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Original Article

Resveratrol inhibited the formation of NLRP3 inflammatory body by activating autophagy signal pathway in atherosclerosis

Yue Wang*, Li Fan*, Junfeng Zhang, Yuqi Fan, Qizhi Chen, Kan Chen, Lin Gao, Huasu Zeng, Changqian Wang#, Zhihua Han#

*Department of Cardiology, No. 9 People's Hospital, School of Medicine, Shanghai Jiao Tong University, 639 Zhizaoju Road, Shanghai 200011, P.R. China. *Equal contributors and co-first authors. #Equal contributors.*

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Abstract: Atherosclerosis (AS) is a common disease and it seriously endangers human health. Studies have found that inflammation theory is a possible pathogenesis of AS. Howevere, the specific mechanism between AS and inflammation remains to be elaborated. Therefore the objective of this study was to explore the association between NLRP3 inflammatory body and autophagy signal pathway in AS and evaluate the therapeutic effect of resveratrol (RSV) on AS and its underlying mechanism. In the study, we detected the function of RSV on the expression of inflammation related genes and activated autophagy related genes in vitro. And we tested RSV's effect on the occurrence and development of AS in vivo. From the results, we found that RSV could inhibit the mRNA expression of SIRT1, NF-kB and NLRP3 and activate the mRNA expression of mTOR, p70S6Kaxl and LC3-I/II with a concentration dependent. Consistently, western bloting showed that RSV could decrease the protein expression of pNF-kB and NLRP3 and increased the protein expression of LC3-II/I, Beclin1 and mTOR with a concentration dependent. In addition, RSV decreased the IL-1β level in serum of ApoE-/- mice. Moreover, the fluorescence intensity of DiI-OX-LDL was significantly enhanced in RSV+ group in Raw cells at per concentration of DiI-OX-LDL, including 6.25, 12.5, 50, 100 μg/ml. And animal experiment also indicated that RSV attenuated AS of ApoE-/- mice. In conclusion, RSV may inhibit the formation of NLRP3 inflammatory body and attenuate inflammation by activating autophagy signal pathway in AS.

Keywords: Resveratrol, NLRP3, inflammation, autophagy, mTOR

Introduction

Atherosclerosis (AS) is a common disease, which seriously endangers human health, and is the main pathological basis of ischemic cardiovascular and cerebrovascular diseases such as coronary heart disease, cerebrovascular disease and thromboembolic disease [1, 2]. So far, the pathogenesis of AS is not yet fully understood. There are a variety of theories, which involve a variety of risk factors, but still lack effective clinical medicine for treatment of AS. A large number of basic and clinical studies and investigations have indicated variety of risk factors for AS, including hyperlipidemia [3], hypertension [4], hyperglycemia (diabetes) [5], hyperfibrinogenemia [6], hyperhomocysteinemia [7], hyperuricemia [8], obesity [8], renin-angiotensin-aldosterone system (RAAS) activation [9], smoking, coagulation hyperthyroidism (tissue factor, thrombin) [10], metabolic disorders of trace elements (iron, copper, zinc, selenium, chromium, manganese, germanium, etc.) [11], autologous bioactive substances (such as serotonin, NO, endothelin-1) [12]. Moreover, the theory of the pathogenesis of AS includes lipid infiltration theory [13], retention theory [14], vascular smooth muscle cell cloning theory [15], oxidative stress theory [16], platelet hyperfunction theory, thrombosis theory [17], Ca^{2+} super Load theory [18], immune dysfunction theory, the theory of shear stress, injury response theory, the inflammation theory, of which, inflammation theory is a relatively novel one for scientific research. However, the specific mechanism between atherosclerosis and inflammation remains to be elaborated.

NLRP3, fully named nucleotide binding and oligomerization domain-like receptor family pyrin domain-containing 3, is a famous inflammation-related gene, which encodeds the key protein of NLRP3 inflammatory body and plays a crucial role in the process of inflammation. And the NLRP3 inflammatory body consists of NLRP3, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and caspase-1 or caspase-5 [19]. Over the two decades, emerging evidences have demonstrated that the NLRP3 inflammatory body was associated with AS [20, 21]. Autophagy is a kind of self-stabilizing mechanism of eukaryotic organisms, which could degrade intracellular dysfunctional organelles, misclassify proteins and other harmful macromolecules to maintain normal function of cells. Autophagy process is regulated by the autophagy-associated gene (ATG), including microtubule associated protein 1 light chain 3 (LC3A and LC3B) and target of rapamycin (TOR), in which, autophagy cell could apply its double - layer membrane structure to wrap the obsolete, damaged proteins or organelles and degrade them after fusing with lysosomes into autophagy lysosomes [22]. Macrophages, endothelial cells (ECs) and smooth muscle cells (SMCs), which are considered as the three types of key cells in formation and stability of AS, were reported to promote the development of AS by regulating the formation of complex regulatory network through the expression of adhesion molecules and secretion of cytokine interactions [23].

In this study, we aimed to explore the association between NLRP3 inflammatory body and autophagy signal pathway in AS and evaluate the therapeutic effect of resveratrol (RSV) on AS and investigate its underlying mechanism.

Materials and methods

Cell culture

RAW264.7 cells and a mouse monocyte/macrophage cell line were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA) and maintained in an atmosphere with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; HyClone, Logan, UT, USA) and 1% antibiotic-antimycotic (Invitrogen, Grand Island, NY, USA). The RAW264.7 cells were maintained by weekly passage, and the cells were utilized for experimentation at 60-80% confluence. In addition, LPS-stimulated RAW-264.7 cells were established by the activation of lipopolysaccharide (LPS).

RSV concentration administration

According to the concentration of RSV, all cells were divided into four groups, including 0 μM, 1 μM, 10 μM, 100 μM group, of which, 0 μM group was wild RAW264.7 cells and the other groups were LPS-stimulated RAW264.7 cells.

Animal study

All the protocol of animal experiment was approved by the Laboratory Animal Administration Committee of Shanghai Jiao Tong University and was carried out in accordance with the Guidelines for Animal Experimentation. A total of 20 ApoE-/- mice (10-week-old males, C57BL/6J background) were randomly divided into two groups (n = 10 per group), including high-fat diet group and high-fat diet +RSV (100) group, were fed a chow diet of 1.25% cholesterol for 20 weeks. 10 wild-type mice (C57BL/6J, 10 week-old males) were served as a control group. After 20 weeks feed, all mice were sacrificed by decapitated and the aortas tissues of mice were isolated and collected for the further investigations. And aortas tissue of per mice was divided into two portions, including the upper (aortic root) portion for histologic analysis and the abdominal/thoracic aorta for mRNA and protein expression analyses. Blood was immediately obtained for analyses.

Histology

The heart and whole aorta were immediately extracted when mice were sacrificed by decapitated. The aorta was embedded in optimal cutting temperature (OCT) embedding medium (Tissue-Tek, Sakura Finetek USA, Torrance, CA). Then hematoxylin-eosin (HE) staining was used to determine the morphology of atherosclerotic plaque. The aorta (except for the aortic root) from each mouse from all the groups were removed and stored in -80°C.

RNA isolation and real-time PCR

In accordance with the manufacturer's instructions, total RNA was isolated from the cells using TRIzol reagent (Qiagen, Valencia, CA, USA). The cDNAs were reverse transcribed from total RNA by Prime Script RT-PCR Kit (Takara, Dalian, China). A 20 μl qRT-PCR system was established, including Forward Primer (0.6 μl),

Table 1. All primer sequences for RT-PCR

Gene	Primer sequences
mTOR	
Forward	5'-TCGGCACATCACTCCCTTCA-3'
Reverse	5'-AACAACGGCTTTCCACCAGA-3'
β-actin	
Forward	5'-GGCACAGTCAAGGCTGAGAATG-3'
Reverse	5'-ATGGTGGTGAAGACGCCAGTA-3'
p70S6Kaxl	
Forward	5'-CTACAGAGACCTGAAGCCGGAGA-3'
Reverse	5'-AATGTGTGCGTGACTGTTCCATC-3'
LC3-II	
Forward	5'-TAGGTACCACTTTATCCCGTTCAC CA-3'
Reverse	5'-ATCTCGAGGCAGGGAGAGAGGAATAA-3'
SIRT1	
Forward	5'-GCAGATTAGTAAGCGGCTTGAGG-3'
Reverse	5'-AGCACATTCGGGCCTCTCCGTA-3'
NF-kB	
Forward	5'-TAGGTACCACTTTATCCCGTTCAC CA-3'
Reverse	5'-ATCTCGAGGCAGGGAGAGAGGAATAA-3'

Reverse Primer (0.6 μl), cDNA (2 μl), ROX Reference Dye II (0.4 μl), SYBR Premix Ex Taq (10 μl), and ddH_2O (6.6 μl), and tested by the DA7600 Real-time Nucleic Acid Amplification Fluorescence Detection System (Bio-Rad). GAPDH was used as internal control in present study. The primers used for mTOR, p70S6Kaxl, LC3-II, IL-1β, SIRT1, NF-kB and NLRP3 in this study were recorded in **Table 1**. The levels of relative expression were quantified using the $2^{-\Delta\Delta CT}$ threshold cycle method.

Quantification of released IL-1β, IFN-γ and TNF-α

Concentration of interleukin (IL)-1β, interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) in mice serum were determined using mouse IL-1β, IFN-γ and TNF-α enzyme-linked immuno sorbent assay (Elisa) kit (4A Biotech, China) according to the manufacturer's protocol.

Western blotting

Cells were collected and lysed by RIPA buffer and BCA assay was used to detect the protein concentration, and equal amount of protein were electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gel (Bio-Rad, USA) and transferred onto poly vinylidene difluoride membranes (PVDF, Millipore). After blocking for non-specific binding by 5% skim milk, the membrane was incubated with specific primary antibodies against mTOR (1:1000; Abcam), beclin-1 (1:1000; Abcam), LC3-I/II (1:1000; Abcam), pNF-kB (1:1000; Abcam) and NLRP3 (1:1000; Abcam) overnight at 4°C. After washing with tris buffered saline Tween (TBST) 3 times, membranes were incubated with secondary antibodies (Abcam) for 1 h at room temperature.

Immunofluorescence assay

Cells were grown on glass slices and fixed in 4% formaldehyde for 10 min, permeabilized through 0.3% Triton X-100. Then the slices were blocked in goat serum for 15 min, 37°C and incubated overnight at 4°C with anti-LC3B (1:80, Bioworld, MN, USA), anti-NLRP3 (1:80, Bioworld, MN, USA). Samples were washed three times before incubated with goat TRITC labeled secondary antibody (1:70, Bioworld, MN, USA) at 37°C for 1 h. DAPI (GenviewInc, Shanghai, China) was used for counterstaining. Then the cells were examined under a laser scanning microscope (TCSSP2-AOBS-MP, Leica Microsystems CMS).

Dil-ox-LDL uptake

Cells were incubated with 1 μg/ml Dil-ox-LDL for 2 hours. Upon completion of incubation, cells were gently washed with 1× PBS three times to remove free Dil-ox-LDL and analyzed using fluorescent microscope (TCSSP2-AOBS-MP, Leica Microsystems CMS).

Statistical analysis

Continuous variables are presented as mean and standard deviation (SD). Data analysis was conducted by SPSS 19.0 software. Statistical analysis was carried out by GraphPad Prism5.0 (San Diego, CA, USA). Comparisons between two groups were made using the Student's t-test. P value <0.05 was considered statistically significant. All data were obtained from at least three independent experiments.

Results

RSV inhibited the mRNA expression of inflammation related genes

As shown in **Figure 1**, compared with that in 0 μM group, the mRNA expression of SIRT1 were significantly inhibited in 0.1 μM, 1 μM and 100 μM group, respectively (0.78±0.12, 0.46±0.18,

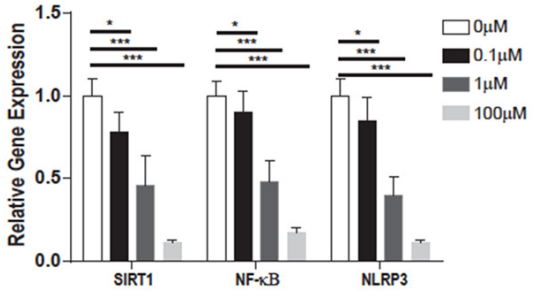

Figure 1. The mRNA expression of inflammation related genes were determined by QPCR after adding RSV. Compared with the 0 mM group, *P<0.05, ***P<0.001, differences were statistically significant.

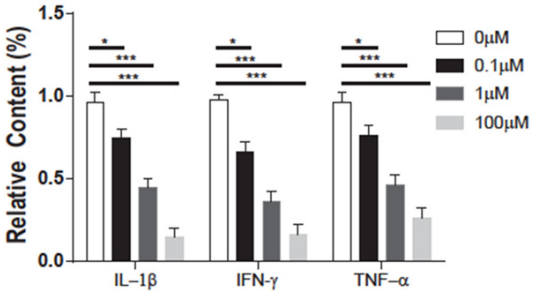

Figure 2. Effect of RSV on IL-1β, IFN-γ, TNF-α serum levels of mice by Elisa. Compared with the 0 mM group, *P<0.05, **P<0.01, ***P<0.001, differences were statistically significant.

0.11±0.02 *vs.* 1.0±0.10, P<0.05, P<0.001, P< 0.001, respectively). Moreover, the mRNA expression of NF-KB were remarkably decreased in 0.1 μM, 1 μM and 100 μM group compared with 0 μM group (0.90±0.13, 0.48±0.13, 0.17± 0.03 vs. 1.0±0.09, P<0.05, P<0.001, P<0.001, respectively), and the mRNA expression of NLRP3 was remarkably decreased in 0.1 μM, 1 μM and 100 μM group compared with 0 μM group (0.85±0.14, 0.40±0.11, 0.11±0.02 vs. 1.0±0.10, P<0.05, P<0.001, P<0.001, respectively). These results indicated that RSV inhibited the mRNA expression of inflammation related genes with a concentration dependent.

RSV decreased the IL-1β, IFN-γ and TNF-α levels in serum of mice

To further investigate the effect of RSV on inflammation, Elisa assay was used to detect the expression levels of IL-1β, IFN-γ and TNF-α in serum of mice. As shown in **Figure 2**, compared with that in 0 μM group, the IL-1β, IFN-γ

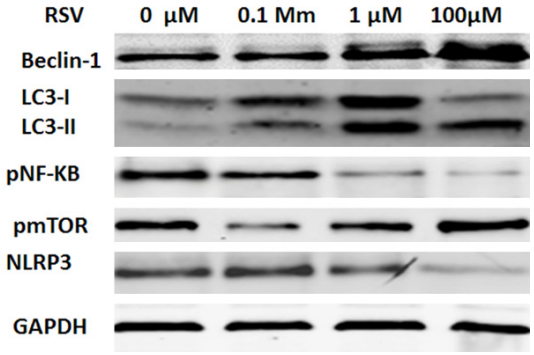

Figure 3. Western Blotting was used to evaluate the function of RSV inhibiting theprotein expression of inflammation related genes and activated autophagy related genes. GAPDH was used as a loading control.

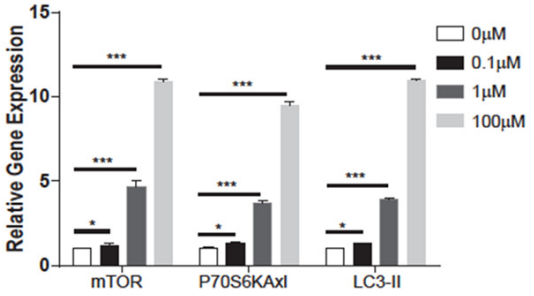

Figure 4. The mRNA expression of autophagy related genes after adding RSV were tested by QPCR. Compared with the 0 mM group, ***P<0.001, differences were statistically significant.

and TNF-α levels were significantly inhibited in 0.1 μM, 1 μM and 100 μM group, respectively. In addition, the IL-1β, IFN-γ and TNF-α levels among 0.1 μM, 1 μM and 100 μM group had an obvious statistically significant (P<0.05). These results also demonstrated that RSV could decrease inflammation with a concentration dependent.

RSV inhibited the protein expression of inflammation related genes

To validate the effect of RSV on protein expression of inflammation related gene, western blot was applied for detecting the protein expression of inflammation related gene, including NF-kB and NLRP3. As shown in Figure 3, compared with that in 0 μM group, the protein expression of NLRP3 were significantly inhibited in 0.1 μM, 1 μM and 100 μM group, respectively (P<0.05). In addition, the expression difference of protein expression of NLRP3 among

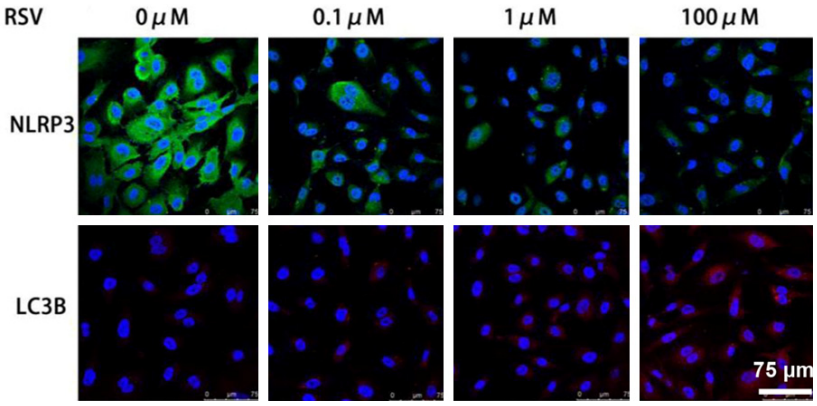

Figure 5. Effects of RSV on autophagy and inflammation related genes were detected by immunofluorescence assay. (Original magnification: ×200).

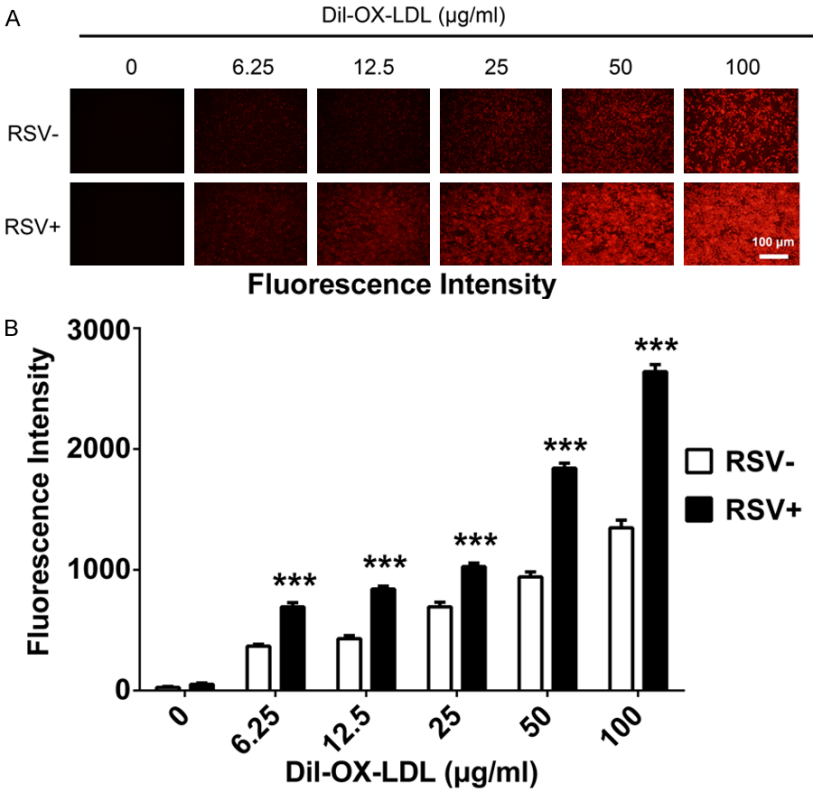

Figure 6. RSV promoted Raw cells to swallow Dil-OX-LDL. Compared with the RSV- group, ***P<0.001, differences were statistically significant. (Original magnification: ×200).

0.1 μM, 1 μM and 100 μM group had an obvious statistically significant (P<0.05). Compared with that in 0 μM group, the protein expression of pNF-kB were significantly inhibited in 0.1 μM, 1 μM and 100 μM group, respectively (P<0.05). In addition, the expression difference of protein expression of pNF-kB among 0.1 μM, 1 μM and 100 μM group had an obvious statistically significant (P<0.05). A consistent conclusion showed that the levels of IL-1β, TNF-α and IFN-γ decreased with the increase in RSV concentration.

RSV activated autophagy in LPS-stimulated RAW264.7 cells

As shown in **Figure 4**, compared with that in 0 μM group, the mRNA expression of mTOR were significantly increased in 0.1 μM, 1 μM and 100 μM group, respectively (1.21±0.12, 4.67±0.34, 10.89±0.19 vs. 1.0±0.02, P<0.05). Moreover, the mRNA expression of p70S6Kaxl was remarkably overexpressed in 0.1 μM, 1 μM and 100 μM group compared with 0 μM group (1.30±0.13, 3.67±0.15, 9.48±0.23 vs. 1.0±0.01, P<0.05), and the expression difference among 0.1 μM, 1 μM and 100 μM group had an obvious statistically significant (P<0.05, P<0.001, P<0.001). In addition, the mRNA expression of LC3-II was remarkably elevated in 0.1 μM, 1 μM and 100 μM group compared with 0 μM group (1.31±0.05, 3.89±0.11, 11.02±0.25 vs. 1.0±0.02, P<0.05, P<0.001, P<0.001). Moreover, we further investigated the effect of RSV on the protein expression of autophagy related genes. As showed in **Figure 3**, compared with that in 0 μM group, the protein expression of mTOR were significantly increased in 0.1 μM, 1 μM and 100 μM group, respectively (P<0.05, P<0.001, P<0.001). Similar results showed in the protein expression of LC3-II/I and Beclin1. In addition, immunofluorescence assay showed that RSV could inhibit

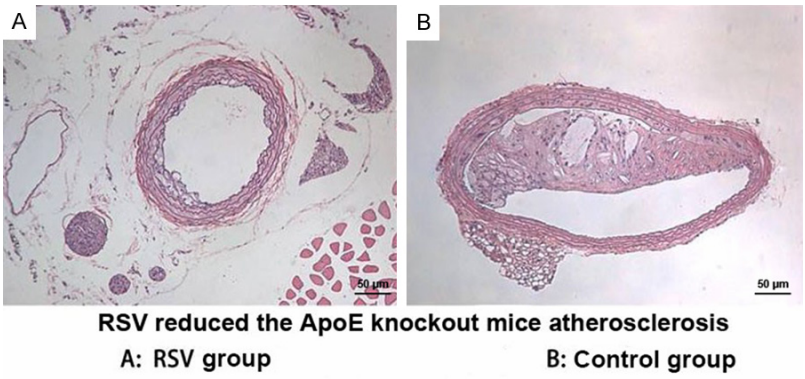

Figure 7. RSV attenuated AS in ApoE-/- mice. (Original magnification: ×200).

the formation of inflammatory body NLRP3 and activate the autophagy protein LC3B, and this effect also had a concentration dependent (**Figure 5**). These results indicated that RSV activated autophagy in LPS-stimulated RAW264.7 cells.

RSV promoted Raw cells to swallow Dil-OX-LDL

As shown in **Figure 6**, compared with that in RSV- group, the fluorescence signal of Dil-OX-LDL was significantly enhanced in RSV+ group in Raw cells at per concentration, including 6.25, 12.5, 25, 50, 100 μg/ml. These results indicated that RSV promoted Raw cells to swallow Dil-OX-LDL.

RSV attenuates AS of ApoE-/- mice

As shown in **Figure 7**, compared with control group, AS was obviously attenuated in RSV group in ApoE-/- mice.

Discussion

As we all known, AS is one type of common disease and seriously threats human health [24]. As the mayor pathological basis of coronary heart disease, cerebrovascular disease and thromboembolic disease, the pathogenesis of AS is not yet fully understood, which leads to the lack of effective clinical targeted medicines [25, 26]. Therefore, it is crucial for us to explore the underlying mechanism of pathogenesis of AS and find more effective medicines for the patients with AS. In this study, we found that there was a close association between inflammation, autophagy and AS, and RSV might inhibit the formation of NLRP3 inflammatory body by activating autophagy signal pathway in AS.

Over the past several decades, pathological examination revealed that AS had the basic characteristics of inflammatory response, such as degeneration, exudation and hyperplasia [27, 28]. According to the different of nature of inflammation and inflammatory substances, inflammatory response related to AS could be divided into biological inflammation, immune inflammation and chemical inflammation, of which, immunological inflammation has a crucial role in the formation of AS [29-31]. Of note, inflammatory response associated with AS is regulated by a series of inflammation-related genes and pathways, including NLRP3 and NF-kB [32-34]. Paramel et al. [35] showed that mRNA expression of CARD8 was significantly overexpressed in AS plaques compared with normal vessels, which could promote inflammation. In our study, we found that RSV could inhibit the mRNA and protein expression of SIRT1, NF-kB and NLRP3 with a concentration dependent. And immunofluorescence assay also showed that RSV could inhibit the formation of inflammatory bodies NLRP3. Moreover, Elisa assay indicated that RSV decreased the IL-1β, IFN-γ and TNF-α levels in serum of ApoE-/- mice. As we all known, IL-1β, IFN-γ and TNF-α were closely associated with incidence and development of AS [36-39]. Similarly, Alarcón et al. [40] found that the strawberry played a protective effect on thromboembolic-related disorders and anti-inflammation through decreasing IL-1β level. In addition, we also found that RSV could obviously attenuate AS of ApoE-/- mice. These results indicated that RSV could attenuate inflammation and plaque formation of AS and play a beneficial role in AS.

Over the past decades, more and more evidences showed that cell autophagy played a crucial role in the progression of AS [41, 42]. Through degradation of intracellular damage structure to adapt to oxidation, inflammation, endoplasmic reticulum and other stress or hypoxia and other environments, autophagy could reduce apoptosis and necrosis, which could protect AS plaque cells from oxidative stress

and other cell damage [43-45]. After knockout of Beclin1 and Atg5 gene, inflammatory markers in the plaque increased significantly, which confirmed that there was a close link between inflammation and autologous deletion [46]. Liao et al. [47] found that inhibition of autophagy by silencing ATG5 enhanced apoptosis and NADPH oxidase-mediated oxidative stress, which increased apoptosis and oxidative stress in advanced lesioned macrophages, promoted plaque necrosis, and worsened lesioned efferocytosis. Moreover, autophagy has been shown to play an important role in the development of AS, which could degrade lipid droplets in the cell and regulate lipid metabolism [48, 49]. Wang et al. [50] found that the deficiency of Pdcd4 gene significantly improved oxidized low-density lipoproteins-impaired autophagy efflux, which could promote autophagy-mediated lipid degradation and prevent macrophage conversion into foam cells. In our study, the results indicated that RSV could increase the mRNA expression of mTOR, p70S6Kaxl and LC3-I/II and protein expression of LC3-II/I, Beclin1 and mTOR with a concentration dependent. Similarly, immunofluorescence assay showed that RSV could activate the autophagy protein LC3B. Moreover, RSV promoted Raw cells to swallow DiI-OX-LDL. These results demonstrated that RSV might attenuate AS by activating autophagy signal pathway in AS.

In conclusion, our study found that there was a close association between inflammation and autophagy in AS, and RSV may inhibit the formation of NLRP3 inflammatory body by activating autophagy signal pathway in AS.

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Disclosure of conflict of interest

None.

Address correspondence to: Zhihua Han and Changqian Wang, Department of Cardiology, No. 9 People's Hospital, School of Medicine, Shanghai Jiao Tong University, 639 Zhizaoju Road, Shanghai 200011, P.R. China. E-mail: changqianwang123@163.com

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