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Original Paper

Effect of Paclitaxel+Hirudin on the TLR4-MyD88 Signaling Pathway During Inflammatory Activation of Human Coronary Artery Smooth Muscle Cells and Mechanistic Analysis

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Key Words
Human coronary smooth muscle cells • TLR4/MyD88/NF-κB pathway • Intra-stent restenosis • Thrombosis • Western blot analysis

Abstract
Background/Aims: Approximately 10%-20% of patients with acute cardiovascular disease who have received coronary intervention suffer restenosis and high inflammation. The stent compound paclitaxel+hirudin was prepared for the treatment of post-intervention restenosis. This study aimed to explore the anti-inflammatory and anti-restenosis mechanisms of paclitaxel+hirudin with regard to the TLR4/MyD88/NF-κB pathway. ***Methods:*** Human coronary artery smooth muscle cells (HCASMCs) at 4-6 generations after *in vitro* culture were used as a model. Lipopolysaccharide (LPS) was used as an inducer to maximally activate the TLR4/MyD88/NF-κB inflammation pathway. After MyD88 knockdown and selective blocking of MyD88 degradation with epoxomicin, the effects of paclitaxel+hirudin stenting on key sites of the TLR4/MyD88/NF-κB pathway were detected using ELISA, Q-PCR, and western blot analysis. ***Results:*** LPS at 1 μg/mL for 48 h was the optimal modeling condition for inflammatory activation of HCASMCs. Paclitaxel+hirudin inhibited the levels of key proteins and the gene expression, except for that of the MyD88 gene, of the TLR4-MyD88 pathway. The trend of the effect of paclitaxel+hirudin on the pathway proteins was similar to that of MyD88 knockdown. After epoxomicin intervention, the inhibitory effects of paclitaxel+hirudin on the key genes and proteins of the TLR4-MyD88 pathway were significantly weakened, which even reached pre-intervention levels. Paclitaxel+hirudin affected the MyD88 protein in a dosage-dependent manner. ***Conclusion:*** The paclitaxel+hirudin compound promotes MyD88 degradation in the TLR4/MyD88/NF-κB pathway to reduce the activity of TLR4 and NF-κB p65 and to weaken the LPS-initiated inflammatory reactions of IL-1β, IL-6, and TNF-α.

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Introduction

Post-operative restenosis and thrombosis in the stent are major challenges in the field of vascular intervention. Although stents, a next-generation treatment, have decreased the incidence of thrombosis to approximately 0.2% [1], they fail to effectively control restenosis, which seriously affects the quality of life and long-term prognosis of patients after intervention. Therefore, minimizing the risk of in-stent restenosis and thrombosis has become the focus of research on and development of intervention apparatuses [2-6].

For post-intervention restenosis, stents/balloons coated with paclitaxel (a microtubular inhibitor) have been extensively applied in clinical practice due to their safety and efficacy. However, after treatment with stents/balloons coated with paclitaxel alone, the restenosis incidence remains as high as 10%-20%; even worse, delayed thrombosis further limits the popularization of their application [7-10]. Therefore, further research and development on drug stents/balloons is needed. With a deepening of their understanding of percutaneous coronary intervention (PCI), scholars have reached a consensus on the positive correction between post-PCI inflammation and restenosis, and methods for restenosis prevention via anti-inflammatory treatment have emerged. Anti-inflammation has become a new strategy for post-PCI restenosis prevention [11-14]. In recent years, an "endothelial-friendly" stent has been developed in which recombinant hirudin and iloprost are combined. The anti-thrombotic effect of this treatment has been proven by animal experiments, and it has also effectively reduced the coronary restenotic area [15]. The use of this compound has inspired an important research idea: using two monomers for balloon/stent coating may have a stronger effect on reducing restenosis and preventing thrombosis after an operation than the use of monomers alone.

Based on this idea as well as new results from pharmacological research, the current team prepared a new compound of paclitaxel and hirudin. Pacilitaxel is a microtubular inhibitor that blocks cell division, and hirudin is a thrombin inhibitor that has confirmed anti-inflammatory and anti-thrombotic effects. These monomers were combined at an optimal ratio and then used for stent/balloon coating. To evaluate the feasibility and safety of paclitaxel+hirudin-coated stents/balloons, the current team implanted microporous paclitaxel+hirudin-loaded stents into the rabbit abdominal aorta and observed the effect of the compounds on the vascular intima. The results showed that the stents decreased the risks for restenosis and in-stent thrombosis, and the paclitaxel+hirudin-eluting balloons also exhibited favorable characteristics, with stable drug release and satisfactory vascular reactivity. Additionally, the safety and effectiveness of this treatment were comparable to those of the paclitaxel-eluting balloons currently available on the market [16, 17]. To further explore the anti-restenosis mechanisms of the paclitaxel+hirudin compound, the current team adopted a cellular co-culture system to simulate the mechanism of action of the compound on rabbit smooth muscle cells and endothelial cells. The effects of paclitaxel+hirudin at different concentrations on the incorporation rate of tritiated thymidine and on the protein level and migration rate of proliferating cell nuclear antigens were observed, and the median effective inhibitory concentrations of the compound on the proliferation and migration of the smooth muscle cells and endothelial cells were determined. Based on these studies, an appropriate combination ratio between paclitaxel and hirudin was determined. Furthermore, these studies showed that the compound blocked the proliferation and migration of smooth muscle cells by inhibiting inflammatory reactions to exert its anti-restenosis effects [18, 19]. However, the mechanism underlying the anti-restenosis effect of the compound remains to be explored.

Increasing studies have shown that tissue damage can induce congenital immune reactions to activate Toll-like receptor protein 4 (TLR4), which stimulates the formation of atherosclerotic plaques and the reconstruction of vascular tunica adventitia [20-22]. Although the signal transduction pathways of most members of the TLR family are MyD88-dependent, TLR4 possesses MyD88-dependent and independent pathways for signal transduction [23-25]. The signal transduction of the MyD88-dependent pathways is primarily intracellular and

results in the transfer of NF-κB to the nucleus; this translocation initiates the transcription of inflammatory cytokines such as TNF-α, IL-1β and IL-6 to amplify inflammatory reactions in a cascade manner, inducing vascular injury and ultimately leading to post-operative restenosis. In contrast, the MyD88-independent pathways are primarily responsible for the expression of lipopolysaccharide (LPS)-induced interferons, interferon-inducible protein-10 and interferon regulatory factor-1 and the maturation of dendritic cells, which involves TRAM and TRIF. In a pilot intervention study on a coronary artery disease model in miniature swine, the current team found that paclitaxel+hirudin-coated stents significantly decreased the expression of TLR4 and notably down-regulated the protein level of MyD88 in local vascular tissue compared with those of the sham operation group; however, this treatment did not show significant inhibitory effects on TRAM and TRIF. These results indicate that the compound inhibits the expression of the downstream NF-κB and related genes by primarily blocking the intracellular MyD88-dependent signal transduction pathways of TLR4, thereby effectively inhibiting inflammatory reactions; this process might be one of the mechanisms underlying the preventive effect of paclitaxel+hirudin on post-PCI restenosis.

Based on the aforementioned findings, this study focused on the classic TLR4-MyD88 inflammatory pathway using human coronary artery smooth muscle cells (HCASMCs) as a model to test the hypothesis that the anti-restenosis effect of the paclitaxel+hirudin compound is mediated by regulating the TLR4-MyD88 pathway. To assess this hypothesis, we established an LPS-induced inflammation activation model of HCASMCs to observe the regulatory effect of the compound on key components of the TLR4-MyD88 pathway. Epoxomicin (a proteasome inhibitor) intervention was then performed to explore the activation status of TLR4-MyD88-associated molecules under different conditions to verify the exact targets of the compound.

Materials and Methods

Materials

HCASMCs at the second generation (art. no. FC-0031) were purchased from Lifeline (Madison, WI, USA). Paclitaxel preservative liquid (100 μl; art. no. KGA8221) was purchased from KeyGEN BioTECH (Nanjing, China), and research-standard natural hirudin lyophilized powder (500AT-U/g) was purchased from Shengtianyu Biotech (Wuhan, China). Epoxomicin was a product of Sigma, St. Louis, MO, USA.

Cell culture in vitro

HCASMCs were cultured in 6 ml pre-heated antibody-free culture medium overnight. The medium was replaced. When confluence reached 90%, cell passaging was performed. The medium was removed, and the cells were washed twice with 5 ml of PBS. Approximately 0.5 ml of EDTA was applied to the culture for 1-2 min. The culture bottle was lightly shaken, and the same volume of complete medium was added to terminate the digestion. The cell suspension was centrifuged at 900 rpm for 5 min. The supernatant was removed, and 2 ml of complete medium was added for resuspension. The cell suspension was cultured with pre-heated medium at 37 °C at a ratio of 1:3. HCASMCs at generations 4-6 were used in later experiments.

Inflammation activation model establishment

Comprehensive techniques were used to determine the optimal modeling conditions for LPS in which LPS-stimulated HCASMC inflammatory activation was high, and the gene expression and protein levels of key components and downstream inflammatory products of the TLR4/MyD88/NF-κB pathway reached maximum values.

MTT method

The non-toxic concentration range of LPS was determined using the MTT method [26, 27]. The cells were divided into 7 groups: the control group (100 μl HCASMC complete medium, without LPS), the LPS-0.01 μg/mL group, the LPS-0.1 μg/mL group, the LPS-0.5 μg/mL group, the LPS-1 μg/mL group, the LPS-10 μg/mL group, and the LPS-100 μg/mL group. Six parallel wells were used for each group. The LPS groups

were cultured with 100 μl LPS at the corresponding concentrations. All groups were cultured for 24 h. Approximately 20 μl of 5 mg/mL MTT solution was applied to each well for a 4-h culture. DMSO at 150 μl was then added. The optical density of each well was read at 492 nm (MK3; Thermo, Waltham, MA, USA). Cell vitality was calculated based on the following formula: cell vitality = the OD value of the LPS intervention group/that of the control group.

Enzyme-linked immunosorbent assays (ELISA)

The effects of LPS-induced inflammatory activation on the protein levels of the downstream inflammatory products IL-6, TNF-α, and IL-1β were detected using the ELISA method. Based on the outcomes of the MTT assays, 5 non-toxic LPS concentrations and 3 intervention time points were selected. Cells were divided into 16 groups: the control group, the 0.01 μg/mL LPS-6 h group, the 0.1 μg/mL LPS-6 h group, the 0.5 μg/mL LPS-6 h group, the 1 μg/mL LPS-6 h group, the 10 μg/mL LPS-6 h group, the 0.01 μg/mL LPS-24 h group, the 0.1 μg/mL LPS-24 h group, the 0.5 μg/mL LPS-24 h group, the 1 μg/mL LPS-24 h group, the 10 μg/mL LPS-24 h group, the 0.01 μg/mL LPS-48 h group, the 0.1 μg/mL LPS-48 h group, the 0.5 μg/mL LPS-48 h group, the 1 μg/mL LPS-48 h group, and the 10 μg/mL LPS-48 h group. The procedures were strictly performed in accordance with the instructions described in the kits (Uscan Life, Wuhan, China). Protein concentrations were calculated using CurveExpert 1.30 software.

Quantitative polymerase chain reaction (q-PCR)

The effects of LPS intervention on the gene expression of TLR4, MyD88, NF-κB p65, IL-6, TNF-α, and IL-1β were detected using q-PCR. Based on the outcomes of MTT assays and ELISAs, the concentration ranges and experimental time points of LPS were further narrowed. In this experiment, two LPS concentrations and two experimental time points were selected. Cells were divided into five groups: the control group, the 0.5 μg/mL LPS-24 h group, the 0.5 μg/mL LPS-48 h group, the 1 μg/mL LPS-24 h group, and the 1 μg/mL LPS-48 h group.

Total RNA was obtained according to the instructions described in the kit (art. no. RP1202; BioTeke, Beijing, China). The primers were designed and synthesized by OligoBio, Beijing, China. The reverse transcription kits used were Promega products (Madison, WI, USA; art. no. A3500). The obtained cDNA was diluted 5 times with 80 μl RNA-free water. The 25 μl reaction system used contained 13 μl PCR Mix, a 2 μl mixture of upstream and downstream primers, and 10 μl of diluted cDNA. The amplification conditions included 95 °C for 10 min followed by 40 cycles of 95 °C for 30 s, 55 °C for 1 min and 72 °C for 1 min. The $2^{-\Delta\Delta Ct}$ method was used to compare the target gene in the LPS group to that in the control group using the following formula: ΔΔCt = (the cycle number of the target gene in the experimental group – that of GAPDH in the same group)/(the cycle number of the target gene in the control group – that of GAPDH in the control group).

Western blot analysis

The effects of LPS-induced inflammatory activation on the protein levels of TLR4, MyD88, and NF-κB p65 were observed using western blot analysis. Based on the outcomes of MTT assays and ELISAs, two LPS concentrations and two intervention times were selected. The cells were divided into five groups: the control, 0.5 μg/mL LPS-24 h, 0.5 μg/mL LPS-48 h, 1 μg/mL LPS-24 h, and 1 μg/mL LPS-48 h groups. Briefly, cells were scraped off, and total protein was routinely extracted. After denaturation, the protein sample was fractionated on SDS-PAGE and electrotransferred to a PVDF membrane. The membrane was treated with 5% skim milk at room temperature for 1 h and incubated with primary antibodies at 4 °C overnight. After the membrane was washed with TBST (10 min × e times), it was incubated with HRP-conjugated goat anti-mouse antibody for IgG at 37 °C for 1 h. After another wash with TBS-T (10 min × 3 times), the membrane was detected by chemiluminescence.

Paclitaxel+hirudin experiment

ELISA, q-PCR, and western blot analysis were used to detect the effects of the compound on the gene expression and protein levels of key components and downstream inflammatory products of the TLR4/MyD88/NF-κB axis to preliminarily identify its anti-inflammatory action sites.

MTT

The non-toxic concentration range of the paclitaxel+hirudin compound for HCASMCs was screened using the MTT method (cell vitality, no lower than 90%). Based on previous studies [28, 29], seven combination ratios of paclitaxel and hirudin were used, and the cells were divided into eight groups: the control (paclitaxel+hirudin-free), 1 μmol/L paclitaxel+0.0125 mg/mL hirudin, 1 μmol/L paclitaxel+0.025 mg/mL hirudin, 1 μmol/L paclitaxel+0.05 mg/mL hirudin, 1 μmol/L paclitaxel+0.1 mg/mL hirudin, 1 μmol/L paclitaxel+0.2 mg/mL hirudin, 1 μmol/L paclitaxel+0.4 mg/mL hirudin, and 1 μmol/L paclitaxel+0.8 mg/mL hirudin groups, with six parallel wells for each group.

ELISA

The effects of the compound on the protein levels of IL-6, TNF-α, and IL-1β were detected using ELISAs. The treatments with the highest and lowest non-toxic concentrations (1 μmol/L paclitaxel+0.2 mg/mL hirudin and 1 μmol/L paclitaxel+0.0125 mg/mL hirudin, respectively) based on the MTT results were used for treatment of the inflammation-activated HCASMCs. The cells were divided into four groups: the control, LPS, LPS+1 μmol/L paclitaxel+0.2 mg/mL hirudin, and LPS+1 μmol/L paclitaxel+0.0125 mg/mL hirudin groups.

q-PCR

The effects of the compound on the gene expression of TLR4, MyD88, NF-κB p65, IL-6, TNF-a, and IL-1β were detected using q-PCR. The cells were divided into four groups: the control, LPS, LPS+1 μmol/L paclitaxel+0.2 mg/mL hirudin, and LPS+1 μmol/L paclitaxel+0.0125 mg/mL hirudin groups.

Western blot analysis

The effects of the compound on the protein levels of TLR4, MyD88, NF-κB p65, IL-6, TNF-a, and IL-1β were observed using western blot analysis. Four groups were established: the control, LPS, LPS+1 μmol/L paclitaxel+0.2 mg/mL hirudin, and LPS+1 μmol/L paclitaxel+0.0125 mg/mL hirudin groups.

Inhibition analyses

MyD88 was knocked down, or MyD88 degradation was selectively inhibited with epoxomicin. The gene expression and protein levels of key components and downstream inflammatory products of the TLR4/MyD88/NF-κB pathway after compound intervention were detected using ELISA, q-PCR, and western blot analysis. The cells were divided into seven groups: the control, LPS, MyD88-knockdown (MyD88 siRNA), LPS+MyD88-knockdown, LPS+compound, LPS+compound+0.1 μM epoxomicin, and LPS+compound+1 μM epoxomicin groups.

Statistical analysis

Homogeneity tests of variance of the experimental data were performed using SPSS 24.0 software. The population means of multiple samples were compared using one-factor analysis of variance. All data are presented as the mean ± standard deviations ($\overline{X} \pm S$). $P<0.05$ was considered statistically significant.

Results

Stable HCASMCs at 4-6 generations

The growth of HCASMCs was observed under an inverted phase contrast microscope. The spindle-shaped cells showed satisfactory adherence and growth, strong refraction, abundant and translucent cytoplasm, and good flexibility. After 3 d of culture, the cells entered the exponential phase of growth: the cells were arranged in a dense pattern without contact inhibition, and when the density was high, the cells overlapped and accumulated (Fig. 1).

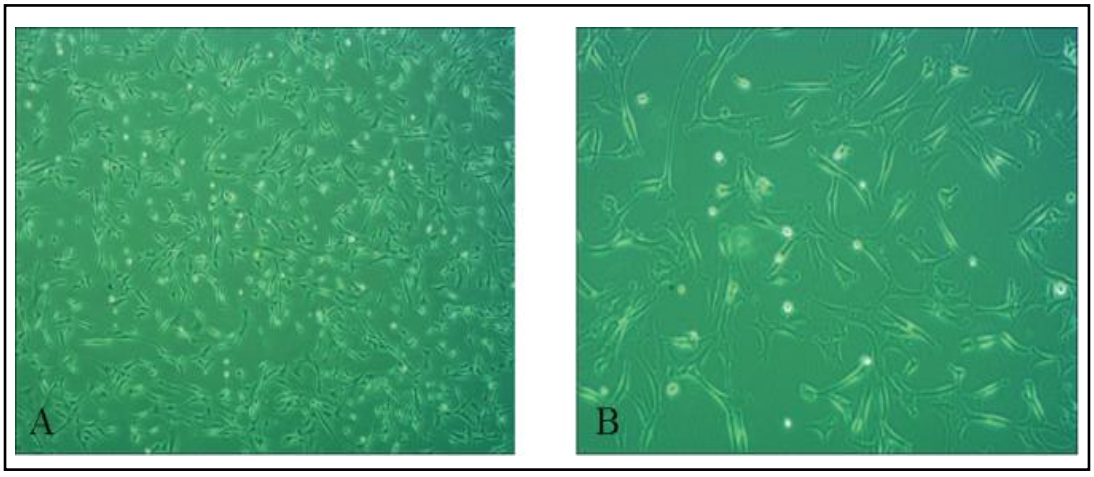

Fig. 1. Cell growth under an inverted phase contrast microscope. A, 40×. B, 100×.

Non-toxic LPS concentration range

MTT results showed that LPS at <100 μg/mL did not significantly inhibit the vitality of HCASMCs. LPS at 100 μg/mL decreased cell vitality to less than 80.1%. Therefore, 0-10 μg/mL was considered the non-toxic concentration range (Table 1).

Table 1. Effects of LPS at different concentrations on the vitality of HCASMCs

LPS concentration (μg/mL)	OD ($\overline{X}\pm S$)	Cell vitality
0	0.552±0.041	1
0.01	0.621±0.124	1.125
0.1	0.635±0.061	1.15
0.5	0.596±0.056	1.079
1	0.588±0.069	1.065
10	0.536±0.030	0.971
100	0.442±0.189	0.801

Effects of LPS on the protein levels of IL-6, TNF-α, and IL-1β

The effects of LPS on the protein levels of IL-6, TNF-α, and IL-1β were detected using ELISAs. LPS at 0.5 μg/mL and 1 μg/mL for 24 and 48 h induced strong excretion of IL-6, IL-1β, and TNF-α, which strongly increased the concentrations of the inflammatory products and the inflammatory activation of HCASMCs (Fig. 2). These results narrowed the concentration screening range for later modeling.

Effects of LPS on the gene expression of key molecules of the TLR4-MyD88 pathway

q-PCR was used to determine the optimal modeling conditions for LPS to maximize the gene expression of TLR4, MyD88, NF-κB p65, IL-6, TNF-α, and IL-1β.

Compared with the control group, the group treated with LPS at 1 μg/mL for 48 h showed significantly increased mRNA expression of TLR4 (P<0.05; Fig. 3A). The mRNA levels of MyD88 and NF-κB p65 increased significantly after treatment with LPS at 1 μg/mL for 24 h and 48 h (P<0.05; Fig. 3B and C). LPS at 0.5 μg/mL and 1 μg/mL induced a rapid increase in the mRNA expression of IL-6 after 48 h of treatment (P<0.05; Fig. 3D) and notably increased the mRNA levels of TNF-α and IL-1β after 24 h and 48 h (P<0.05; Fig. 3E and F).

Effects of LPS on the protein levels of TLR4, MyD88, and NF-κB p65

Western blotting was used to analyze the optimal modeling conditions for LPS to maximize the gene expression of TLR4, MyD88, and NF-κB p65.

In normal HCASMCs, the protein level of MyD88 was high, whereas the protein levels of TLR4 and NF-κB p65 were extremely low. After LPS stimulation, the protein levels of TLR4 and NF-κB p65 increased to various degrees. At 48 h after stimulation with 1 μg/mL LPS, all key protein molecules of the TLR4-MyD88 pathway were activated, which was demonstrated by the high protein levels of TLR4, MyD88, and NF-κB p65 (Fig. 4).

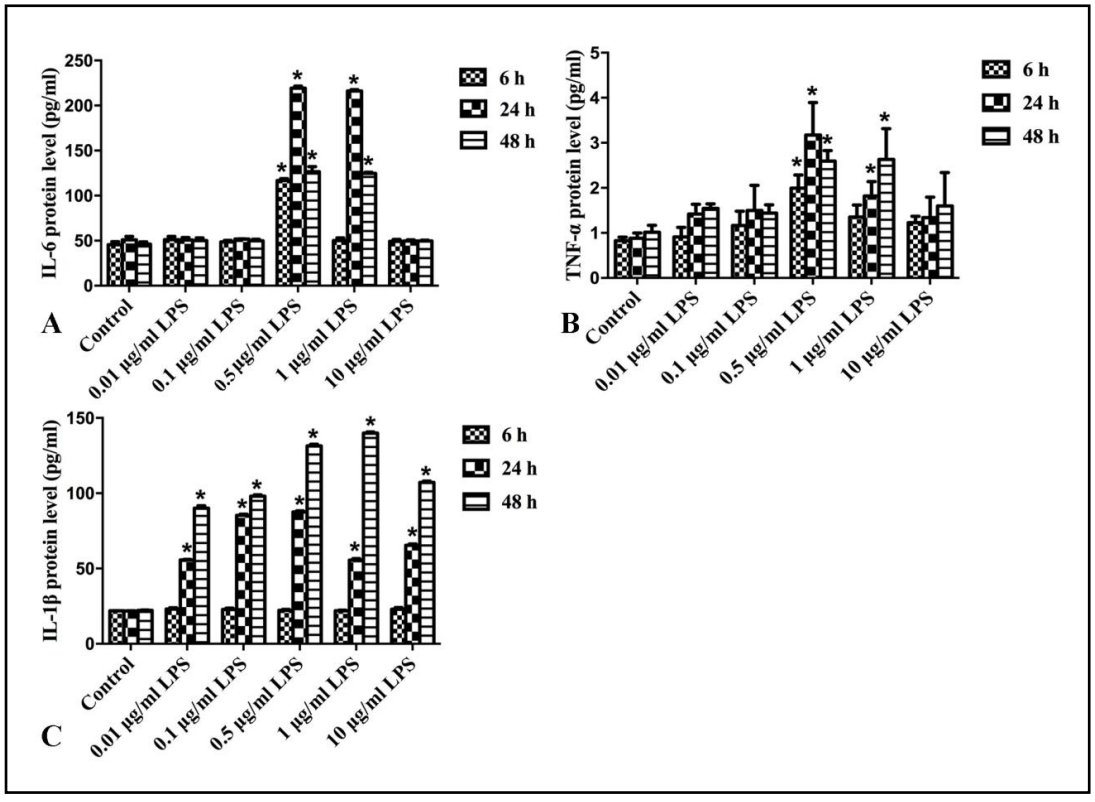

Fig. 2. Effects of LPS on the protein levels of IL-6, TNF-α, and IL-1β according to ELISAs. *P< 0.05 vs. the control group.

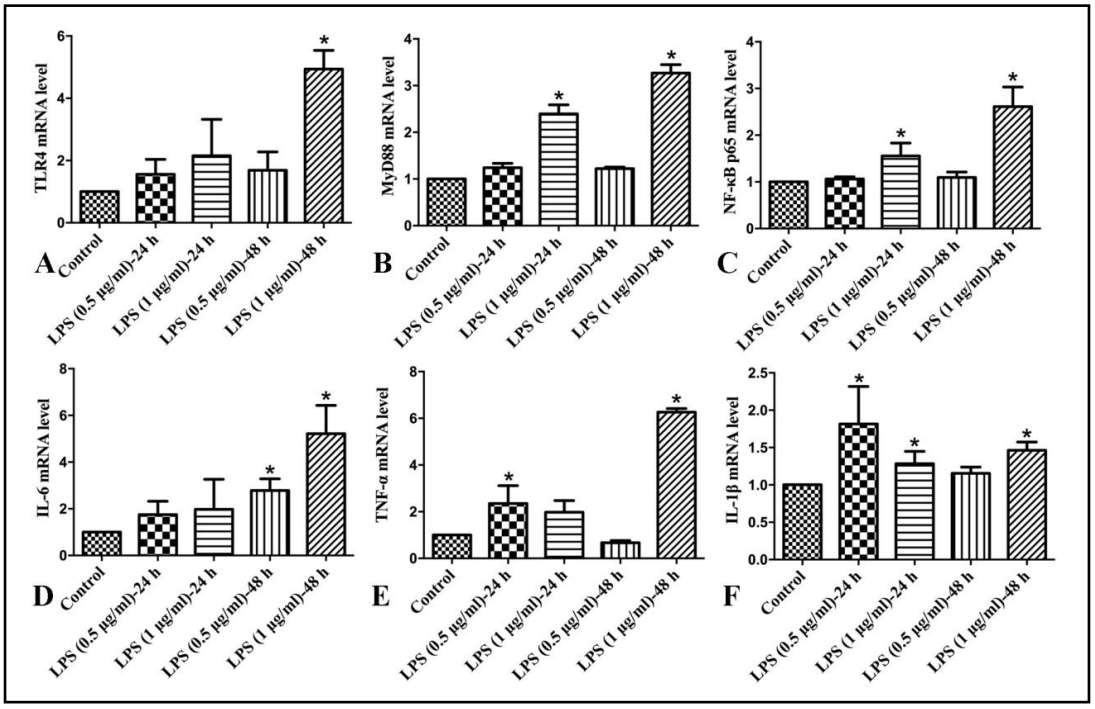

Fig. 3. Effects of LPS on the gene expression of key molecules of the TLR4-MyD88 pathway according to q-RCR. A, TLR4. B, MyD88. C, NF-κB p65. D, IL-6. E, TNF-α. F, IL-1β. *P<0.05 vs. the control group.

Non-toxic paclitaxel+hirudin concentration range

MTT results showed that the combination of 1 μmol/L paclitaxel and 0.8 mg/mL hirudin had a noticeable inhibitory effect on HCASMCs compared with that of the control group (P<0.05; Table 2). Based on these results, the maximal and minimal non-toxic concentrations of the compound were 1 μmol/L paclitaxel+0.2 mg/mL hirudin and 1 μmol/L paclitaxel+0.0125 mg/mL hirudin, respectively.

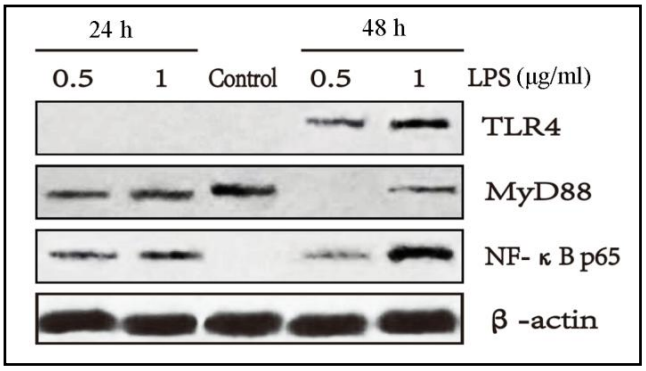

Fig. 4. Effects of LPS on the protein levels of TLR4, MyD88, and NF-κB p65 determined using western blot analysis.

Table 2. Effects of different combination ratios of paclitaxel and hirudin on the vitality of HCASMCs. *P<0.05, vs. the control group

Paclitaxel (μmol/L)	Hirudin (mg/mL)	OD ($\bar{X}\pm S$)	Cell vitality
0	0	0.272±0.027	1
1	0.0125	0.297±0.042	1.092
1	0.025	0.278±0.059	1.022
1	0.05	0.256±0.065	0.941
1	0.1	0.255±0.043	0.938
1	0.2	0.250±0.058	0.919
1	0.4	0.214±0.042	0.787
1	0.8	0.212±0.028*	0.779

Effects of paclitaxel+hirudin on the protein levels of IL-6, TNF-α, and IL-1β

ELISA results showed that the protein levels of IL-6, TNF-α, and IL-1β significantly increased in the LPS group compared with those in the control group (P<0.05), indicating that a successful model was established. Compared with the LPS group, both the maximal and minimal paclitaxel+hirudin groups showed a significant decrease in the protein levels of the downstream inflammatory products (P<0.05; Fig. 5).

Effects of paclitaxel+hirudin on the gene expression of TLR4, MyD88, NF-κB p65, IL-6, TNF-α, and IL-1β

q-PCR showed that LPS activated the gene expression of TLR4, MyD88, NF-κB p65, IL-6, TNF-α, and IL-1β simultaneously (P<0.05), indicating that a successful model was established. Compared with the LPS group, both paclitaxel+hirudin groups showed a significant decrease in the gene expression of TLR4, NF-κB p65, IL-6, TNF-α, and IL-1β (P<0.05; Fig. 6). However, no significant difference in the gene expression of MyD88 was observed.

Effects of paclitaxel+hirudin on the protein levels of TLR4, MyD88, and NF-κB p65

Western blot analysis showed that the protein levels of TLR4, MyD88, and NF-κB p65 significantly increased in the LPS group compared with those in the control group (P<0.05). The protein levels of these components were significantly decreased in both the maximal and minimal paclitaxel+hirudin groups compared with those in the LPS group (P<0.05; Fig. 7), with a better anti-inflammatory effect observed in the maximal concentration group.

Effects of MyD88 knockdown/epoxomicin intervention on the inhibitory effect of the compound

The effects of MyD88 knockdown/epoxomicin intervention on the inhibitory effect of the compound on the gene expression of TLR4, MyD88, NF-κB p65, IL-6, TNF-α, and IL-1β were detected using q-PCR (Fig. 8). In this study, MyD88 gene expression was knocked down via liposome transfection in the LPS-induced HCASMC inflammation model. Compared with the LPS group, the MyD88 siRNA group showed significant decreases in the gene expression

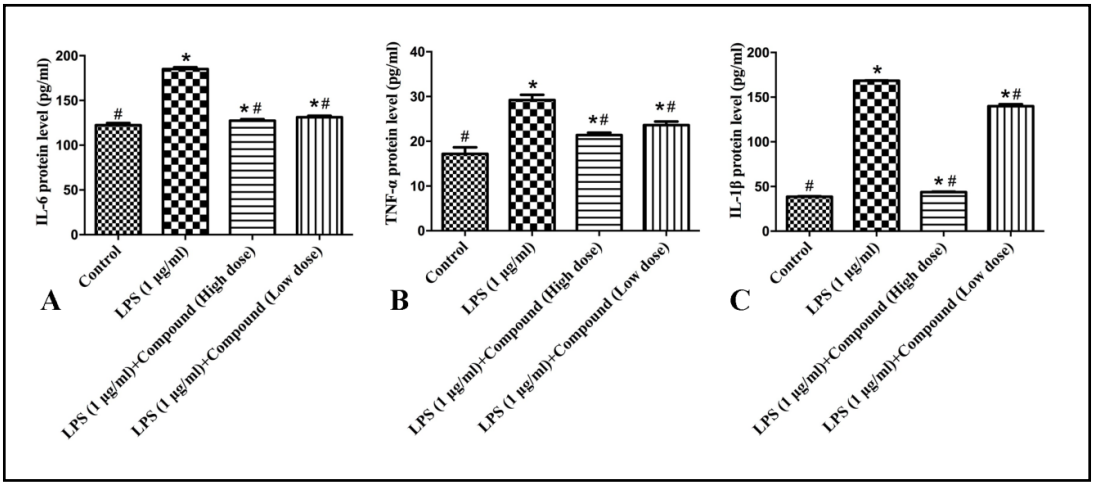

Fig. 5. Effects of paclitaxel+hirudin on the protein levels of IL-6, TNF-α, and IL-1β. A, IL-6. B, TNF-α. C, IL-1β. *P<0.05 vs. the control group. #P<0.05 vs. the LPS group.

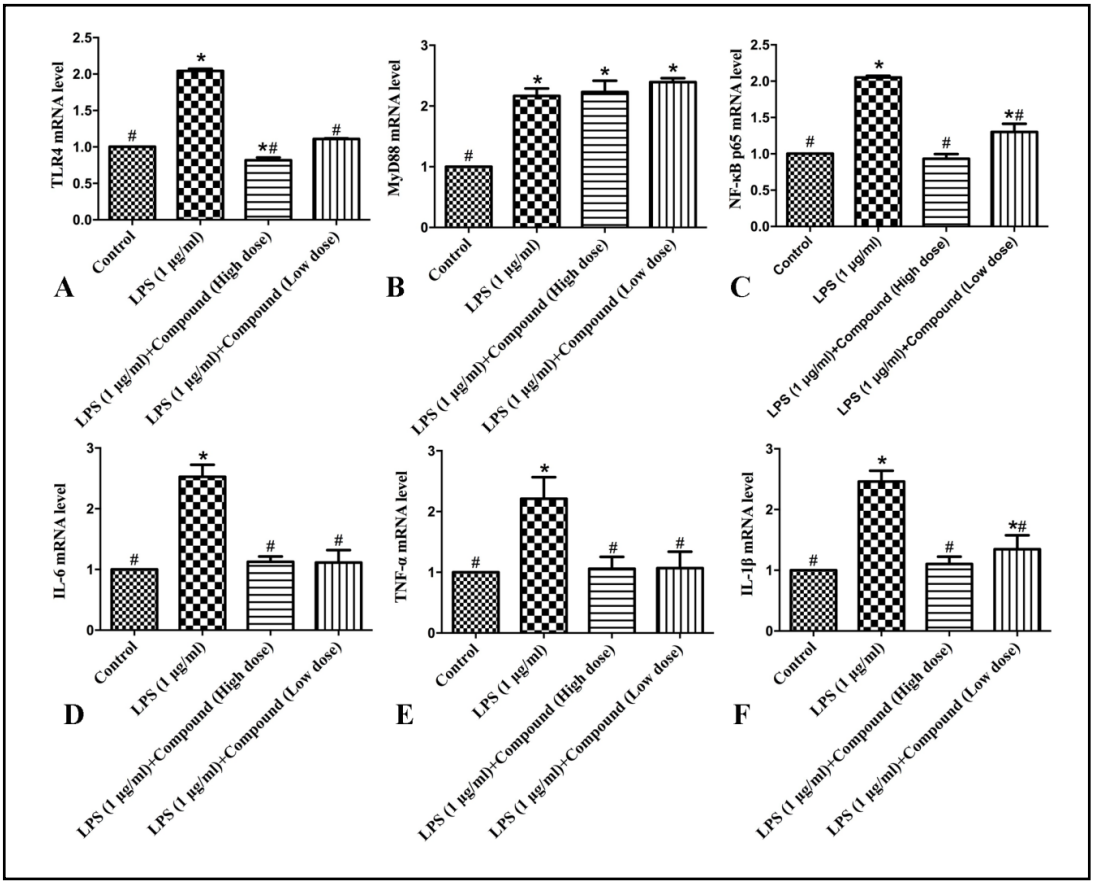

Fig. 6. Effects of paclitaxel+hirudin on the gene expression of TLR4, MyD88, NF-κB p65, IL-6, TNF-α, and IL-1β. A, TLR4. B, MyD88. C, NF-κB p65. D, IL-6. E, TNF-α. F, IL-1β. *P<0.05 vs. the control group. # P<0.05 vs. the LPS group.

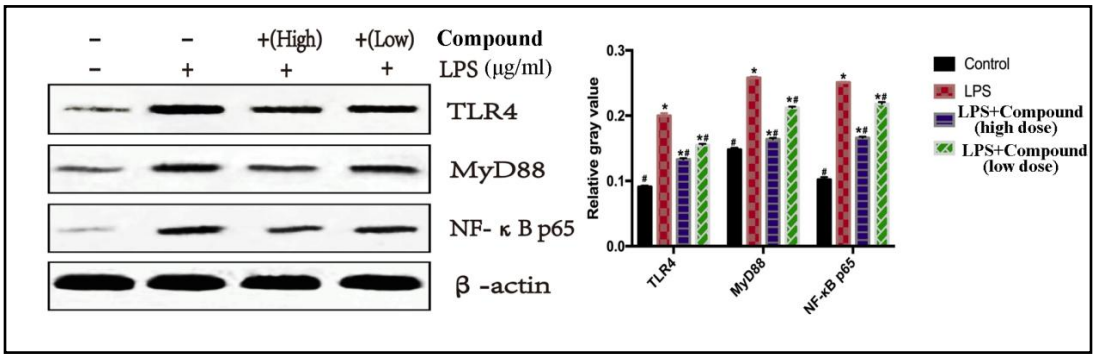

Fig. 7. Effects of paclitaxel+hirudin on the protein levels of TLR4, MyD88, and NF-κB during LPS-induced inflammatory activation according to western blot analysis. *P<0.05 vs. the control group. # P<0.05 vs. the LPS group.

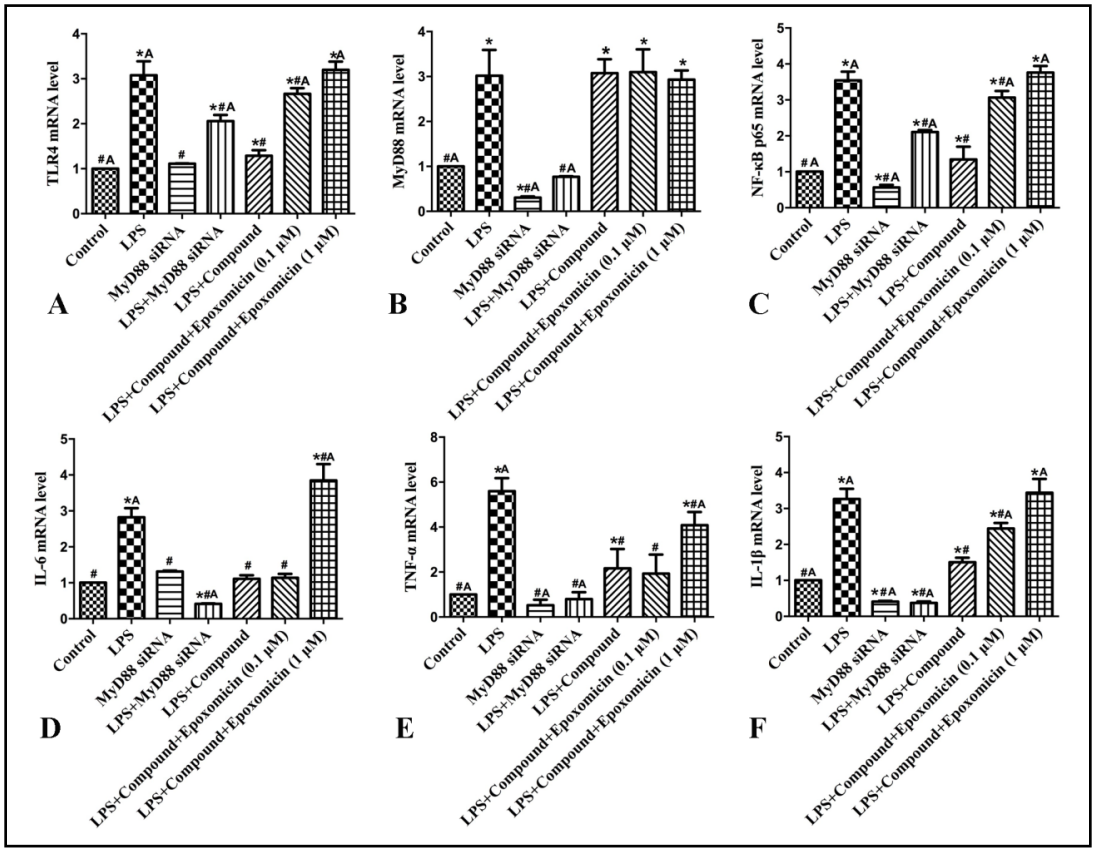

Fig. 8. Effects of MyD88 knockdown/epoxomicin intervention on the inhibitory effects of the compound on the gene expression of TLR4, MyD88, NF-κB p65, IL-6, TNF-α, and IL-1β using q-PCR. A, TLR4. B, MyD88. C, NF-κB p65. D, IL-6. E, TNF-α. F IL-1β. *P<0.05 vs. the control group. #P<0.05 vs. the LPS group. AP<0.05 vs. the LPS+compound group.

of TLR4, NF-κB p65, IL-6, TNF-α, and IL-1β (P<0.05), which exhibited a trend similar to that of the LPS+compound group. Epoxomicin, particularly at a high concentration, increased the gene expression of TLR4, NF-κB p65, IL-6, TNF-α, and IL-1β, which showed significant differences compared with those of the LPS+compound group (P<0.05). Compared with LPS, epoxomicin did not influence the gene expression of MyD88. However, this inhibitor weakened the anti-inflammatory effect of paclitaxel+hirudin (Fig. 8).

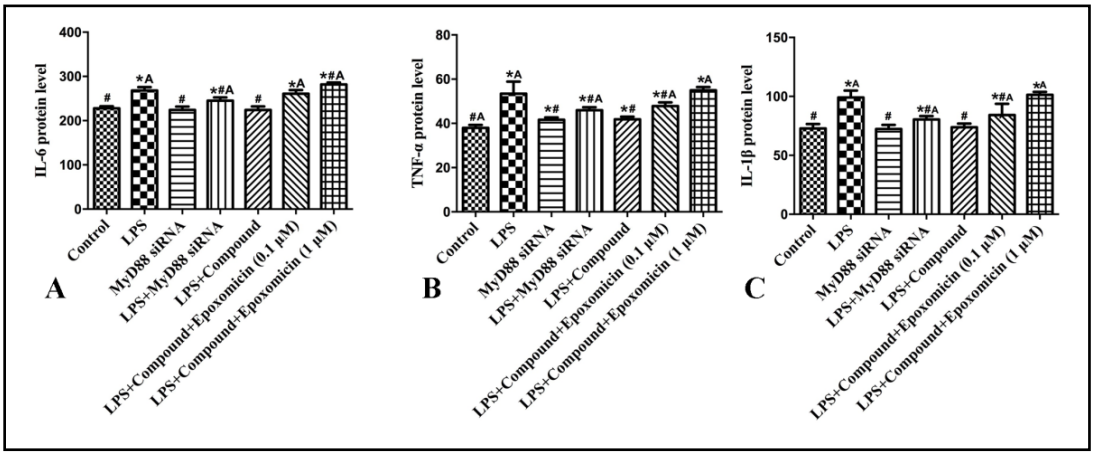

Fig. 9. Effects of MyD88 knockdown/epoxomicin intervention on the inhibitory effects of the compound on the protein levels of IL-6, TNF-α, and IL-1β using ELISAs. A, IL-6. B, TNF-α. C, IL-1β. *P<0.05 vs. the control group. #P<0.05 vs. the LPS group. AP<0.05 vs. the LPS+compound group.

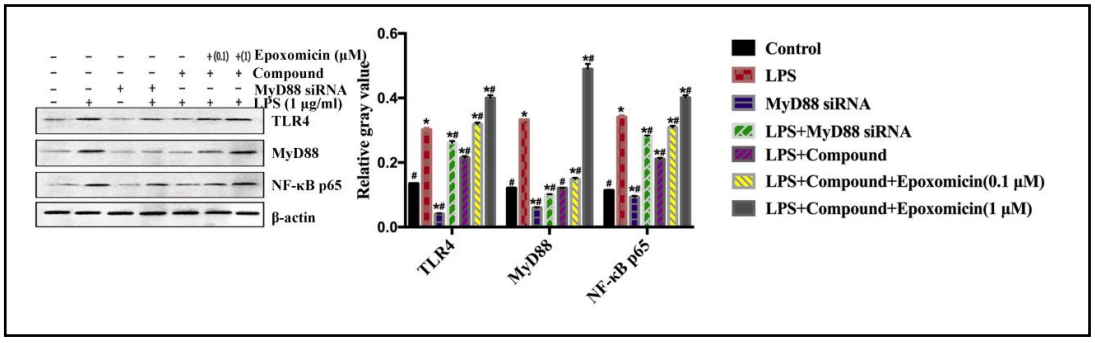

Fig. 10. Effects of MyD88 knockdown/epoxomicin intervention on the inhibitory effects of the compound on the protein levels of TLR4, MyD88, and NF-κB p65 using western blot analysis. *P<0.05 vs. the control group. # P<0.05 vs. the LPS group.

The effects of MyD88 knockdown/epoxomicin intervention on the inhibitory effect of the compound on the protein levels of IL-6, TNF-α, and IL-1β were detected using ELISAs. Compared with the LPS group, the MyD88 siRNA group showed significant decreases in the protein levels of IL-6, TNF-α, and IL-1β (P<0.05), and this change exhibited a trend similar to that in the LPS+compound group. Epoxomicin noticeably weakened the inhibitory effect of paclitaxel+hirudin on IL-6, TNF-α, and IL-1β (P<0.05), particularly epoxomicin at a high concentration (Fig. 9).

The effects of MyD88 knockdown/epoxomicin intervention on the inhibitory effects of the compound on the protein levels of TLR4, MyD88, and NF-κB p65 were observed using western blot analysis (Fig. 10). Compared with the LPS group, the MyD88 siRNA group showed significant decreases in the protein levels of TLR4, MyD88, and NF-κB p65 (P<0.05). Epoxomicin significantly weakened the inhibitory effects of paclitaxel+hirudin on TLR4, MyD88, and NF-κB p65 (P<0.05), particularly epoxomicin at a high concentration (P<0.05), and MyD88 demonstrated the most representative changes.

Discussion

The pathophysiological mechanisms underlying post-PCI restenosis are complex and involve immunity, inflammation, oxidative stress, and vascular remodeling. In recent years, the role of the microenvironment in post-PCI inflammation of restenosis has attracted

increasing attention from scholars. Local inflammatory activation of vascular endothelial cells, smooth muscle cells, and macrophages has been recognized as a post-operative factor that promotes restenosis, and the strong proliferation and migration of activated smooth muscle cells are key factors that aggravate restenosis progression [30, 31].

Smooth muscle cells, as the main component of the intima media of the vessel wall, play an important physiological role in maintaining the structure and contractility of vessels and stabilizing blood flow and pressure [32]. In a normal state, highly differentiated smooth muscle cells are characterized by low proliferation, migration and secretion. However, when the vessel wall is damaged by physical or chemical factors or under long-term inflammatory stimulation, a rapid phenotypic change from contractility to a secretion type will occur in smooth muscle cells, which participate in revascularization [33]. The cells show increased activity and differentiate into other types of cells. In addition, secretory smooth muscle cells synthesize and secrete high levels of various cytokines and adhesion molecules, which promote the early occurrence of local inflammatory reactions of the damaged vessel and form an extremely complicated inflammation network control system via the sequential activation of multiple overlapping intracellular signal pathways. Moreover, this process causes the chemotaxis of inflammatory cells and inflammatory cell aggregation and adhesion, aggravates inflammatory reactions, induces local lesion of the vessel, and promotes post-intervention restenosis [34, 35]. Therefore, further research on inflammation-activated smooth muscle cells is important for the prevention and treatment of restenosis.

In the inflammatory microenvironment, smooth muscle cells of the intima media of the vessel wall are characterized by the cascaded activation of intracellular inflammatory pathways. The TLR family, particularly TLR4, binds rapidly with their ligands, which is the initiating factor for the activation of inflammatory pathways and the synthesis and secretion of high levels of inflammatory mediators [36]. TLR4 is expressed in nearly all cells and is one of the most acute receptors of the TLR family. TLR4 can recognize endogenous ligands and certain risk signals released after histiocyte damage. After binding with endogenous ligands, such as heat shock protein 60, intracellular fibronectin and oxygenized low-density lipoprotein, this molecule can be activated immediately and then transfer activation signals to downstream inflammatory pathways to mediate inflammatory reactions and induce cell proliferation and differentiation [37]. TLR4 can be activated by the exogenous ligand LPS, causing self-dimerization and exerting its functions via MyD88-dependent and independent channels [38, 39]. The MyD88-dependent channel binds with interleukin-1 receptor-associated kinase (IRAK) via its death domain to cause self-phosphorylation of IRAK, activate tumor necrosis factor receptor-associated factor 6, activate c-Jun N-terminal protein kinase to induce the latter to activate I-κB α and β kinase, promote the activation of NF-κB and its transfer into the nucleus to initiate transcription, synthesize and secrete downstream inflammatory factors, and ultimately aggravate systemic and local inflammatory reactions. The MyD88-independent channel activates NF-κB via interactions of MyD88 adapter proteins to indirectly regulate the syntheses of inflammatory factors and downstream products. Currently, as a classic inflammation pathway, the TLR4/MyD88/NF-κB pathway has become a hotspot of research. This pathway plays an important role in inflammatory activation, proliferation and migration and restenosis of vascular smooth muscle cells. Restenosis requires the acquisition of an inflammatory phenotype of smooth muscle cells, and TLR4/MyD88/NF-κB is a critical promoting factor for the phenotypic acquisition of the cells [40-42]. Therefore, activation of the TLR4/MyD88/NF-κB pathway is closely associated with restenosis, and the LPS-induced HCASMC inflammatory activation model can serve as a reliable cell model for research on the restenosis mechanism.

The paclitaxel+hirudin stent-coating compound prepared by the current team is a further innovation based on a paclitaxel coating already available on the market. Paclitaxel-coated stents not only notably reduce the incidence of post-PCI restenosis but also substantially weaken the influence of major cardiovascular adverse events [43, 44]. These effects of paclitaxel are associated with its inhibition of mitosis in the late G2-M phase to block the cell cycle [45]. Compared with conventional paclitaxel-coated stents, paclitaxel elution balloons

show major advantages. High liposolubility indicates that paclitaxel is more suitable than other drugs for balloon coating: the paclitaxel on the surface of a balloon can be rapidly taken up by local vascular cells for drug transfer at the time of balloon expansion, which continuously inhibits the proliferation and migration of vascular smooth muscle cells [46, 47]. Among various paclitaxel elution balloon products, the most representative are Braun SeQuent®Please balloons (Germany); the anti-restenosis effect of these products has been proven by a series of studies [48, 49]. Thus, Braun SeQuent®Please balloons have become the first choice in the treatment of post-operative restenosis. To further strengthen the anti-restenosis and anti-thrombosis effects of paclitaxel, the current team combined hirudin and paclitaxel for stent coating in an optimal ratio. Hirudin is a polypeptide of 65 amino acids. Hirudin's direct inhibition/inactivation of thrombin is independent of antithrombin III, heparin cofactor II, protein C and tissue factor pathway inhibitors, and hirudin can simultaneously affect the active sites and substrate recognition sites of thrombin. Compared with heparin, hirudin is not inactivated by platelets. Moreover, this molecule can inhibit thrombin-induced platelet aggregation and thus exhibits satisfactory anti-blood coagulation and anti-thrombosis effects. Bivalirudin, a recombinant derivative of hirudin, shows excellent anti-coagulation and anti-thrombosis effects [50].

In the current study, an HCASMC inflammation model was established by selecting the classic inflammation pathway TLR4/MyD88/NF-κB as the pathway of interest and LPS as the *in vitro* inducer to maximally activate the TLR4/MyD88/NF-κB pathway as well as its downstream inflammatory factor network. The pathological state of HCASMCs in a post-PCI inflammatory microenvironment was simulated.

First, we explored the non-toxic concentration range of LPS. Based on the literature, we expanded the concentration range of LPS as much as possible and used LPS at 0.01, 0.1, 0.5, 1, 10, and 100 μg/mL for 24-h intervention. MTT results showed that LPS at 100 μg/mL had a major influence on cell vitality. Therefore, we used 0-10 μg/mL LPS to construct the HMASMC model. We further screened the modeling concentrations of LPS using ELISAs by detecting the contents of inflammatory factors after different intervention times of LPS at a non-toxic concentration. Thus, we initially identified the concentration and intervention time of LPS that maximized the protein levels of IL-6, IL-1β, and TNF-α (e.g., 0.5 μg/ml and 1 μg/ml; 24 h and 48 h). Then, we screened the LPS modeling conditions in depth using q-PCR and western blot analysis. The outcomes showed that 1 μg/mL LPS for a 48-h continuous induction maximized the gene expression and protein levels of TLR4, MyD88, NF-κB p65, IL-6, IL-1β, and TNF-α in the cells and that these conditions resulted in a continuous high inflammation status of the HCASMCs and maximal activation of the TLR4-MyD88 pathway.

Based on the abovementioned results, we conducted a mechanistic analysis of the effect of the compound on the inflammatory activation cell model, and the results showed that the compound inhibited key proteins of the TLR4-MyD88 pathway in the activated HCASMCs. Specifically, the protein levels of the upstream nodes TLR4 and MyD88 were decreased, the activity of NF-κB p65 was reduced, and the LPS-initiated inflammatory reactions represented by TNF-α, IL-6, and IL-1β were decreased. Further analysis showed that the compound significantly down-regulated the gene expression of components of the pathway, except for that of MyD88. This finding suggests that the anti-inflammatory effect of the compound may be associated with its regulatory effect on the MyD88 protein level. Therefore, we focused on MyD88 in further experiments and investigated the role of MyD88 in the regulatory effect of the compound on the TLR4-MyD88 pathway to explore the anti-restenosis effect of the compound at the protein level. MyD88 knockdown down-regulated the expression of TLR4-MyD88 inflammatory pathway-associated proteins and genes after LPS-induced inflammatory activation. The changes showed a trend similar to that of the changes caused by addition of the compound to the inflammatory cell model, with the latter showing a more noticeable inhibitory effect. Therefore, there must be other pathways through which the compound exerts an anti-inflammatory effect, in addition to MyD88. After epoxomicin was applied, the anti-inflammatory effect of the compound was weakened to various degrees. Specifically, epoxomicin at a high concentration nearly counteracted the inhibitory effect of

the compound on the MyD88 protein level. The effect of epoxomicin on the decrease in the MyD88 protein level induced by the paclitaxel+hirudin compound was dosage-dependent; this finding suggests that the negative effect of the compound on the MyD88 protein level is associated with its promotion of the degradation of MyD88 in the pathway of interest. In addition, pretreatment with epoxomicin significantly weakened the inhibitory effect of the compound on the gene expression and protein levels of the upstream molecules and downstream inflammatory products of the TLR4-MyD88 pathway, and some of the values even returned to those observed before intervention. These findings indicate that blocking the inhibitory effect of the paclitaxel+hirudin compound on TLR4-MyD88 can reverse the compound-mediated blockade of the cascading activation of the TLR4-MyD88 pathway. This finding confirms that the anti-inflammatory effect of the paclitaxel+hirudin compound is associated with its degradation-promoting effect of MyD88 in the inflammatory pathway.

This study has some limitations. First, this study was conducted *in vitro*. Microenvironmental changes *in vivo* may alter the results. Therefore, *in vivo* studies should be conducted to further contribute to the development of combined monomers for stent/balloon coating. In addition, this study employed IL-1β, IL-6 and TNF-α as the representative indices, whereas other inflammatory mediators, such as ifn, vcam-1, icam-1 and mcp-1, were not assessed. This decision was based on the findings that ifn, vcam-1, icam-1 and mcp-1 reflect cell adhesion and aggregation and that cell adhesion and aggregation-induced restenosis have an indirect effect on vascular smooth muscle cells [51, 52]. To further complement the outcomes of the current study, the current team is exploring the pathomechanisms of intraductal thrombosis-induced restenosis due to vascular endothelial cell damage, in which ifn, vcam-1, icam-1 and mcp-1 will be used as the core indices of the growth and adhesion of endothelial cells.

Conclusion

The molecular mechanism of paclitaxel+hirudin in preventing post-intervention restenosis is associated with the anti-inflammatory effect of the compound. This compound acts on MyD88 in the TLR4/MyD88/NF-κB pathway to promote MyD88 degradation, thereby down-regulating the activity of TLR4 and NF-κB p65 and weakening LPS-initiated inflammatory reactions represented by IL-1β, IL-6, and TNF-α.

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Disclosure Statement

The authors declare no conflicts of interest.

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