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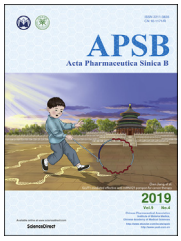

ORIGINAL ARTICLE

Aspirin alleviates endothelial gap junction dysfunction through inhibition of NLRP3 inflammasome activation in LPS-induced vascular injury

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Abstract The loss of endothelial connective integrity and endothelial barrier dysfunction can lead to increased vascular injury, which is related to the activation of endothelial inflammasomes. There are evidences that low concentrations of aspirin can effectively prevent cardiovascular diseases. We hypothesized that low-dose aspirin could ameliorate endothelial injury by inhibiting the activation of NLRP3 inflammasomes and ultimately prevent cardiovascular diseases. Microvascular endothelial cells were stimulated by lipopolysaccharide (2 μg/mL) and administrated by 0.1–2 mmol/L aspirin. The wild type mice were stimulated with LPS (100 μg/kg/day), and 1 h later treated with aspirin (12.5, 62.5, or 125 mg/kg/day) and dexamethasone (0.0182 mg/kg/day) for 7 days. Plasma and heart were harvested for measurement of ELISA and immunofluorescence analyses. We found that aspirin could inhibit NLRP3 inflammasome formation and activation *in vitro* in dose-dependent manner and has correlation between the NLRP3 inflammasome and the ROS/TXNIP pathway. We also found that low-concentration aspirin could inhibit the formation and activation of NLRP3 inflammasome and restore the expression of the

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endothelial tight junction protein zonula occludens-1/2 (ZO1/2). We assume that aspirin can ameliorate the endothelial layer dysfunction by suppressing the activation of NLRP3 inflammasome.

1. Introduction

The dysfunction of endothelial cells can result in blood vessel injury, alter vascular homeostasis and eventually cause vascular diseases[1]. Vascular endothelial cells (VECs), the inner lining of blood vessels, serve as an anti-thrombotic and anti-inflammatory barrier and are critically involved in the maintenance of vascular homeostasis, regulating vascular movement and participating in life activation[2]. Endothelial cell dysfunction (ECD) is the fundamental of cardiovascular diseases[3]. Endothelial dysfunction leads to endothelium inflammation, and further impairs endothelial integrity. Therefore, it is very significant to protect endothelial permeability and integrity. It is necessary to find a drug that can maintain the homeostasis of endothelial junction proteins and endothelial cells in the early stage of disease development and ameliorate endothelial dysfunction.

Aspirin is a typical non-steroidal anti-inflammatory drug (NSATD); however, it has a certain advantage over other NSATDs, such as a lowered risk of occlusive cardiovascular event[4]. In traditional concept, NSATDs are known for their analgesic, anti-pyretic, and anti-inflammatory effect, and have characteristic side effects, including gastric intolerance and depression of blood clotting through inhibiting platelet activation[5]. Giving low-dose of aspirin has fewer disadvantages than giving other NSATDs. For example, low-dose aspirin can prevent thrombotic cardiovascular events with minimum adverse effects in the gastric epithelium[6,7]; and it has been shown to be highly effective in the secondary prevention of cardiovascular diseases. Mainstream concept believes that the effect of aspirin against cardiovascular diseases is mainly based on its anticoagulant effect[8]. Otherwise, the endothelial cell plays an important role in the pathological process of cardiovascular diseases which is closely related to inflammasomes. It is still unclear whether the pharmacological effects of aspirin in prevention of cardiovascular diseases are related to the endothelial inflammasome.

Inflammasomes are important signaling platform of the innate immune system, poised to detect a wide range of molecular signatures including pathogens and sterile agents. They are multiprotein complexes response of the innate immune system to a noxious stimulus, including infections or tissue damage. Recent studies have found the NOD-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome play an important role in the development of cardiovascular diseases[9,10]. Several molecular signaling pathways are shown to mediate the activation of the NLRP3 inflammasome, such as releasing of reactive oxygen species (ROS), lysosome leakage and activation of cathepsin B or the modifications in K^+ efflux[11–16]. Then the activation of NLRP3 inflammasome triggers a series of downstream inflammatory factors, leading to vascular endothelial dysfunction and the ultimate occurrence or development of cardiovascular diseases.

In this study, we demonstrated a novel role of aspirin in LPS-induced inter-endothelial junction disruption for the first time. Our findings showed that aspirin protected the expression of inter-endothelial junction proteins including zonula occludens-1 (ZO-1) and zonula occludens-2 (ZO-2) by inhibiting NLRP3 inflammasome pathway. The binding of LPS with TLR4 increases ROS release and NLRP3 inflammasome activation, leading to pyroptosis and tissue damages[17,18]. NLRP3 inflammasome activation is mediated by thioredoxin-interacting protein (TXNIP)[19]. And recent work demonstrated that NLRP3 inflammasome induced the release of high mobility group box 1 (HMGB1) to cause endothelial dysfunction[20,21]. In addition, aspirin was proved to play a key role in interdicting redox signaling and inhibiting NLRP3 inflammasome, thereby reduce endothelial injury and vascular endothelial dysfunction. Our study implicates the clinical potential of aspirin for the prevention of chronic vasculopathy on the early stage.

2. Materials and methods

2.1. Animal procedures

All experiments were done with C57BL/6 J mice age-matched 8–12-week old (20 to 25 g), and housed with water and food *ad libitum* in a 12 h/12 h reverse light/dark cycle (lights on at 7:00 A.M. and off at 7:00 P.M.). All mice were bred from breeding pairs from Nanjing Biomedical Research Institute (Nanjing, China). All protocols were approved by the Institutional Animal Care and Use Committee of Guangzhou University of Chinese Medicine (Guangzhou, China). PBS (100 μL) containing LPS (100 μg/kg; Eco-LPS, L4130, sigma, Darmstadt, Germany) was administered by intraperitoneal injection (i.p.) to stimulate the vascular inflammation model. Forty-six mice were randomly assigned to 6 groups: control group ($n = 6$), LPS group ($n = 8$), dexamethasone group ($n = 8$), aspirin low concentration group ($n = 8$), aspirin medium concentration group ($n = 8$), and aspirin high concentration group ($n = 8$). Mice were pretreated i.p. with 0.1 mL of 0.5% CMC-Na (Sigma, C5678), dexamethasone (0.0182 mg/kg; Sigma, D1756), or aspirin (12.5 mg/kg, 62.5 mg/kg, or 125 mg/kg; Sigma, A5376) 1 h after LPS administration. After a week, mice were humanely sacrificed after fasting for 12 h. Blood was centrifuged for 20 min at 3000 rpm and 4 °C in refrigerate centrifuge (Sigma, 3K15), and plasma was collected. Heart tissue and plasma samples were kept at −80 °C until analyzed for inflammatory markers.

2.2. Cell culture

The mouse vascular endothelial cells (MVECs) line EOMA was purchased from ATCC (Shanghai, China). MVECs was cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, 11995, Rockford, IL, USA), containing 10% of fetal bovine serum (Gibco, 16140-071) and 1% penicillin–streptomycin (Gibco, 15140-122). The

cells were cultured in a humidified incubator at mixture at 37 °C with 5% CO_2 and 95% air. Cells were passaged by trypsinization (0.25 trypsin/EDTA; Gibco, 25200-056), followed by dilution in DMEM medium containing 10% fetal bovine serum. The cells were seeded in 6-well plates at a density of 5×10^5 cells/mL, cultured in DMEM media with 10% FBS for 24 h. Mouse carotid arterial endothelial cells (MVEC) were cultured and treated with 0.1–3 mmol/L of aspirin in response to LPS (2 μg/mL) stimuli.

2.3. Cell proliferation assay

We assayed EOMA in real time by microscope monitoring in real time. Endothelial were seeded in the 96-well plates at the density of 5000 cells/well, then given corresponding stimulation and drug interference after the cells adhered to the wall. The 96-well plate was put in the IncuCyte ZOOM Real-Time Live-Cell Imaging System (Essen Bioscience, Ann Arbor, MI, USA), after which the cell state was monitored in 24 h under the program set up. The effect of cell proliferation was analyzed by comparing the growth rate of each component.

2.4. Western blot analysis

Harvested cells were lysed in radio immunoprecipitation assay (RIPA) buffer (Themor Scientific, Rockford, MI, USA) containing protease inhibitor (Roche, 04693132001, Basel, Switzerland). The amount of total extracted protein was determined by BCA protein assay kit (Beyotime, Beijing, China) and denatured with 5 × protein loading buffer (sodium dodecyl sulfate polyacrylamide gel electrophoresis, SDS-PAGE; Beyotime) in metal bath for 5 min, followed by cooling on ice for another 5 min. Equal amounts of the protein samples were separated by 12% SDS-PAGE and transferred onto 0.2 μm polyvinylidene fluoride membranes. The membrane was blocked with 5% non-fat milk for 1 h at room temperature. The blocked membrane was incubated with the indicated primary antibodies at 4 °C overnight and then treated with anti-rabbit IgG (1:2000; Cell Signaling Technology, Danvers, MA, USA) or anti-mouse IgG (1:2000; CST) for 2 h at room temperature. The primary antibodies were anti-NLRP3 (1:1000; CST), anti-caspase-1 (8:5000; Santa Cruz, Dallas, TX, USA), anti-TXNIP (1:2000; CST) and anti-ASC (8:5000; Santa Cruz). The anti-β-actin (1:1000; CST) was used as an internal control. Protein bands were visualized using the Western blotting detection system (Tanon, Shanghai, China) according to the manufacturer's instructions and analyzed using Image J software (NIH, Bethesda, USA).

2.5. Immunofluorescence microscopy

To confirm inflammasome activation in EOMA and the endothelium of mouse coronary arteries, immunofluorescence co-localization method was adapted. Goat anti-NLRP3 antibody (1:200, Abcam, Cambridge, MA, USA), mouse anti-caspase-1 antibody (1:200; Santa Cruz), rabbit anti-ASC antibody (1:200; Santa Cruz), rabbit anti-ZO2 antibody(1:250; Invitrogen, Calif, CA, USA), rabbit anti-TXNIP antibody (1:200; Abcam), mouse anti-COX2 antibody (1:500; Santa Cruz), rabbit anti-HMGB1 antibody (1:200; Santa Cruz), and sheep anti-VWF antibody (1:200; Abcam) were used for these experiments. The cells were seeded in culture dish at a density of 5×10^5 cells/mL. To measure cell tightness, immunofluorescence was selected to detect endothelial junction proteins ZO1/ZO2, and the cells were seeded in culture dish at a density of 10×10^5 cells/mL fixed with 4% paraformaldehyde or frozen slides were fixed in acetone for 15 min, then incubated overnight at 4 °C with primary antibody, and the fluorescence-conjugated secondary antibody were co-incubated for 1.5 h at room temperature. Then, the dishes or slides were washed, mounted, and visualized through sequentially scanning on an Olympus laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany). Co-localization in vein or cells was analyzed by Image Pro Plus software (Media Cybernetics, Rockville, MD, USA), and the colocalization coefficient was represented by Pearson's correlation coefficient.

2.6. ROS detection

DCFH-DA can be freely penetrated into the cell membrane, but the DCFH does not, making it a probe easy to be loaded into the cell. The active oxygen in the cell can oxidize the fluorescence free DCFH to produce the fluorescent DCF. The fluorescence of DCF can be detected by the level of intracellular reactive oxygen species. Well-treated cells in the 96-well plate were added the ROS probe (20 nmol/L, 1:5000, Nanjing Jiancheng, Nanjing, China), and incubated for 30 min at 37 °C in cell incubator, and then washed by PBS 2–3 times at least. Finally, cells in each pore of 96-well plate added 150 μL PBS was placed and detected in the IncuCyte ZOOM Real-Time Live-Cell Imaging System for half an hour.

2.7. Cytokine analysis by ELISA

Supernatants from cell culture or plasma were assayed for mouse HMGB1, according to the manufacturer's instructions with the microplate reader (Biotex SYNERGY HTX, VT, USA). The reference standard and sample (100 μL) were added to the 96-well plates and incubated for 2 h followed by triple washes with buffer solution. Wells were dried and 200 mL of substrate (tetramethyl benizidine) were added to each well for 20 min in the dark at room temperature, after which the absorbance was measured at 450 nm. HMGB1 levels were compared using a standard curve generated from the standard solutions supplied by the manufacturer.

2.8. Endothelial permeability measurement

Endothelial permeability was assessed by measurement of fluorescein isothiocyanate–dextran flux across monolayers of cultured endothelial cells. Cells were plated on top of trans-well chambers in 24-well plates (0.4-μm pore size) and grown to confluence. Corresponding stimulation and drug interference were given after the cells adhered to the wall. After FITC–dextran 40 kDa (Sigma) was added to the upper chamber for 1 h, paracellular flux was assessed by taking 100 μL aliquots from both chambers to measure real-time changes of permeability across endothelial cell monolayers. Fluorescence was measured in those samples using a fluorescent plate reader and emission wavelengths of 485 and 530 nm, respectively[22,23].

2.9. Determination of trans endothelial electric resistance (TEER)

Trans endothelial electrical resistance (TEER) was measured using an assembly containing current-passing and voltage measuring electrodes. Cell treating is the same as steps in detecting cell permeability[24,25]. MVECs were also seeded on top of transwell

chamber in 24 wells (0.4-μm pore size). TEER was measured using an assembly containing current-passing and voltage measuring electrodes (EVOM2, WPI, FL, USA).

2.10. Single-guide RNA (gRNA) transfection

Nlrp3 was knocked down in CAECs by gRNA, which targeted stable expressing endothelial g*Nlrp3*. gRNA sequences for CRISPR/Cas9 gene editing of coding genes were designed by the CRISPR Design tool (gttp://crispr.mit.edu/). gRNA sequences were synthesized and then inserted into the *Bbsl*-digested px459 plasmid. *gNlrp3* sequences were 5′-GACGAGTGTCCGTTGC AAGC-3′. Gene editing in CAECs was performed by Lipofectamine 3000 transfection (Invitrogen, Carlsbad, CA, USA) according to manufacturer's guidelines. The transfected cells were incubated in the media with 2.5 μg/mL puromycin to screen out the gRNA plasmid-containing cells.

2.11. Statistical analysis

All results were expressed as mean ± SEM of four independent experiments with each experiment including triplicate sets *in vitro*. The Student's *t*-test was used to evaluate the differences between two groups. One-way ANOVA analysis and *post hoc* tests were applied when there are more than two groups in the independent variable. The level of significance was set at a P value of 0.05.

3. Results

3.1. Aspirin alleviates LPS-induced disruption of tight junction proteins in MVECs

Endothelial dysfunction occurs in the early stage of cardiovascular diseases with various coronary risk factors[8]. Endothelial cell damage may eventually lead to endothelial permeability change, endothelial integrity destruction, and endothelial function loss[23]. Tight junction proteins and adhesion junction proteins play critical role in mediating the permeability of solutes between adjacent endothelium cells. Disruption of junction proteins, including ZO-1 and ZO-2, increases endothelial cells permeability during vascular dysfunction[26]. As shown in Fig. 1A and B, MCVEs stimulated with LPS markedly decrease the expression of tight junction proteins ZO-1/ZO-2, which was obviously restored by aspirin, while the classic anti-inflammatory drug dexamethasone had no effect on the recovery of ZO1/ZO2. The results of the endothelial permeability and TEER are consistent with the expression of ZO1/ZO2 (Fig. 1C and D), which proved that dexamethasone did not have effect of protecting the tight junction proteins for endothelium, but the concentrations of aspirin at 1 and 2 mmol/L are effective and stable.

3.2. Aspirin suppressed the formation and activation of NLRP3 inflammasomes and HMGB1 expression in MVECs with LPS stimulation

The NLRP3 inflammasome is best characterized as a type of inflammasome in mammalian cells that consists of a proteolytic complex formed by NLRP3, ASC, and pro-caspase-1[9]. To biochemically assess the anti-inflammatory elevation effect of aspirin, we detected NLRP3 inflammasome protein expression and inflammasome formation. LPS-induced inflammasome activation (Fig. 2A and B) was reversed after aspirin treatment. Aspirin treatment dramatically downregulated the expression of NLRP3 protein but had little effect on ASC (Fig. 2C and D). Based on these experimental results, we selected three effective and slightly concentrations (0.5, 1, and 2 mmol/L) for immunofluorescence colocalization experiments. The immunofluorescence colocalization method was used to detect the recruitment of inflammasome (Fig. 2E and F). Moreover, these three concentrations of aspirin significantly inhibited the recruitment of NLRP3 inflammasome. To confirm the activation of NLRP3 inflammasome after recruitment, we detected caspase-1 inflammasome protein expression, containing pro-caspase-1, which relates to the recruitment of NLRP3 inflammasomes, and cle-caspase-1, which relates to the activation of NLRP3 inflammasomes (Fig. 2G and H). Aspirin clearly decreased pro-caspase-1 expression and caspase-1 cleavage in LPS-treated MECs in a concentration-dependent reduction. NLRP3 inflammasomes are activated to produce a number of inflammasome products including the HMGB1, which serves as a novel vascular permeability factor to mediate vascular hyperpermeability and promote endothelial cell-mediated vascular remodeling[21]. As shown in Fig. 4C and D, we found that regardless of aspirin intervention or LPS stimulation, the HMGB1 expression of intracellular proteins was basically unchanged. Since HMGB1 protein is a nuclear protein, it will be transferred to cytoplasm when being stimulated, and further stimulation would cause HMGB1 released from cytoplasm to extracellular cells to induce pyroptosis[27]. It was found that the expression of cytoplasmic protein had a significant change—the expression of HMGB1 is increased with the stimulation of LPS and the expression level is decreased after administration.

3.3. Aspirin abolishes LPS-induced disassembly of tight junction proteins via the NLRP3 pathway in MVECs

To prove that aspirin can protect the endothelial function by inhibiting NLRP3 inflammasomes, the gene of *Nlrp3* was knocked down in MVCEs (gRNA) and MCVEs stably expressing g*Nlrp3* were established. The silence efficiency was shown by Western blot analysis shown in Fig. 3A. Compared with the scramble group, the LPS stimulation of the knockdown group showed no obvious inhibition on the expression of ZO1/ZO2 protein, and there was no significant change after aspirin administration (Fig. 3B, C, E, and F).

3.4. Aspirin-restrained activation of NLRP3 inflammasomes is associated with ROS/TXNIP dependent pathway

There are many signal pathways activating NLRP3 inflammasome, such as the activation of NLRP3 inflammasome caused by LPS that induces the increase in ROS release[28]. We hypothesized that aspirin could inhibit the activation of the inflammasome by inhibiting the ROS because of its antioxidant effect. To verify our conjecture, we examined ROS level in cells (Fig. 4A and B). Aspirin can significantly inhibit intracellular ROS production induced by LPS. Under conditions of high ROS levels and oxidative stress, TXNIP dissociates from thioredoxin to associate with NLRP3, activating the inflammasomes to mediate inflammatory signaling[29]. We detected that TXNIP activated NLRP3 through the ROS signaling pathway. As shown in Fig. 4C and D, we found that aspirin obviously decreased TXNIP expression in

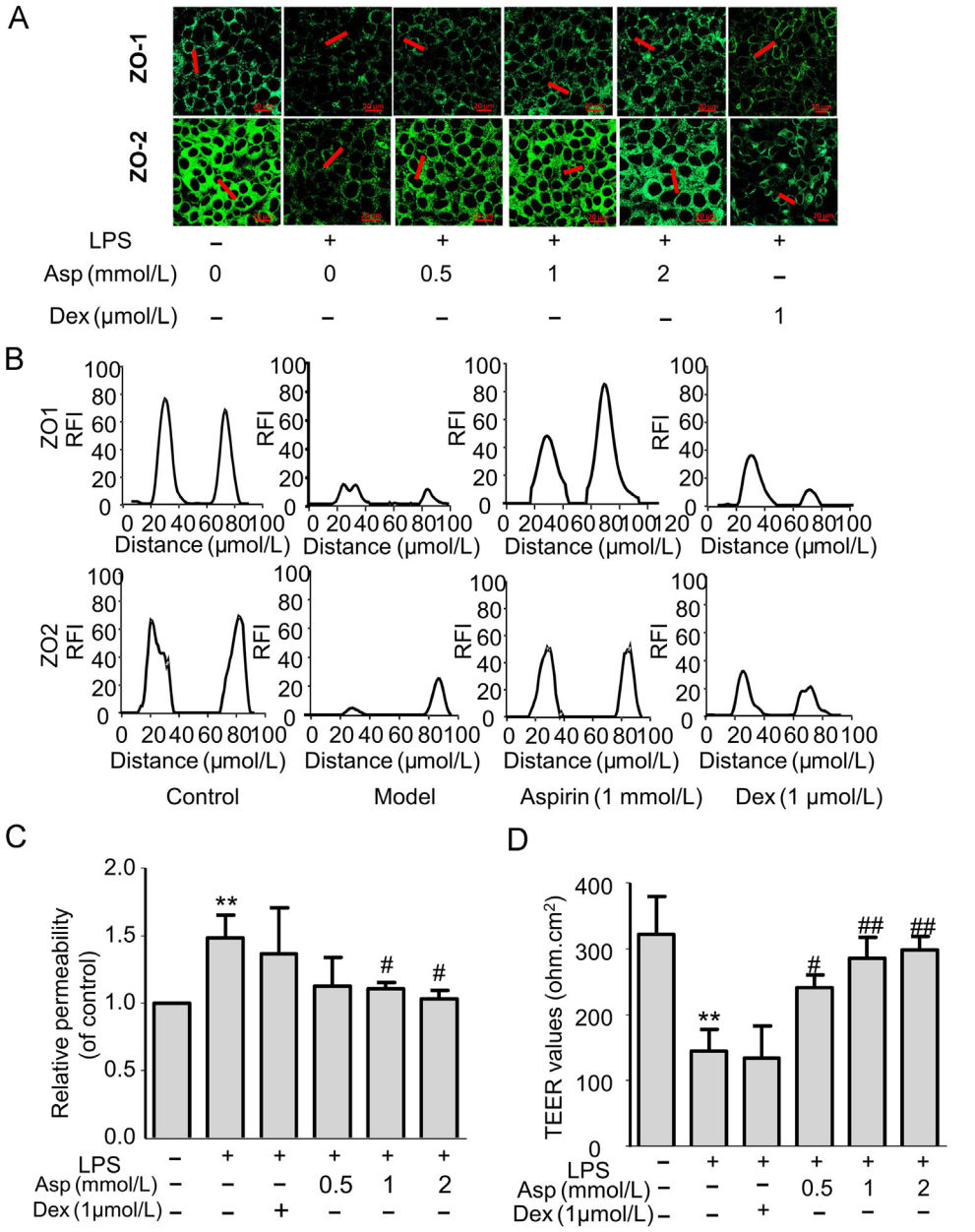

Figure 1 Aspirin repairs the integrity of the endothelium. (A) and (B) The expression of ZO1/ZO2 in endothelial cells was detected by fluorescence microscopy to determine the integrity of endothelial cells ($n = 4$). (C)The integrity of endothelial cells was determined by detecting changes in endothelial cell permeability ($n = 4$). (D) The integrity of endothelial cells was determined by detecting changes in trans endothelial electric resistance ($n = 4$). $^{*}P < 0.05$, LPS *vs*. control, $^{\#}P < 0.05$, aspirin or dexamethasone *vs*. LPS. Scale bar: 20 μm.

a concentration dependent manner. The immunofluorescence colocalization method was used to detect the merge of TXNIP and NLRP3 (Fig. 4E and F), and aspirin obviously decreased the NLRP3 and TXNIP colocalization.

3.5. Aspirin prevents LPS-induced disassembly of tight junction proteins in coronary arterial endothelium

Mice were fed normal chow diet for 8 weeks, and the experiment lasted for a week. After LPS (100 μg/kg) administering for 1 h, aspirin (12.5, 62.5 and 125 mg/kg) was injected intraperitoneally. To determine the success of the vascular endothelial injury model, the damage of endothelial was determined by examining the changes in the gap protein and the permeability of the cell membrane. As shown in Fig. 5A–D, we found that low-concentration and high-concentration aspirin increase expression ZO1/ZO2 after LPS stimulate; that is, these concentrations repair endodermal permeability and inhibit the destruction of the endothelium.

3.6. Aspirin inhibited LPS-induced inflammasome activation and HMGB1 expression in coronary arterial endothelium

Through colocalizing NLRP3/caspase-1 by immunofluorescence, we found that aspirin at low concentration and high concentration

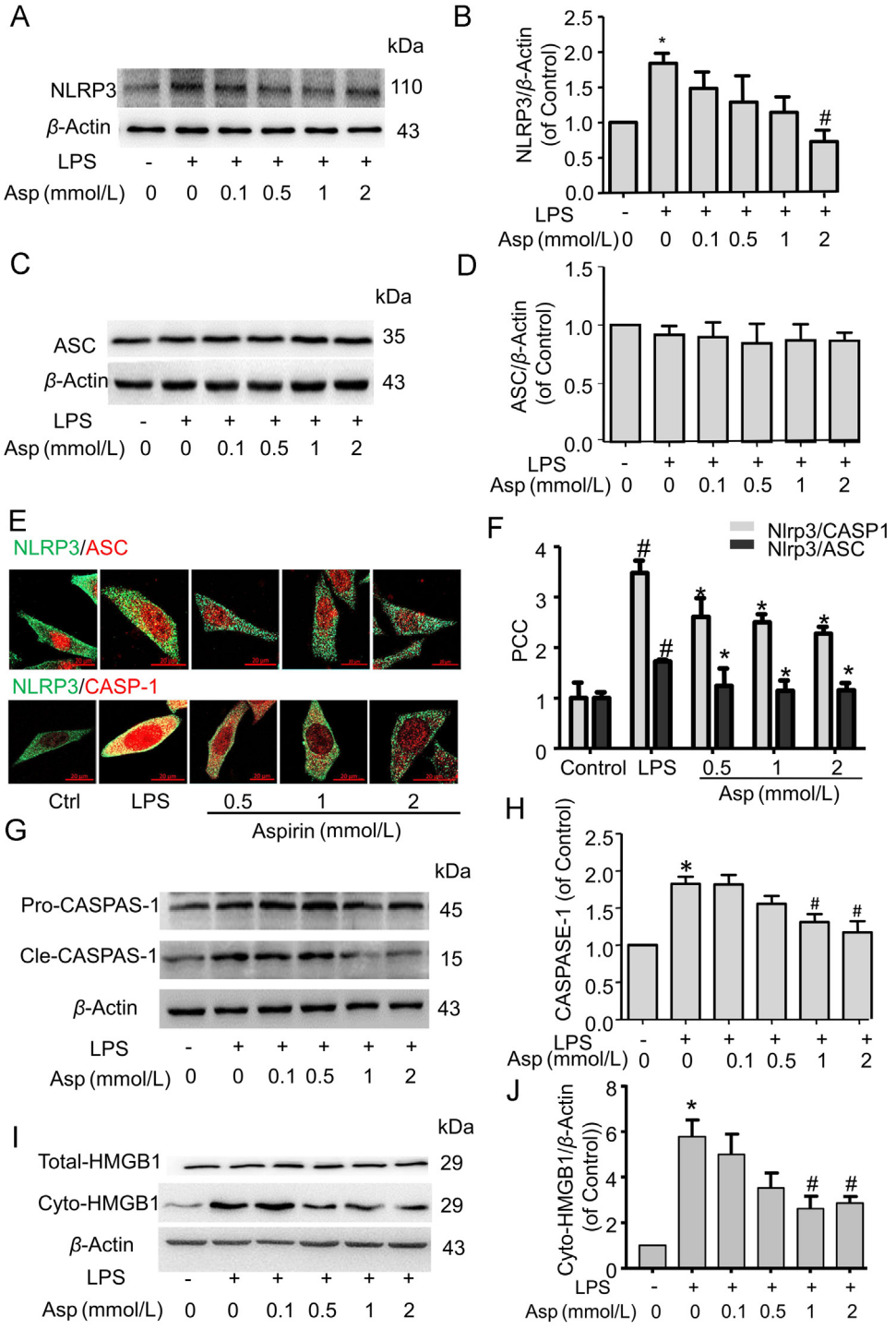

Figure 2 Aspirin inhibits the recruitment and activation of NLRP3 inflammasome. (A) and (B) Western blot analysis of NLRP3 expression in different aspirin concentrations (0.1, 0.5, 1, 2, and 3 mmol/L) after LPS-induced ($n = 4$). (C) and (D) Western blot analysis of ASC expression ($n = 4$). (E) and (F) NLRP3/CASPASE-1 and NLRP3/ASC were identified by confocal microscopy to determine the formation of inflammasome ($n = 4$). (G) and (H) Western blot analysis of pro-caspase-1 and cle-caspase-1 expression ($n = 4$). (I) and (J) Western blot analysis of HMGB1 expression in cells and supernatant ($n = 4$). $^{*}P < 0.05$, LPS *vs.* control, $^{\#}P < 0.05$, aspirin *vs.* LPS.

inhibited the formation of NLRP3 inflammasome, but the effects of dexamethasone and aspirin at middle concentration were not obvious (Fig. 6A and B). The activation of inflammasomes in each group after stimulation and administration was shown in Fig. 6C and D. Similarly, we found that aspirin at low concentration and high concentration inhibited the activation of inflammation in coronary arterial endothelium (Fig. 6C). When serum HMGB1 was detected by ELISA, low concentration was still effective, while the other two concentrations were basically ineffective, but dexamethasone showed a different trend (Fig. 6E).

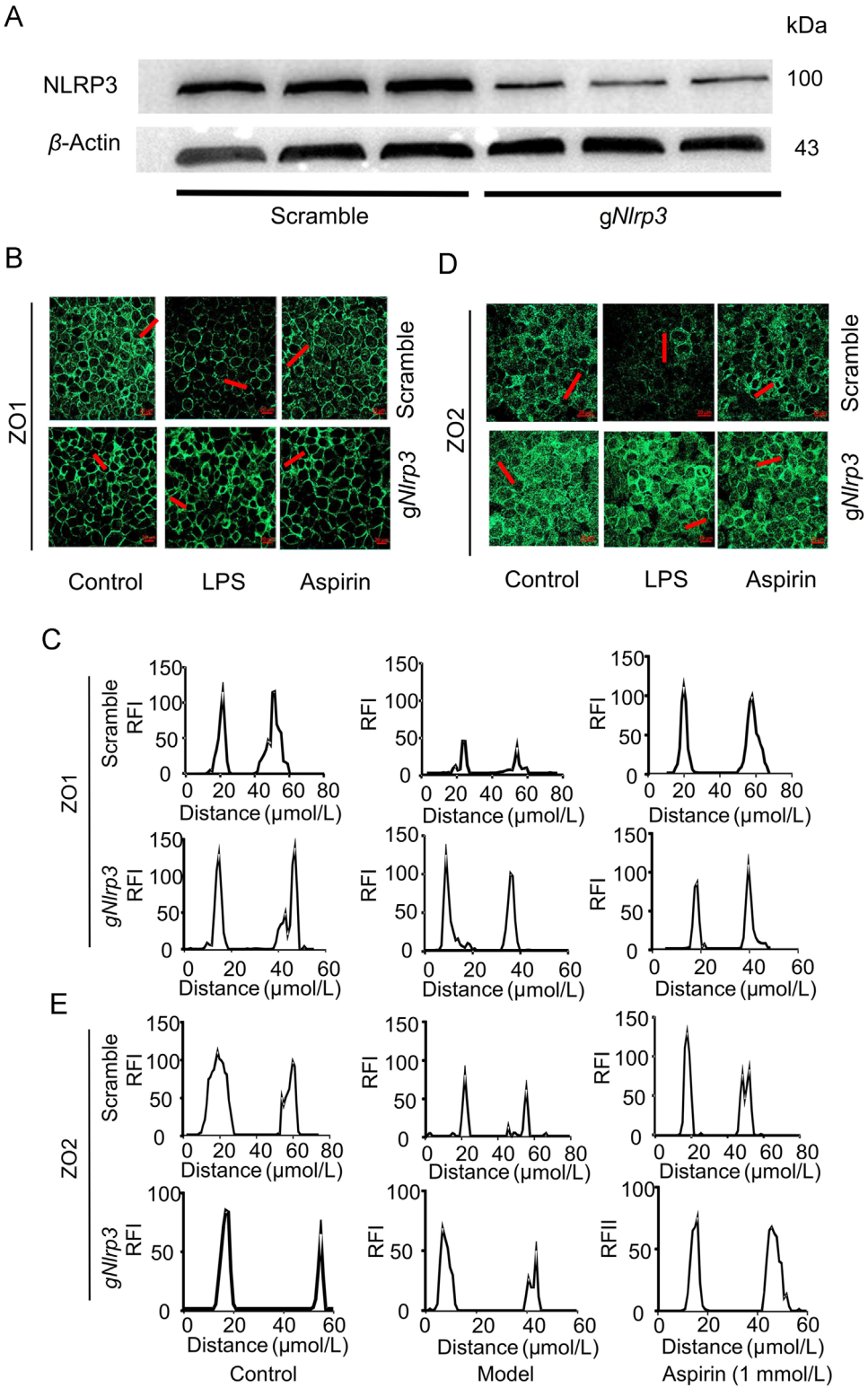

Figure 3 Aspirin has no therapeutic effect on gRNA *Nlrp3* cells. (A) *Nlrp3* gene knockdown succeeded. (B) and (C) Detecting the connection proteins ZO1 of g*Nlrp3* endothelial cells, the expression was unchanged regardless stimulation or administration ($n = 4$). (D) and (E) Detecting the connection proteins ZO2 of g*Nlrp3* endothelial cells, the expression was unchanged regardless stimulation or administration ($n = 4$). Aspirin was determined to protect endothelial integrity by inhibiting NLRP3 inflammasome.

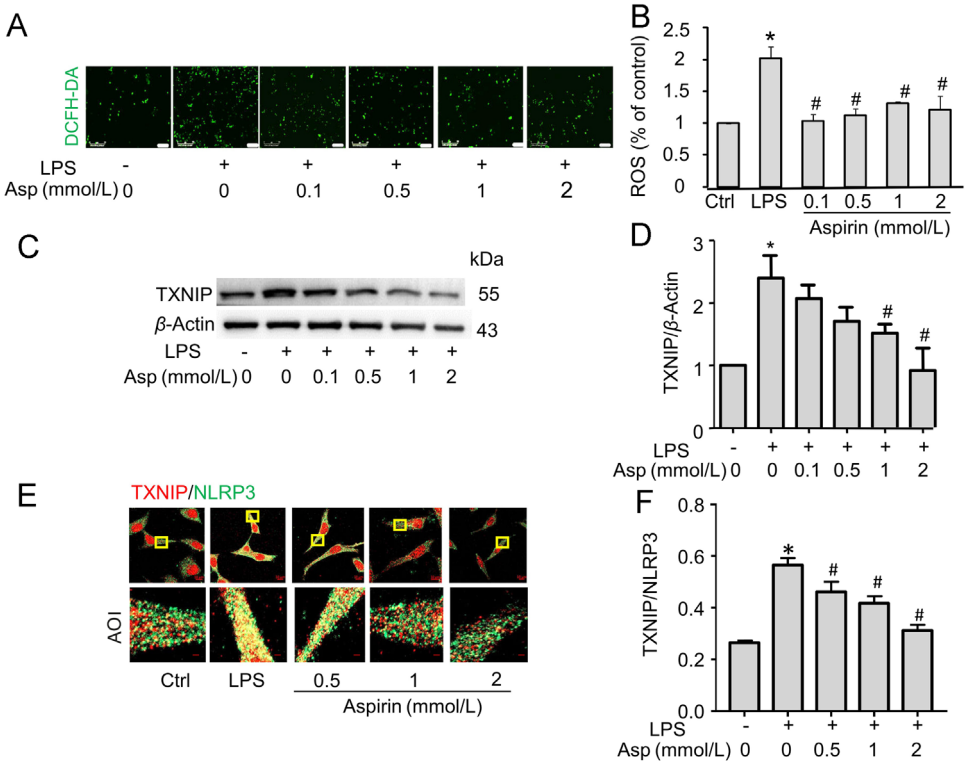

Figure 4 Aspirin inhibits the activation of NLRP3 inflammasome by depressed the ROS/TXNIP pathway. (A) and (B) Intracellular ROS release was detected by Time Live-Cell Imaging System ($n = 4$). (C) and (D) Western blot analysis of TXNIP expression ($n = 4$). (E) and (F) NLRP3/TXNIP were identified by confocal microscopy to determine the recruitment of inflammasome ($n = 4$). $^{*}P < 0.05$, LPS *vs*. control, $^{\#}P < 0.05$, aspirin *vs*. LPS.

4. Discussion

The present study demonstrates that inflammasomes participate in endothelial dysfunction[30,31], and ECD is the bedrock of diverse cardiovascular, renal, and metabolic diseases. Therefore, we believe the prevention and treatment of endothelial cell is the best choice to treatment cardiovascular diseases at the early stage. Clinically, it has proven that the long-term use of low-concentration aspirin can effectively prevent and treat cardiovascular diseases[32]. But there are debates about low-dose aspirin treatment with cardiovascular protection. The effects of aspirin on risks of vascular events have been recently described[33]. And one common adverse effect is bleeding. More significant side effects include gut bleeding, stomach ulcers, and stomach bleeding. Bleeding risk is greater among those who are older, drink alcohol, take other NSAIDs, or are on other blood thinners[34,35]. At the same time, aspirin is also recommended to treat cardiovascular disease if you are at high risk of factor, such as gender, toxin and assessments of cholesterol, blood pressure and other health indicators[36–38]. Our study also explored that aspirin can repair the integrity of the endothelial cytomembrane under the highly risk factors stimulation, thus can prevent and cure cardiovascular diseases. These researches suggest that appropriate dose aspirin used for prevention against secondary cardiovascular event can result in improved patient. As a simple and inexpensive prophylactic measure for cardiovascular event prevention, aspirin application should be carefully considered in all appropriate at-risk adult patients[39]. So it is meaningful to research the mechanism of aspirin.

Endothelial inflammasome was induced by LPS to confirm whether aspirin can protect endothelial integrity or not. We demonstrated that aspirin can alleviate the vascular endothelial damage by inhibiting the activation of NLRP3 inflammasome. We also found that aspirin can interdict the ROS/TXNIP pathway to inhibit activation of the NLRP3 inflammasome.

Clinically, it has been verified that long-term use of low-dose aspirin mitigates cardiovascular diseases, but the signaling pathways is still unclear. Aspirin, the same as the majority of NSAIDs, is thought to exert its anti-inflammatory effect by inhibition of cyclooxygenase (COX) enzymes that regulate the production of prostaglandins[40]. We found that the low-concentration aspirin has the significant effect *in vitro* on restoring the endothelial junction protein ZO1/ZO2 (Fig. 1A). The tight junction proteins can create a paracellular barrier to protect vascular from the external environment. But dexamethasone, a classic anti-inflammatory drug, has no effect on the recovery of the endothelial junction protein. Endothelial membrane permeability and TEER have the same results as junction proteins (Fig. 1C and D). The results of endothelial cell permeability, membrane resistance and endothelial junction proteins attest that aspirin has the effect on restoring tight junction damage. Endothelial junction proteins have closely related with the NLRP3 inflammasome, so we also detected the NLRP3 inflammasome formation and activation. We believe that aspirin is superior to other anti-inflammatory drugs in ameliorating endothelial dysfunction. As the data shown, we confirmed that aspirin can effectively inhibit the formation and activation of NLRP3 inflammasome (Fig. 2). When we research the NLRP3 inflammasome, we should first confirm that it is activated. Since caspase-1 is an important marker of NLRP3 inflammasome activation, it is necessary to detect cle-caspase-1. In this study, we used three methods to detect the cle-caspase-1 activation. As the data shown (Fig. 2G), Western blot not only detected the

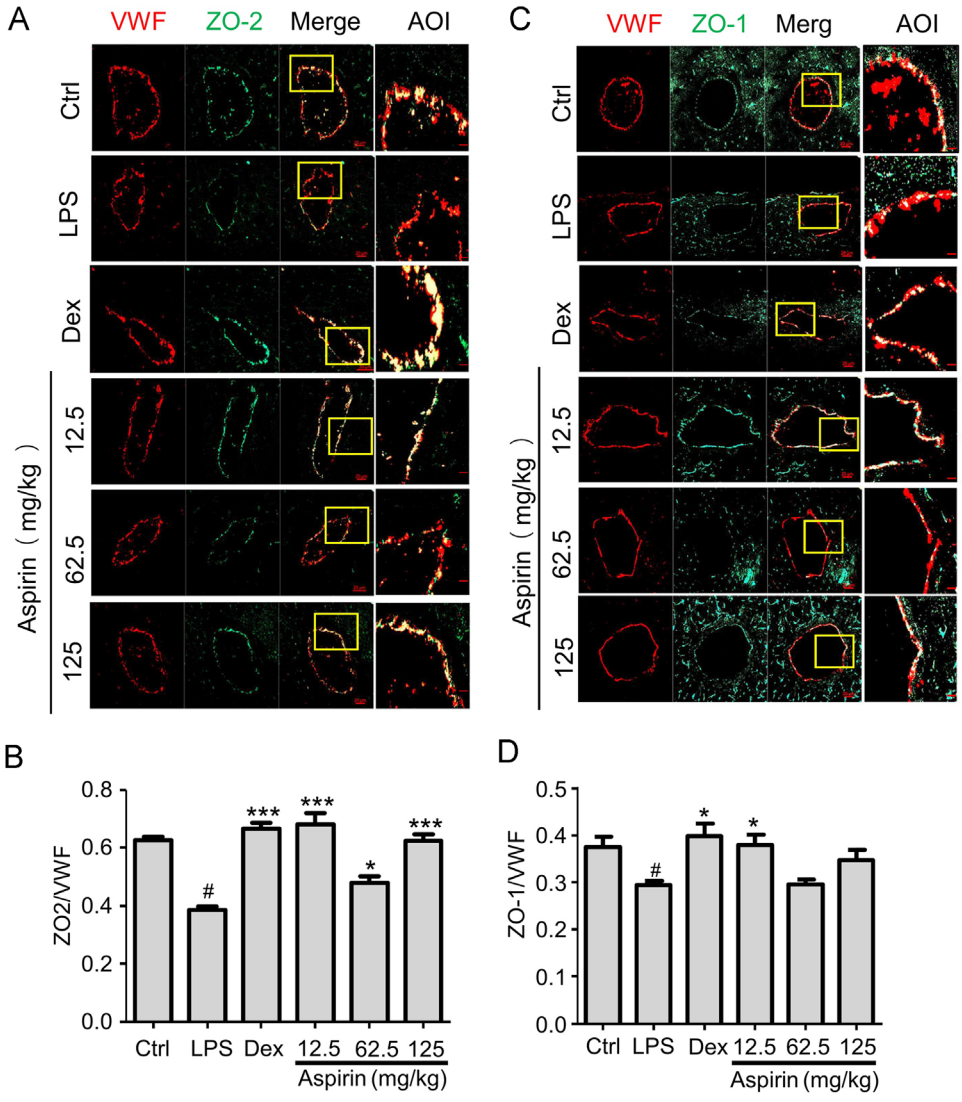

Figure 5 Aspirin protects the integrity of the endothelium *in vivo*. (A) ZO2/VWF was identified by confocal microscopy with Frozen sections of mouse hearts, the merged images displayed yellow dots or patches indicating the colocalization of ZO2 (green) with VWF (red). (B) ZO1/VWF was identified by confocal microscopy. (C) Summarized data showing the colocalization coefficient of ZO2 with VWF ($n = 6$). (D) Summarized data showing the colocalization coefficient of ZO1 with VWF ($n = 6$). $^{*}P < 0.05$, LPS *vs*. control, $^{\#}P < 0.05$, aspirin *vs*. LPS.

activated caspase-1, but also the precursor caspase-1. The second method used the ELISA Kit (Bio Vision, K110, USA) to recognize the sequence YVAD, and YVAD is the specific recognition sequence for IEC/caspase-1. The last method is immunofluorescence, in which FAM FLICA™ caspase-1 assay kit (Immuno Chemistry Technologies, USA) was used to detect active caspase-1 by immunofluorescence. The FLICA is a specific green fluorescent probe to detect activation caspase-1. Three different methods were used to detect the same index from different angles, and we got the results in the same trend. As these data shown (Fig. 2G and Supporting Information Fig. S1A and B), the activation of caspase-1 is increased by LPS stimulation, but is concentration dependently decreased after aspirin administrated. HMGB1 is primarily located in the nucleus of most cells, including macrophage and endothelial cells. Cle-caspase-1 is one of the inducers of HMGB1 release. When stimulated, HMGB1 transferred from the nucleus to the cytoplasm and released to extracellular. Based on the results, we found that aspirin can significantly inhibit the expression of HMGB1 in the cytoplasm, which means that low-concentration aspirin can significantly inhibit the activation of HMGB1. But once the cells die, the contents of the cytoplasm disperse outside the cell, greatly increasing inflammation[41]. In addition, it has been reported that HMGB1 can activate the receptor for advanced glycosylated end products (RAGE) on the cell membrane. And HMGB1–RAGE axis could lead to the decrease of the tightness of endothelial junction protein ZO1/ZO2, and ultimately, the damage to endothelial integrality[42]. Our previous study has showed that NLRP3 inflammasome resulted in disruption of endothelial tight junction and endothelial permeability, which was dependent on the increased release of HMGB1[43]. Changes about HMGB1 were consistent with recent findings that endothelial tight junction disruption is closely related to the HMGB1–RAGE axis[44]. In the present work, our data confirm that aspirin decreases HMGB1 release from endothelial cells and NLRP3 inflammasome activation, which could lead to secretion of HMGB1 (Fig. 2I). Other

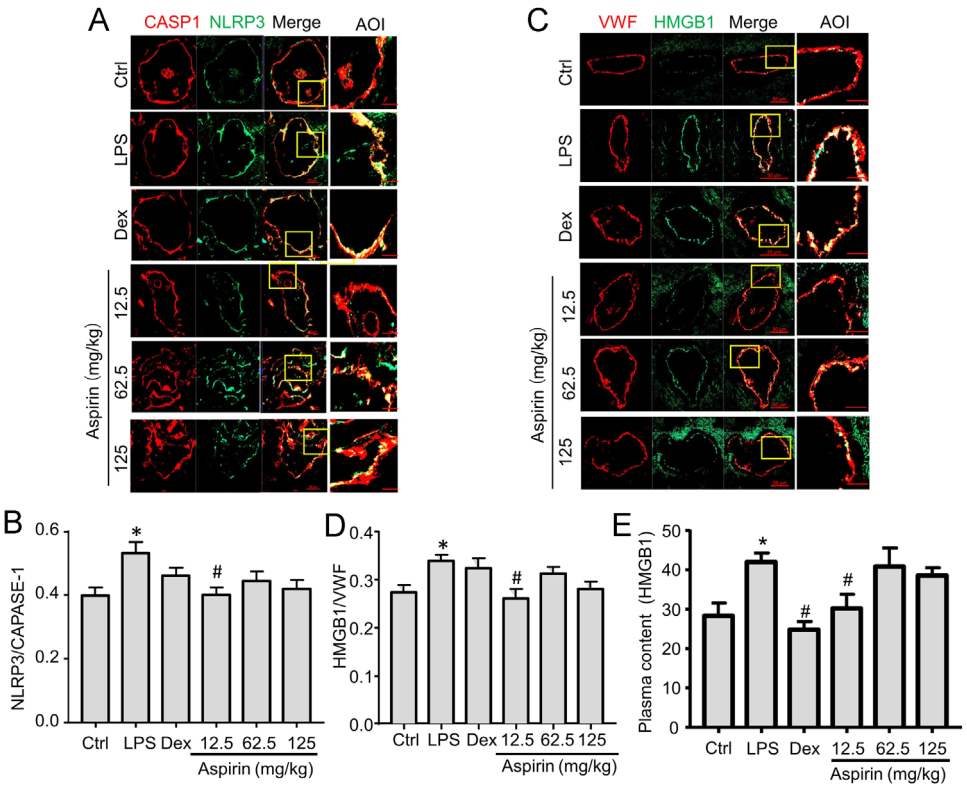

Figure 6 Aspirin suppressed LPS-induced activation of NLRP3 inflammasome *in vivo*. (A) and (B) NLRP3/CASPASE-1 was identified by confocal microscopy to determine the formation of inflammasome *in vivo* ($n = 6$). (C) Frozen sections of mouse hearts were stained for HMGB1, and Alexa555-conjugated antibodies against an endothelial cell marker VWF in coronary arteries. The merged images displayed yellow dots or patches indicating the colocalization of HMGB1 (green) with VWF (red). (D) Summarized data showing the colocalization coefficient of HMGB1 with VWF ($n = 6$). (E) The contents of HMGB1 in serum were detected by ELISA kit ($n = 6$). $^{*}P < 0.05$, LPS *vs.* control, $^{\#}P < 0.05$, aspirin *vs.* LPS.

studies also demonstrate that the activation and release of HMGB1 are related to the activation of caspase-1[21]. When HMGB1 is released, it would bind with RAGE to form the activated HMGB1–RAGE axis[45], then the axis further destroys the endothelial junction protein, resulting in endothelial dysfunction. For further proof that the HMGB1 is the key internal molecular mechanism for this hypothesis, we added a set of experiment (Supporting Information Fig. S2). As the data shown, the expression of RAGE increased after LPS stimulation, while it decreased with the increase of the concentration of aspirin administered. HMGB1 could affect RAGE and eventually change the HMGB1–RAGE axis. When the gene of *RAGE* was knocked down (Fig. S2B), the expression of endothelial junction protein ZO1 did not changed, either by LPS stimulation or by aspirin administer. Additionally, the junction of endothelium was complete (Fig. S2C). The data further explains the relationship between HMGB1–RAGE axis and endothelial junction protein, and the activation of HMGB1–RAGE axis causes damage to the integrity of endothelial junction proteins.

To further elucidate the role of NLRP3 inflammasome on LPS-induced endothelial dysfunction in low-concentration aspirin group, we knocked down the *Nlrp3* gene and detected the junction protein. We demonstrated that low-concentration aspirin inhibits the NLRP3 signaling pathway to alleviate endothelial dysfunction (Fig. 3), because ZO1/ZO2 of g*Nlrp3* cells has no significant change in the normal group, model group, or administration group. Due to the antioxidant and anti-inflammatory properties of the aspirin, we hypothesized that the aspirin suppresses the NLRP3 inflammasome by inhibiting the ROS/TXNIP pathway, so we selected LPS as the stimulus. Further, the experimental results also prove our hypothesis (Fig. 4).

In our cell experiments, we found that dexamethasone can restore cell proliferation by weakening the cytotoxicity that LPS stimulated (data no shown), but it did not ameliorate endothelial dysfunction. We hypothesized that it is not related to NLRP3 inflammasome signaling pathway. *In vivo*, we also found that the positive control dexamethasone did not have a significant effect on the inhibition of the formation and activation of inflammasome; however, there was an obvious protective effect on the integrity of the endothelium (Figs. 5 and 6). Dexamethasone is a hormone anti-inflammatory drug. Its function is to regulate inflammatory cells *in vivo*, which may be the reason why dexamethasone does not protect endothelial integrity *in vitro*, but does *in vivo*.

We found an interesting phenomenon that high concentration of aspirin has different results compared with low concentration of aspirin on cell proliferation and cytotoxicity (data no shown). Low concentrations of aspirin can significantly restore the inhibition of cell proliferation induced by LPS; however, high concentrations promoted cell death (data not shown). It has been reported that high concentrations of aspirin could be used to treat cancer by promoting apoptosis[40,46]. As a nonselective inhibitor of COX enzyme, the inhibitory function of aspirin was different at different concentrations. COX1 is expressed constitutively in most tissues and regulates basal levels of prostaglandins, which control platelet activation and protect the lining of the gastrointestinal tract[46]. In contrast, COX2 is inducible and responsible for releasing prostaglandins after an infection or injury or in cancer development[47].

There are evidences that low concentration of aspirin prevents cardiovascular diseases due to the inhibition of COX1 and the effect of anticoagulation[48]. However, in our study, low concentration of aspirin protected the endothelial function by inhibiting the formation and activation of inflammasomes. We also detected the expression of COX2 and found that low-concentration aspirin could decrease its expression (data not shown). Therefore, we believe that it has a closely relationship between aspirin and its anti-inflammatory effect in the treatment of cardiovascular diseases. Given different concentrations of aspirin, we found that low and high concentrations of aspirin were effective, but medium concentration was ineffective (Figs. 5 and 6). Because cyclooxygenase has a dual character, being proinflammatory and anti-inflammatory, we assume that the medium concentration did unselectively inhibit COX, leading to its main proinflammatory role; or its anti-inflammatory effects were weaker than its proinflammatory effects, leading to the failure to exert the effect of vascular endothelium protection. The low-concentration aspirin rarely causes cell death, but it also has a significant anti-inflammatory effect (Fig. 6). In the detection of HMGB1, we found that the low-dose aspirin was effective and had the best effect in both the vascular endothelium and serum. The ineffective concentration was still the medium-level doses, and the high concentration was effective but not obvious. Although it has been shown that the expression of COX2 increases during endothelial cell inflammation, the association with the NLRP3 signaling pathway remains unclear. The aspirin treatment dose-dependently inhibits LPS-induced the NLRP3 inflammasome formation and activation and enhance ZO1/2 expression *in vitro*. It means aspirin is effective in protecting vascular endothelium (Figs. 1 and 2). Previous studies have also suggested that low-dose aspirin has anti-inflammatory effect[49–51]. And one of the interesting phenomena is the low-dose and high-dose aspirin both can restore the endothelial dysfunction, but the low dose can inhibit the activation of NLRP3 inflammasome, the high doses of aspirin have no effect. There are evidences that low-dose aspirin is as effective as high-dose aspirin in treating vascular dysfunction[52], and high-dose aspirin still commonly prescribed after coronary events due to concerns about the efficacy of lower doses[53]. Although they are effective for vascular dysfunction, the mechanism of different concentration is different *in vivo*[54]. Our data shows that the mechanism of protecting blood vessels by low-dose aspirin is the effect of anti-NLRP3 inflammasome activation. As we know, there are factors to affect the activation of NLRP3 inflammasome *in vivo*. The precision and complexity of body can complicate the activation factors that affect the NLRP3 inflammasome such as the tissues and tissues interactions, cells and cells communication, all of which could lead to the progress of inflammasome activation. That may be the reason why there is no dose-dependence *in vivo*. But low-dose aspirin could ameliorate the dysfunction of the endothelial layer and restore the expression of the endothelial junction protein ZO1/2. Meanwhile, the efficacy of aspirin in inhibiting the activation of NLRP3 inflammasomes and restoring endothelial homeostasis by inhibiting NLRP3 inflammasomes has been confirmed *in vitro* and *in vivo*. The destruction of endothelial homeostasis is also a prerequisite for cardiovascular diseases, so we considered that aspirin has significant advantage in the early prevention and treatment of cardiovascular diseases.

5. Conclusions

Aspirin was explored to repair the integrity of the endothelial cytomembrane. It turns out that the action involves its own anti-inflammatory and antioxidant effects. By inhibiting the release of ROS, aspirin can depress the signaling pathway of ROS/TXNIP to activate NLRP3 inflammasome, thereby inhibiting the activation and release of cytokine HMGB1 and thus restoring the tight junction proteins and permeability. However, it is necessary to further study whether it has a relationship between the inhibitory effect of aspirin on activation of NLRP3 inflammasome and other mechanisms associated with NLRP3 inflammasomes to prevent the cardiovascular diseases. It is the ultimate aim of aspirin research to expand the efficacy of its potential action target and reduce its adverse reactions.

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Appendix A. Supporting information

Supporting data associated with this article can be found in the online version at https://doi.org/10.1016/j.apsb.2019.02.008.

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