ORIGINAL RESEARCH

Dapagliflozin Attenuates NLRP3/Caspase-1 Signaling Pathway-Mediated Pyroptosis of Vascular Smooth Muscle Cells by Downregulating CTSB

Hui Li, MD; Quanwei Zhao, MM; Danan Liu, MD; Bo Zhou, MD; Caiwei Gong, MM; Guangjian Zhao, MM

ABSTRACT

Background • Atherosclerosis is a chronic inflammatory disease. Pyroptosis triggers and amplifies the inflammatory response and plays an important role in atherosclerosis. Cathepsin B (CTSB) can promote atherosclerosis and activate NOD-like receptor protein 3 (NLRP3) to mediate pyroptosis. Dapagliflozin (DAPA) can inhibit cell pyroptosis to improve atherosclerosis. This study aimed to explore the effect of DAPA on oxidized low-density lipoprotein (ox-LDL)-induced pyroptosis of vascular smooth muscle cells (VSMCs) and its underlying mechanism.

Objective • We aimed to investigate the effect of DAPA on ox-LDL-induced pyroptosis of VSMCs in mice and its underlying mechanism.

Methods • VSMCs were transfected with CTSB-overexpressing and -silencing lentiviral vectors. VSMCs were treated with different concentrations of ox-LDL (0, 50, 100 and 150 μg/ml). Then, Hoechst 33342/PI double staining, interleukin (IL)-1β and lactate dehydrogenase (LDH) release assay were used to detect cell pyroptosis. Western blotting was used to detect pyroptosis indicators protein, based on which the appropriate concentration of ox-LDL was selected. After VSMCs were treated with different concentrations of DAPA (0.1 μM, 1.0 μM, 5.0 μM, 10 μM, 25 μM and 50 μM), the proliferative activity of VSMCs was detected using Cell Counting Kit-8 (CCK8) assay. After VSMCs were pretreated with different DAPA concentrations (0.1 μM, 1.0 μM, 5.0 μM and 10 μM) for 24 hours and then treated with 150 μg/mL ox-LDL for 24 hours, the effects of different concentrations of DAPA on pyroptosis of VSMCs were detected, based on which the appropriate DAPA concentration was selected. After lentivirus transfected VSMCs were treated with 150 μg/mL ox-LDL for 24 hours, the effects of overexpression and silencing of CTSB in pyroptosis were observed. On the basis of DAPA (0.1 μM)- and ox-LDL(150 μg/mL)-treated VSMCs, overexpression and silencing of CTSB were used to observe the effects of DAPA and CTSB on ox-LDL-mediated VSMCs pyroptosis.

Results • (1) VSMCs stably transfected with CTSB-overexpressing and -silencing lentiviruses were obtained; 150 μg/mL was the optimal concentration of ox-LDL for inducing pyroptosis of VSMCs, and 0.1 μM was the optimal concentration of DAPA for ameliorating pyroptosis of VSMCs. (2) Ox-LDL-induced pyroptosis of VSMCs was worsened by CTSB overexpression but suppressed by CTSB silencing. (3) DAPA attenuated ox-LDL-induced pyroptosis of VSMCs through downregulating CTSB and NLRP3. (4) Overexpression of CTSB based on DAPA intervention aggravated ox-LDL-induced pyroptosis of VSMCs.

Conclusion • DAPA attenuates NLRP3/caspase-1 pathway-mediated pyroptosis of VSMCs through downregulating CTSB. (*Altern Ther Health Med.* 2023;29(6):384-392).

Hui Li, MD, Attending doctor; **Quanwei Zhao, MM,** Resident doctor; **Danan Liu, MD,** Chief physician; **Bo Zhou, MD,** Associate chief physician; **Caiwei Gong, MM,** Resident doctor; **Guangjian Zhao, MM,** Resident doctor; Department of Cardiovascular Medicine, Affiliated Hospital of Guizhou Medical University, Institute of Medical Sciences, Guizhou Medical University, Guiyang, Guizhou, China.

Corresponding author: Danan Liu, MD
E-mail: liudanan2000@126.com

INTRODUCTION

Atherosclerosis is a chronic inflammatory disease of the large- and medium-sized arteries, and its pathophysiological process involves endothelial cell injury and dysfunction, macrophage inflammatory infiltration, lipid core formation, proliferation and migration of vascular smooth muscle cells (VSMCs) and phenotypic transformation.[1] Due to the great harm it causes, atherosclerosis remains the leading cause of death worldwide.[2] VSMCs are a major component of atherosclerotic plaques, and transcriptomics and genetic lineage tracing studies have shown that VSMC-derived cells

account for 30% to 70% of atherosclerotic plaques.[3] In the early stage of atherosclerosis formation, VSMCs change from contractile to synthetic phenotypes under the stimulation of inflammation,oxidative stress and blood flow shear stress, enhancing proliferation, and also migrate from media to intima to synthesize the extracellular matrix, thus promoting the formation of atherosclerotic plaques. During the development of atherosclerosis, VSMCs contribute to the inflammatory infiltration of mononuclear macrophages by secreting pro-inflammatory and pro-proliferation factors. Moreover, VSMCs take up lipids through membrane protein receptors on the cell membrane surface, promote the foaming of smooth muscle cells and participate in the lipid core formation of atherosclerotic plaques, worsening arterial vascular inflammation and leading to vascular remodeling.[3,4] VSMCs and extracellular matrix synthesized by them are the main components of the protective fibrous cap, and the fibrous cap can become thinner due to VSMC death and macrophage inflammatory infiltration. The expansion of the lipid core and the thinning of the fibrous cap are the major causes of plaque instability and rupture.[5] Arterial thrombosis will result from plaque rupture, promoting the incidence of clinical cardio-cerebrovascular events such as acute coronary syndrome and stroke.

Pyroptosis is a recently discovered pro-inflammatory programmed cell death mode. The classical pyroptosis pathway is dependent on the activation of caspase-1 by inflammasomes. NOD-like receptor protein 3 (NLRP3), a member of the NLR family, is the most important inflammasome involved in the activation of caspase-1. Activated caspase-1 enhances the maturation of inflammatory mediators interleukin (IL)-18 and IL-1β and cleaves gasdermin D (GSDMD) into GSDMD-N, which destroys the integrity of the cell membrane and promotes the release of inflammatory mediators.[6,7] The mechanism of NLRP3 inflammasome activation has not been fully elucidated, but direct agonist activation, lysosomal damage and oxidative stress are considered the 3 major pathways of NLRP3 inflammasome activation.[8,9] Cathepsin B (CTSB) is a cysteine cathepsin, which is an important member of the papain family located in lysosomes, and is widely expressed in human tissues.[10] As shown in previous studies, CTSB is implicated in the pathogenesis of atherosclerosis by enhancing the pyroptosis of endothelial cells and VSMCs and promoting the formation of foam cells.[11-13] Existing evidence suggests that CTSB is involved in NLRP3 inflammasome activation-mediated pyroptosis.[14] Although CTSB and pyroptosis have been confirmed to participate in the pathological process of atherosclerosis, whether CTSB can regulate the NLRP3 inflammasome activation-mediated pyroptosis of VSMCs and the role of pyroptosis in atherosclerosis remain unclear.

Sodium-glucose cotransporter-2 (SGLT2) inhibitor (SGLT2i) is a novel oral hypoglycemic drug that acts on the SGLT2 receptor in the brush border membrane of the S1 and S2 segments of the renal proximal convoluted tubule, which reduces renal tubular reabsorption of glucose, thus increasing urinary glucose excretion and lowering blood glucose.[15] Dapagliflozin (DAPA) is an SGLT2i first marketed in 2012. Clinical benefits of DAPA in major adverse cardiovascular events (cardiovascular death, myocardial infarction and stroke), all-cause mortality and heart failure hospitalization risk have been verified in randomized controlled trials such as DAPA-HF and DECLARE-TIMI 58 trials.[16,17] As recent studies have shown, DAPA has an anti-atherosclerosis effect, and its mechanism may be related to improving endothelial relaxation function, inhibiting the formation of foam cells and relieving arterial wall inflammation.[18-20]

However, the underlying mechanism of DAPA in resisting atherosclerosis remains unclear, and whether DAPA can reduce NLRP3 activation-mediated pyroptosis of VSMCs by regulating CTSB has not been reported.

In our study, pyroptosis of VSMCs was induced by oxidized low-density lipoprotein (ox-LDL), and VSMCs were transfected with CTSB-overexpressing and -silencing lentiviruses. It was confirmed that CTSB was associated with ox-LDL-induced pyroptosis of VSMCs, and overexpression of CTSB could promote NLRP3 inflammasome activation and enhance the release of IL-18, IL-1β and lactate dehydrogenase (LDH), thus worsening VSMC pyroptosis. Then DAPA was used to intervene with the pyroptosis model, so that the inhibitory effect of SGLT2i DAPA on pyroptosis of VSMCs was confirmed. DAPA was also used to intervene with CTSB-overexpressing and -silencing lentivirus-transfected VSMCs, so that the mechanism of DAPA reducing the NLRP3/caspase-1 signaling pathway activation-mediated pyroptosis of VSMCs by inhibiting CTSB was further clarified.

This study aims to improve the understanding of the mechanism of pyroptosis of VSMCs and to identify potential therapeutic targets for ameliorating pyroptosis and antagonizing atherosclerosis.

MATERIALS AND METHODS

Main Materials and Reagents

The following materials and reagents were used: VSMCs derived from the murine aortic vascular smooth muscle cell line (MOVAS) (Shanghai Fuheng Biotechnology Co., Ltd., Shanghai, China), lentiviral overexpression vector pcSLenti-EF1-EGFP-F2A-Puro-CMV-Ctsb-WPRE and lentiviral silencing vector pSLenti-U6-shRNA(Ctsb)-CMV-EGFP-F2A-Puro-WPRE (OBiO Technology [Shanghai] Corp., Ltd., Shanghai, China), ox-LDL (Guangzhou Yiyuan Biotech Co., Ltd. Guangzhou, China), DAPA (MedChemExpress, Monmouth Junction, New Jersey USA), TRIzol and dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, Missouri USA), Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) (GIBCO, Rockville, Maryland USA), Reverse Transcription Kit and SYBR Premix Ex Taq™ kit (TaKaRa, Beijing, China), 1% (v/v) penicillin-streptomycin mixture, Hoechst 33342/PI Double Stain Kit, LDH Activity Assay Kit and IL-1β ELISA Kit (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China), puromycin (Beyotime

Biotechnology, Shanghai, China), Cell Counting Kit-8 (CCK-8) (Dojindo, Shanghai, China), and antibodies against CTSB, NLRP3, cleaved-caspase-1, ASC, GSDMD-N, IL-1β, IL-18 and glyceraldheyde 3-phosphate dehydrogenase (GAPDH) (Affinity, Cincinnati, Ohio USA). Primer synthesis and sequencing were conducted by Nanjing Tsingke Biotechnology Co., Ltd. (Nanjing, China)

METHODS

Cell culture

VSMCs of the 10^{th}-25^{th} generations were used for all experiments. VSMCs were cultured with complete DMEM containing 10% FBS and 1% (v/v) penicillin-streptomycin mixture in a 95% air/5% CO_2 incubator at 37°C.

Lentiviral transfection and screening of VSMCs stably transfected with CTSB-overexpressing and -silencing lentiviruses

Construction and packaging of CTSB-overexpressing and -silencing lentiviruses were completed by OBiO Technology (Shanghai) Corp., Ltd. At 24 hours before lentiviral transfection, VSMCs were inoculated into a 24-well plate at 5×10^4 cells/well and cultured in a 95% air/5% CO_2 incubator at 37°C until the cell confluence reached 30% to 40%. With the multiplicity of infection (MOI) set to 20, 40, 80 and 100, polybrene (5 μg/mL) and lentiviral vectors were added to each well, and the viral load (μL) in each well = MOI × number of cells when infected/titer × 10^3. The old medium was replaced with a fresh one after 12 h of viral transfection, and the transfection efficiency was observed after 72 h of viral transfection. Meanwhile, the cells were screened with the selection medium containing puromycin (10 mg/mL). After 6 days of screening, the gene and protein expressions of CTSB in the transfected cells were detected. Finally, the stably transfected cells were obtained for subsequent experiments.

Cell viability assay

The effects of different concentrations of DAPA on the viability of VSMCs were detected using CCK-8 assay. Specifically, VSMCs were inoculated in a 96-well plate at 5×10^3 cells/well, and DAPA dissolved in DMSO (0.1 μM, 1.0 μM, 5.0 μM, 10 μM, 25 μM and 50 μM in each well) was added, and the blank control and 0.1% DMSO control were set up, with 6 replicates in each group. After culture in an incubator for 24 h, 10 μL of CCK-8 buffer was added to each well, and the cells were cultured for another 3 h. Finally, the absorbance was measured at a wavelength of 450 nm.

Effects of different concentrations of ox-LDL on pyroptosis of VSMCs

VSMCs were inoculated in a 6-well plate at 1×10^5 cells/well and cultured in an incubator for 24 h. Following treatment of cells with different concentrations of ox-LDL (0, 50, 100 and 150 μg/mL) for 24 h, the pyroptosis model was established and used for subsequent experiments.

Establishment of a pyroptosis model with DAPA intervention

VSMCs were inoculated in a 6-well plate at 1×10^5 cells/well and cultured in an incubator for 24 h. Then. the medium was discarded and the cells were washed with phosphate buffered saline (PBS) 3 times and pretreated with a complete medium containing different concentrations of DAPA (0.1 μM, 1.0 μM, 5.0 μM and 10 μM) for 24 h, with a DAPA-free control group set up. After pretreatment the medium was discarded, and the cells were washed with PBS 3 times and cultured with fresh complete medium and ox-LDL (150 μg/mL) for another 24 h to induce pyroptosis of VSMCs.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

The total RNA was extracted from cells using TRIzol, and reversely transcribed into complementary deoxyribose nucleic acid (cDNA), followed by RT-qPCR using the SYBR Premix Ex Taq™ kit (Applied Biosystems, Foster City, California USA). The reaction conditions were as follows: pre-denaturation at 95°C for 1 min, 40 cycles of denaturation at 95°C for 15 seconds and annealing at 60°C for 1 min. Non-specific amplification was excluded by melting curve and amplification curve analysis, and the reaction was repeated 3 times for each sample. With GAPDH as an internal reference, the relative expression level of target gene was calculated by the delta-delta Ct ($2^{-\Delta\Delta Ct}$) method. The primer sequences were as follows: GAPDH F: 5'-TTCACCACCATGGAGAAGGC-3', R: 5'-TGAAGTCGCAGGAGACAACC-3'; target gene CTSB F: 5'-TCCTTGATCCTTCTTTCTTGCC-3', R: 5'-ACAGTGCCACACAGCTTCTTC-3'.

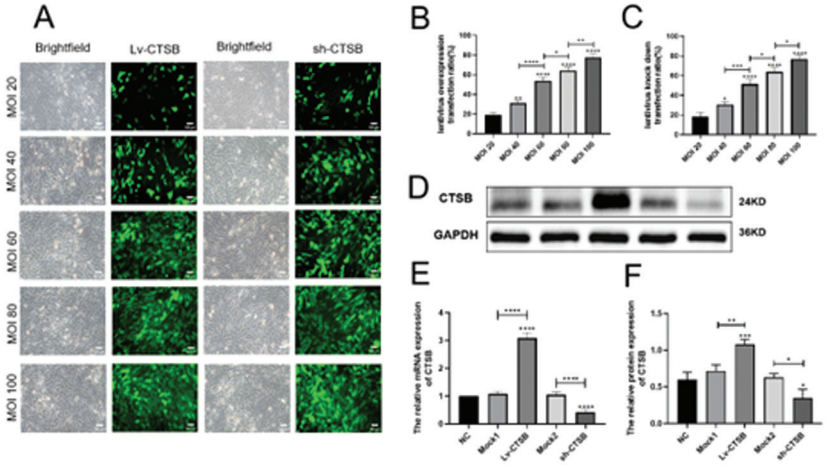
Western blotting

The total protein was extracted from cells with radioimmunoprecipitation assay (RIPA) lysis buffer and 1% (v/v) protease inhibitor and quantified by bicinchoninic acid (BCA). After boiling with sodium dodecyl sulphate (SDS) loading buffer, the same volume of samples was separated by 10% or 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, Massachusetts USA), blocked with 5% skim milk for 1 h at room temperature and washed with tris buffered saline-tween (TBST) for 30 min, followed by incubation with primary antibodies overnight at 4°C. After washing with TBST for 30 min, the samples were incubated with secondary antibodies for 1 h at room temperature. Finally, the protein bands were detected using an ECL system (ECL Systems LLC, Bangladesh, Pakistan) and analyzed using ImageJ (National Institutes of Health, Bethesda, Maryland USA).

Hoechst 33342/PI fluorescent double staining

VSMCs were inoculated and cultured in a 6-well plate at 1×10^5 cells/well and treated correspondingly. After the medium was discarded, the cells were washed with PBS 3 times, added with 1 mL of cell staining buffer, 5 μL of

Figure 1. VSMCs transfected with CTSB-overexpressing and -silencing lentiviruses and CTSB mRNA and protein expressions detected by RT-qPCR and Western blotting. (**1A**) With the MOI of 20, 40, 60, 80 and 100, CTSB-overexpressing and -silencing lentiviruses and polybrene (5 μg/mL) were added in each group. After 72 h of transfection, green fluorescence could be observed in VSMCs successfully transfected with CTSB-overexpressing and -silencing lentiviruses under an inverted fluorescence microscope since the lentivirus contained EGFP, while VSMCs not successfully transfected displayed no green fluorescence. The transfection efficiency could be preliminarily estimated by observation under the inverted fluorescence microscope: transfection efficiency = number of green fluorescent cells/total number of cells × 100%. (**1B**) Statistical results of transfection efficiency on VSMCs transfected with CTSB-overexpressing lentivirus at different MOI. (**1C**) Statistical results of transfection efficiency on VSMCs transfected with CTSB-silencing lentivirus at different MOI. (**1D**) CTSB protein expression detected by Western blotting in VSMCs transfected with CTSB-overexpressing and -silencing lentiviruses (MOI=100). (**1E** and **1F**) Transfection effect verified by RT-qPCR and Western blotting.



*$P < .05$
**$P < .01$
***$P < .001$ and
****$P < .0001$

Note: n = 3; Bar = 100 μm

Abbreviations: CTSB, cathepsin B; MOI, multiplicity of infection; mRNA, messenger RNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; VSMCs, vascular smooth muscle cells

Hoechst 33342 stain solution and 5 μL of PI stain solution to each well, and subjected to an ice bath for 20 min. Then the stain solution was washed away with PBS, the cells were observed and photographed under an inverted fluorescence microscope, and the percentage of PI-positive cells was evaluated with ImageJ (NIH, USA).

LDH release assay

LDH is released as the integrity of the cell membrane is destroyed due to pyroptosis, and the severity of pyroptosis can be reflected by LDH release assay combined with Hoechst 33342/PI staining. After the cells were treated accordingly, the supernatant was collected, and the level of LDH was measured using an LDH Assay Kit (Solarbio, China) according to the manufacturer's instructions.

Enzyme-linked immunosorbent assay (ELISA)

After the cells were treated accordingly, the supernatant was harvested, and the level of IL-1β was measured using an ELISA kit (Solarbio, Beijing, China) according to the manufacturer's instructions.

Statistical Analysis

Each experiment was repeated independently at least 3 times. GraphPad Prism 9 (Dotmatics, La Jolla, California USA) was used for statistical analysis. The experimental data were described by mean ± standard deviation ($\bar{x} \pm s$) and compared between groups by one-way ANOVA and Newman-Keuls test. The abnormal distribution data were compared via Dunnett's T3 test. $P < .05$ was considered statistically significant.

RESULTS

Lentiviral Transfection of VSMCs

When the MOI was 20, only a small amount of green fluorescence was found in VSMCs transfected with CTSB-overexpressing and -silencing lentiviruses, and the transfection efficiencies of CTSB-overexpressing lentivirus and CTSB-silencing lentivirus were (19.02 ± 3.12)% and (18.45 ± 4.27)%, respectively. When the MOI was 40, more green fluorescence could be observed, and the transfection efficiencies of CTSB-overexpressing lentivirus and CTSB-silencing lentivirus were (31.22 ± 2.82)% and (30.63 ± 2.99)%, respectively. When the MOI was 60, the transfection efficiencies were further enhanced, which were (53.78 ± 3.66)% and (51.44 ± 4.60)%, respectively, for CTSB-overexpressing lentivirus and CTSB-silencing lentivirus. When the MOI was 80, most of the VSMCs emitted green fluorescence, and the transfection efficiencies of CTSB-overexpressing lentivirus and CTSB-silencing lentivirus were (64.10 ± 3.56)% and (64.02 ± 4.23)%, respectively. When the MOI was 100, most of the VSMCs emitted green fluorescence, and the transfection efficiencies of CTSB-overexpressing lentivirus and CTSB-silencing lentivirus were (77.80 ± 3.77)% and (76.78 ± 3.81)%, respectively (Figure 1A-1C). Therefore, VSMCs were transfected with viruses under an MOI of 100, and those stably transfected with CTSB-overexpressing and -silencing lentiviruses were screened using puromycin (1 μg/mL).

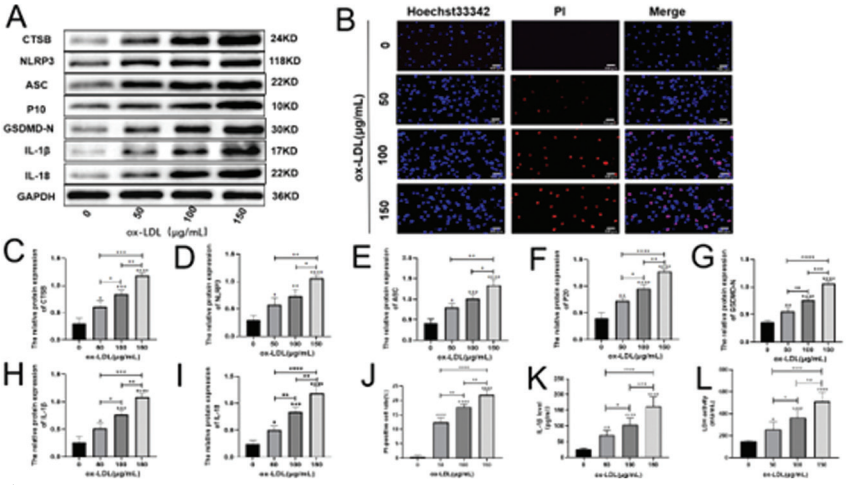
mRNA and Protein Expressions of CTSB Detected by RT-qPCR and Western Blotting

Following screening by puromycin, VSMCs stably transfected with CTSB-overexpressing and -silencing lentiviruses were subjected to RT-qPCR and Western blotting. The results of RT-qPCR showed that there was no difference in CTSB mRNA expression in the Mock1 group (1.082 ± 0.095) and Mock2 group (1.058 ± 0.096) compared with in the NC group ($P > .05$). It was significantly higher in the lv-CTSB group (3.086 ± 0.199) than in the Mock1 group ($P < .0001$), and significantly lower in the sh-CTSB group (0.419 ± 0.100) than in the Mock2 group ($P < .0001$) (Figure 1E). Western blotting revealed that CTSB protein expression showed no difference in the Mock1 (0.718 ± 0.085) and Mock2 groups (0.631 ± 0.052) compared with the NC group (0.597 ± 0.103) ($P > .05$). It was significantly higher in the lv-CTSB group (1.082 ± 0.067) than in the Mock1 group ($P < .01$), and lower in the sh-CTSB group (0.345 ± 0.123) than in the Mock2 group ($P < .05$) (Figure 1D and 1F). These results suggest that VSMCs were successfully transfected with CTSB-overexpressing and -silencing lentiviruses, and the stably transfected cells were successfully constructed.

Effects of Different Concentrations of Ox-LDL on Pyroptosis of VSMCs

VSMCs were treated with different concentrations (0, 50, 100 and 150 μg/mL) of ox-LDL for 24 h. It was observed with Hoechst 33342/PI staining that there were a few PI-stained cells in red in the 0 μg/mL group [(0.50 ± 0.440)%]. The number of PI-stained cells in red was increased in the 50 μg/mL group [(12.453 ± 1.581)%], further increased in the 100 μg/mL group [(17.673 ± 1.001)%], and a large number of PI-stained cells in red were seen under the microscope in the 150 μg/mL group [(21.897 ± 1.728)%] (Figure 2B and 2J). The Western blotting results revealed that the protein expressions of CTSB, NLRP3, ASC, cleaved-caspase-1 (p10), IL-1β, IL-18 and GSDMD-N rose in the 50, 100 and 150 μg/mL groups compared with the 0 μg/mL group ($P < .05$), and increased with the increase in the ox-LDL concentration (Figure 2A, 2C-2I). Moreover, the ELISA results showed that IL-1β activity was higher in the 50, 100 and 150 μg/mL groups than in the 0 μg/mL group ($P < .05$), and increased with the increase in the ox-LDL concentration, which was also verified by the results of the LDH release assay (Figure 2K and 2L). Compared with the 0 μg/mL group, the 50, 100 and 150 μg/mL groups had statistically significant differences in the number of propidium iodide (PI)-positive cells, expressions of pyroptosis-related proteins, LDH activity and IL-1β activity ($P < .05$), especially in the 150 μg/mL group. Therefore, the pyroptosis model was induced by 150 μg/mL ox-LDL.

Figure 2. Effects of different concentrations of ox-LDL on pyroptosis of VSMCs. VSMCs were treated with 0, 50, 100 and 150 μg/mL ox-LDL for 24 h. (**2A**) Protein expressions of CTSB, NLRP3, ASC, cleaved-caspase-1 (p10), IL-1β, IL-18 and GSDMD-N in VSMCs detected by Western blotting in each group. (**2B**) Hoechst 33342/PI fluorescent double staining (blue: Hoechst 33342 staining, red: PI staining). (**2C**) Statistical results of CTSB protein expression in each group. (**2D**) Statistical results of NLRP3 protein expression in each group. (**2E**) Statistical results of ASC protein expression in each group. (**2F**) Statistical results of p10 protein expression in each group. (**2G**) Statistical results of GSDMD-N protein expression in each group. (**2H**) Statistical results of IL-1β protein expression in each group. (**2I**) Statistical results of IL-18 protein expression in each group. (**2J**) Statistical results of Hoechst 33342/PI fluorescent double staining in each group. (**2K**) Statistical results of IL-1β activity in each group. (**2L**) Statistical results of LDH activity in each group.



*$P < .05$
**$P < .01$
***$P < .001$
****$P < .0001$

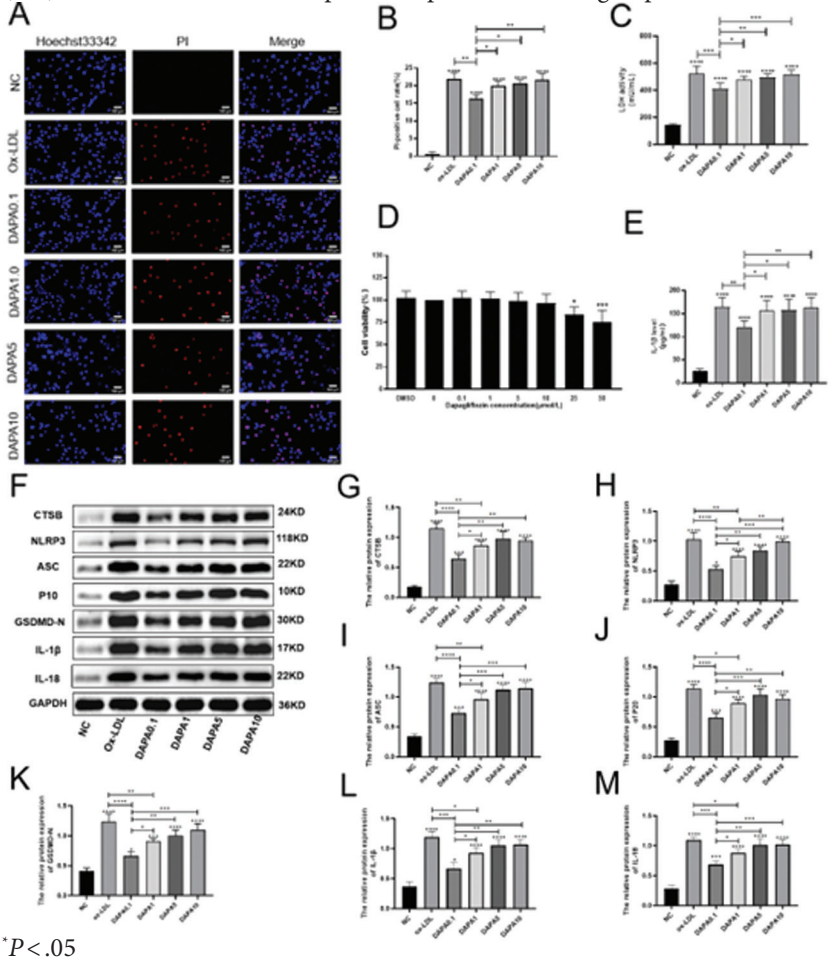
Note: n = 3; Bar = 100 μm

Abbreviations: CTSB, cathepsin B; GSDMD, gasdermin D; IL-1β, interleukin 1 beta; LDH, lactate dehydrogenase; NLRP3, NOD-like receptor protein 3; ox-LDL, oxidized low-density lipoprotein; PI, propidium iodide; VSMCs, vascular smooth muscle cells.

Effects of Different Concentrations of DAPA on Proliferative Activity of VSMCs

A solution of 0.1% DMSO and different concentrations of DAPA (0.1 μM, 1.0 μM, 5.0 μM, 10 μM, 25 μM and 50 μM) were used for intervention with VSMCs. The results manifested that compared with the NC group, cell viability at 24 h after culture was not significantly different from that in the DMSO group [(102.449 ± 7.538)%], the 0.1 μM DAPA group [(102.011 ± 8.007)%], 1.0 μM DAPA group [(101.572

Figure 3. Effects of different concentrations of DAPA on proliferative activity of VSMCs and on ox-LDL-induced pyroptosis of VSMCs. After pretreatment with DAPA at corresponding concentrations for 24 h in each group, the cells were treated with 150 μg/mL ox-LDL for another 24 h. (**3A**) Hoechst 33342/PI fluorescent double staining (blue: Hoechst 33342 staining, red: PI staining). (**3B**) Statistical results of Hoechst 33342/PI fluorescent double staining in each group. (**3C**) Statistical results of LDH activity in each group. (**3D**) Proliferative activity of VSMCs measured by CCK-8 assay after pretreatment with DAPA at corresponding concentrations for 24 h in each group. (**3E**) Statistical results of IL-1β activity in each group. (**3F**) Protein expressions of CTSB, NLRP3, ASC, cleaved-caspase-1 (p10), IL-1β, IL-18 and GSDMD-N in VSMCs detected by Western blotting in each group. (**3G**) Statistical results of CTSB protein expression in each group. (**3H**) Statistical results of NLRP3 protein expression in each group. (**3I**) Statistical results of ASC protein expression in each group. (**3J**) Statistical results of p10 protein expression in each group. (**3K**) Statistical results of GSDMD-N protein expression in each group. (**3L**) Statistical results of IL-1β protein expression in each group. (**3M**) Statistical results of IL-18 protein expression in each group.



*$P<.05$
**$P<.01$
***$P<.001$
****$P<.0001$

Note: n=3; Bar=100 μm

Abbreviations: CCK8, Cell Counting Kit-8; CTSB, cathepsin B; DAPA, dapagliflozin; GSDMD, gasdermin D; IL-1β, interleukin 1 beta; LDH, lactate dehydrogenase; NLRP3, NOD-like receptor protein 3; ox-LDL, oxidized low-density lipoprotein; PI, propidium iodide; VSMCs, vascular smooth muscle cells.

±7.682)%], 5.0 μM DAPA group [(99.362 ± 8.906)%] and the 10 μM DAPA group [(96.539±10.137)%] ($P>.05$), and declined in the 25 μM DAPA group [(83.891 ± 8.375)%] ($P<.05$) and 50 μM DAPA group [(75.314±13.216)%] ($P<.001$). The results suggested that 25 μM and 50 μM DAPA could affect the proliferative activity of VSMCs, and the optimal concentration of DAPA for intervention with VSMCs was 0.1-10 μM (Figure 2D).

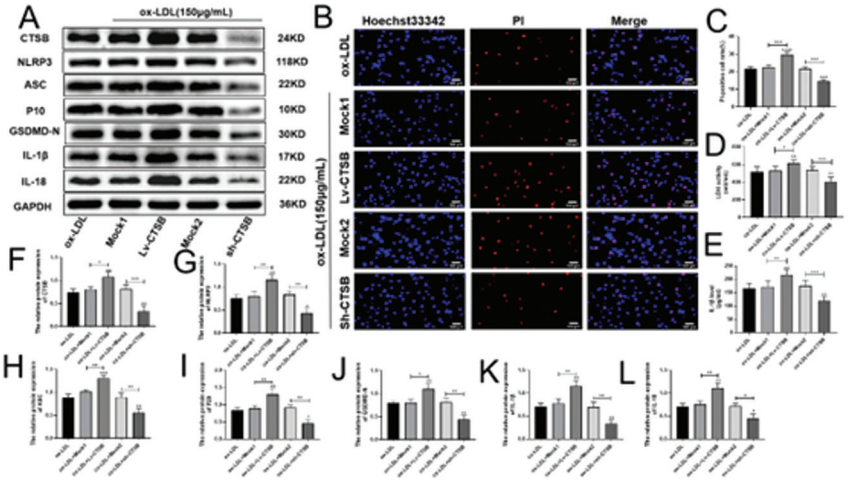
Optimal Concentration of DAPA for Ameliorating Pyroptosis of VSMCs

To explore the optimal concentration of DAPA for ameliorating the ox-LDL-mediated pyroptosis of VSMCs, VSMCs were pretreated with different concentrations of DAPA (0 μM, 0.1 μM, 1 μM, 5 μM and 10 μM) for 24 h, and then treated with 150 μg/mL ox-LDL for 24 h to induce the pyroptosis model. Then, Hoechst 33342/PI fluorescent double staining was performed on the pyroptosis model, and the expressions of pyroptosis-related proteins, LDH activity and IL-1β activity were detected by Western blotting, LDH release assay and ELISA, respectively. The results showed that in the ox-LDL group, the number of PI-stained cells in red significantly increased [(21.897 ± 1.728)%] compared with the NC group [(0.657±0.584)%] (Figure 3A and 3B), the protein expressions of CTSB, NLRP3, ASC, cleaved-caspase-1 (p10), IL-1β, IL-18 and GSDMD-N rose, with statistically significant differences ($P<.05$) (Figure 3F-3M), and the activity of LDH and IL-1β was enhanced, showing statistically significant differences ($P<.05$) (Figure 3C and 3E). Compared with ox-LDL group, 0.1 μM DAPA group had significantly decreased number of PI-stained cells in red (Figure 3A and 3B), expressions of pyroptosis-related proteins (Figure 3F-3M) and activity of LDH and IL-1β (Figure 3C and 3E) ($P<.05$). With the increase in DAPA concentration, however, the number of PI-stained cells in red, expressions of pyroptosis-related proteins and activity of LDH and IL-1β all increased, with statistically significant differences ($P<.05$). To sum up, DAPA could ameliorate the pyroptosis of VSMCs, and the optimal concentration was 0.1 μM.

Effects of Overexpression and Silencing of CTSB on Ox-LDL-Mediated Pyroptosis of VSMCs

To explore the effects of CTSB overexpression and silencing on ox-LDL-mediated pyroptosis of VSMCs, lentivirus-transfected VSMCs were treated with 150 μg/mL ox-LDL for 24 h to induce pyroptosis. Then, Hoechst 33342/PI fluorescent double staining was performed on the pyroptosis model, the protein expressions of CTSB, NLRP3, cleaved-caspase-1 (p10), ASC, IL-1β, IL-18 and GSDMD-N were detected by Western blotting, and the activity of LDH and IL-1β was detected by LDH release assay and ELISA, respectively. The results revealed that there were no statistically significant differences in the PI-positive cell rate (Figure 4B and 4C), CTSB protein expressions, NLRP3, ASC, cleaved-caspase-1 (p10), IL-1β, IL-18 and GSDMD-N (Figure 4A, 4F-4L) and LDH and IL-1β activity (Figure 4D and 4E) in the Mock1 and Mock2 groups compared with the ox-LDL group ($P > .05$). The PI-positive cell rate (Figure 4B and 4C), protein expressions of CTSB, NLRP3, ASC, cleaved-caspase-1 (p10), IL-1β, IL-18 and GSDMD-N (Figure 4A, 4F-4L), and activity of LDH and IL-1β (Figure 4D and 4E) all significantly rose in the lv-CTSB group compared with the Mock1 group, while they declined in sh-CTSB group compared with the Mock2 group, all showing statistically significant differences ($P < .05$).

Figure 4. Effects of overexpression and silencing of CTSB on ox-LDL-mediated pyroptosis of VSMCs. The cells in the ox-LDL group, ox-LDL + Mock1 group, ox-LDL + lv-CTSB group, ox-LDL + Mock2 group and ox-LDL + sh-CTSB group were treated with 150 μg/mL ox-LDL for 24 h. (**4A**) Protein expressions of CTSB, NLRP3, ASC, cleaved-caspase-1 (p10), IL-1β, IL-18 and GSDMD-N in VSMCs detected by Western blotting in each group. (**4B**) Hoechst 33342/PI fluorescent double staining (blue: Hoechst 33342 staining, red: PI staining). (**4C**) Statistical results of Hoechst 33342/PI fluorescent double staining in each group. (**4D**) Statistical results of LDH activity in each group. (**4E**) Statistical results of IL-1β activity in each group. (**4F**) Statistical results of CTSB protein expression in each group. (**4G**) Statistical results of NLRP3 protein expression in each group. (**4H**) Statistical results of ASC protein expression in each group. (**4I**) Statistical results of p10 protein expression in each group. (**4J**) Statistical results of GSDMD-N protein expression in each group. (**4K**) Statistical results of IL-1β protein expression in each group. (**4L**) Statistical results of IL-18 protein expression in each group.



n = 3; Bar = 100 μm

$^{*}P < .05$
$^{**}P < .01$
$^{***}P < .001$
$^{****}P < .0001$

Abbreviations: CTSB, cathepsin B; DAPA, dapagliflozin; GSDMD, gasdermin D; IL-1β, interleukin 1 beta; LDH, lactate dehydrogenase; NLRP3, NOD-like receptor protein 3; ox-LDL, oxidized low-density lipoprotein; PI, propidium iodide; VSMCs, vascular smooth muscle cells.

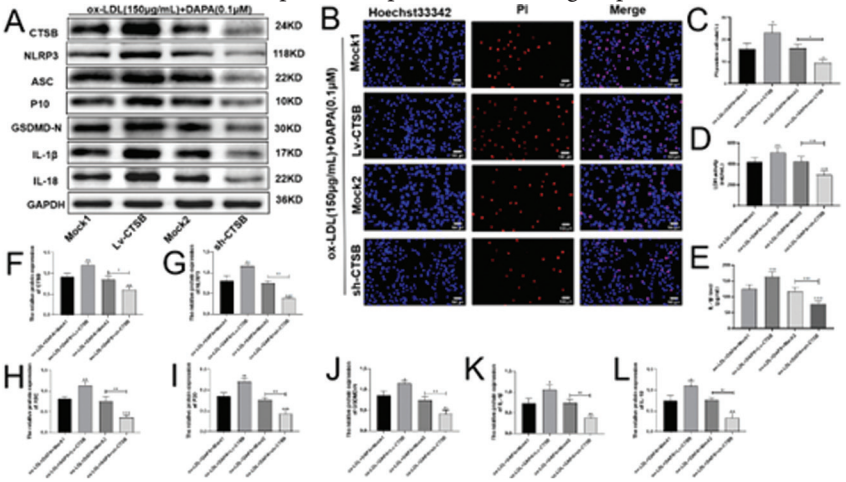
Effects of Overexpression and Silencing of CTSB on Ox-LDL-Induced Pyroptosis of VSMCs After DAPA Intervention

To explore the molecular mechanism by which DAPA ameliorates VSMC pyroptosis, VSMCs were pretreated with 0.1 μM DAPA for 24 h and then with 150 μg/mL ox-LDL for 24 h to induce pyroptosis of VSMCs. Then, Hoechst 33342/PI fluorescent double staining was performed on the pyroptosis model, the protein expressions of CTSB, NLRP3, cleaved-caspase-1 (p10), ASC, IL-1β, IL-18 and GSDMD-N were detected by Western blotting, and the activity of LDH and IL-1β was detected by LDH release assay and ELISA, respectively. The results revealed that the PI-positive cell rate (Figure 5B and 5C), protein expressions of CTSB, NLRP3, ASC, cleaved-caspase-1 (p10), IL-1β, IL-18 and GSDMD-N (Figure 5A and 5F-5L), and activity of LDH and IL-1β (Figure 5D and 5E) all significantly rose in the ox-LDL + DAPA + lv-CTSB group compared with the ox-LDL + DAPA + Mock1 group ($P < .05$), while they declined in the ox-LDL + DAPA + sh-CTSB group compared with the ox-LDL + DAPA + Mock2 group, all showing statistically significant differences ($P < .05$).

DISCUSSION

In this study, CTSB was overexpressed or silenced in VSMCs by lentiviral transfection, and then pyroptosis of VSMCs was induced by ox-LDL. Meanwhile, DAPA was used for intervention with the pyroptosis model to explore the molecular mechanism by which DAPA alleviates pyroptosis of VSMCs. It was found that ox-LDL could mediate pyroptosis of VSMCs by activating the NLRP3/caspase-1 signaling pathway and increase the pyroptosis in a concentration-dependent manner, and CTSB was associated with the

Figure 5. Effects of overexpression and silencing of CTSB on ox-LDL-induced pyroptosis of VSMCs after DAPA intervention. The cells in the ox-LDL + DAPA + Mock1 group, ox-LDL + DAPA + lv-CTSB group, ox-LDL + DAPA + Mock2 group and ox-LDL + DAPA + sh-CTSB group were pretreated with 0.1 μM DAPA for 24 h and then with 150 μg/mL ox-LDL for 24 h. (**5A**) Protein expressions of CTSB, NLRP3, ASC, cleaved-caspase-1 (p10), IL-1β, IL-18 and GSDMD-N in VSMCs detected by Western blotting in each group. (**5B**) Hoechst 33342/PI fluorescent double staining (blue: Hoechst 33342 staining, red: PI staining). (**5C**) Statistical results of Hoechst 33342/PI fluorescent double staining in each group. (**5D**) Statistical results of LDH activity in each group. (**5E**) Statistical results of IL-1β activity in each group. (**5F**) Statistical results of CTSB protein expression in each group. (**5G**) Statistical results of NLRP3 protein expression in each group. (**5H**) Statistical results of ASC protein expression in each group. (**5I**) Statistical results of p10 protein expression in each group. (**5J**) Statistical results of GSDMD-N protein expression in each group. (**5K**) Statistical results of IL-1β protein expression in each group. (**5L**) Statistical results of IL-18 protein expression in each group.



Note: n = 3; Bar = 100 μm

*$P < .05$
**$P < .01$
***$P < .001$
****$P < .0001$

Abbreviations: CTSB, cathepsin B; DAPA, dapagliflozin; GSDMD, gasdermin D; IL-1β, interleukin 1 beta; LDH, lactate dehydrogenase; NLRP3, NOD-like receptor protein 3; ox-LDL, oxidized low-density lipoprotein; PI, propidium iodide; VSMCs, vascular smooth muscle cells.

severity of pyroptosis of VSMCs. Ox-LDL-induced VSMC pyroptosis could be worsened by overexpression of CTSB but reduced by DAPA intervention or CTSB silencing. Therefore, it was speculated that DAPA may reduce the NLRP3/caspase-1 signaling pathway activation-mediated pyroptosis of VSMCs by inhibiting CTSB.

To test this hypothesis, CTSB was overexpressed in VSMCs based on DAPA intervention. The results showed that overexpression of CTSB recovered pyroptosis of VSMCs that was attenuated by DAPA before, proving that DAPA reduces NLRP3/caspase-1 signaling pathway activation-mediated pyroptosis by inhibiting CTSB. In addition, CTSB was also silenced based on DAPA intervention. As a result, a further decrease in NLRP3 expression and pyroptosis was seen, indicating that DAPA could not only suppress CTSB but also reduce NLRP3 expression.

Some studies have revealed that NLRP3 inflammasome activation-mediated pyroptosis of VSMCs is implicated in and exacerbates atherosclerosis. Pan, et al.[21] found that ox-LDL can increase pyroptosis of VSMCs in a concentration-dependent manner, and AIM2 can enhance NLRP3 inflammasome activation, thus worsening pyroptosis of VSMCs and promoting atherosclerosis occurrence. Li, et al.[22] also found that ox-LDL can promote pyroptosis of VSMCs, and VX-765, by specifically blocking caspase-1, inhibits ox-LDL-induced pyroptosis of VSMCs and delays atherosclerosis in ApoE$^{-/-}$ mice fed a high-fat diet. He, et al.[23] showed that VX-765 can not only suppress pyroptosis of VSMCs but also reduce formation of smooth muscle-derived foam cells. In our study, we found that ox-LDL could activate NLRP3 inflammasomes to promote pyroptosis of VSMCs in a concentration-dependent manner, consistent with previous findings.

The mechanism of NLRP3 inflammasome activation has not been fully clarified, but direct agonist activation, lysosomal damage and oxidative stress have been recognized as relevant pathways. CTSB is one of the important members of the papain family located in lysosomes. Due to lysosomal damage, CTSB is released from lysosomes into the cytoplasm and extracellular matrix to participate in NLRP3 inflammasome activation-mediated pyroptosis.[24,25] As demonstrated in a previous study, CTSB can directly bind to the leucine-rich repeats (LRR) domain of NLRP3 inflammasomes, thereby activating NLRP3 inflammasomes and inducing pyroptosis.[26]

In our study, we found that ox-LDL-mediated NLRP3 inflammasome activation and pyroptosis were associated with CTSB, and the expression of CTSB rose with the increasing severity of pyroptosis. Moreover, after CTSB was overexpressed and silenced in VSMCs by lentiviral transfection, we found that CTSB regulated the NLRP3 inflammasome activation and was involved in the NLRP3/caspase-1 signaling pathway-mediated pyroptosis of VSMCs.

DAPA is a novel oral hypoglycemic drug that reduces renal tubular reabsorption of glucose and increases urinary glucose excretion by selectively inhibiting SGLT2, thereby lowering blood glucose. As demonstrated in animal experiments, DAPA

can ameliorate atherosclerosis in ApoE$^{-/-}$ mice by improving endothelium-dependent relaxation and reducing formation of macrophage-derived foam cells.[19, 20] According to recent *in vitro* studies, DAPA can reduce polarization of pro-inflammatory M1 macrophages and expressions of pro-inflammatory miRNAs.[27] In our study, we found that DAPA could attenuate ox-LDL-induced NLRP3 inflammasome activation and lower the expression of IL-18 and IL-1β, and also reduce the activation of NLRP3/caspase-1 signaling pathway by inhibiting CTSB, thereby ameliorating pyroptosis. Then, CTSB was overexpressed in the ox-LDL-induced pyroptosis model based on DAPA intervention. The results revealed that overexpression of CTSB recovered pyroptosis of VSMCs that was attenuated by DAPA, activated the NLRP3/caspase-1 signaling pathway and enhanced the expressions of IL-18 and IL-1β and the release of LDH, thereby worsening the ox-LDL-induced pyroptosis of VSMCs. This suggests that DAPA suppresses NLRP3/caspase-1 signaling pathway-mediated pyroptosis by inhibiting CTSB. In addition, silencing of CTSB in the ox-LDL-induced pyroptosis model based on DAPA intervention further reduced NLRP3 expression and pyroptosis. Therefore, we speculated that DAPA can not only reduce the activation of NLRP3 by inhibiting CTSB, but also synergistically reduce the expression of NLRP3. In a study on DAPA and reactive oxygen species (ROS), it was found that DAPA can weaken the activation of the NLRP3/caspase-1 signaling pathway by reducing macrophage ROS production, thereby ameliorating atherosclerosis in ApoE$^{-/-}$ mice with type 2 diabetes fed a high-fat diet.[28]

Combined with the findings in this study, we speculated that silencing of CTSB in the ox-LDL-induced pyroptosis model based on DAPA intervention further reduces NLRP3 inflammasome activation, which may be related to the reduction of ROS by DAPA. Although CTSB can activate NLRP3 inflammasomes, it is not the only pathway of NLRP3 inflammasome activation. Whether DAPA can also reduce the activation of NLRP3 inflammasomes by PAMPs and DAMPs and whether DAPA can inhibit downstream factors such as ASC, caspase-1 and GSDMD remains unclear, so further study is needed.

At present, research on the pathogenesis of atherosclerosis mainly focuses on endothelial cells and macrophages, and the role of VSMCs in the pathogenesis of atherosclerosis is often underestimated. However, the proliferation and migration of VSMCs, macrophage-like phenotypic transformation, osteoblast-like phenotypic transformation, synthetic phenotypic transformation and formation of smooth muscle-derived foam cells all play important roles in the occurrence and development of atherosclerosis. Hence, there is reason to believe that clarifying the role of VSMCs in atherosclerosis is of great significance for the prevention and treatment of atherosclerosis-related diseases.

CONCLUSION

This study provides a theoretical basis for the effect of SGLT2i DAPA in reducing NLRP3/caspase-1 signaling pathway-mediated pyroptosis of VSMCs by inhibiting CTSB. These findings are of great significance for elucidating the role of pyroptosis of VSMCs in the pathogenesis of atherosclerosis and offer a theoretical foundation for the use of SGLT2i in the prevention and treatment of atherosclerosis and related diseases. Moreover, new ideas and targets are provided for inhibiting CTSB-mediated NLRP3 inflammasome activation and reducing pyroptosis.

CONFLICT OF INTERTEST

None.

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