Mechanism of berberine regulating ox-LDL induced endothelial pyroptosis based on TXNIP / NLRP3 / GSDMD pathway

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Research Article

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Abstract

This study aimed to explore the regulatory effect of berberine on ox LDL induced HUVECs pyroptosis, and reveal the possible molecular mechanism and target of berberine in preventing and treating coronary atherosclerosis. The experiment used 50μg/mL ox-LDL to stimulate HUVECs to construct an in vitro inflammatory injury model, and the protective effect of berberine at a concentration of 20 μmol/L on HUVECs. Pathway phase proteins and cytokines were detected using methods such as ELISA, scanning electron microscopy, Western blotting, and RT-qPCR. We found that ox-LDL can induce pyroptosis in HUVECs, including pyroptosis related proteins NLRP3, Caspase-1, GSDMD, and downstream products IL-18 and IL-1β. The levels of LDH were significantly increased, and berberine intervention can reduce the expression levels of these indicators, indicating that BBR may inhibit ox-LDL induced HUVECs pyroptosis by downregulating the NLRP3/Caspase-1/GSDMD pathway. We further infected HUVECs with TXNIP Lentivirus to construct overexpression of TXNIP. The results showed that TXNIP can regulate NLRP3 mediated pyroptosis, while berberine can inhibit the expression of TXNIP. The specific molecular mechanism may be realized by downregulating the TXNIP/NLRP3/GSDMD pathway.

Introduction

In recent years, the incidence rate and mortality rate of atherosclerosis (AS) continue to increase. It is reported that about 20 million people worldwide die of AS every year, which is a progressive disease that seriously endangers human health [1]. The pathogenesis of AS is quite complex, often accompanied by pathological features such as oxidative stress, mitochondrial dysfunction, endothelial damage, cell proliferation, lipid deposition, and abnormal immune inflammatory response [2–3]. Endothelial cells play a crucial role in maintaining vascular blood flow tension, inhibiting vascular inflammation, and oxidative stress. When vascular homeostasis is disrupted, endothelial damage and dysfunction become the starting factors for AS [4–5]. Endothelial pyroptosis is one of the key pathways leading to vascular endothelial dysfunction [6]. NOD like receptor protein 3 (NLRP3) inflammasome dependent pyroptosis is an important pathogenic factor in the pathogenesis of AS [7]. Under pathological conditions, such as oxidative stress, high glucose, dyslipidemia, inflammation, and hyperhomocysteinemia, NLRP3 inflammasome can be activated in endothelial cells, triggering pyroptosis [8,9,10]. Although the role of pyroptosis in AS has been widely studied, its specific molecular mechanism is still unclear and needs further exploration.

The formation of NLRP3 inflammasome is one of the important characteristics of Caspase-1 dependent classical pyroptosis pathway [11]. NLRP3 inflammasome is a multi protein complex composed of NLRP3, Caspase-1, and ASC. The activation of inflammasomes leads to the self catalytic maturation of Po Caspase-1 and its cleavage into active Caspase-1 [12,13,14]. Activated Caspase-1, on the one hand, cleaves Gasdermin (GSDMD) into GSDMD-N, which can insert into the cell membrane and form membrane pores, leading to a series of inflammatory reactions such as cell swelling, plasma membrane lysis, cell death, and intracellular substance release [15]. On the other hand, Caspase-1 activates the precursors of interleukin-1β (IL-1β) and interleukin-18 (IL-18) into active IL-1β and IL-18, and releases
other cellular contents such as IL-18 and IL-1β, lactate dehydrogenase (LDH), etc. outside the cells through GSDMD cell membrane pores, recruiting more inflammatory cells and expanding the inflammatory response [16,17]. There are more and more studies on pyroptosis and AS, but the mechanism is more complex. We need to find safe and effective targets and molecular pathways, especially understanding the regulatory relationship between upstream and downstream proteins, which will help us understand the mechanism of pyroptosis in the progression and prognosis of AS and provide strategies for the treatment of AS.

The thioredoxin (TRX) system is one of the most important redox systems in the human body, playing an important role in promoting cell proliferation, anti apoptosis, and homeostasis regulation [18]. Thioredoxin interacting protein (TXNIP) is an endogenous oxidative stress regulatory molecule that interacts with TRX and is a negative regulatory factor of TRX. It directly interacts with two cysteine residues in the catalytic center of TRX to form a mixed disulfide bond, inhibiting TRX activity and expression, and altering cellular redox status [19,20]. It can regulate intracellular mitochondrial oxidative phosphorylation, fatty acid β oxidation, autophagy, apoptosis, participate in oxidative stress, inflammatory reaction, etc. Research has shown that some traditional Chinese medicines have therapeutic effects on diseases by inhibiting the expression of TXNIP [21,22,23]. However, there is relatively little research on the regulatory effect of berberine on TXNIP, especially in the field of AS.

Berberine (BBR), also known as berberine, is a isoquinoline alkaloid extracted from plants, which is the main active ingredient of traditional Chinese medicine Coptis chinensis. It has various pharmacological effects such as lowering blood sugar, regulating blood lipid metabolism, anti-inflammatory, antioxidant stress, anti fibrosis, anti arrhythmia, and vasodilation [24,25,26]. BBR can combine different biological targets based on different disease causes, and this biological function may be related to its multi pathway and multi target effects [27, 28]. BBR can reduce AS in multiple dimensions by reducing blood lipids, protecting vascular endothelium, inhibiting inflammatory cell infiltration and foam cell formation, activating macrophage autophagy, regulating the proliferation and migration of vascular smooth muscle, reducing platelet aggregation and regulating intestinal microbiota [29,30,31]. At present, the specific mechanism of berberine on AS is not fully understood, especially the mechanism of BBR on pyroptosis is not deep enough. It is necessary to explore the mechanism of BBR regulating NLRP3 inflammasome and its upstream and downstream from the perspective of molecular biology and pharmacokinetics.

In this study, HUVECs were stimulated by ox LDL, the key pathogenic factor of AS, to construct a model of vascular endothelial pyroptosis. ELISA, scanning electron microscopy, Western blotting, RT qPCR and other experimental methods were used to explore the inhibitory effect of BBR on ox LDL induced vascular endothelial pyroptosis. On this basis, HUVECs were infected by TXNIP Lentivirus to construct overexpression of TXNIP, further verify the influence of berberine on TXNIP/NLRP3/GSDMD classic pyroptosis pathway, improve the molecular regulation mechanism of berberine to protect vascular endothelial function, and elaborate that BBR plays an anti AS role by inhibiting TXNIP, the upstream molecule of pyroptosis, to reduce inflammatory cascade reaction, indicating that berberine may be a potential drug for effective treatment of AS.
Materials and Methods

Cell Culture

Human umbilical vein endothelial cells (HUVECs, Sciencell, USA, batch number 8000): Cells were cultured in ECM culture medium containing 5% FBS, 1% ECGS, and 1% P/S in a constant temperature incubator at 37 °C and 5% CO. According to different treatment factors, HUVECs are divided into 7 groups: (1) Control group: normal control group HUVECs without special treatment. (2) Model group (ox-LDL group): induced HUVECs at a working concentration of 50µg/mL ox-LDL for 24 hours. (3) Berberine group (BBR group): after pretreatment with the optimal concentration of 20µmol/L berberine for 1 hour, continue to intervene with 50µg/mL ox-LDL in HUVECs for 24 hours. (4) Empty body control group (LV-eGFP group): After successful transfection of HUVECs with empty body containing green fluorescent protein, continue to culture in complete medium for 24 hours. (5) TXNIP overexpression group (LV-eGFP + TXNIP group): after the successful transfection of TXNIP Lentivirus into HUVECs, continue to culture in complete medium for 24 hours. (6) TXNIP overexpression + ox LDL group (LV-eGFP + TXNIP + ox LDL group): after successful transfection of HUVECs with TXNIP Lentivirus, the working concentration of 50µg/mL ox-LDL was used to stimulate for 24 hours. (7) TXNIP overexpression + ox- LDL + BBR group (LV-eGFP + TXNIP + ox LDL + BBR group): after TXNIP Lentivirus was successfully transfected into HUVECs, it was pretreated with 20µmol/L berberine for 1 hour, and then continued to intervene with 50µg/mL ox-LDL for 24 hours.

TXNIP Lentivirus transfection

TXNIP titer in this experiment: 1×10^8 TU/mL, blank titer: 2.33×10^8 TU/mL Use 3–5 generation HUVECs for Lentivirus infection, adjust before infection and ensure that cells are in good condition. Inoculate the HUVECs into a 6well plate with 10^5 cells per well and 2 mL per well. When the cell density is between 20% and 30% 24 hours later, according to the operating instructions provided by the reagent manufacturer (Tongyong Biol, V1322637, China), calculate the required volume of Lentivirus according to MOI = 10, and infect the HUVECs. After 48 to 72 hours, observe the fluorescence effect, take photos and record, collect cells, extract RNA, and detect the expression of target genes at mRNA level for subsequent experiments.

Scanning electron microscopy observation of cell morphology

Inoculate HUVECs into a 6 well plate at 5×10^5 cells per well, with 2 mL per well. After incubation in a 37 °C, 5% CO₂ incubator for 24 hours, different intervention factors were added according to the grouping. After 24 hours of cultivation, 2 mL of electron microscope fixative containing 4% glutaraldehyde was added to each well, and fixed in a dark place at room temperature. Rinse the fixed sample 3 times with 0.1 M PBS buffer for 15 minutes each time. 1% citric acid was fixed at room temperature in dark for 1–2 hours, and PBS rinsing was repeated 3 times. Perform gradient dehydration with 30%, 50%, 70%, 90%, 95%, and 100% ethanol for 15 minutes each time. Place the sample in a critical dryer for drying, spray gold on the sample with MC1000 ion sputtering instrument for 30 seconds, observe with a scanning electron microscope, and collect images for analysis.
LDH vitality testing

Inoculate HUVECs into 96 well plates at a rate of 104 cells per well. After treatment with different factors, collect the supernatant and transfer it to a centrifuge tube at 4°C, 14000g. Centrifuge for 5 minutes and store at -80°C. According to the manufacturer’s instructions for the LDH activity assay kit (Elabscience, E-BC-K046-M, China), perform the operation, use an enzyme-linked immunosorbent assay to detect the OD value at a wavelength of 450 nm, create a standard curve, and calculate the content to be measured for each group.

Enzyme-linked immunosorbent assay (ELISA)

According to the manufacturer’s instructions, the levels of inflammatory factors IL-18 and IL-1β in the serum of HUVECs were detected using the human IL-18 ELISA kit (Elabscience, E-EL-H0253c) and the human IL-1β high sensitivity ELISA kit (Lianke Biology, 70-EK101BHS, China), respectively. The OD value at a wavelength of 450 nm was detected using an enzyme-linked immunosorbent assay and calculated according to the formula.

Western blotting analysis

The HUVECs were inoculated into a 6 well plate with 10⁵ cells per well. Cells were lysed with a mixture of RIPA high efficiency lysate and protease inhibitor to extract 14000g of protein. After centrifugation for 20 min, the supernatant was collected into a centrifuge tube. Use the BCA protein concentration assay kit to determine protein concentration. The protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Then transfer the protein at a constant current of 300 mA for 30 minutes to a polyvinylidene fluoride PVDF membrane, rinse with 1×TBST three times, and seal at room temperature for 30 minutes with 5% skimmed milk. Incubate the PVDF membrane at 4 °C with prepared antibodies TXNIP (1:2000, AB188865, UK), NLRP3 antibody (1:1000, AB263899, UK), GSDMD (1:1000, AB210070, UK), and GAPDH (1:1000, AB263962, UK), Caspase1 p10 (GeneTex, GTX134551, USA), and shake the bed slowly overnight. Wash the PVDF membrane with 1×TBST three times and place it on a decolorization shaker for rapid elution, each time lasting for 5 minutes. Add horseradish peroxidase coupled secondary antibody (HRP) (1:5000, Servicebio GB23303) and incubate at room temperature for 30 minutes, followed by rapid elution of 1×TBST for 5 minutes and repeated 3 times. Follow the instructions to use the ECL reagent kit (Servicebio, G2020, China) for chemiluminescence development, save the original image in TIFF format, and perform data analysis using Image J software. Using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal reference.

Real time quantitative polymerase chain reaction RT-qPCR

Use the Total RNA Extraction Kit (TianGen, DP419, China) to extract total RNA from each group of HUVECs, and measure RNA concentration using a nucleic acid protein quantification instrument. Using TB Green® Premix Ex Taq™ II (Takara, RR820A, Japan) reverse transcriptional cDNA, Using Prime Script™ RT reagent Kit with gDNA Eraser (Takara, RR047A, Japan) was carried out in Roche LC480 fluorescence quantitative PCR instrument for quantitative polymerase chain reaction (qPCR), Calculate the Ct value,
calculate the relative mRNA expression using the $2^{△△Ct}$ method, and use glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the internal reference. The primers were designed by Shenggong (Shanghai, China) and the sequences are shown in Table 1.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Primer sequence 5'-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>TXNIP</td>
<td>Forward primer : CCCTGGTAATTGGCAGCAGA</td>
</tr>
<tr>
<td></td>
<td>Reverse primer : TGCAGGGATCCACCTCAGTA</td>
</tr>
<tr>
<td>NLRP3</td>
<td>Forward primer : AGGGATGAGAGTGTTGTGTGAAACG</td>
</tr>
<tr>
<td></td>
<td>Reverse primer : GCTTCTGTTGCTGAGGAC</td>
</tr>
<tr>
<td>Caspase 1</td>
<td>Forward primer : GAAGAAACACTCTGAGCAAGTC</td>
</tr>
<tr>
<td></td>
<td>Reverse primer : GATGATGATCACCTTCGGTTT</td>
</tr>
<tr>
<td>GSDMD</td>
<td>Forward primer : GCCTCCACAAACTCCTGAGCATG</td>
</tr>
<tr>
<td></td>
<td>Reverse primer : GGTCCTCACCCTCGCCGTAG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward primer : CATTGGTCGTATTGGGGCGC</td>
</tr>
<tr>
<td></td>
<td>Reverse primer : TTCCGTTCTCAGCCTTGTCA</td>
</tr>
</tbody>
</table>

**Statistical Analysis**

Perform statistical analysis on the data using SPSS 28.0 software, and collect data from at least three independent experiments. Measurement data is expressed as mean ± standard deviation ( $x \pm s$ ). If the population conforms to normal distribution, single factor analysis of variance is used, and the variance is homogeneous. LSD test is used for comparison between multiple groups; The variance is uneven and Dunnett’s T3 test is used. If the normal distribution is not met, the nonparametric Wilcoxon rank sum test is used. There is a statistical difference in the test level with $P < 0.05$.

**Results**

**Berberine inhibits ox-LDL induced endothelial cell injury**

To evaluate the protective effect of berberine on ox-LDL induced damage to HUVECs. We used 50µg/mL ox-LDL to induce endothelial cell inflammatory damage and 20µmol/L BBR for drug intervention. Detect the activity of LDH and the content of inflammatory factors IL-18 and IL-1β in the supernatant of HUVECs, as shown in Fig. 1 (a-c). Compared with the control group, the content of LDH, IL-18 and IL-1β in the supernatant of the ox-LDL group was significantly increased. Compared with the ox-LDL group, BBR could significantly downregulate the secretion of IL-18 and IL-1β in the supernatant of HUVECs. The results indicate that berberine can reduce the expression levels of LDH, IL-18, and IL-1β in ox-LDL induced
oxidative damage in HUVECs. BBR has the effect of reducing inflammatory response and inhibiting endothelial damage.

Next, we used electronic scanning electron microscopy (d-e) to analyze the morphological changes of HUVECs. The control group had long spindle shaped cells, smooth surfaces without swelling, no obvious protrusions, intact cell membranes without cracks, and overall regular boundaries. In ox-LDL group, there were round and oval cells with rough surface and a large number of folds, which showed cell swelling and bulge, cell membrane rupture, bubble like protrusions and pore formation, and obvious pseudopodia, consistent with the typical morphological changes of pyroptosis. The surface pores and folds of the ox-LDL + BBR group cells were significantly reduced compared to the ox-LDL group, with a slightly rough surface and a small amount of pseudopodia visible. The damage to HUVECs was significantly reduced after BBR treatment, indicating that BBR can protect endothelial cell damage induced by ox-LDL and has an inhibitory effect.

**BBR inhibits ox-LDL induced pyroptosis in HUVECs**

After activation of NLRP3 inflammasomes, pro-capase-1 activation can be induced to cleave into Caspase-1 composed of Caspase-1 p10 and p20 subunits, and mature Caspase-1 cleaves GSDMD. To verify the effect of BBR on ox LDL induced endothelial pyroptosis, we used Western Blotting to quantitatively analyze the expression levels of pyroptosis related proteins NLRP3, Caspase1 p10, and GSDMD. As shown in Fig. 2(a-b), compared with the control group, the expression of NLRP3, Caspase1 p10, and GSDMD proteins in the ox-LDL group was significantly increased, indicating that ox-LDL can induce HUVECs to pyroptosis. Compared with the ox-LDL group, after BBR intervention, the expression level of pyroptosis protein in the ox-LDL + BBR group was significantly downregulated. We further used RT-qPCR to detect the relative mRNA expression of NLRP3, Caspase-1, and GSDMD, and obtained results similar to Western Blotting (Fig. 2c-e). ox-LDL can induce the scorch of HUVECs. After BBR treatment, the relative mRNA expression of NLRP3, Caspase-1, and GSDMD was significantly reduced compared with the ox-LDL group, indicating that BBR can inhibit pyroptosis. The results suggest that berberine can inhibit the apoptosis of endothelial pyroptosis by down regulating NLRP3/GSDMD.

**Berberine downregulates the expression of TXNIP**

TXNIP can directly bind to NLRP3 and activate NLRP3 inflammasomes, in order to demonstrate the regulatory effect of BBR on TXNIP. We used Western blotting and RT-qPCR to detect its protein expression level and relative mRNA expression level. As shown in Fig. 3, compared with the control group, the protein level and relative mRNA expression of TXNIP in the ox-LDL group were significantly increased after 24 hours of ox-LDL induction. Compared with the ox-LDL group, after BBR intervention, the protein expression and mRNA level of TXNIP in the ox-LDL + BBR group were significantly reduced. The results suggest that BBR can inhibit the expression of TXNIP in HUVECs pyroptosis induced by ox-LDL, and TXNIP may be the target of BBR's inhibition of HUVECs pyroptosis.
TXNIP transfection effect evaluation

We infected HUVECs with Lentivirus TXNIP (MOI = 10) labeled with green fluorescent protein, and constructed the overexpression of TXNIP in HUVECs. Observe the transfection efficiency under a fluorescence microscope. Under the microscope, it can be seen that the transfection efficiency of the LV-eGFP group is greater than 80%, while the transfection efficiency of the LV-eGFP-TXNIP group is about 60% (Figure. 4a-b). The total RNA of cells was extracted and the transfection effect was verified. Compared with the control group, the expression of TXNIP in the LV-eGFP group was roughly the same, and there was no obvious change. Compared with the LV-eGFP group, the mRNA expression in the LV-eGFP-TXNIP group was about 7–8 times that in the LV-eGFP group (Fig. 4c), suggesting that the transfection effect of Lentivirus was good, and follow-up experiments could be continued.

BBR inhibits IL-18, IL-1β, and LDH levels by downregulating TXNIP

We infected HUVECs with Lentivirus TXNIP, constructed the overexpression of TXNIP, and then treated them with different intervention factors for 24 hours. The supernatant of each group was collected, and the secretion of IL-18, IL-1β and LDH in the supernatant of HUVECs was detected. As shown in Fig. 5, the levels of IL-18, IL-1β, and LDH in the supernatant of the LV-eGFP + TXNIP group were significantly higher than those of the LV-eGFP group, indicating that overexpression of TXNIP can promote the release of inflammatory factors. Compared with the LV-eGFP + TXNIP + ox-LDL group, the LV-eGFP + TXNIP + ox-LDL + BBR group significantly reduced the content of IL-18, IL-1β, and LDH, indicating that BBR can inhibit the expression levels of IL-18, IL-1β, and LDH by inhibiting TXNIP levels.

BBR inhibits ox-LDL induced HUVECs pyroptosis by downregulating TXNIP levels

In order to investigate the specific molecular mechanism of BBR regulation of TXNIP, Western blotting and RT qPCR were used to detect the protein content and relative mRNA expression of pathway related molecules, and similar results were obtained. As shown in Fig. 5, after Lentivirus infection with HUVEC, the level of TXNIP in LV-eGFP-TXNIP group was higher than that in LV-eGFP group, and the related pathway molecules NLRP3, Caspase1, GSDMD, protein expression and mRNA expression were significantly up-regulated, indicating that TXNIP could aggravate the focal death of HUVECs. After BBR treatment, the above results can be weakened. BBR can not only inhibit the expression of TXNIP, but also reduce the levels of NLRP3, Caspase-1 and GSDMD by inhibiting TXNIP, playing a role in inhibiting pyroptosis. The results suggest that BBR inhibits ox-LDL induced HUVECs pyroptosis by downregulating the TXNIP/NLRP3/GSDMD pathway.

Discussion
AS is a chronic inflammatory disease that can be caused by endothelial damage and lipid deposition, leading to inflammatory reactions, thrombosis, thickening of blood vessel walls, and arteriosclerosis. Inflammatory response is a driving factor for AS and runs through the entire process. Endothelial cell damage and cellular dysfunction are classic markers and predictive factors for the occurrence of AS [5,32]. When endothelial cells are injured or dysfunctional, inflammatory cytokines and cell adhesion molecules are secreted in large quantities. The secreted chemokines and other effector proteins lead to the aggregation of a large number of inflammatory cells, the enhancement of inflammatory reaction of vascular wall, the destruction of the integrity of tunica intima, and the aggravation of the pathological process of AS [33–34]. In this study, we used ox-LDL to induce inflammatory damage in HUVECs. The previous research team searched for literature and combined it with CCK8 to select the optimal concentrations of ox-LDL and BBR for intervention in HUVECs, which were 50mg/mL and 20µmol/L, respectively. When the vascular endothelium is damaged and the cell membrane ruptures, inflammatory cells can secrete IL-18 and IL-1\(\beta\) and a large number of inflammatory factors such as LDH are released into the extracellular space. This study found that the secretion of LDH in the supernatant of HUVECs suggests an increase, and LDH is one of the markers of cell membrane rupture, and its release to some extent reflects the degree of cell damage [4]. At the same time, ELISA was used to detect the protein content of inflammatory factors IL-18 and IL-1\(\beta\) in the supernatant, and the results showed a significant increase, indicating the presence of cell damage in HUVECs. This is consistent with the results of previous literature suggesting cell inflammation damage. Firstly, it was determined that the construction of a cell inflammation damage model was successful.

Endothelial pyroptosis is one of the main ways of endothelial cell injury, and NLRP3 inflammatory body dependent Caspase-1 is the classic way of pyroptosis [35]. The accumulation of ox-LDL is one of the ways to trigger endothelial pyroptosis. When ox-LDL accumulates in the vascular wall, it triggers the activation of NLRP3 inflammasome, and the expression levels of NLRP3, Caspase-1 and related inflammatory factors are significantly increased, through NLRP3/Caspase-1/IL-1\(\beta\) Signal pathways promote endothelial cell activation and dysfunction [36,37]. In arterial plaque, pyroptosis caused by NLRP3 inflammasome is highly correlated with plaque rupture and vasculitis, and pyroptosis related molecules, NLRP3, ASC, Caspase-1, and GSDMD also show a trend of high expression in unstable plaque [38].

In this study, scanning electron microscope was used to find that after ox LDL stimulated HUVEC for 24 hours, cells swelled, membrane ruptured, accompanied by a large number of bubble like projections and pore formation, and pseudopodia was obvious, which was consistent with the typical morphological changes of pyroptosis. After stimulating HUVECs with ox-LDL, we found an increase in NLRP3 levels, indicating activation of NLRP3 inflammasomes. Activated Caspase-1 is composed of its subunits p10 and p20 heterotetramer, which are both effective components of Caspase-1. The formation of its subunits p10 and p20 not only reflects the activation level of Caspase-1, but also reflects the degree of pyroptosis [39]. Therefore, we used RT-qPCR and WB to detect Caspase-1 and Caspase-1 p10 respectively, and the results were higher than those of the normal group, suggesting that Caspase-1 was activated, and the expression level of GSDMD was up-regulated. GSDMD is an executive protein of pyroptosis, and
a marker of pyroptosis [40]. In addition, activated Caspase-1 can induce IL-1β and IL-18 precursors to become active IL-1β and IL-18, allowing inflammatory substances to be released into the extracellular space through membrane pores [41], while explaining the results of inflammatory factors in the previous experiment. Ox-LDL can induce the pyroptosis of HUVECs, which has been verified in this experiment.

Berberine, as a traditional Chinese herbal medicine, has powerful effects in anti-inflammatory, lipid-lowering, improving vascular endothelial function, inhibiting inflammatory cell infiltration and smooth muscle proliferation, migration, and stabilizing plaques [2,42]. It has been found that berberine can treat digestive system, respiratory system, nervous system and other related diseases by inhibiting the classic pyroptosis pathway mediated by NLRP3 inflammasome [43,44]. However, the regulatory mechanism of berberine on NLRP3 inflammasome mediated pyroptosis is still unclear. We demonstrated whether BBR could inhibit ox-LDL induced pyroptosis of HUVECs. Before ox-LDL induced endothelial pyroptosis, HUVECs were pretreated with 20µmol/L BBR 1 hour in advance. Observation of cell morphology under scanning electron microscopy showed that the cell surface of the drug group was slightly rough. After BBR treatment, the pores and folds on the cell membrane surface were significantly reduced compared to the model group. BBR can alleviate endothelial cell damage and inhibit the pyroptosis of HUVECs induced by ox-LDL. The WB and RT qPCR results suggest that BBR can reduce the expression of NLRP3 mediated pyroptosis protein molecules, and downregulate the relative expression of NLRP3, Caspase-1, GSDMD mRNA, while IL-18 and IL-1β The secretion of LDH in the cell supernatant was also reduced. We can conclude that BBR can inhibit NLRP3 inflammasome mediated endothelial pyroptosis, and its mechanism may be that BBR can reduce endothelial cell damage by downregulating NLRP3/Caspase-1/GSDMD pathway, intervening in inflammatory cascade reaction, reducing the production of IL-18, IL-1β, LDH and other downstream products.

TXNIP is the second signal activated by NLRP3 and can directly bind to NLRP3. When TXNIP is activated, it immediately separates from TRX and acts directly on NLRP3 [45]. After the activation of NLRP3 inflammasomes, the expression of Caspase-1 and GSDMD proteins increased, and the release of inflammatory factors IL-18 and IL-1β increased. After gene knockout of TXNIP, the activation of NLRP3 inflammasomes was affected, and downstream molecules were significantly reduced [46]. Multiple research results have shown that many drugs, such as Tangshen Formula and Taurine, are involved in regulating TXNIP to achieve the goal of treating related diseases [47,48]. However, there are few studies on the regulatory effect of BBR on TXNIP, especially in the AS field. Therefore, the research team studied the regulatory mechanism of berberine on TXNIP, and further elaborated the mechanism of BBR's effect on NLRP3 inflammatory body mediated endothelial pyroptosis by regulating the level of TXNIP.

We infected HUVECs with Lentivirus TXNIP to construct a TXNIP overexpression system. We found that after overexpression of TXNIP, the expression levels of NLRP3, Caspase-1 and GSDMD were significantly increased, the levels of IL-18 and IL-1β inflammatory factors were increased, and the content of LDH in the supernatant was increased. Therefore, overexpression of TXNIP could aggravate NLRP3 mediated pyroptosis. After overexpression of TXNIP, we pretreated HUVECs with BBR for 1 hour, and then continued to interfere with HUVECs with ox LDL for 24 hours. The results showed that the expression levels of
NLRP3, Caspase-1, and GSDMD were inhibited, and the levels of downstream products IL-18 and IL-1β were reduced, and the LDH release was reduced, indicating that BBR can offset the pyroptosis effect caused by overexpression of TXNIP to a certain extent, indicating that BBR can inhibit endothelial pyroptosis by inhibiting the overexpression of TXNIP. It can be seen that TXNIP may be the target of BBR, and its mechanism may be through down-regulation of TXNIP/NLRP3/GSDMD signaling pathway to inhibit ox LDL induced endothelial pyroptosis, thus playing a role in alleviating endothelial cell damage and delaying the occurrence and development of AS.

The mechanism of pyroptosis and AS is complex, and the research is not thorough. How NLRP3 inflammatory bodies drive AS needs to be further explored. The characteristic of berberine action is multiple pathways and targets, and the mechanism is relatively complex. Although the research group has done some research at the cell level, the cell type is single, and the pyroptosis pathway is limited, which cannot represent the in vivo experiment, and needs further systematic improvement. In this study, the mechanism of berberine inhibiting pyroptosis has not been verified in clinical practice, and there is a lack of relevant clinical data. Therefore, more in vivo studies and clinical experiments are needed to clarify the molecular pathway and specific mechanism of berberine on pyroptosis, so as to provide a basis for the development of AS target inhibitors.

Declarations

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Figures
Figure 1

Effect of berberine on ox-LDL induced damage to HUVEC. (a) LDH release in the supernatant of HUVECs. (b) IL-18 release in HUVEC supernatant. (c) IL-1β release in the supernatant of HUVECs. (d) low power lens, ×1500, scale: 30.0μm. (e) High power lens ×6000, scale:5.0μm. The data of each group is expressed as mean ± standard deviation (x±s), n=7-9. Compared with the control group, * P<0.05; Compared with the ox-LDL group, # P<0.05.
Figure 2

The effect of BBR on the pyroptosis of HUVECs induced by ox-LDL. (a) Western blotting detection of related pyrolytic protein levels. (b) Pyrolysis protein level; (c) Relative expression level of NLRP3 mRNA. (d) Relative expression level of Caspase1 mRNA. (e) The relative expression level of GSDMD mRNA. The data of each group is expressed as mean ± standard deviation (x±s), n=4-8. Compared with the control group, *P<0.05; Compared with the ox-LDL group, #P<0.05.
Figure 3

The impact of BBR on TXNIP. (a) Western blotting was used to detect the expression level of TXNIP. (b) The protein level of TXNIP (c) is the relative expression level of TXNIP mRNA. The data of each group is expressed as mean ± standard deviation (x±s), n=5. Compared with the control group, *P<0.05; Compared with the ox-LDL group, # P<0.05.
Figure 4

Transfection effect of TXNIP Lentivirus. (a-b) Observing green fluorescent cells under an inverted fluorescence microscope ×100, scale: 200μm. (c) RT-qPCR detection of TXNIP relative mRNA expression in HUVECs. The data of each group is expressed as mean ± standard deviation (x±s), n=8. Compared with the control group, *P<0.05 compared to the LV-eGFP group, &P<0.05.

Figure 5

Effect of BBR on the expression levels of LDH, IL-18, and IL-1β in the supernatant of HUVECs. (a) The expression level of LDH. (b) The expression level of IL-18. (c) The expression level of IL-1β. The data of each group is expressed as mean ± standard deviation (x±s), n=8. Compared with LV-eGFP, &P<0.05; compared with LV-eGFP-TXNIP group, ▽P<0.05; compared with LV-eGFP-TXNIP+ox-LDL group, $P<0.05.
The effect of BBR on the pyrolytic pathway molecules of HUVECs. (a) Western blotting detected the expression level of TXNIP pathway after overexpression. (b) The protein levels after overexpression of TXNIP. (c) Relative expression level of TXNIP mRNA. (d) Relative expression level of NLRP3 mRNA. (e) Relative expression level of Caspase1 mRNA. (f) The relative expression level of GSDMD mRNA. The data of each group is expressed as mean ± standard deviation (\(\bar{x} \pm s\), n=4-8. Compared with LV-eGFP, &\( P<0.05\), compared with LV-eGFP-TXNIP group, ▼\( P<0.05\), compared with LV-eGFP-TXNIP+ox-LDL group, $\ P<0.05\).