CTRP9 prevents atherosclerosis progression through changing autophagic status of macrophages by activating USP22 mediated-de-ubiquitination on Sirt1

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Abstract

**Background:** Atherosclerosis (AS) is commonly regarded as a key driver accounted for the leading causes of morbidity and mortality among cardiovascular and cerebrovascular diseases. A growing body evidence indicates that autophagy in macrophages involved in AS might be a potential therapeutic target. C1q/TNF-related protein 9 (CTRP9) has been proved to delay the progression of cardiovascular diseases. However, the relations among CTRP9 and Sirt1 either with its effects on macrophages autophagy has not been fully explored.

**Methods:** Macrophages were differentiated from the mononuclear cells collected from the peripheral blood samples of healthy donors. The *in vitro* AS model were constructed by ox-LDL treatment. Cell viability was determined by CCK-8 assay. Immunofluorescence assay of LC3 was implemented for evaluating autophagy activity. Oil Red O staining was performed for lipid accumulation detection. ELISA, cholesterol concentration assay and cholesterol efflux analysis was conducted using commercial kit. Cycloheximide assay was implemented for revealing protein stability. RT-qPCR was used for mRNA expression detection, and western blotting was performed for protein level monitoring.

**Results:** CTRP9 attenuated impaired cell viability, autophagy inhibition and increased lipid accumulation induced by ox-LDL. Moreover, CTRP9 maintained Sirt1 protein level through enhancing its stability by de-ubiquitination, which was mediated by upregulated USP22 level. CTRP9 exerted its protective role in promoting autophagy and reducing lipid accumulation through USP22/Sirt1 axis.

**Conclusion:** Collectively, CTRP9 alleviates lipid accumulation and facilitated the macrophages autophagy through upregulating USP22 level and maintaining Sirt1 protein expression, thereby exerting a protective role in AS progression.

Introduction

Atherosclerosis (AS) has been regarded as one of the most prevalent cardiovascular diseases, and it was predicted that AS would become the leading cause of the disease burden around the world (1). AS was a chronic inflammatory disease, which could be characterized by the lipid deposition in the artery and the gradual development of atherosclerotic plaques (2). Anti-inflammatory treatments were the main therapeutic methods for AS in the past few decades. It was reported that inhibitors like HMG-CoA reductase, phospholipase A2, glucagon-like peptide-1, and suppressing the inflammatory cytokines expression using TNF-α blockers might be used for alleviating the inflammation in AS (3). Moreover, new drug carriers like dexamethasone or prednisolone phosphate-loaded liposomes were used to ameliorate the inflammatory response in rabbits with hyperlipidemia (4). However, the current treatments were mainly systemic approaches instead of local approaches, which lacked efficacy and might cause side effects. Besides inflammatory responses, the progression of AS was a complicated process that included the differentiation, apoptosis and autophagy of vascular endothelial cells, smooth muscle cells and
Therefore, targeting the molecular pathways in these biological processes might be an alternative approach for the clinical treatment of AS.

Macrophages participated in the different stage of AS. The macrophages were formed by circulating monocytes, which further converted into form cells by oxygenized low-density lipoproteins (ox-LDL) by endocytosis (6). Autophagy was a lysosome-based self-protecting mechanism that mediated the degradation of misfolded proteins or injured organelles, regulating the progression of tumor growth, neurodevelopment, and cardiovascular disease (7). Recent studies revealed that autophagy could prevent macrophages from apoptosis and defective efferocytosis, which decreased the necrotic area in plaques and alleviated inflammatory responses (5). Moreover, the suppressed autophagy was found, and increased inflammatory cytokines and dysregulated LC3 and SQSTM1 expression was demonstrated in macrophages during AS progression (8). The autophagy of macrophages was vital for the stability of plaques and inflammatory responses, indicating that targeting this process might contribute to the improvement of AS.

C1q/TNF-related proteins 9 (CTRP9) belonged to CTRP family, which were paralogs of adiponectin and enriched in the adipose tissues or cardiovascular system (9). Recently, it was reported that CTRP9 dramatically promoted the efferocytosis of macrophages and activated its metabolism, and further enhanced p-DRP1 levels and facilitated mitochondrial fission in various cardiovascular disease (10). Moreover, CTRP9 was found to suppress the formation of lipid droplets and decrease the cholesteryl ester expression, which subsequently enhanced LC3-II levels and induced cell autophagy, thus prevent the differentiation of macrophages into foam cells (11). Sirtuin 1 (Sirt1) was NAD+-dependent de-acetylating enzymes that was revealed to correlate with macrophages autophagy and polarization (12). However, the relations between CTRP9 and Sirt1 have not been fully explored in AS progression.

In the present study, we demonstrated that CTRP9 upregulated the expression of USP22, a kind of deubiquitinating enzymes, which further suppressed the degradation of Sirt1 protein for triggering macrophages autophagy, thus attenuating AS progression. The data provided solid reference for understanding the biological processes regarding cardiovascular diseases, and indicated potential molecular target for the clinical treatment of AS.

### Methods

#### Cell model

Macrophages were obtained as previously described (13). Briefly, the mononuclear cells were collected from the peripheral blood samples of human donors signed the written informed consent and preserved in a -80°C freezer. Then the mononuclear cells were cultured in RPMI 1640 medium (Thermo Scientific, Carlsbad, CA, USA) supplemented with 10% FBS and 50 ng/mL M-CSF (Solarbio, Beijing, China) at 37°C for one week, after which the differentiated macrophages were isolated. For induction of *in vitro* AS models, different concentration of ox-LDL (0, 50, 100, 150, 200 µg/ml) (R&D Systems, Minneapolis, MN,
USA) was added into RPMI 1640 medium of macrophages for 24 h. Then different concentration of CTRP9 (0, 0.3, 1, 3 µg/ml) (R&D Systems, Minneapolis, MN, USA) was added for 24 h to explore its role in AS progression. The optimal concentrations of ox-LDL and CTRP9 were determined for the subsequent experiments.

**Cell transfection**

To validate the roles of various signaling pathways, si-CTRP9, si-USP22, and pcDNA3.1 containing the coding sequence of CTRP9, were obtained from GenePharma (Shanghai, China). The si-NC or empty vectors were served as controls. Cell transfection was performed through Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) as per the manufacturer’s protocols.

**Cell viability**

Briefly, the cells were inoculated into a 96-well plate at a density of 2×10^4 cells per well, and then the cells were treated under various conditions, added with 10 µL of CCK-8 solution (Solarbio, Beijing, China) and incubated for 2 h at 37°C. The absorbance at 450 nm was measured using a microplate reader (PerkinElmer, Shanghai, China).

**Immunofluorescence**

Briefly, the cells were pre-treated with 4% paraformaldehyde, and then the cells were washed with PBS and maintained with 5% FBS at 37°C for 60 min. Thereafter, the samples were incubated with primary antibodies including anti-LC3 (ab192890, 1:1000) at 4°C overnight, followed by incubating with fluorescence-conjugated secondary antibodies (ab150077, 1:1000) in the dark for 60 min. The results were observed and analyzed by a BX53 fluorescence microscope (Olympus, Tokyo, Japan).

**Oil Red O staining**

After treated under various conditions, the cells were collected, pre-treated with 4% paraformaldehyde, and washed with PBS. Then the cells were maintained with 60% isopropanol for 5 min and stained with premixed Oil Red O (Solarbio, Beijing, China) for 30 min and hematoxylin for 30 s. Then results were observed by a CHBS phase-contrast microscope (Olympus, Tokyo, Japan).

**Cholesterol concentration assay**

The content of cholesterol was determined using the commercial kit (Solarbio, Beijing, China) as previously described (14). The colorimetric analysis was implemented to determine the concentration of total cholesterol (TC), cholesterol ester (CE) and free cholesterol (FC).

**ELISA**

The concentration of CTRP9 in the supernatant of cells were monitored using Enzyme-linked immunosorbent assay (ELISA, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocols.
**Cholesterol efflux analysis**

Briefly, the fluorescence-labeled cholesterol was added into cells and incubated in the dark at 37°C overnight. After treated under various conditions, the supernatant was collected, the cell monolayer was lysed and mixed by pipetting, and the absorbance was detected at 482/515 nm. The cholesterol efflux was calculated as fluorescence intensity of supernatant / (fluorescence intensity of supernatant + cell lysate) * 100%.

**Cycloheximide assay**

Briefly, the cells were treated with 10 µg/ml cycloheximide (CHX) for the indicated times (0, 2, 4, 6 and 8 h). Then the level of Sirt1 protein was detected by western blotting and quantified by quantity-one software.

**RT-qPCR**

The total RNA was isolated using TRizol reagent (Invitrogen, Carlsbad, CA, USA). According to the instructions of reverse transcription reagent kit (Sangon Biotech, Shanghai, China), the cDNA was obtained, and the amplification was implemented on the ABI quantitative PCR system (Applied Biosystems, Waltham, MA, USA) for detecting RNA expression. The PCR conditions were as follows: denaturation at 95°C for 10 min, followed by 35 cycles of denaturation at 94°C for 20 s, annealing at 60°C for 30 s and extension at 72°C for 30 s. The relative expressions of mRNA were calculated using $2^{-\Delta\Delta Ct}$ method. The primers were list as follows:

- **USP22-F**: 5'- CTCCTGTCTGGTCTGTGAGATG -3'
- **USP22-R**: 5'- CAGCAACTTATACGGGATGTGA -3'
- **Sirt1-F**: 5'- CATAGACACGCTGGAAACAGG -3'
- **Sirt1-R**: 5'- GCAGATGAGGCAAAGGTT -3'
- **GAPDH-F**: 5'- GACAAGATGGTGAAGGTCGG -3'
- **GAPDH-R**: 5'- CATGGACTGTGGTCATGAGC -3'

**Western blotting**

The total proteins were extracted using RIPA lysis buffer (Solarbio, Beijing, China). After centrifugation, the supernatant was obtained, and the concentration of proteins was detected by BCA Assay Kit (Sangon Biotech, Shanghai, China). Then proteins were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE, Sangon Biotech, Shanghai, China) gel. The proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA), and blocked with 5% non-fat milk. The samples were incubated with primary antibodies that included anti-USP22 (ab195289, Abcam, 1:1000), anti-Sirt1 (ab110304, Abcam, 1:1000), anti-LC3I/II (ABC929, Millipore, 1:1000), anti-SQSTM1 (ab109012,
Abcam, 1:1000), anti-tubulin (ab7291, Abcam, 1:1000) at 4°C overnight. Next, the membrane was incubated with HRP-conjugated rabbit anti-mouse IgG secondary antibody (ab6728, 1:1000) for 2 h at room temperature. The band density was quantified using the ImageJ software (US National Institutes of Health, Bethesda, MD, USA).

**Statistical analysis**

The quantitative data were presented as the mean ± standard deviation (SD). All statistical analyses were performed using GraphPad Prism 7.0. Student's t test was conducted for comparison between two groups. One-way analysis of variance followed by Tukey's post hoc test for comparison among three or more groups. P<0.05 were statistically significant. All the experiments were performed in triple.

**Results**

**CTRP9 attenuated impaired cell viability, autophagy inhibition and increased lipid accumulation induced by ox-LDL**

The in vitro studies were performed using primary human macrophages isolated from peripheral blood samples. As illustrated in Fig. 1A, different concentration of CTRP9 (0, 0.3, 1, 3 µg/ml) was used to treat cells, and the data showed that CTRP9 had no effect on cell viability. However, the data in Fig. 1B revealed that ox-LDL (0, 50, 100, 150, 200 µg/ml) decreased cell viability of macrophages in a dose-dependent manner. For the subsequent experiments, 1 µg/ml of CTRP9 and 50µg/ml of ox-LDL was used. The results in Fig. 1C indicated that CTRP9 significantly reversed the inhibitory effects of ox-LDL on cell viability, while addition of 3-MA, a kind of autophagy inhibitor, dramatically reversed the protective effects of CTRP9. Then the expression of LC3 was determined using immunofluorescence, and the data indicated that ox-LDL reduced LC3 expression, which was reversed by CTRP9 treatment (Fig. 1D). Similarly, the effects of CTRP9 were lost by 3-MA pretreatment. Compared with control group, ox-LDL induced lipid accumulation and increased CE content as well as the ratio of CE/TC, whereas it was significantly reduced after CTRP9 treatment, but this effect was again reversed after 3-MA pretreatment (Fig. 1E&1F). The above data revealed that CTRP9 played a protective role in ameliorating impaired cell viability, autophagy inhibition and increased lipid accumulation induced by ox-LDL.

**CTRP9 maintained Sirt1 protein level through enhancing its stability**

To elucidate the role of CTRP9 on Sirt1-related autophagy, subsequent experiments were conducted. As presented in Fig. 2A, the treatment of ox-LDL significantly decreased the Sirt1 mRNA level. However, overexpression of CTRP9 exhibited no effects on Sirt1 mRNA expression, as well as the addition of 3-MA. Moreover, the western blotting analysis revealed that ox-LDL reduced the Sirt1 protein level and LC3II/LC3I ratio, and enhanced SQSTM1 protein level (Fig. 2B). Overexpression of CTRP9 in contrast
increased Sirt1 protein level and LC3II/LC3I ratio, and decreased SQSTM1 protein level, whereas pretreatment of 3-MA reversed the effects of CTRP9 (Fig. 2B). Furthermore, it was demonstrated that different concentration of CTRP9 had no effects on Sirt1 mRNA expression but enhanced the Sirt1 protein expression in a dose-dependent manner (Fig. 2C&2D). The overexpression or knockdown model of CTRP9 was constructed by pcDNA-CTRP9 or si-CTRP9 and verified by ELISA (Fig. 2E). To validate the effects of CTRP9 on the stability of Sirt1, CHX was used to treat cells. As shown in Fig. 2F, transfection of pcDNA-CTRP9 significantly alleviated the degree of Sirt1 degradation. Then, MG132, a proteasome inhibitor, was added, and the results indicated that silencing CTRP9 decreased Sirt1 protein level, while MG132 reversed the effects of si-CTRP9 (Fig. 2G). The evidence suggested that CTRP9 maintained Sirt1 protein level through enhancing its stability.

**CTRP9 triggered the de-ubiquitination of Sirt1 via up-regulating USP22 expression**

The expression of USP22, a ubiquitin specific peptidase, was detected to reveal the change on ubiquitination of Sirt1. As illustrated, both mRNA and protein expression of USP22 was upregulated by CTRP9 treatment in a dose-dependent manner (Fig. 3A&3B). Moreover, the ox-LDL treatment dramatically decreased both mRNA and protein level of USP22, while overexpression of CTRP9 had the reversed effects (Fig. 3C&3D). Then the knockdown model of USP22 was constructed by si-USP22 and verified by RT-qPCR (Fig. 3E). Depletion of USP22 exhibited no effects on Sirt1 mRNA level, but significantly inhibited Sirt1 protein level (Fig. 3F&3G). Western blotting analysis further proved that knockdown of USP22 dramatically attenuated CTRP9-induced Sirt1 de-ubiquitination (Fig. 3H). Collectively, these results revealed that CTRP9 promoted USP22 expression, which removed the conjugated poly-ubiquitin chains from Sirt1 and enhanced the stabilization of Sirt1 protein.

**CRTP9 exerted its protective role through USP22/Sirt1 axis**

As shown in Fig. 4A, CTRP9 treatment significantly attenuated the ox-LDL-induced cell viability inhibition, whereas depletion of USP22 or usage of EX527, a Sirt1 inhibitor, significantly reversed the effects of CTRP9. Similarly, immunofluorescence analysis on LC3 expression demonstrated that depletion of USP22 or usage of EX527 dramatically reversed the effects of CTRP9 on ameliorating the suppressed cell autophagy triggered by ox-LDL (Fig. 4B). Moreover, depletion of USP22 or usage of EX527 reduced the Sirt1 protein level and LC3II/LC3I ratio, and enhanced SQSTM1 protein level compared with CTRP9 treatment (Fig. 4C). The overexpression of CTRP9 decreased lipid accumulation, elevated the cholesterol efflux levels of HDL and apoA-1, whereas depletion of USP22 or usage of EX527 reversed its effects (Fig. 4D&4E). These results indicated that CRTP9 might exert its protective role through USP22/Sirt1 axis.

**Discussion**

During the progression of AS, the lipid-containing plaques were formed and deposited on the vessel wall of arteries, resulting in the enhanced morbidity and mortality rates (15). The destabilization of
Atherosclerotic plaques might lead to plaque rupture, which subsequently induced multiple syndromes that included stroke and myocardial infarction (16). Recently, it was reported that autophagy played a vital role in regulating the stability of atherosclerotic plaques and influencing AS development. The cytoplasmic substances were engulfed by autophagosomes and degraded into biomolecules by the lysosomal hydrolases (17). It was demonstrated that autophagy mainly occurred in macrophages, vascular smooth muscle cells and endothelial cells during the progression of AS (18). The activated autophagy promoted enhanced the survival rate of macrophages and promoted the efflux of free cholesterol from foam cells (19). Therefore, the present study explored the potential mechanisms of autophagy in AS, aiming at providing new therapeutic approaches for the clinical treatments.

The protective roles of CTRP in ameliorating inflammatory cascade and cardiovascular systems has been reported in recent years. It was reported that increasing CTRP9 expression would alleviate diabetic cardiovascular injury through suppressing abnormal platelet activity in diabetic mice (20). Kim et al. revealed that the knockdown of CTRP9 in mice enhanced the content of atherosclerotic lesions, whereas transplanting the gut microbiota of the wild-type control mice significantly decreased the occurrence (21). A clinical study demonstrated that CTRP9 was upregulated in patients with type 2 diabetes and chronic kidney disease, and positively correlated with intima-media thickness in those without chronic kidney disease (22). CTRP9 also induced the conversion of LC3 and degradation of P62 via phosphorylated AMPK, which further promoted autophagy in human primary hepatocytes and alleviated hepatic steatosis (23). Similarly, the present study showed that overexpression of CTRP9 reversed the inhibitory effects of ox-LDL on cell viability and autophagy, elucidating the protective role of CTRP9.

With respect to Sirt1, it was reported that it was involved in the process of attenuating inflammatory status and atherosclerotic plaques (24). The inhibited Sirt1 expression was found in endothelial cells and macrophages, and contributed to forming foam cells, activating inflammatory cascades, and impairing autophagy (25). Yuan et al. reported that laminar flow enhanced Sirt1 expression, which further induced YAP deacetylation and downregulated the proinflammatory cytokine levels, and enhanced LC3II/LC3I ratio to suppress the formation of atherosclerotic plaques (26). USP22 was also reported to remove the poly-ubiquitin chains that was conjugated on Sirt1, thus increased the stability of Sirt1 and facilitated the development of mouse embryonic (27). Ao et al. indicated that USP22 inhibited the acetylation of STAT3 via its de-ubiquitination effects on Sirt1 (28). Consistently, in this study, we firstly demonstrated the effects of USP22 on Sirt1 in AS progression, and newly proved the relations between CTRP9 and USP22. We found that CTRP9 increased the expression of USP22, which triggered the de-ubiquitination of Sirt1 and promoted cell autophagy.

In conclusion, the present study demonstrated that CTRP9 alleviated lipid accumulation, facilitated the macrophages autophagy, and played a protective role in AS progression through upregulating USP22 level and maintaining Sirt1 protein expression (Fig. 5). These results provided potential molecular targets for the clinical treatment of AS. However, further in vivo and clinical experiments are needed to demonstrate the effects of CTRP9, and other biological pathways are needed to investigate for comprehensively understanding the mechanisms in AS.
Declarations

Ethical Approval

This study has obtained approval from the Ethics Committee of Hainan Affiliated Hospital of Hainan Medical University and the patient's written informed consent.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Min Zeng and Xin Wei contributed to the conception of the study.

Min Zeng, Ziyan Wang, Xiuyang Zhao and Dianshu Zhu performed the experiment.

Mengdi Wang and Yue Chen contributed significantly to analysis and wrote the original draft.

Xiuyang Zhao and Xin Wei prepared figures.

All authors edited and reviewed the main manuscript.

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Availability of data and material

All data generated or analyzed during this study are available on request to the corresponding author.

References


Figures
Figure 1

**CTRP9 attenuated impaired cell viability, autophagy inhibition and increased lipid accumulation induced by ox-LDL.** (A) CCK8 assay to detect cell viability of macrophages. Macrophages were treated with different concentrations of CTRP9 (0, 0.3, 1, 3 μg/ml) for 12h. (B) CCK8 assay to detect cell viability of macrophages. Macrophages were treated with different concentrations of ox-LDL (0, 50, 100, 150, 200 μg/ml) for 24h. (C) CCK8 assay to detect cell viability. Macrophages were treated with ox-LDL, CTRP9 and 3-MA. (D) Immunofluorescence detected the LC3 expression. (E) Oil Red O staining for determining lipid accumulation. (F) Cholesterol content was detected to assess the lipid deposition. *P<0.05, **P<0.01, ***P<0.001.
Figure 2

Sirt1 served as a downstream effector of CTRP9 through enhancing its stability and suppressing its degradation. (A) The mRNA expression of Sirt1 measured by RT-qPCR. (B) The protein expression of Sirt1, LC3II/ LC3I, SQSTM1 determined using western blotting. (C) The mRNA expression of Sirt1 measured by RT-qPCR. (D) The protein expression of Sirt1 determined using western blotting. (E) ELISA verified the effects of the pcDNA-CTRP9 or CTRP9 siRNA transfection on CTRP9 secretion. (F) Treatment of
cycloheximide to indicate the effects of CTRP9 on Sirt1 protein stability. (G) Treatment of MG132 to reveal the role of proteasome in CTRP9-mediated inhibition of Sirt1 degradation. *P<0.05, **P<0.01, ***P<0.001.

Figure 3
CTRP9 triggered the de-ubiquitination of Sirt1 via up-regulating USP22 expression. (A) The mRNA expression of USP22 measured by RT-qPCR. (B) The protein expression of USP22 determined using western blotting. (C) The mRNA expression of USP22 measured by RT-qPCR. (D) The protein expression of USP22 determined using western blotting. (E) The mRNA expression of USP22 measured by RT-qPCR. (F) The mRNA expression of Sirt1 measured by RT-qPCR. (G) The protein expression of USP22 and Sirt1 determined using western blotting. (H) Western blotting verified that knockdown of USP22 dramatically attenuated CTRP9-induced Sirt1 de-ubiquitination. *P<0.05, **P<0.01, ***P<0.001.

Figure 4

CTRP9 exerted its protective role through USP22/Sirt1 axis. (A) CCK8 assay to detect cell viability. (B) Immunofluorescence detected the LC3 expression. (C) The protein expression of Sirt1, LC3II/LC3I, SQSTM1 determined using western blotting. (D) Oil Red O staining for determining lipid accumulation. (E) Cholesterol efflux analysis. *P<0.05, **P<0.01, ***P<0.001.
Figure 5

A graphic image of CTRP9 exerting inhibitory effects on the formation of macrophage foam cell via regulating autophagic status.