CTRP13 Mitigates Endothelial Cell Ferroptosis via the AMPK/KLF4 Pathway: Implications for Atherosclerosis Protection

Background: C1q/tumor necrosis factor-related protein 13 (CTRP13) preserves endothelial function and possesses anti-oxidation activity. However, its effects on ferroptosis of human umbilical vein endothelial cells (HUVECs) remain unclear. We investigated the effects of CTRP13 on HUVEC ferroptosis induced by oxidized low-density lipoprotein (ox-LDL) and explored the underlying mechanisms of CTRP13 against ferroptosis via the AMPK/KLF4 pathway.

Material/Methods: Cell Counting Kit-8 assay was used to evaluate cell viability. Lactate dehydrogenase activity and malondialdehyde content analysis were performed to evaluate the cell membrane integrity and lipid peroxidation. Mitotracker, JC-1, and 2',7'-dichlorofluorescein di-acetate were used to evaluate the biological activity of mitochondria, mitochondrial membrane potential, and reactive oxygen species (ROS) in endothelial cells. The ferroptosis indicator expressions, recombinant solute carrier family 7, member 11, glutathione peroxidase 4 (GPX4), and acyl-CoA synthetase long-chain family member 4 were examined using real-time reverse transcription-polymerase chain reaction and Western blot. Immunofluorescence staining detected GPX4 location in endothelial cells.

Results: The results demonstrate that CTRP13 (450 ng/mL) prevented HUVEC ferroptosis by inhibiting ROS overproduction and mitochondrial dysfunction, and CTRP13 accelerated antioxidant enzyme expression levels, such as heme oxygenase 1, superoxide dismutase 1, and superoxide dismutase 2, compared with the ox-LDL (100 µg/mL) group for 48 h. Additionally, CTRP13 treatment increased p-AMPK/AMPK expression by 47.65% (P<0.05) while decreasing Krüppel-like factor 4 expression by 37.43% (P<0.05) in ox-LDL-induced HUVECs and elucidated the protective effect on endothelial dysfunction from ferroptosis.

Conclusions: These findings provide new insights for understanding the effects and mechanism of CTRP13 on preventing endothelial cell ferroptosis.

Keywords: Atherosclerosis • C1Q13 Protein, Human • Endothelial Cells • Ferroptosis • KLF4 Protein, Human

Abbreviations: CTRP13 – C1q/TNF-related protein-13; ox-LDL – oxidized low-density lipoprotein; AMPK – AMP-activated protein kinase; KLF4 – Krüppel-like factor 4; GPX4 – glutathione peroxidase 4; Fer-1 – ferrostatin-1; ACSL4 – acyl-CoA synthetase long-chain family member 4

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/942733
Atherosclerosis cardiovascular disease has become a global public health concern because it causes the death of more people than all cancers combined and is one of the leading causes of morbidity and mortality worldwide [1]. Atherosclerosis characteristics include lipid substance accumulation, pro-inflammatory response in macrophages, endothelial cell injury, and arterial wall sclerosis. Recent evidence demonstrates that atherosclerosis begins long before clinical manifestations, with onsets that can be traced back to adolescence or early adulthood [2]. New anti-atherosclerosis agents are required to supplement our current arsenal of anti-atherosclerosis drugs and provide therapeutic options for all patients at high risk for atherosclerosis.

The mechanism of atherosclerosis remains to be established. Some scholars believe hyperlipidemia and inflammation-induced endothelial dysfunction are crucial initiating factors and key links to atherosclerosis development. Extensive research has demonstrated that endothelial dysfunction is a common feature of atherosclerosis. Endothelial dysfunction results from increased oxidative stress and nitric oxide (NO) reduction [3-5]. Ferroptosis is a new type of regulatory cell death characterized primarily by lipid peroxidation and iron dependence but lacks the typical apoptosis and necrosis manifestations [6]. Ferroptosis participates in various pathological processes, including cancers, atherosclerosis, and cardiomyopathy. Our previous studies revealed that high glucose or oxidized low-density lipoprotein (ox-LDL) stimulation reduces endothelial NO synthase (eNOS)/NO bioavailability and inhibits the AMPK pathway in cardiomyocytes and human umbilical vein endothelial cells (HUVECs) [7,8]. However, little is known about how ferroptosis impacts HUVECs, particularly under ox-LDL conditions.

In contrast to other cell death pathways, ferroptosis has shrunk mitochondria, increased membrane density, and decreased or disappeared mitochondrial ridges. Ferroptosis is a non-apoptotic cell death characterized by an imbalance of iron metabolism and lipid peroxidation [9]. Recent evidence suggests elevated extracellular glutamate levels present in atherosclerosis-inhibited system xc transportation (xCT), triggering ferroptosis [10]. Abundant experimental data support that ferroptosis inhibition can attenuate lipid peroxidation and endothelial dysfunction in endothelial cells. Lv et al indicated that treating HUVECs with ox-LDL or the ferroptosis inducer erastin and estradiol inhibited iron death in HUVECs by activating the NRF2 pathway [11]. Liu et al found that HUVECs phagocytized large qualities of γ-Fe₂O₃ and Fe₃O₄ nanoparticles, increasing intracellular iron levels. They further observed the disrupted cystine/glutamate reverse transporter and GPX4 signaling in γ-Fe₂O₃ and Fe₃O₄ nanoparticle-challenged HUVECs. The γ-Fe₂O₃ and Fe₃O₄ nanoparticles could also cause mitochondrial fusion and fission dysregulation and activate lipid peroxidation and iron metabolism-related genes in a PS3-dependent manner [12]. Bai et al demonstrated that ferroptosis inhibition alleviates atherosclerosis by attenuating lipid peroxidation and endothelial dysfunction in mouse aortic endothelial cells [13]. Vascular endothelial cells play an essential role, forming the barrier between blood and tissues, regulating blood vessel function, and maintaining a stable internal environment [14]. Oxidative stress and mitochondrial dysfunction can cause endothelial dysfunction in the initial atherosclerosis stage. Moreover, iron overload causes endothelial dysfunction by aggravating the abnormal mitochondria structure and oxidation reaction in endothelial cells [13]. Whether ferroptosis is related to endothelial dysfunction is an interesting remaining question. Hence, in this study, we investigated the regulatory mechanism and important role of ferroptosis on endothelial dysfunction.

C1q/tumor necrosis factor-related protein 13 (CTRP13), an adipokine secreted by adipocytes, has many systemic effects and important roles in developing atherosclerosis and cardiovascular diseases. CTRP13 has a restored mitochondrial calcium ion balance, lowering reactive oxygen species (ROS) production [15]. Recent studies have discovered that CTRP13 significantly recovered NO production and eNOS coupling in the aorta of diabetic mice and high glucose-treated HUVECs. These findings showed that CTRP13 treatment reversed the down-regulation of GCH1 and the ratio of eNOS dimers to monomers in the renal arteries of patients with diabetes [4]. Soudabeh et al demonstrated that lower circulating levels of CTRP13 increased the risk of coronary artery disease and type 2 diabetes mellitus [16]. To the best of our knowledge, the molecular mechanism and pharmacological action of CTRP13 in endothelial cell ferroptosis remains uninvestigated.

Krüppel-like factor 4 (KLF4) is a zinc finger-containing transcription factor, the expression of which is connected with growth arrest. KLF4 participates in the regulation of processes including cell proliferation, differentiation, invasion, and migration [17,18]. Studies have shown that the knockdown of KLF4 by siRNA enhances the proliferation inhibitory effect of Polyphyllin III by promoting ferroptosis in breast cancer [19]. However, it remains unknown whether KLF4 mediates endothelial cell ferroptosis. AMPK is a serine/threonine kinase [20,21]. Accumulating evidence indicates that AMPK acts as an important regulator in the pathogenesis of cardiovascular diseases [22,23]. Kurabayashiet al indicated that dysregulation of Elov6-driven long-chain fatty acid metabolism induces phenotypic switching of vascular smooth muscle cells via ROS production and AMPK/KLF4 signaling, which leads to growth arrest and downregulation of vascular smooth muscle cell marker expression [24].
This study aimed to investigate the effects of CTRP13 on HUVECs ferroptosis induced by ox-LDL and to explore the underlying mechanisms of CTRP13 against ferroptosis via activating the AMPK/KLF4 pathway.

Material and Methods

Cell Culture and Treatment

HUVECs were obtained from Science Cell Research Laboratories (Carlsbad, CA, USA), and the purity of HUVECs was more than 98.67%, which was confirmed by immunofluorescence identification (iCell Bioscience Inc, Shanghai, China). Cells were cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (Gibco, Life Technologies, Waltham, MA, USA), 100 U/mL penicillin, and 0.1 mg/mL streptomycin (Gibco, Grand Island, NY, USA). The cells were cultured in an incubator under 5% CO2 at 37°C, and the culture medium was exchanged every 2 days. The cells within 7 passages were used for in vitro study. The cells were treated with trypsin (Beyotime Biotechnology, Shanghai, China) and seeded in plates for reagent intervention. Ox-LDL (YB-002) was obtained from Yiyuan Biotechnology, Guangzhou, China. HUVECs were cultured in DMEM containing ox-LDL (0-200 μg/mL) for ox-LDL treatment for 24 or 48 h [25].

Chemicals and Reagents

CTRP13 (purity >99.37%) was purchased from R&D Systems (9115-TN) and reconstituted at 500 μg/mL in water. Ferrostatin-1 (Fer-1) was purchased from Sigma (SML0583), and a 5-mmol/L stock solution was prepared in dimethyl sulfoxide (Sigma, St. Louis, MO). APTO (HY-16291, MedChem Express) was used as an inducer of KLF4. Primary antibodies against GPX4, recombinant soluble carrier family 7 member 11; HO1 – heme oxygenase 1.

Reverse Transcription-Quantitative Polymerase Chain Reaction

Total cellular RNA was extracted using TRIzol reagent (Invitrogen, CA, USA). Real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed using the SYBR Premix reagent (cat# 1725125, BIO-RAD) and ABI Fast 7500 Real-time PCR System (Applied Biosystems, South San Francisco, CA). The 2(-ΔΔCt) method was used to calculate relative fold changes with GAPDH as the internal control. Primers for real-time RT-PCR are listed in Table 1.

Table 1. Primers for qRT-PCR.

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SC7A11 – recombinant solute carrier family 7 member 11; HO1 – heme oxygenase 1.

Transmission Electron Microscopy

HUVECs were seeded in 6-well plates. After treatment, the cells were digested with trypsin and re-suspended in phosphate-buffered saline (PBS) twice. The samples were obtained by centrifuging at 3000 rpm. The supernatant was removed and fixed with 2.5% glutaraldehyde overnight at 4°C. Finally, the mitochondria ultrastructure was observed using transmission electron microscopy.

Hoechst/Propidium Iodide Staining

Hoechst/propidium iodide (PI) staining was performed according to the manufacturer’s instructions. HUVECs (1×10^6 cells/well) were cultured in a 12-well plate to detect cell membrane

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integrity. The cells were washed twice with PBS and stained with Hoechst staining (5 µg/mL; staining for all cell nuclei) and PI (2 µg/mL; staining for membrane-damaged cells) at 4°C for 30 min. Finally, cells were washed with ice-cold PBS once and observed under a fluorescence microscope (Olympus, Japan).

**Measurement of Lactate Dehydrogenase Activity**

The culture supernatant was obtained after centrifugation and assayed for lactate dehydrogenase (LDH) activity (Beyotime, Shanghai, China, C0017). Then, 120 µL of supernatant from each well was added to the corresponding well of a new 96-well plate. The absorbance was measured at 490 nm.

**Iron Content Assay**

Intracellular iron ion levels were examined using an iron assay kit (TC1015, Leagene, Beijing, China) according to the manufacturer’s instructions. Briefly, the supernatant was centrifuged at a low speed, mixed, and incubated at 37°C for 10 min. Then, the absorbance was measured at 562 nm.

**Measurement of Intracellular Oxidative Stress Levels**

Total ROS production was detected using the fluorescence probe DCFH-DA (S0033S, Beyotime, Shanghai, China) according to the manufacturer’s instructions. DCFH-DA was diluted in serum-free medium at 1: 1000 to a final concentration of 10 mM and incubated in the cells at 37°C for 20 min. Then, the absorbance was measured at 488 and 525 nm. A commercially available malondialdehyde (MDA) assay kit (Beyotime Institute of Biotechnology) was used to measure lipid peroxidation parameters by detecting MDA content, following the instructions for when cells undergo oxidative stress. The γ-glutamyl-cysteinyl-glycine (GSH) level was measured according to the manufacturer’s instructions (Beyotime, China).

**Immunofluorescence**

The HUVECs were fixed with 4% paraformaldehyde for 15 min and blocked with 5% bovine serum albumin for 30 min. Then, the cells were incubated overnight at 4°C with anti-GPX4 antibody (1: 200; Abcam; Cambridge, MA, USA), followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody (Amresco) for 1 h at room temperature in the dark. The nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI) for 1 h. The blots were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1: 1000) and anti-β-actin (1: 1000), and with the following primary antibodies from Abcam: anti-GPX4 (1: 5000), anti-SLC7A11 (1: 1000), anti-ACSL4 (1: 10000), anti-HO-1 (1: 2000), anti-KLF4 (1: 1000), and anti-ACSL4 (1: 1000). Then, the blots were incubated at 4°C overnight with the following primary antibodies from Cell Signaling Technology: anti-p-AMPK (1: 1000), anti-AMPK (1: 1000), anti-SOD-1 (1: 1000), and anti-AMPK (1: 1000). Then, the blots were incubated with HRP-conjugated secondary antibodies (1: 5000) for 1 h at room temperature. Subsequently, blots were developed using enhanced chemiluminescence (Model 6600; Tanon, Shanghai, China).

**Statistical Analysis**

The data were analyzed using GraphPad Prism version 7.0 (GraphPad Software Inc. San Diego, CA, USA). Differences between 2 groups were analyzed using the t test, whereas differences between multiple groups were analyzed using one-way analysis of variance, followed by Tukey’s test. All experiments were performed at least thrice. All data are presented as mean±SEM. A P value of 0.05 or less was statistically significant.

**Results**

**Ox-LDL Induced HUVECs Ferroptosis in a Dose-Dependent Manner**

We used ox-LDL (0-200 µg/mL) for 24 or 48 h to induce endothelial cell ferroptosis and to explore the effect of ox-LDL on HUVECs. The CCK8 results showed that treatment with ox-LDL in a dose- and time-dependent manner markedly reduced the cell viability in HUVECs (Figure 1A, 1B). Hoechst/PI staining was
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used to evaluate the integrity of the cellular membrane. The results indicated that the number of PI-positive cells was higher in the ox-LDL group (100 µg/mL) for 48 h than in the control group (Figure 1C). HUVEC mitochondria of the ox-LDL-induced group (100 µg/mL) for 48 h showed morphological characteristics of ferroptosis, such as shrinkage, membrane density increasing, and mitochondrial ridges decreasing or disappearing (Figure 1D). These results indicated that the cellular viability of HUVECs was significantly decreased following treatment with 100 µg/mL ox-LDL at 48 h. Our results revealed that the ferroptosis protein ACSL4 expression level was increased after ox-LDL stimulation, compared with the control (Figure 1E, 1F). Meanwhile, GPX4 and SLC7A11 expressions were reduced in the ox-LDL group (Figure 1G, 1H). These results suggested that ox-LDL induced HUVECs ferroptosis.

CTRP13 Suppressed Ox-LDL-Induced HUVEC Ferroptosis

We treated HUVECs with different CTRP13 concentrations for 48 h to further confirm whether CTRP13 was involved in ferroptosis responses. CTRP13 had no significant toxic effect on HUVECs at concentrations below 1200 ng/mL, compared with the control group (Figure 2A). HUVECs had approximately 45% viability at 1200 ng/mL after being cultured with 100 µg/mL ox-LDL (Figure 2B). Viability at 150, 300, and 450 ng/mL CTRP13 was consistent with a previous report [26]. Then, we treated HUVECs with 100 µg/mL ox-LDL at indicated time points. The results revealed that 450 ng/mL CTRP13 unregulated GPX4/SLC7A11 protein and mRNA levels, compared with an ox-LDL group (Figure 2C-F, 2H, 2I). Conversely, CTRP13 treatment of 450 ng/mL significantly blocked ACSL4 signal activity.
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in the ox-LDL group (Figure 2G). The results indicated that 450 ng/mL CTRP13 significantly inhibited HUVEC ferroptosis.

**CTRP13 Inhibited Ox-LDL-Induced HUVEC Lipid Peroxidation and Mitochondrial Dysfunction**

We detected LDH release in the supernatant to explore whether CTRP13 pre-treatment could ameliorate cellular membrane integrity. The results indicated that ox-LDL significantly increased LDH release, and CTRP13 attenuated this effect (Figure 3A). Moreover, CTRP13 treatment inhibited MDA and GSH (Figure 3B, 3C). The iron content was lower in the CTRP13 treatment group (Figure 3D) than in the ox-LDL group. ROS-mediated lipid peroxidation was the key step that induced ferroptosis. The CTRP13 and Fer-1 group significantly decreased ROS levels, compared with the ox-LDL group (Figure 3E, 3F). Also, we detected a dramatic improvement in mitochondrial membrane potential. The mito-tracker results reduced many mitochondrial membranes and fissions, compared with the control, while CTRP13 and Fer-1 alleviated this effect (Figure 3G, 3H).

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Figure 3. C1q/tumor necrosis factor-related protein 13 (CTRP13) inhibited oxidized low-density lipoprotein (ox-LDL)-induced human umbilical vein endothelial cell (HUVEC) lipid peroxidation and mitochondrial dysfunction. (A) Lactate dehydrogenase (LDH) level in cell supernatant was evaluated using a cytotoxicity detection LDH kit. (B, C) Total γ-glutamyl-cysteinyl-glycine (GSH) and malondialdehyde (MDA) indicators were measured using assay kits in endothelial cells. (D) The iron levels in endothelial cells were measured using commercial kits. (E) Representative fluorescent images present reactive oxygen species (ROS) levels. (F) Quantification of intracellular ROS levels. (G) Mito-tracker for labeling mitochondria in endothelial cells. (H) Quantification of fragmented mitochondria levels. A representative image from 3 separate experiments was illustrated. The images were captured at ×100 magnification. * P<0.05 vs the control group. # P<0.05 vs the ox-LDL group. Fluorescent images were evaluated using Olympus fluorescence microscopy with cellSens Dimension software (Version 1.3 rev, Olympus, Tokyo, Japan), and the positive cells were measured by Image Pro Plus 6.0 (Media Cybermetics, Rockville, MD, USA). GraphPad Prism 9.0 software (La Jolla, CA, USA) was used to analyze the data.
CTRP13 Reversed the Downregulation of Multifarious Antioxidant Proteins

CTRP13 accelerated antioxidant enzyme expression levels, such as HO-1, SOD-1, and SOD-2, compared with the ox-LDL group (Figure 4A-4E). Moreover, JC-1 staining revealed that adding CTRP13 restored mitochondrial membrane potential (Figure 4F, 4G). The immunofluorescence images indicated an extensive increase of GPX4 mean intensity in aggregates with CTRP13 and Fer-1, compared with the ox-LDL group (Figure 4H, 4I).

Ferroptosis Protective Effect of CTRP13 was Attenuated by Inhibiting AMPK/KLF4 Pathway

We examined the AMPK phosphorylation levels and KLF4 expression to elucidate the molecular ferroptosis mechanism induced by CTRP13. The p-AMPK/AMPK expression ratio decreased, and KLF4 expression increased in the HUVECs treated with ox-LDL (Figure 5A-5C). However, these changes were more pronounced in the HUVECs treated with ox-LDL or compound C (CC, 5 μmol/L), a potent and selective AMPK inhibitor. Ferroptosis biomarker proteins GPX4 and SLC7A11 expressions were decreased in the compound C group. In contrast, compound C groups had significantly increased ACSL4 and KLF4 levels, compared with the ox-LDL group. APTO (5 μmol/L), a KLF4 activator, did not affect AMPK phosphorylation, while APTO attenuated the anti-ferroptosis effect of CTRP13 (Figure 5D-5F).

CTRP13 Restored Mitochondrial Functioning by Counteracting Against ox-LDL Effects on the AMPK/KLF4 Pathway

Next, we examined whether the AMPK/KLF4 pathway was responsible for the mitochondrial functioning of CTRP13 by inhibiting AMPK, the upstream activator of KLF4. DCFH and JC-1 staining indicated that compound C and APTO increased ROS and mitochondrial dysfunction (Figure 6A-6D). Expectedly, immunofluorescence staining revealed compound C and APTO diminished GPX4 expression (Figure 6E, 6F).
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They discovered the elevation of intracellular iron levels, lipid peroxidation, and cell death in a dose- and time-dependent manner [28]. Therefore, it is important to discover an anti-ferroptosis drug to treat endothelial dysfunction diseases. This study revealed, for the first time, that CTRP13 prevented endothelial cell ferroptosis effectively. CTRP13 could significantly reduce LDH and MDA levels, whereas CTRP13 increased GSH content in ox-LDL-induced HUVECs. Additionally, iron assay results suggested that CTRP13 could decrease the iron content levels in ox-LDL-induced HUVECs. However, in the present experiment, we used only ox-LDL to model changes in HUVECs experiment, we used only ox-LDL to model changes in HUVECs. We will focus on the protective effect and mechanism of CTRP13 against atherosclerosis caused by other factors in the future.

**Discussion**

This study provided promising evidence that CTRP13 suppressed endothelial dysfunction by inhibiting HUVEC ferroptosis via improving mitochondrial function and ROS levels, which provides a new insight into the mechanisms of CTRP13 in alleviating atherosclerosis. In ox-LDL-treated HUVECs to induce endothelial cell ferroptosis, CTRP13 reduced lipid peroxidation and mitochondrial dysfunction via regulating the AMPK/KLF4 pathway.

Endothelial cells have been demonstrated to play important roles in regulating blood vessel tone, thrombogenicity, homeostasis, monocyte recruitment, and hormone transport, and dysfunctions of endothelial cells have been related to the development of cardiovascular diseases [26]. Ferroptosis has been proposed as a feasible trial that regulates endothelial cell death and dysfunction. For example, inhibition of ferroptosis could suppress ox-LDL-induced lipid peroxidation and endothelial dysfunction in mouse aortic endothelial cells in vitro [27]. Moreover, Qin et al discovered that zinc oxide nanoparticles could cause ferroptosis in HUVECs and EA.hy926 cells. They discovered the elevation of intracellular iron levels, lipid peroxidation, and cell death in a dose- and time-dependent manner [28]. Therefore, it is important to discover an anti-ferroptosis drug to treat endothelial dysfunction diseases. This study revealed, for the first time, that CTRP13 prevented endothelial cell ferroptosis effectively. CTRP13 could significantly reduce LDH and MDA levels, whereas CTRP13 increased GSH content in ox-LDL-induced HUVECs. Additionally, iron assay results suggested that CTRP13 could decrease the iron content levels in ox-LDL-induced HUVECs. However, in the present experiment, we used only ox-LDL to model changes in HUVECs in atherosclerotic plaques, which did not reflect other factors that cause atherosclerosis, such as smoking and diabetes. This is one of the limitations of this study. We will focus on the protective effect and mechanism of CTRP13 against atherosclerosis caused by other factors in the future.

**Figures**

**Figure 4.** C1q/tumor necrosis factor-related protein 13 (CTRP13) reversed the downregulation of multifarious antioxidant proteins. (A-C) RT-PCR of the heme oxygenase 1 (HO-1), superoxide dismutase 1 (SOD-1), and superoxide dismutase 2 (SOD-2) mRNA levels. (D) Protein expression levels of antioxidant enzymes were measured using Western blot analysis. (E) Quantitative analysis of antioxidant-associated protein expression. (F) Representative fluorescent images for testing the mitochondrial membrane potential in HUVECs, aggregates shown in green, and monomers shown in red (scale bars=100 μm). (G) Quantitative analysis of the aggregates to monomers ratio. (H, I) Intracellular glutathione peroxidase 4 (GPX4) levels were illustrated by representative immunofluorescence images. The data were expressed as the mean±SD (n=3–4 per group). Statistical power=0.982987, effect size(r)=0.918048. * P<0.05 vs the control group. # P<0.05 vs the ox-LDL group. Fluorescent images were evaluated using Olympus fluorescence microscopy with cellSens Dimension software (Version 1.3 rev, Olympus, Tokyo, Japan), and the positive cells were measured by Image Pro Plus 6.0 (Media Cybernetics, Rockville, MD, USA). The relative protein levels were quantified by Image J software (NIH, Bethesda, MD, USA). GraphPad Prism 9.0 software (La Jolla, USA) was used to analyze the data.

Hence, these results suggested that CTRP13 most likely protected ox-LDL-induced HUVEC ferroptosis by activating the AMPK/KLF4 pathway and upregulating various antioxidant enzymes (**Figure 7**).

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Figure 5. The ferroptosis protective effect of C1q/tumor necrosis factor-related protein 13 (CTRP13) was attenuated by inhibiting the AMPK/KLF4 pathway. (A) Protein expression levels were measured using Western blotting. The following groups were assessed: (1) Control; (2) oxidized low-density lipoprotein (ox-LDL); (3) ox-LDL+CTRP13; and (4) ox-LDL+Fer-1. (B, C) Quantitative analysis of phosphorylated AMP-activated kinase (p-AMPK), AMP-activated kinase (AMPK), and Krüppel-like factor 4 (KLF4). (D-F) The cells were treated with a combination of CTRP13 (450 ng/mL) and ox-LDL (100 µg/mL) for 24 h and subsequently incubated with compound C (5 μmol/L) or inducer of Krüppel-like factor 4 (APTO, 5 μmol/L) for another 24 h. Western blotting results (left panel) and quantitative data (right panel) for ferroptosis-associated protein and p-AMPK, AMPK, and KLF4 are presented. The data are expressed as the mean±SD (n=3-4 per group). Statistical power=0.933291, effect size(ρ)=0.847233. * P<0.05 vs the control group. # P<0.05 vs the ox-LDL group. The relative protein levels were quantified by Image J software (NIH, Bethesda, MD, USA). GraphPad Prism 9.0 software (La Jolla, USA) was used to analyze the data.
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B

![Bar graph showing mean fluorescence intensity (DCFH) for Control, CTRP13, Compound-C, and ATOO.](image)

C

![Images showing fluorescent microscopy results for ox-LDL, ox-LDL+APTO, ox-LDL+Compound-C, Control, and ox-LDL+CTRP13.](image)
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D

![Graph showing the ratio of Jc-1 (aggregate/monomer) for different treatments: Control, CTRP13, ComC, and APTO.]

E

Indexed images showing fluorescence microscopy of cells treated with ox-LDL, CTRP13, and Compound-C.

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that CTRP13 supplementation reduces macrophage foam cell formation and inflammation reactions, thus reducing atherosclerotic plaque development. BH4 availability impacts redox balance in GCH1 overexpressing cells sensitivity to ferroptosis [31]. Thus, we explored the effects of CTRP13 in endothelial cell ferroptosis in the present study.

Oxidative stress triggers and mediates ferroptosis, an iron-dependent form of cell death [6]. Oxidative damage can arise when there is an imbalance between the generation of free radicals and the body’s ability to neutralize or eliminate their harmful effects via antioxidants. ROS-mediated lipid peroxidation is the key step that drives ferroptosis. Mitochondria play a key role in regulating cell energy and cell death signal transduction. In

Figure 6. C1q/tumor necrosis factor-related protein 13 (CTRP13) restored mitochondrial functioning by counteracting oxidized low-density lipoprotein (ox-LDL) effects on the AMPK/KLF4 pathway. The following groups were assessed: (1) Control; (2) ox-LDL; (3) ox-LDL + CTRP13; (4) ox-LDL + CTRP13 + compound C; and (5) ox-LDL + CTRP13 + APTO. (A) Representative fluorescent images of DCFH-DA probe and (B) quantification of intracellular reactive oxygen species (ROS) levels in 5 cell groups. (C) Representative fluorescent JC-1 images for testing the mitochondrial membrane potential in human umbilical vein endothelial cells (HUVECs), aggregates shown in green, and monomers shown in red (scale bars=100 μm). (D) Quantitative analysis of the aggregates to monomers ratio. (E, F) Intracellular glutathione peroxidase 4 (GPX4) levels were depicted by representative immunofluorescence images. The data are expressed as the mean±SD (n=3-4 per group). Statistical power=0.972987, effect size(r)=0.914731. * P<0.05 vs the control group. * P<0.05 vs the ox-LDL group. Fluorescent images were evaluated using Olympus fluorescence microscope with cellSens Dimension software (Version 1.3 rev, Olympus, Tokyo, Japan), and the positive cells were measured by Image Pro Plus 6.0 (Media Cybernetics, Rockville, MD, USA). GraphPad Prism 9.0 software (La Jolla, USA) was used to analyze the data.

Figure 7. A schematic of the proposed mechanism by which C1q/tumor necrosis factor-related protein 13 (CTRP13) inhibited oxidized low-density lipoprotein (ox-LDL)-induced ferroptosis. Signal I: ox-LDL depresses the amino acid antiporter solute carrier family 7 member 11 (SLC7A11/ xCT/system xc-), resulting in oxidative damage-induced ferroptosis, while this effect was suppressed by CTRP13 pretreatment. Signal II: ox-LDL activates the Krüppel-like factor 4 (KLF4) pathway and attenuates mitochondrial functioning due to increased production of reactive oxygen species (ROS), while ultimately, CTRP13 treatment inhibits this effect.
addition to producing adenosine triphosphate, mitochondria are the primary source of ROS production. Mitochondrial ROS production primarily occurs during oxidative phosphorylation in the electron transport chain located on the mitochondrial inner membrane [6,32]. Inhibiting ferroptosis can protect against ox-LDL-induced endothelial dysfunction and improve postmenopausal atherosclerosis progression [11]. We found that CTRP13 attenuated ROS overproduction and reversed the decrease of antioxidant molecules, such as SOD and GSH. Also, the fragmented mitochondria increased significantly in an ox-LDL group compared with in the control group, while the percentage of fragmented mitochondria decreased after CTRP13 or Fer-1 treatment. Our data showed that CTRP13 could significantly reduce oxidative damage in HUVECs. Intracellular ROS level regulates endogenous antioxidants, such as HO-1, SOD1, and SOD2.

In our study, CTRP13 significantly increased HO-1 and SOD1, with a slight increase in SOD2. These results suggest that CTRP13 can stimulate the HO-1/SOD anti-oxidative pathway. The JC-1 stain revealed a significant decrease in the red/green staining ratio in ox-LDL-stimulated HUVECs, indicating that ox-LDL treatment disrupts the mitochondrial membrane potential, which CTRP13 or Fer-1 treatment prevents. The reduced ROS and the elevated GPX4 levels, an antioxidant defense enzyme active in repairing oxidative damage to lipids, suggest ferroptosis inactivation after treatment with CTRP13 or Fer-1.

Emerging evidence suggests that the 2 major regulators of the antioxidant response, AMPK and KLF4, are related to the progression of endothelial cell ferroptosis. After being stimulated with ox-LDL, AMPK and p-AMPK expressions were diminished in HUVECs, while CTRP13 pretreatment increased AMPK phosphorylation. AMPK was regarded as a key regulator of cellular energy metabolism. AMPK is also key in regulating mitochondrial homeostasis and biological energy metabolism [33]. Evidence shows that AMPK can be activated in response to ROS signaling under metabolic stress, including mitochondrial dysfunction and oxidative stress. A recent study has demonstrated that salidroside ameliorates acetonophen-induced acute liver injury through the inhibition of endoplasmic reticulum stress-mediated ferroptosis by activating the AMPK/SIRT1 pathway [34]. Another study provided energy-stress-mediated AMPK activation-inhibited ferroptosis through regulating acetyl-CoA carboxylase phosphorylation mechanisms [35]. Song et al discovered that AMPK-mediated Beclin 1 phosphorylation promotes ferroptosis by inhibiting SLC7A11-mediated cystine transport [36]. Our present study linking AMPK to ferroptosis also proposes an interesting question: whether CTRP13 regulation of ferroptosis plays an important role in endothelium dysfunction. It revealed that CTRP13 treatment increased p-AMPK/AMPK expression while decreasing KLF4 expression in ox-LDL-induced HUVECs. However, the Fer-1 treatment reversed these effects. Compound C is a potent and selective AMPK inhibitor, and p-AMPK, GPX4, and SLC7A11 expressions significantly decreased after co-culturing with compound C. KLF4, an important transcription factor in eukaryotes, participates in the regulation of cell proliferation, differentiation, and embryo development [18]. A recent study demonstrated that KLF4-mediated xCT upregulation serves as negative feedback during ferroptosis progression [19]. APTO, a KLF4 activator, did not affect AMPK phosphorylation but attenuated the anti-ferroptosis effect of CTRP13. Compound C blocked ox-LDL-induced ROS generation, and CTRP13 inhibited mitochondria membrane potential by JC-1 staining. Similarly, GPX4 immunofluorescence was attenuated after co-treatment of ox-LDL with compound C. Our results revealed that CTRP13 upregulated the AMPK/KLF4 signaling pathway, thereby protecting endothelial dysfunction from ferroptosis. However, further studies are required to evaluate whether CTRP13 could inhibit the ferroptosis of endothelial cells, thereby reducing vulnerable plaques and cardiovascular adverse events by activating the AMPK/KLF4 pathway in vivo.

Study Limitations

This study had potential limitations. First, using HUVECs instead of human aortic endothelial cells may be less reflective of actual aortic endothelial cell activity. Second, in vitro and in vivo experiments with endothelial cells are required for future studies to confirm the vascular protective effect of CTRP13 on ox-LDL-induced ferroptosis.

Conclusions

Our study showed that CTRP13 inhibited HUVEC ferroptosis induced by ox-LDL. CTRP13 depressed ROS production and decreased cell ferroptosis. We elucidated the molecular basis of the AMPK/KLF4-mediated induction of CTRP13 expression that inhibits ferroptosis. From a therapeutic perspective, these results provide a preliminary understanding of the pharmacological action of CTRP13 and can have important translational value in the treatment of endothelial dysfunction-accelerated atherosclerosis.

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