Dapagliozin Attenuates NLRP3/Caspase-1 Signaling Pathway-Mediated Pyroptosis of Vascular Smooth Muscle Cells by Down-regulating CTSB

Hui Li
Affiliated Hospital of Guizhou Medical University, Guizhou Medical University

Quanwei Zhao
Affiliated Hospital of Guizhou Medical University, Guizhou Medical University

Danan Liu (liudanan2000@163.com)
Affiliated Hospital of Guizhou Medical University, Guizhou Medical University

Bo Zhou
Affiliated Hospital of Guizhou Medical University, Guizhou Medical University

Caiwei Gong
Affiliated Hospital of Guizhou Medical University, Guizhou Medical University

Guangjian Zhao
Affiliated Hospital of Guizhou Medical University, Guizhou Medical University

Research Article

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Abstract

Background

• Atherosclerosis is a chronic inflammatory disease. Pyroptosis triggers and amplifies the inflammatory response and plays an important role in AS. Cathepsin B (CTSB) can promote AS and activate NLRP3 to mediate pyroptosis. Dapagliflozin (DAPA) can inhibit cell pyroptosis to improve AS. 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.

Methods

• VSMCs were transfected with cathepsin B (CTSB)-overexpressing and -silencing lentiviral vectors. Then, VSMCs were treated with different concentrations of ox-LDL (0, 50, 100, 150 µg/ml), Hoechst33342/PI double staining and LDH release assay were used to detect cell pyroptosis. After VSMCs were treated with different concentrations of DAPA (0.1 µM, 1.0 µM, 5.0 µM, 10 µM, 25 µM, 50 µM), the proliferation activity of VSMCs was detected by CCK8 method. On the basis of ox-LDL-induced VSMCs pyroptosis, DAPA intervention, 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 overexpression of CTSB but suppressed by silencing of CTSB. 3) DAPA attenuated ox-LDL-induced pyroptosis of VSMCs through down-regulating 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 down-regulating CTSB.

Background

Atherosclerosis (AS) is a chronic inflammatory disease of 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. With great harm, AS remains the leading cause of death in the world. VSMCs are a major component of AS plaques, and transcriptomics and genetic lineage tracing studies have shown that VSMC-derived cells account for 30–70% in AS plaques. In the early stage of AS formation, VSMCs change from contractile to synthetic phenotypes under the stimulation of some factors, enhancing proliferation, and also migrate from media to intima to synthesize extracellular matrix, thus promoting the formation of AS plaques. During the development of AS, 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 AS plaques, worsening arterial vascular inflammation and leading to vascular remodeling. 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 lipid core and the thinning of fibrous cap are the major causes of plaque instability and rupture. Arterial thrombosis will be caused following plaque rupture, thus promoting the incidence of clinical cardio-cerebrovascular events such as acute coronary syndrome and stroke.

Pyroptosis is a pro-inflammatory programmed cell death mode newly discovered in recent years. 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-18 (IL-18) and IL-1β and cleaves gasdermin D (GSDMD) into GSDMD-N, which destroys the integrity of cell membrane and promotes the release of inflammatory mediators. The mechanism of NLRP3 inflammasome activation has not been fully elucidated, but direct agonist activation, lysosomal damage and oxidative stress are considered the three major pathways of NLRP3 inflammasome activation. 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. As shown in previous studies, CTSB is implicated in the pathogenesis of AS by enhancing the pyroptosis of endothelial cells and VSMCs and promoting the formation of foam cells. Existing evidence suggests that CTSB is involved in NLRP3 inflammasome activation-mediated pyroptosis. Although CTSB and pyroptosis have been confirmed to participate in the pathological process of AS, whether CTSB can regulate the NLRP3 inflammasome activation-mediated pyroptosis of VSMCs and the role of pyroptosis in AS 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 S1 and S2 segments of the renal proximal convoluted tubule, which is used to reduce renal tubular reabsorption of glucose, thus increasing urinary glucose excretion and lowering blood glucose. Dapagliozin (DAPA) is an SGLT2i 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. As shown in recent studies, DAPA has an anti-AS effect, and its mechanism may be related to improving endothelial
relaxation function, inhibiting the formation of foam cells and relieving arterial wall inflammation.\textsuperscript{18–20} However, the underlying mechanism of DAPA in resisting AS remains unclear, and whether DAPA can reduce NLRP3 activation-mediated pyroptosis of VSMCs by regulating CTSB has not been reported.

In this 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 the NLRP3 inflammasome activation, and enhance the release of IL-1\textsubscript{8}, IL-1\textsubscript{β} and lactate dehydrogenase (LDH), thus worsening pyroptosis of VSMCs. 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 on the mechanism of pyroptosis of VSMCs and to identify potential therapeutic targets for ameliorating pyroptosis and antagonizing AS.

**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, NJ, USA), TRIzol and dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA), Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) (GIBCO, Rockville, MD, 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-1\textsubscript{β} 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-1\textsubscript{β}, IL-18 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Affinity, USA). Primer synthesis and sequencing were conducted by Nanjing Tsingke Biotechnology Co., Ltd. (Nanjing, China)

**Methods**

**Cell culture**

VSMCs of the 10th -25th 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\textsubscript{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. Twenty-four 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% CO₂ incubator at 37°C until the cell confluence reached 30–40%. 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 d 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 added with DAPA dissolved in DMSO (0.1 µM, 1.0 µM, 5.0 µM, 10 µM, 25 µM and 50 µM in each well), 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. The reaction conditions were as follows: pre-denaturation at 95°C for 1 min, 40 cycles of denaturation at 95°C for 15 s 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 $2^{\Delta \Delta CT}$ method. The primer sequences were as follows: GAPDH F: 5'-TTCACCACCATGGAGAAGGC-3', R: 5'-TGAAGTGCAGAGACAAACC-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, MA, 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 and analyzed using ImageJ.

Hoechst 33342/PI fluorescent double staining

VSMCs were inoculated and cultured in a 6-well plate at $1 \times 10^5$ 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 Hoechst 33342 stain solution and 5 µL of PI stain solution in 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, Bethesda, MD, 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, Beijing, 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 9.0 (La Jolla, CA, USA) was used for statistical analysis. The experimental data were described by mean ± standard deviation (χ ± s), and compared among groups by one-way ANOVA and Newman-Keuls test. The data of abnormal distribution were compared by Dunnett's T3 test. $P<0.05$ 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 (Fig. 1A-C). Therefore, VSMCs were transfected with viruses under the 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 the CTSB mRNA expression had no difference in Mock1 group (1.082 ± 0.095) and Mock2 group (1.058 ± 0.096) compared with that in NC group ($P>0.05$), it was significantly higher in lv-CTSB group (3.086 ± 0.199) than that in Mock1 group ($P<0.0001$), and it was significantly lower in sh-CTSB group (0.419 ± 0.100) than that in Mock2 group ($P<0.0001$) (Fig. 1E). It was found by Western blotting that the CTSB protein expression had no difference in Mock1 group (0.718 ± 0.085) and Mock2 group (0.631 ± 0.052) compared with that in NC group (0.597 ± 0.103) ($P>0.05$), it was significantly higher in lv-CTSB group (1.082 ± 0.067) than that in Mock1 group ($P<0.01$), and it was lower in sh-CTSB group (0.345 ± 0.123)
than that in Mock2 group (P < 0.05) (Fig. 1D&F). The above results suggested 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. Then it was observed by Hoechst 33342/PI staining that there were a few PI-stained cells in red in 0 µg/mL group [(0.50 ± 0.440)\%], the number of PI-stained cells in red was increased in 50 µg/mL group [(12.453 ± 1.581)\%] and further increased in 100 µg/mL group [(17.673 ± 1.001)\%], and a large number of PI-stained cells in red were seen under the microscope in 150 µg/mL group [(21.897 ± 1.728)\%] (Fig. 2B&J). The results of Western blotting revealed that the protein expressions of CTSB, NLRP3, ASC, cleaved-caspase-1 (p20), IL-1\(\beta\), IL-18 and GSDMD-N rose in 50, 100 and 150 µg/mL groups compared with those in 0 µg/mL group (P < 0.05), and they increased with the increase in the ox-LDL concentration (Fig. 2A, C-I). Moreover, the results of ELISA showed that the activity of IL-1\(\beta\) was higher in 50, 100 and 150 µg/mL groups than that in 0 µg/mL group (P < 0.05), and it increased with the increase in the ox-LDL concentration, which was also verified by the results of LDH release assay (Fig. 2K&L). Compared with 0 µg/mL group, 50, 100 and 150 µg/mL groups had statistically significant differences in the number of PI-positive cells, expressions of pyroptosis-related proteins, LDH activity and IL-1\(\beta\) activity (P < 0.05), especially 150 µg/mL group. Therefore, the pyroptosis model was induced by 150 µg/mL ox-LDL.

**Effects of different concentrations of DAPA on proliferative activity of VSMCs**

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 that in NC group, the cell viability at 24 h after culture had no differences in DMSO group [(102.449 ± 7.538)\%], 0.1 µM DAPA group [(102.011 ± 8.007)\%], 1.0 µM DAPA group [(101.572 ± 7.682)\%], 5.0 µM DAPA group [(99.362 ± 8.906)\%] and 10 µM DAPA group [(96.539 ± 10.137)\%] (P > 0.05), and it declined in 25 µM DAPA group [(83.891 ± 8.375)\%] (P < 0.05) and 50 µM DAPA group [(75.314 ± 13.216)\%] (P < 0.001). 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 (Fig. 3D).

**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, activity of LDH and activity of IL-1β were detected by Western blotting, LDH release assay and ELISA, respectively. The results showed that in ox-LDL group, the number of PI-stained cells in red significantly increased [(21.897 ± 1.728)%] compared with that in NC group [(0.657 ± 0.584)%] (Fig. 3A&B), the protein expressions of CTSB, NLRP3, ASC, cleaved-caspase-1 (p20), IL-1β, IL-18 and GSDMD-N rose, with statistically significant differences (P < 0.05) (Fig. 3F-M), and the activity of LDH and IL-1β was enhanced, showing statistically significant differences (P < 0.05) (Fig. 3C&E). Compared with ox-LDL group, 0.1 µM DAPA group had significantly decreased number of PI-stained cells in red (Fig. 3A&B), expressions of pyroptosis-related proteins (Fig. 3F-M) and activity of LDH and IL-1β (Fig. 3C&E) (P < 0.05). 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 < 0.05). To sum up, DAPA could ameliorate the pyroptosis of VSMCs, and its 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 (p20), 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 (Fig. 4B&C), protein expressions of CTSB, NLRP3, cleaved-caspase-1 (p20), ASC, IL-1β, IL-18 and GSDMD-N (Fig. 4A, F-L), and activity of LDH and IL-1β (Fig. 4D&E) in Mock1 and Mock2 groups as compared to those in ox-LDL group (P > 0.05). The PI-positive cell rate (Fig. 4B&C), protein expressions of CTSB, NLRP3, ASC, cleaved-caspase-1 (p20), IL-1β, IL-18 and GSDMD-N (Fig. 4A, F-L), and activity of LDH and IL-1β (Fig. 4D&E) all significantly rose in lv-CTSB group compared with those in Mock1 group, while they declined in sh-CTSB group compared with those in Mock2 group, all showing statistically significant differences (P < 0.05).

**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 pyroptosis of VSMCs, 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 (p20), 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 (Fig. 5B&C), protein expressions of CTSB, NLRP3, ASC, cleaved-caspase-1 (p20), IL-1β, IL-18 and GSDMD-N (Fig. 5A, F-L), and activity of LDH and IL-1β (Fig. 5D&E) all significantly rose in ox-LDL + DAPA + lv-CTSB group compared with those in ox-
LDL + DAPA + Mock1 group ($P < 0.05$), while they declined in ox-LDL + DAPA + sh-CTSB group compared with those in ox-LDL + DAPA + Mock2 group, all showing statistically significant differences ($P < 0.05$).

**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 through activating the NLRP3/caspase-1 signaling pathway and increase the pyroptosis in a concentration-dependent manner, and CTSB was associated with the severity of pyroptosis of VSMCs. Ox-LDL-induced pyroptosis of VSMCs 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 the NLRP3 expression and pyroptosis was found, indicating that DAPA could not only suppress CTSB but also reduce the NLRP3 expression.

Some studies have revealed that NLRP3 inflammasome activation-mediated pyroptosis of VSMCs is implicated in and exacerbates AS. Pan et al.\(^2^1\) 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 occurrence of AS. Li et al.\(^2^2\) also found that ox-LDL can promote pyroptosis of VSMCs, and VX-765, through specifically blocking caspase-1, inhibits ox-LDL-induced pyroptosis of VSMCs and delays AS in ApoE\(^{-/-}\) mice fed with a high-fat diet. He et al.\(^2^3\) showed that VX-765 can not only suppress pyroptosis of VSMCs but also reduce formation of smooth muscle-derived foam cells. In this study, it was 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.\(^2^4,2^5\) As demonstrated in a previous study, CTSB can directly bind to the LRR domain of NLRP3 inflammasomes, thereby activating NLRP3 inflammasomes and inducing pyroptosis.\(^2^6\) In this study, it was 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, it was 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 AS in ApoE\(^{-/-}\) mice by improving endothelium-dependent relaxation and reducing formation of macrophage-derived foam cells.\(^{19,20}\) According to recent \textit{in vitro} studies, DAPA can reduce polarization of pro-inflammatory M1 macrophages and expressions of pro-inflammatory miRNAs.\(^{27}\) In this study, it was found that DAPA could attenuate ox-LDL-induced NLRP3 inflammasome activation and lower the expressions of IL-18 and IL-1\(\beta\), and it also reduced 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-1\(\beta\) and the release of LDH, thereby worsening the ox-LDL-induced pyroptosis of VSMCs, which suggested 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 the NLRP3 expression and pyroptosis. Therefore, it is 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 ROS, it was found that DAPA can weaken the activation of NLRP3/caspase-1 signaling pathway by reducing macrophage ROS production, thereby ameliorating AS in type 2 diabetic ApoE\(^{-/-}\) mice fed with high-fat diet.\(^{28}\) Combined with the findings in this study, it is 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 remain unclear, so further study is needed. At present, research on the pathogenesis of AS mainly focuses on endothelial cells and macrophages, and the role of VSMCs in the pathogenesis of AS 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 AS. Hence, there is reason to believe that it is of great significance to clarify the role of VSMCs in AS for the prevention and treatment of AS-related diseases.

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

**Declarations**

**Ethics approval and consent to participate**

Not applicable

**Consent for publication**

Not applicable

**Availability of data and materials**

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

**Competing interests**

The authors declare that they have no competing interests

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**Authors' contributions**

Hui Li and Danan Liu designed the study and performed the experiments, Quanwei Zhao and Bo Zhou collected the data, Caiwei Gong and Guanjian Zhao analyzed the data, Hui Li and Danan Liu prepared the manuscript.

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Not applicable

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Figures
VSMCs transfected with CTSB-overexpressing and -silencing lentiviruses and CTSB mRNA and protein expressions detected by RT-qPCR and Western blotting. (A) 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%. (B) Statistical results of transfection efficiency on VSMCs transfected with CTSB-overexpressing lentivirus at different MOI. (C) Statistical results of transfection efficiency on VSMCs transfected with CTSB-silencing lentivirus at different MOI. (D) CTSB protein expression detected by Western blotting in VSMCs transfected with CTSB-overexpressing and -silencing lentiviruses (MOI=100). (E&F) Transfection effect verified by RT-qPCR and Western blotting. n=3; *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001; Bar=100 μm.
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. (A) Protein expressions of CTSB, NLRP3, ASC, cleaved-caspase-1 (p20), IL-1β, IL-18 and GSDMD-N in VSMCs detected by Western blotting in each group. (B) Hoechst 33342/PI fluorescent double staining (blue: Hoechst 33342 staining, red: PI staining). (C) Statistical results of CTSB protein expression in each group. D) Statistical results of NLRP3 protein expression in each group. (E) Statistical results of ASC protein expression in each group. F) Statistical results of p20 protein expression in each group. (G) Statistical results of GSDMD-N protein expression in each group. (H) Statistical results of IL-1β protein expression in each group. I) Statistical results of IL-18 protein expression in each group. (J) Statistical results of Hoechst 33342/PI fluorescent double staining in each group. (K) Statistical results of IL-1β activity in each group. (L) Statistical results of LDH activity in each group. n=3; *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001; Bar=100 μm.
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. (A) Hoechst 33342/PI fluorescent double staining (blue: Hoechst 33342 staining, red: PI staining). (B) Statistical results of Hoechst 33342/PI fluorescent double staining in each group. (C) Statistical results of LDH activity in each group.
(D) Proliferative activity of VSMCs measured by CCK-8 assay after pretreatment with DAPA at corresponding concentrations for 24 h in each group. (E) Statistical results of IL-1β activity in each group. (F) Protein expressions of CTSB, NLRP3, ASC, cleaved-caspase-1 (p20), IL-1β, IL-18 and GSDMD-N in VSMCs detected by Western blotting in each group. (G) Statistical results of CTSB protein expression in each group. (H) Statistical results of NLRP3 protein expression in each group. (I) Statistical results of ASC protein expression in each group. (J) Statistical results of p20 protein expression in each group. (K) Statistical results of GSDMD-N protein expression in each group. (L) Statistical results of IL-1β protein expression in each group. (M) Statistical results of IL-18 protein expression in each group. n=3; *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001; Bar=100 μm.

**Figure 4**

Effects of overexpression and silencing of CTSB on ox-LDL-mediated pyroptosis of VSMCs. The cells in 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. (A) Protein expressions of CTSB, NLRP3, ASC, cleaved-caspase-1 (p20), IL-1β, IL-18 and GSDMD-N in VSMCs detected by Western blotting in each group. (B) Hoechst 33342/PI fluorescent double staining (blue: Hoechst 33342 staining, red: PI staining). (C) Statistical results of Hoechst 33342/PI fluorescent double staining in each group. (D) Statistical results of LDH activity in each group. (E) Statistical results of IL-1β activity in each group. (F) Statistical results of CTSB protein expression in each group. (G) Statistical results of NLRP3 protein expression in each group. (H) Statistical results of ASC protein expression in each group. (I) Statistical results of p20 protein expression in each group. (J) Statistical results of GSDMD-N protein expression in each group. (K)
Figure 5

Effects of overexpression and silencing of CTSB on ox-LDL-induced pyroptosis of VSMCs after DAPA intervention. The cells in 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. (A) Protein expressions of CTSB, NLRP3, ASC, cleaved-caspase-1 (p20), IL-1β, IL-18 and GSDMD-N in VSMCs detected by Western blotting in each group. (B) Hoechst 33342/PI fluorescent double staining (blue: Hoechst 33342 staining, red: PI staining). (C) Statistical results of Hoechst 33342/PI fluorescent double staining in each group. (D) Statistical results of LDH activity in each group. (E) Statistical results of IL-1β activity in each group. (F) Statistical results of CTSB protein expression in each group. (G) Statistical results of NLRP3 protein expression in each group. (H) Statistical results of ASC protein expression in each group. (I) Statistical results of p20 protein expression in each group. (J) Statistical results of GSDMD-N protein expression in each group. (K) Statistical results of IL-1β protein expression in each group. L) Statistical results of IL-18 protein expression in each group. n=3; *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001; Bar=100 μm.