shown to inhibit P65 and pyroptosis. They all have the potential to treat ISR by improving pyroptosis and P65 activity [35,36]. Moreover, other monomer compounds, which have been shown to inhibit pyroptosis nd P65 activity, also have the potential to treat ISR [15].

In this study, we identified that ECs pyroptosis may be a potential mechanism of ISR, and inhibition of ECs pyroptosis may serve as a novel opinion to prevent the occurrence of ISR. Our results suggest that IL-18 and IL-1β, the key factors of pyroptosis, may be potential biomarkers of ISR, and intervention of pyroptosis may be a potential target for the prevention and treatment of ISR in the future. However, inhibition of P65 activity and pyroptosis may affect the body's immune system, which is a challenge for clinical application. Subsequently, targeted drug delivery to endothelial cells may be the key to solving this problem.

Our results suggest that pyroptosis may be involved in ISR and that pyroptosis may be a potential intervention target for ISR. Future studies should be carried out based on small molecule compounds of P65 and NLRP3 inflammasome activity. In addition, targeted methods to inhibit endothelial pyroptosis should be further explored.

Limitation

There are some limitations that need to be mentioned in the current study. First, we only studied the role and mechanism of endothelial pyroptosis in ISR only at the animal level. Our conclusion lacks pathological data from clinical patients to support it. Second, in the current study, we only used one type of coronary stent. Third, in the present study, evidence for plasma inflammatory cytokines in patients with ISR was lacking. Our results may not be applicable to other types of coronary stents, which also need to be confirmed by further studies. Fourth, our results lack a large cohort of patient samples to verify, and larger prospective studies should be carried out in the future. Fifth, due to the difficulty in obtaining NLRP3 knockout rabbits, the involvement of pyroptosis in the occurrence of ISR cannot be fully confirmed. Sixth, rabbits were used in this study, and their genetic background, food intake, and details of the surgical procedure may have potential effects on the results. Seventh, at present, the number of animals used in the manuscript is small, which should be confirmed by larger rabbits in the future. In addition, there are many differences between rabbits and humans, and whether the current results apply to humans still needs to be further verified. Currently commercialized antibodies are difficult to achieve immunofluorescence in rabbits, and we did not colocate CD31 and caspase1 in tissues.

Conclusions

EC pyroptosis mediated by P65/NLRP3 inflammasome axis may promote ISR, our results provide a potential intervention target for the treatment of ISR.

Availability of Data and Materials

All original data are available from corresponding authors upon reasonable request.

Author Contributions

BH and JH designed the research study, BH and GW performed the research. BH writed the manuscript. All authors read and approved the final manuscript. All authors contributed to editorial changes in the manuscript. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

Ethics Approval and Consent to Participate

The present study was approved by the ethics review board of the Harbin Medical University (SYDW 2019-253).

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.59958/hsf.6845.

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