Introduction

Atherosclerotic cardiovascular disease is one of the leading causes of death\(^1\). Moreover, the epidemiology of atherosclerosis has significantly changed in recent years\(^2\). With advancements in medical technology and the use of anti-inflammatory drugs and vaccines, patients with atherosclerosis could be living longer, forcing them to encounter the long-term consequences of atherosclerosis, such as myocardial infarction, ischemic cardiomyopathy (one of the most common causes of heart failure), and stroke (often causing the loss of cognitive function and activity ability)\(^3^-^5\). Acute destruction of unstable atherosclerotic cardiovascular disease, often leading to myocardial infarction or stroke. High uric acid (HUA) is associated with the increasing risk of cardiovascular events and death. However, the mechanism by which HUA promotes atherosclerosis and whether HUA affects plaque stability are still unclear.

Methods:

We constructed an atherosclerotic \(\text{Apoe}^{-/-}\) mouse model with HUA. The progression of atherosclerosis and plaques was determined by Oil Red O staining, hematoxylin and eosin (H&E) staining, and Masson staining. TdT-mediated dUTP nick-end labeling assay and immunohistochemistry were used to observe the changes of apoptosis and autophagy in plaques, respectively. Then, we validated the \textit{in vivo} results with RAW 264.7 cell line.

Results:

HUA promoted atherosclerosis and exacerbated plaque vulnerability, including significantly increased macrophage infiltration, lipid accumulation, enlarged necrotic cores, and decreased collagen fibers. HUA increased cell apoptosis and inhibited autophagy in plaques. \textit{In vitro} results showed that HUA decreased cell viability and increased cell apoptosis in foam cells macrophages treated with oxidized low-density lipoprotein. An activator of autophagy, rapamycin, can partially reverse the increasing apoptosis.

Conclusion:

HUA promoted atherosclerosis and exacerbated plaque vulnerability, and HUA facilitates foam cell apoptosis by inhibiting autophagy.

Key words: High uric acid, Atherosclerosis, Plaque instability, Autophagy, Apoptosis

Abbreviations: MACE: major adverse cardiovascular events, UA: uric acid, HUA: high uric acid, SCD: standard chow diet, HFD: high fat diet, ox-LDL: oxidized lipoprotein

High Uric Acid Promotes Atherosclerotic Plaque Instability by Apoptosis Targeted Autophagy

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atherosclerotic plaques is a cause of these major adverse cardiovascular events (MACE)\(^6\).

Several epidemiological studies have indicated that the change of uric acid (UA) is associated with the risk of cardiovascular and all-cause death\(^7\)\(^-\)\(^9\). For every 1 mg/dl increase in UA, cardiovascular and overall mortality increase by 12% and 20%, respectively\(^10\). Some UA-lowering drugs can effectively protect against the occurrence of MACE in patients with diabetes and gout\(^9\)\(^,\)\(^11\)\(^-\)\(^13\). Although many studies have confirmed the relationship between the high uric acid (HUA) and atherosclerosis, the mechanism within HUA and increasing cardiovascular mortality is still unclear.

Decades of research have established atherosclerosis as a chronic inflammatory disease\(^14\). Macrophages engulf lipoprotein and transform into foam cells, which undergo intricate kinds of death and impact the plaque stability\(^15\)\(^-\)\(^17\). Accumulating evidence suggests that defective autophagy has a detrimental effect on cardiovascular diseases\(^18\).

Additionally, autophagy may regulate apoptosis, which is important for atherosclerosis\(^18\). However, the role of HUA in this process remains unclear.

In this study, we aimed to determine whether the HUA contributes to atherosclerosis progression and plaque vulnerability. A preliminary exploration of HUA in foam cell autophagy and apoptosis was conducted.

\section*{2. Methods}

\subsection*{2.1 Animals and \textit{in vivo} Treatments}

Male 8-week-old \textit{ApoE}/- mice were purchased from Xiamen University Laboratory Animal Center (XMULAC), raised in a standard housing environment (free access to food and water in a temperature and humidity-controlled, 12 h light:12 h darkness cycle environment). Mice were randomly divided into three groups: standard chow diet (SCD) group, high fat diet (HFD) group, and HFD + HUA group. The HFD group was maintained for 17 weeks, and in the last 3 weeks, the HFD + HUA group received intraperitoneal injections of hypoxanthine (100 mg/kg) and oteracil potassium (150 mg/kg) every other day. The HFD provided 37% kcal in fat, 43.3\% kcal in carbohydrates, and 19.7\% kcal in protein (HD012a; Botai, China). All experimental procedures of animals in this study were approved by the Institutional Animal Care and Committee of Xiamen University, China (Animal Ethics no. XMULAC20200122).

\subsection*{2.2 Measurement of UA in Mice}

Two methods are used to determine the UA level in mice. First, the blood sample was collected from the tail vein, and UA was measured with the UA detection instrument (EA-11; Sinocare, China). This approach was used to determine the change in blood UA over time. Second, blood was collected at the end of 24 weeks. Separating the serum and UA level was determined with a uric acid test kit (C012-2-1; Nanjing Jiancheng, China). Then, 50 μl serum and 500 μl protein precipitant were mixed uniformly and centrifuged to obtain the supernatant. The three reagents were combined in proportions of supernatant:chromogenic solution:buffer=1.6:0.5:0.5, and the absorbance value was measured at a detection wavelength of 690 nm (Multiskan Sky; Thermo Fisher, Waltham, MA, USA).

\subsection*{2.3 Assessment and Histopathology of Atherosclerotic Lesions}

The animals were anesthetized and euthanized before performing surgical procedures. After PBS buffer and 4\% paraformaldehyde perfusion, the hearts and aortas of mice were carefully removed and then pinned on black pans. After soaking in isopropyl alcohol, the arteries were stained with Oil Red O dyeing solution at room temperature (25°C-27°C) overnight. The top half of the heart was separated for making the frozen slices. Samples were embedded in OCT for cryosectioning (7 μm). Sections were stained with Oil Red O staining (G1262; Solarbio, China), H&E staining (C02-04004; Bioss, China), Masson staining (no. 20190829; Solarbio), and Sirius Red staining (PH1098; Phycene, China) for lesion analysis according to the manufacturer’s instructions. The image was captured by a bright-field microscope (Olympus CX33; Olympus, Japan). Image-Pro Plus 6.0 software was used for subsequent quantitative image analysis.

\subsection*{2.4 TdT-Mediated dUTP Nick-End Labeling Assay}

To observe apoptotic cells \textit{in situ}, sections of aortic roots were stained with a TdT-mediated dUTP nick-end labeling (TUNEL) kit (C1090; Beyotime, China). The slices were fixed with paraformaldehyde for 60 min and then washed with PBS twice for 10 min. Incubating with PBS containing 0.5\% Triton X-100 at room temperature (25°C-27°C) for 5 min. 5 μl TdT Enzyme and 45 μl fluorescent labeling solution were mixed uniformly. Drop the 50μl mixed liquid on each sample and incubate it at room temperature in the dark for 60 min. And then washing with PBS for three times. The nuclei were labeled with DAPI. The image was captured by a laser scanning
2.5 Tissue Immunochemistry
For staining of intracellular proteins, slides were permeabilized with 0.1% Triton X-100 in TBS. Sections were blocked at room temperature with 0.3% H2O2 in methanol, 10% goat serum, and 1% BSA in TBS. Then, sections were incubated overnight at 4°C with anti-LC3B rabbit antibody (A19665; ABclonal, China) and anti-P62 rabbit antibody (A19700; ABclonal). The secondary antibody HRP-conjugated goat anti-rabbit IgG was added and incubated at room temperature for 20 min. Staining was visualized using a DAB kit (DAB-0031; Fuzhou Manxin, China).

2.6 Blood Lipid Determination
Serum total cholesterol, triglyceride, high-density lipoprotein cholesterol, and low-density lipoprotein cholesterol levels were measured by using biochemical detection reagent kits (A111-2-1/A110-1-1/A112-1-1/A113-1-1; Nanjing Jiancheng, China) according to the manufacturer’s instructions. The measurement of the absorbance value was detected using a microplate reader (Multiskan Sky; Thermo Fisher).

2.7 Cell Culture and Treatment
RAW 264.7 cells were obtained from the American Type Cell Collection and cultured in DMEM medium (Gibco, USA) containing 10% fetal bovine serum. Cells were incubated in DMEM for 24 h and then exposed to oxidized low-density lipoprotein (ox-LDL) (100 μg/ml), followed by coincubation with UA (15 mg/dl) for 24 h. Cells were pretreated with Rapa (10 μM) for 0.5 h, and then ox-LDL and/or UA were added for another 24 h.

2.8 Cell Viability Assays
Cells (2 × 103/ml) were incubated overnight in 96-well plates (100 μl/well) and then treated with 0, UA, ox-LDL, or UA with ox-LDL for 24 h, respectively. Then, 10 μl CCK8 solution (#K1018; APEBIO, Houston, TX, USA) was added to each well for 2 h before termination. The optical density was measured at 450 nm with a microplate reader (Multiskan Sky; Thermo Fisher).

2.9 Analysis of Apoptosis by Flow Cytometry
The cell apoptosis was measured with the Annexin V-FITC kit (C1062S; Beyotime). Briefly, RAW 264.7 cells were incubated with DMEM for 24 h, then exposed to ox-LDL (100 μg/ml), and coincubated with UA (15 mg/dl) for another 24 h. After collection, cells were resuspended with 195 μl binding solution. Then, 5 μl Annexin V-FITC and 10 μl PI were added, and the cells were incubated at room temperature for 15 min. The percentage of apoptosis was measured with flow cytometry (Beckman Cytoflex LX, USA) and analyzed with FlowJo.

2.10 Western Blot Analysis
Cellular lysates were extracted with RIPA on ice in the presence of protease inhibitors, separated in 10%–15% SDS-PAGE, and transferred to PVDF membranes. After blocking with 5% skim milk, membranes were incubated with primary antibodies against LC3 (CST), P62 (CST), cleaved caspase-3 (ABclonal), and GAPDH (ABclonal) with 1:1000 dilution in 4°C overnight followed by a secondary antibody with 1:3000 dilution at room temperature for 1 h. Immunoblots were detected with ECL and analyzed with Image J.

2.11 Statistical Analysis
All experiments were performed at least three times independently. Quantitative data are presented as mean ± SD, and Student’s two-tailed t-test or one-way analysis of variance was used for analysis. A P-value <0.05 was considered statistically significant. Statistical parameters can be found in the figure legends.

3. Results
3.1 HUA Promotes Atherosclerosis in Apoe−/− Mice
To investigate the impact of HUA on the progression of atherosclerosis, we established an atherosclerotic Apoe−/− mouse model with acute elevation of UA by intraperitoneal injection (Fig. 1A). After injection, UA peaked at 1 h and started to decline thereafter, and this relatively high level can be maintained for 6–8 h (Fig. 1B). There was no significant difference in body weight between the HFD + HUA group and the HFD group at the beginning of 21 weeks, but there was a slight decrease in body weight in the HFD + HUA group at the end of 24 weeks (Fig. 1C). A significant increase in UA was observed in the HFD + HUA group after modeling (Fig. 1D). There was no difference in total triglycerides and total cholesterol levels between the two groups (Fig. 1E–1F). Among them, the composition of cholesterol changed; the high-density lipoprotein slightly increased, and the low-density lipoprotein slightly decreased, but the very low-density lipoprotein did not change significantly (Fig. 1G–1I). In summary, this change in lipids did not cause an increase in the degree of atherosclerosis. However, we observed that the atherosclerosis plaque burden in the

Advance Publication Journal of Atherosclerosis and Thrombosis
Accepted for publication: October 16, 2022 Published online: November 26, 2022
and the smooth muscle fibers remained unchanged, but the necrotic core volume increased (Fig. 3C–3G). These observations suggested that HUA increases plaque instability. Furthermore, the HFD HUA group had fewer collagen fibers and an uneven internal distribution, with a greater number of fibers at the plaque’s basement and fewer at the surface and body. In conclusion, these results suggest that HUA exacerbates plaque vulnerability in Apoe−/− mice.

3.3 Cell Apoptosis and Autophagy Dysfunction

Cell death and programmed cell clearance are closely linked with the establishment of necrotic cores in advanced plaques. We used in situ TUNEL labeling to determine plaque stability, we analyzed macrophage infiltration, collagen fiber content, smooth muscle, lipid content, and necrotic core size. In the HFD + HUA group, we observed an increase in macrophage infiltration within the plaques (Fig. 3A–3B). Comparing the HFD + HUA group to the HFD group, we found that the lipid content of the plaque increased, the collagen fibers decreased, and the smooth muscle fibers remained unchanged, but the necrotic core volume increased (Fig. 3C–3G). These observations suggested that HUA increases plaque instability. Moreover, the HFD + HUA group had fewer collagen fibers and an uneven internal distribution, with a greater number of fibers at the plaque’s basement and fewer at the surface and body. In conclusion, these results suggest that HUA exacerbates plaque vulnerability in Apoe−/− mice.

3.3 Cell Apoptosis and Autophagy Dysfunction may be Involved in the Progression of HUA-Induced Atherosclerosis and Plaque Vulnerability

Cell death and programmed cell clearance are closely linked with the establishment of necrotic cores in advanced plaques. We used in situ TUNEL labeling...
to evaluate the apoptosis in plaques. In comparison to the HFD group, the HFD + HUA group displayed remarkably enhanced red fluorescence, suggesting an increased number of apoptotic cells in the plaque (Fig. 4A–4B). Because autophagy is associated with apoptosis, we assessed changes of cellular autophagy with LC3 and p62 in plaques. We found that HUA inhibited the autophagy of cells in plaques (Fig. 4C–4D). Overall, these results suggest that HUA promotes apoptosis and inhibits autophagy in atherosclerotic plaques.

3.4 HUA Increases Apoptosis in Macrophage-Derived Foam Cells

The in vivo results were validated in vitro since macrophage-derived foam cells contribute significantly to plaque formation. Both UA and ox-LDL produce reactive oxygen species intracellularly, and their superposition effect has been shown (Fig. 5A–5B). Additionally, we found that UA alone did not result in cell death. However, the coexistence of UA and ox-LDL can cause foam cell death (Fig. 5C). To further investigate the presence of apoptosis, we labeled cells with Annexin V-FITC. The results indicated that UA might induce apoptosis in foam cells, with early apoptosis, late apoptosis, and necrotic apoptosis increased (Fig. 5D–5E). Subsequently, we determined that the level of expression of apoptosis-related proteins cleaved caspase-3 in cells and discovered that generation increased (Fig. 5F–5G). In brief, HUA boosts the apoptosis of macrophage-derived foam cells.

3.5 HUA Inhibits Autophagy in Macrophage-Derived Foam Cells

Autophagy has an inseparable relationship with apoptosis, and autophagy plays a protective role in advanced atherosclerosis. To observe autophagy, we used RAW 264.7 cells for electron microscopy. Under normal conditions, there were no autophagic bodies in the cells; when ox-LDL was added, the cells produced a significant number of autophagic bodies. When we continued to co-culture cells with AU and LDL-beef, production of autophagosomes was inhibited (Fig. 6A–6B). Then, we extracted proteins from cells and verified LC3 and p62 in the autophagy signaling pathway by western blot analysis. HUA could effectively inhibit the autophagy comparing with both the ox-LDL group and the control group, with an obvious inhibition of the LC3 conversion,
cytometry that activating autophagy can reduce UA-induced apoptosis of foam cells (Fig. 7D–7E).

Overall, we suppose that activating autophagy can prevent the increase in apoptosis induced by HUA.

4. Discussion

In the present study, we demonstrated that HUA promotes atherosclerosis and exacerbates plaque vulnerability in Apoε/− mice. In in vivo experiments, we found that HUA changed the microstructure of plaques, including an increase in necrotic cores, a decrease in collagen fibers, and an increase in lipids. Therefore, HUA causes plaques to become unstable. As necrotic cores increased, autophagy levels in the LC3-I to LC3-II (Fig.6C–6D). However, the change of p62 is not obvious. Therefore, HUA inhibits autophagy in macrophage-derived foam cells.

3.6 Activation of Autophagy can Partially Reverse the Foam Cell Apoptosis Induced by HUA

We observed that apoptosis was increased and autophagy was inhibited in the presence of HUA both in vivo and in vitro. Furthermore, we investigated whether HUA-induced apoptosis can be ameliorated by activating autophagy. We added rapamycin, an activator of autophagy, to the cell culture medium (Fig.7A–7B). Autophagy activated by rapamycin could partly offset the increasing cell death caused by UA (Fig.7C). Moreover, we confirmed by flow cytometry that activating autophagy can reduce UA-induced apoptosis of foam cells (Fig.7D–7E). Overall, we suppose that activating autophagy can prevent the increase in apoptosis induced by HUA.

Fig. 3. Phenotype changes in atherosclerotic plaques in mice with HUA
(A) Representative macrophage content in plaques (scale bar=500 μm). (B) Quantification of macrophage content in plaques for each genotype (n=5). (C) Oil Red O staining, Sirius staining, Masson staining, and H&E staining of plaques. (D) Quantification of lipid content of Oil Red O staining (n=5). (E) Quantification of collagen fiber content of Sirius Red staining (n=5). (F) Quantification of smooth muscle of Masson staining (n=5). (G) Quantification of necrotic cores content of H&E staining (n=5). Data are presented as mean ± SD. *P<0.05, **P<0.01, ****P<0.0001; ns, not significant.
plaque decreased. We later demonstrated that HUA increases apoptosis of foam cells by inhibiting autophagy in vitro using RAW 264.7 mouse monocyte cell line. These findings indicated that the HUA might increase foam cell apoptosis by inhibiting autophagy and then exacerbate plaque vulnerability (Fig. 8).

Several clinical and epidemiological studies have established a link between HUA and atherosclerosis. There have also been some basic studies studying the possible mechanisms behind HUA and atherosclerosis and involving a variety of cells, vascular endothelial cells, immune cells (e.g., macrophages, T cells), and vascular smooth muscle cells. The mechanisms involved in these studies are diverse and include inflammation, oxidative stress, and cell death, among others. Our experiments focused on the aspect of macrophage death for initial elaboration. Consistent with previous studies, we also found that HUA can exacerbate the course of atherosclerosis.

Because HUA levels were significant and independent risk factors for all-cause and cardiovascular disease mortality, this increase in mortality did not show gender differences. Several articles have been published showing that the occurrence of MACE is associated with increased vulnerability of plaques. On this basis, we further observed the microstructure of the plaques. We found that the HUA makes the plaque vulnerable to damage. We conducted a search for relevant articles. However, there is very limited clinical evidence as to whether the elevation in MACE is due to increased plaque instability caused by HUA. Therefore, the clinical significance of our study still needs to be explored in more clinical trials. We suspected that the reason for the small number of these studies is likely to be the fact that sampling is not easy and some may require autopsy. Moreover, atherosclerosis with a single hyperuricemia is not common, and there are many confounding factors that can influence the outcome.

We found that the effect of HUA on plaque structure is complex, involving necrotic core, collagen fibers, and lipid accumulation, among others. The specific mechanisms involved behind any of these points are likely to be complex. In this article, we have only taken a preliminary look at the enlargement of
In conclusion, HUA facilitates foam cell apoptosis by inhibiting autophagy. Furthermore, elevated blood UA exacerbates atherosclerosis and increases plaque instability in mice.

Acknowledgements

This work was supported by the Natural Science Foundation of Fujian Province (2020J01018) and National Natural Science Foundation of China (82260163).

Conflicts of Interest

This work was supported by the Natural Science Foundation of Fujian Province (2020J01018) and National Natural Science Foundation of China (82260163).
Fig. 6. HUA inhibits the autophagy of foam cells
(A) Electron micrograph of RAW 264.7 cells. The red arrows indicate autophagy bodies. (B) Quantification of the number of autophagosomes per field (n=6). (C) Western blot analysis of protein levels of LC3, p62, and Gapdh. (D) Quantification of LC3 and p62 protein expression relative to Gapdh level (n=3). Data are presented as mean ± SD. *P<0.05, **P<0.01, ***P<0.001; ns, not significant.

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The authors declare that they have no conflict of interest.

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Fig. 7. Activation of autophagy can partially reverse the foam cell apoptosis induced by HUA
(A) RAW 264.7 cell lysates were assayed by western blot analysis for protein expression of LC3 and Gapdh. (B) Quantification of western blot analysis of LC3 protein expression relative to Gapdh (n = 3). (C) CCK-8 cell counting after treatment of RAW 264.7 cells (n = 4). (D) Apoptosis was detected by flow cytometry with Annexin V-FITC. (E) Quantification of flow cytometry apoptosis results (n = 3). Data are presented as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001; ns, not significant.

Fig. 8. Schematic diagram of the mechanisms by which HUA promotes atherosclerosis and instability
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