Title: Su2bstitution of the SERCA2 Cys^{674} reactive thiol accelerates atherosclerosis by inducing endoplasmic reticulum stress and inflammation

Running title: Substitution of SERCA2 thiol accelerates atherosclerosis

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Author contributions

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Conflict of interest

We declare that we do not have any commercial or associative interest that represent a conflict of interest in connection with the work submitted.

Declaration of transparency and scientific rigor

This declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigor of preclinical research as stated in the *British journal of pharmacology* guide-

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Abstract

BACKGROUND AND PURPOSE

The cysteine^{674} (C674) thiol of Sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase 2 (SERCA2) is easily and irreversibly oxidized under atherosclerotic conditions. However, contribution of the C674 thiol redox status in the development of atherosclerosis remains unclear. Our goal was to elucidate the possible mechanism involved.

EXPERIMENTAL APPROACH

Heterozygous SERCA2 C674S knock-in (SKI) mice in which half of the C674 was substituted by serine^{674} were used to mimic removal of the reactive C674 thiol which occurs under pathological conditions. The whole aorta and aortic root were isolated for histological analysis. Bone marrow derived macrophages (BMDMs) and a cardiac endothelial cell line were used for intracellular Ca^{2+}, macrophage adhesion and protein expression analysis.

KEY RESULTS

SKI mice developed more severe atherosclerotic plaque and macrophage accumulation. Cell culture studies suggest the partial substitution of SERCA2 C674 increased intracellular calcium levels and ER stress in both BMDMs and ECs. The release of pro-inflammatory factors and macrophage adhesion increased in SKI BMDMs. In normal ECs, the overexpression of C674S mutant induced endothelial inflammation and promoted macrophage recruitment. Additionally, 4-phenyl butyric acid (4-PBA), an ER stress inhibitor, prevented the increased atherosclerosis observed in SKI mice, and alleviated ER stress and inflammatory responses in BMDMs and ECs exposed to 4-PBA.

CONCLUSIONS AND IMPLICATIONS

The substitution of SERCA2 C674 thiol accelerates the development of atherosclerosis by inducing ER stress and inflammation. Our findings highlight the importance of SERCA2 C674 redox status in the context of atherosclerosis, and open up a novel therapeutic strategy to combat atherosclerosis.

KEYWORDS

SERCA2, atherosclerosis, macrophages, endoplasmic reticulum stress, inflammation

Summary

'What is already known'

Macrophages and ECs are the primary cellular participants in the initiation and progression of atherosclerosis.

Both aortic vascular smooth muscle cells and renal proximal tubule cells isolated from SKI mice show upregulated abundance of ER stress markers compared with WT mice.

SERCA2 C674 controls angiogenic response to ischemia / hypoxia through coordinated macrophage and ECs function by regulating ER oxidoreductin-1α.

'What this study adds'

The redox state of SERCA2 C674 is an essential regulator of the development of atherosclerosis.

The role of SERCA2 C674 in the inflammatory response in macrophages and ECs is mediated by ER stress and participates in atherosclerosis.

'Clinical significance'

The reactive thiol of SERCA2 C674 plays a vital role in both macrophages and ECs, the irreversible oxidation of which triggers ER stress and inflammatory response to accelerate atherosclerosis.

Inhibiting ER stress can alleviate atherosclerosis by decreasing the inflammatory response.

Abbreviations

SERCA2, sarcoplasmic / endoplasmic reticulum calcium ATPase 2; C674, Cys^{674}; S674, Ser^{674}; ER, endoplasmic reticulum; SR, sarcoplasmic reticulum; 4-PBA, 4-phenyl butyric acid; ECs, endothelial cells; SKI, SERCA2 C674S knock-in mice; WT, wild type.

Introduction

Atherosclerotic plaque formation results from a passive lipid-driven inflammation of the large and mid-sized arteries orchestrated by a complex interaction of various cell types, including endothelial cells (ECs), smooth muscle cells (SMCs) and macrophages (Zhang *et al*., 2014; Chappell *et al*., 2016; Luchetti *et al*., 2017). Specific signal and communication between ECs and macrophages are essential for cardiovascular homeostasis and atherogenesis (Roth Flach *et al*., 2015b; Zhang *et al*., 2020). Diverse environmental stimuli, such as oxidative stress, hypoxia and injury, cause endothelial dysfunction and promote the accumulation of plasma lipoproteins at sites of disturbed blood flow (Daiber *et al*., 2017). The oxidized lipids and ox-LDL trigger an atherogenic inflammatory cascade by inducing the expression of adhesion molecules and chemokines by ECs, which then drives leukocyte infiltration (Schnitzler *et al*., 2020). The leukocytes including neutrophils, monocytes and T cells, then develop into macrophages through lipid uptake and release of various pro-inflammatory factors that augment macrophage activation and intimal recruitment as well as cause extensive endothelial inflammation (Ruparelia *et al*., 2017). Despite numerous studies that have set out to address the individual contributions of each cell type in atherogenesis, the inflammatory interplay between macrophages and ECs appears to play the most important role but is poorly understood.

Intracellular calcium (Ca^{2+}) homeostasis is pivotal in maintaining the normal physiological processes of cells, and previous research identified disrupted Ca^{2+} regulation is associated with metabolic disorders, diabetes, atherosclerosis and ageing (Sturek, 2011; Zhu *et al*., 2019; Jiang *et al*., 2020; Olgar *et al*., 2020). Sarco / endoplasmic reticulum calcium ATPase (SERCA) is the only key player that actively transports cytoplasmic Ca^{2+} into the sarcoplasmic or endoplasmic reticulum (ER) and exerts a pivotal role in maintaining the high Ca^{2+} concentration of ER (Wuytack *et al*., 2002). SERCA2 is the main subtype of SERCA in the cardiovascular system, and the thiol of cysteine^{674} (C674) has been identified as the main redox-sensitive site. This thiol can be S-glutathiolated (GSH-) by reactive oxygen and nitrogen species (ROS/RNS) and regulates SERCA2 activity (Adachi *et al*., 2004a). However, SERCA2 C674 is also easily attacked by chronic augmentation of ROS / RNS, and the irreversible oxidation of C674 is closely associated with various cardiovascular diseases. We previously demonstrated the oxidative inactivation of

the SERCA2 C674 thiol in human patients and mouse aortic aneurysms (Q*ue et a*l., 2020). Because serine lacks only the thiol of the cysteine structure, a heterozygote SERCA2 C674S knock-in (SKI) mouse was used to partially remove the reactive thiol C674 under atherosclerosis-prone conditions. We found impaired Ca^{2+} handling in ECs and SMCs from SKI mice (M*ei et a*l., 2014a; Q*ue et a*l., 2020), indicating the importance of SERCA2 C674 for maintaining calcium homeostasis. Importantly, substitution of SERCA2 C674 leads to ER stress both in macrophages and ECs by regulating ER oxidoreductin-1α (ERO1) induction under hypoxic conditions, and the interaction of SERCA 2 C674 and ERO1 promotes macrophage adhesion in ECs, increases the secretion of vascular endothelial growth factor in macrophages during hypoxia, and in turn promotes angiogenesis (M*ei et a*l., 2014a). However, despite the significant irreversible oxidation of C674 observed in human and rabbit atherosclerotic aorta (Adac*hi et a*l., 2002; Adac*hi et a*l., 2004b), the role of SERCA2 C674 redox status on atherosclerosis development has not been characterized.

The objective of this study was to investigate the molecular mechanism of the redox-reactive SERCA2 C674 thiol on atherosclerosis by determining the effect of SERCA2 C674 substitution on ER stress and inflammatory response, particularly with a focus on the interaction between macrophages and ECs. Moreover, we used heterozygous SKI mice for an *in vivo* investigation of the therapeutic potential of preventing SERCA2 C674 substitution in the context of atherosclerosis.

Methods

2.1 Animals Experiments

All animal care and study protocols were complied with the guidelines of the ethical use of animals and approved by the Animal Care and Use Committee (Third Military Medical University, Chongqing, China). All efforts were made to minimize animal suffering and to reduce the number of animals used. All experiments involving animals or animal tissues were reported in compliance with the guidelines of ARRIVE and British Journal of Pharmacology (Kilken*ny et a*l., 2010; Curt*is et a*l., 2015a). Experimental protocols and design adhere to British Journal of Pharmacology guidelines (Curtis et al., 2015a).

Mice used in this study were C57BL/6J background (RRID: IMSR_JAX:000664, https://www.-

jax.org/strain/000664) and originally obtained from Jackson Laboratory (Bar Harbor, USA). SERCA2 C674S knock-in mice (RRID:MGI:5780876) were generated as previously described (Thompson et al., 2014). For atherosclerosis studies, SKI mice were backcrossed into C57BL/6J low-density lipoprotein receptor-deficient (LDLR $^{-/-}$, the Jackson Laboratory, RRID: IMSR_JAX:002207, https://www.jax.org/strain/002207) mice to obtain SKI LDLR $^{-/-}$ mice, and the littermate WT LDLR $^{-/-}$ mice served as controls. Starting at 8-12 weeks of age, mice (25-30 g) were fed with a Western diet (16.4% milk fat, 2% cholesterol, Beijing Keao Xieli Feed Corporation, China) for 8 weeks to induce atherosclerosis in WT LDLR$^{-/-}$ and SKI LDLR $^{-/-}$ mice (12 mice per group) (Engin et al., 2010). In another set of experiments, SKI LDLR $^{-/-}$ mice were fed on a Western diet and administrated with the ER stress inhibitor 4-phenyl butyric acid (4-PBA) (Sigma-Aldrich, Cat# P21005) 1g/kg/day in drinking water for 8 weeks and mice fed with double distilled water served as control (n=12 per group). Mice were then anesthetized with 2,2,2-tribromoethanol (250 mg/kg, Sigma-Aldrich, Cat# T48402) and killed to obtain tissues. Mice were kept in open polypropylene cages with clean chip bedding. The animal room was maintained at a 12 h cycle of light and dark, with controlled temperature of 22 ± 3 °C and relative humidity of 55-60%. Four to five mice in each cage were free to drink water and fed with regular diet unless otherwise indicated.

2.2 Assessment of cholesterol and triglycerides

Blood was harvested from the ventricles of mice, supplemented with 10% heparin, and centrifuged for 10 min at 2000g. Plasma was then isolated carefully from the supernatant, and stored at -80°C until used. Infinity Cholesterol and Triglycerides measurement kits (Thermo Scientific, USA, Cat# TR13421, Cat# TR22421) were used for the detection of cholesterol and triglycerides, and the absorbance of each sample was normalized to the values of a standard solution.

2.3 Assessment of glucose tolerance test

Glucose tolerance and insulin tolerance tests were performed according to the literature (Vin*ué et a*l., 2015). Briefly, Fasting blood glucose test (FBG): mice were fasted overnight, 3-5 μL blood was then collected from the end of mouse tail, and the blood glucose was obtained with a blood glucose meter (Sinocare, Changsha, China). Glucose solution (20 mg/kg) was then supplied, and the blood glucose was tested at 30, 60, 90, 120 minutes' point with a blood glucose meter for glu-

cose tolerance test (GTT).

2.4 Analysis of aorta and aortic root atherosclerotic lesions

The whole aorta was dissected, cut opened longitudinally and stained with oil red O (Sigma-Aldrich, Cat# O1516) for morphometric lesion analysis. Briefly, the whole aorta was dissected and fixed in 4% paraformaldehyde for 48 h and 30% sucrose for 24 h then rinsed with distilled water, treated with 85% propylene glycol for 2 min and 0.5% oil red O for 30 min, followed by rinsing with 85% propylene glycol and distilled water. Pictures were taken using a microscope (Olympus BX43 or SZX16, Tokyo, Japan) and percentage of the positive stained lesional area was measured following CellSens standard software (RRID:SCR 014551, Olympus) instructions.

2.5 Histology and immunohistochemistry

Tissue samples were dissected and fixed in 4% paraformaldehyde for 48 h and 30% sucrose for 24 h, followed by embedding in OCT compound for the preparation of serial frozen sections with 7 μm. The sections were stained with hematoxylin/eosin (H&E) or oil red O, which were then co-stained with hematoxylin (Solarbio, Cat# H8070) for 2 min at room temperature. Images were acquired with a fluorescence microscope (Olympus BX43, Tokyo, Japan). The ratio of the oil red O positive staining area and necrotic core area to the lesion area were quantified with CellSens standard software (RRID: SCR 014551, Olympus).

The antibody-based procedures used in this study complied with the recommendations made by *British Journal of Pharmacology* (Alexander *et al*., 2018). For the immunohistochemistry of the aortic root, sections were heated for antigen retrieval in 10 mM citrate acid buffer before being blocked for 60 minutes with 5% BSA containing 0.1% Tween-20. The sections were then incubated with the primary antibodies against CD68 (Proteintech, Cat# 28058-1-AP, RRID: AB_10695760) overnight at 4 °C in a wet box according to the manufacture's instructions with HRP-DAB kit (CWBIO, Cat# CW0125M). Non-immune IgG was used as a negative control. The positive stained area (Kilkenny *et al*.) was scored by five individuals blinded to the experimental group using a scale of 0 to 4, where 0 equals negative intensity, 1 is weak intensity, 2-3 stands for medium intensity, and 4 is strong intensity. Because the aorta of the $LDL^{-/-}$ background mice were stained with oil red O and were not suitable for immunofluorescence, immunofluorescence was performed on frozen sections of the aorta of C57BL/6J background mice stained

against intercellular cell adhesion molecule 1 (ICAM1) and VCAM1, Sections were pretreated for antigen retrieval and incubated with the primary antibodies against VCAM-1 (Proteintech, Cat# 11444-1-AP, RRID: AB_2214232) and ICAM-1 (Engene, Cat# E301187, RRID: AB_2861238) overnight at 4 °C in a wet box, followed by the incubation with secondary antibody Alexa Fluor 488 goat anti- rabbit IgG (H + L) (Jackson ImmunoResearch, Cat# 115-545-144) for 120 min at room temperature. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, Solarbio, Cat# C0065) for 10 min at room temperature. The fluorescence images were captured by a microscope (Leica DM6, Germany) and analyzed using the Las X software (Leica, Germany).

2.6 Bone marrow-derived macrophage (BMDM) isolation and culture

Isolation and culture of bone marrow-derived macrophages (BMDM) was previously reported (Pineda-Tor*ra et a*l., 2015) and were cultured in high glucose DMEM supplemented with 10% FBS (ExCell Bio, Cat# FSP500), 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. BMDM phenotype was confirmed by CD68 immunostaining. Because the macrophage LDL receptor plays an important role in cell lipid metabolism(Overt*on et a*l., 2007), all macrophages used were isolated from C57BL/6J background mice. Briefly, 8-12 week old mice were euthanized, and the bone marrow (BM) cells were harvested via flushing the femurs and tibiae with high glucose DMEM medium (Hyclone, Cat# SH30023.01). Red cells were then lysed with red blood cell lysis buffer (Solarbio, Cat# R1010) and the freshly isolated BM cells were differentiated in high glucose DMEM containing 15% FBS (ExCell Bio, Cat# FSP500) and 20% L929-conditioned media (Assouv*ie et a*l., 2018). Fresh medium was supplied every 3 days, and after 6-7 days of differentiation, cells were harvested for further use. In some experiments, the cells were exposed to 5 mM 4-PBA for 48 h before macrophage adhesion assay or being collected for protein expression assessment.

2.7 Adenoviral infection of endothelial cells

To further investigate the effect of SERCA2 C674 in ECs during atherosclerosis, primary aortic-derived ECs were isolated from the hearts of 4-10 week old LDLR $^{-/-}$ mice (Thompson et al., 2014), and transfected with adenoviral human SERCA2 S674, while the empty adenoviral vector was used as a negative control. Cells were infected with the adenovirus at 50 MOI/cell in FBS

free low glucose DMEM in the absence of antibiotics for 6 h. Then, fresh media containing 2% FBS was supplied and the cells were cultured for another 2 days. In some experiments, the cells were exposed to 1 mM 4-PBA for 48 h before macrophage adhesion assay or protein expression assessment.

2.8 Macrophage adhesion assay

ECs were cultured in 12-well plates at a density of 10^6 cells/well in low glucose DMEM containing 0.2% FBS then infected with adenovirus SERCA2 S674 or empty adenovirus and cultured overnight until confluent. Macrophages (5×10^5 cells/well) were added to ECs and incubated for 1 h, then the media was removed and rinsed 3 times with PBS to remove the unbound macrophages. In some experiments, ECs infected with adenovirus SERCA2 S674 were pre-treated with 4-PBA (1mM) for 48 h before macrophage adhesion assay, while double distilled water acted as solvent control. At least five images of macrophages adherent to ECs in each well were obtained with an inverted microscope (Olympus, STX43, Tokyo, Japan), and the number of bound macrophages was counted per image area.

2.9 Intracellular calcium measurements.

Cells were seeded on glass cover slides at a density of 10^4 cells/well and cultured overnight in a 24-well plate. Intracellular calcium was quantitated by Fluo-4 AM (Solarbio, Cat# F8500) staining according to the manufacturer's instructions. Briefly, the cells were incubated with 4 μM Fluo-4 AM in HBSS (Hyclone, Cat# SH30030.02) containing 0.02% pluronic F12 in the dark at 37°C for 20 min. Five volumes of HBSS containing 1% FBS was then added and maintained at 37°C for another 40 min. Cells were washed gently with HEPES buffer (10 mM HEPES, 1 mM $CaCl_2$, 1 mM Na_2HPO_4, 137 mM NaCl, 5 mM KCl, 0.5 mM $MgCl_2$, 5 mM glucose, 0.1% BSA, pH 7.4) at least 3 times. Fluorescence photographs were collected with a laser scanning confocal microscopy (Leica TCS SP8) and the mean fluorescence intensity (MFI) was quantitated by Las X software (Leica, Germany).

2.10 Western blot

Macrophages and ECs were cultured in DMEM containing 0.2% FBS overnight and lysed using RIPA buffer (Enogene, Cat# E1WP106). Proteins were subjected to 10% SDS-PAGE, transferred to PVDF membrane and immunoblotted with specific antibodies against SERCA2 (C498 antibody was a custom polyclonal antibody from Bethyl Laboratories, Inc.110 kDa), p-PERK

264(Thr981, Santa Cruz, Cat# sc-32577, 130 kDa, RRID:AB_2293243), BIP (Proteintech, Cat#
26511587-1-AP, 78 kDa, RRID:AB_2119855), ATF6 (Proteintech, Cat# 24169-1-AP, 100 kDa,
266RRID:AB_2876891), CHOP (Proteintech, Cat# 15204-1-AP, 27 kDa, RRID:AB_2292610),
267Phospho-NF-κB(Ser536, CST, Cat# 3033, 65 kDa, RRID:AB_331284), VCAM-1 (Proteintech,
268Cat# 11444-1-AP, 78 kDa, RRID:AB_2214232), MCP-1 (Proteintech, Cat# 66272-1-Ig, 19 kDa,
269RRID:AB_2861337) and β-actin (Proteintech, Cat#20536-1-AP, 45 kDa, RRID:AB_10700003),
270followed by the incubation with HRP-conjugated secondary antibody (Sino Biological Inc., Cat#
271SSA003, RRID:AB_2814815) for 1 h at room temperature. Proteins were visualized with
272ChemiDoc™ Touch System (Bio-Rad, USA). Bands density were quantified by NIH Image J
273software (RRID:SCR 003070, https://imagej.net/) and the expression levels of protein were nor-
274malized to β-actin.

2752.11 Statistical analysis

276The data and statistical analysis complied with the recommendations on the experimental design
277and analysis in *British Journal of Pharmacology* (Curt*is et a*l., 2018). All experimental groups
278were randomized with equal size, and analyzed blinded. The sample size in each group subjected
279to statistical analysis were determined based on our previous studies, preliminary results and
280power analysis (Curt*is et a*l., 2015b). Statistical analysis was undertaken only using these inde-
281pendent values with $n \geq 5$, and n stands for the number of independent experimental samples / in-
282dividuals. To avoid the larger variation among different experiments, the results for SKI are nor-
283malized to that of the control in the same experiment. Therefore, no variance in the control was
284observed, and the data was analyzed using a non-parametric statistical test. For other experi-
285ments, the mean values of the control group were normalized to 1 and the data was analyzed by
286parametric statistics. Statistical analysis was performed using GraphPad Prism 7.0
287(RRID:SCR_002798, http://www.graphpad.com/). *P<0.5 was considered as a statistically sig-
288nificant difference. No data were excluded from any of the experiments.

2892.12 Nomenclature of targets and ligands

290Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://
291www.guidetopharmacology.org. The common portal for data was from the IUPHAR/BPS Guide
292to PHARMACOLOGY (RRID:SCR 013077) (Hardi*ng et a*l., 2018) and permanently archived in

the Concise Guide to PHARMACOLOGY 2019/20 (Alexander *et al*., 2019).

3. Results

3.1. Genetic substitution of SERCA2 C674 with S674 aggravates atherosclerotic lesions in LDLR $^{-/-}$ genetic background mice

To illustrate the role of the redox-active thiols on SERCA2 C674 during atherogenesis, the SKI mouse line was used to partially substitute C674 with structurally similar serine which lacks only the cysteine thiol. SKI and WT littermates were placed on a Western diet for 8 weeks (Stanic *et al*., 2006). The body weights, plasma concentrations of cholesterol and triglyceride, GTT were firstly assessed, but no significant differences were obtained between the two experimental groups (Figure 1). Aortas were then harvested and stained with oil red O to visualize atherosclerotic plaque. *En face* analysis showed that SKI mice developed a 1.58-fold (Figure 2a) increase of plaque in the whole aorta, and also a significant increase (~1.7 fold) of the lesion was observed in the oil red O stained aortic root cross-sections when compared with WT mice (Figure 2b). Importantly, atherosclerotic lesions formed preferentially in the arch of the aorta consistent with previous studies (Hewing *et al*., 2017; Vion *et al*., 2017). Moreover, H&E staining showed that SKI mice displayed obviously elevated percentage of the lesion necrotic core in the aortic root (~1.8 fold) (Figure 2b). Taken together, these results demonstrate that the chronic substitution of SERCA2 C674 accelerated atherosclerosis progression.

Macrophages are considered as one of the primary cellular participants during atherogenesis (Glass *et al*., 2001). Indeed, a significant increase of macrophage accumulation within atherosclerotic lesion in the aortic root isolated from SKI mice was observed (Figure 2b), suggesting that the SERCA2 C674 thiol plays a key role in macrophage infiltration in the lesion area during atherogenesis.

3.2. Substitution of the SERCA2 C674 thiol provokes ER stress and the inflammatory response in macrophages

To better understand the mechanism by which chronic decreases in the SERCA2 C674 thiol promotes lesional macrophage deposition, BMDMs from WT and SKI mice were isolated and cultured. Despite of the similar protein amounts of SERCA2 in WT and SKI (Figure 3a), SKI BMDMs exhibited elevated levels of cytoplasmic Ca^{2+} (Figure 3b), indicating substitution of the C674 thiol inhibits the Ca^{2+} regulating function of SERCA2 in BMDMs without affecting its ex-

pression.

ER is a primary intracellular Ca^{2+} reservoir and the predominant organelle for protein synthesis and modification. Abnormities in protein processing, Ca^{2+} homeostasis and lipid metabolism may lead to ER stress and then activate inflammatory pathway, and both of which closely relate to the initiation and progression of atherosclerosis (Dong et al., 2016; Ozcan et al., 2016). We have reported that decreased SERCA2 C674 thiol depletes ER calcium stores and induces ER stress in ECs, SMCs and renal proximal tubule cells (M*ei et a*l., 2014b; Jia*ng et a*l., 2020; L*iu et a*l., 2020). As shown in Figure 3a, in BMDMs, substitution of SERCA2 C674 upregulated the protein expression of ER stress markers, including PRKR-like endoplasmic reticulum kinase protein kinase (p-PERK), activating transcription factor 6 (ATF6), binding immunoglobulin protein (BIP) and C / EBP homologous protein (CHOP), confirming that the redox state of SERCA2 C674 is critical in controlling ER function. NFκB is the major driver of pro-inflammatory adhesion molecules, chemokines, and cytokines (Y*ang et a*l., 2015; *Li et a*l., 2017), including vascular cell adhesion molecule-1 (VCAM-1) and monocyte chemotactic protein 1 (MCP-1). As shown in Figure 3a, the expression levels of active form of NFκB (p-p65 NFκB), VCAM-1, and MCP-1 increased in SKI BMDMs compared with WT. Correspondingly, the SERCA2 C674S substitution resulted in enhanced macrophage adhesion to ECs (Figure 3b). These results prove that substitution of SERCA2 C674 thiol induced ER stress and activated inflammatory response in BMDMs.

3.3. 4-PBA ameliorates ER stress and the inflammatory response in SKI BMDMs

To further verify if SERCA2 C674 substitution could trigger ER stress and activate inflammatory responses, therefore, an ER stress inhibitor, 4-PBA was applied. As data not shown, 4-PBA intervention had no effect in WT BMDMs. However, in SKI BMDMs, administration of 4-PBA downregulated the protein expression of ER stress markers (p-PERK, ATF-6, BIP, and CHOP) and inflammatory reaction markers (p-p65, VCAM-1, and MCP-1), (Figure 4a). Furthermore, 4-PBA treatment inhibited SKI BMDMs adhesion to ECs (Figure 4b). These results suggest that the induction of persistent ER stress by SERCA2 C674S substitution is responsible for increased inflammatory response in SKI macrophages.

3.4. Substitution of SERCA2 C674 thiol increased the expression of ICAM-1 and VCAM-1 in

aortic endothelium

Healthy arterial endothelium, consisted of a layer of ECs, is crucial in maintaining vascular homeostasis. In the context of atherosclerosis, abnormal lipid metabolism causes the activation of ECs, and triggers inflammatory response, accompanied by the induction of adhesion molecules, which further facilitates the cellular permeability to lipids and macrophage recruitment in local sites (Laclaustra *et al*., 2016; Al-Sharea *et al*., 2019). It is worth mentioning that EC metabolic perturbation is a predominant pathogenetic contributor, which then evokes vascular inflammation and chronic macrophages activation during atherosclerosis (Roth Flach *et al*., 2015a; Jiang *et al*., 2020). We previously found S-glutathiolation of SERCA2 C674 regulates ECs function and participates in angiogenesis after ischemia (Mei *et al*., 2014a), revealing the redox control of the SERCA2 C674 thiol throughout the body. Since C674S substitution increased the inflammatory response in SKI BMDMs, we stained intercellular cell adhesion molecule 1 (ICAM-1) and VCAM-1 in the aorta. As shown in Figure 5, immunofluorescence staining showed that the mean fluorescence intensity (MFI) of ICAM-1 and VCAM-1 in the aortic endothelial line of SKI mice were significantly increased compared with that of WT mice, suggesting elevated inflammation in local aorta.

3.5. Substitution of SERCA2 C674 thiol caused ER stress and promoted inflammation in ECs

Next, we explored the specific contribution of ECs to deteriorate atherosclerosis in SKI mice. We have attempted to isolate primary aortic ECs, however, the cells were always in poor condition and yields were unacceptably low. As an alternative, cardiac endothelial cells from WT mice that we isolated previously were used in *in vitro* experiments. Adenoviral SERCA2 S674 overexpression was applied in ECs to represent SKI ECs and empty adenoviral vector was used as a control. As shown in Figure 6a, Western blotting examination depicted the adenoviral overexpression of SERCA2 S674 in ECs induced prolonged ER stress and increased inflammatory protein levels of p-P65 and VCAM-1. Fluo-4 AM was then utilized to detect intracellular Ca^{2+} fluctuations, and the ECs overexpressing SERCA2 S674 showed elevated intracellular Ca^{2+} levels compared with control group (Figure 6b), confirmed the importance of SERCA2 C674 in maintaining Ca^{2+} homeostasis in ECs. Furthermore, SERCA2 S674 overexpressed ECs also resulted in enhanced number of adherent macrophages to ECs (Figure 6b), reflecting its significant pro-

inflammatory effects.

3.6. 4-PBA administration ameliorates ER stress and inflammatory response in ECs overexpressing SERCA2 S674

Moreover, 4-PBA administration suppressed SERCA2 S674 overexpression triggered ER stress in ECs by inhibiting the phosphorylation of PERK and reducing the protein levels of BIP and CHOP, while SERCA2 abundance was not affected (Figure 7a). Correspondingly, the sustained inflammatory response as well as macrophages adhesion to ECs were obviously ameliorated by 4-PBA pretreatment (Figure 7b). Taken together, these observations proved that the substitution of SERCA2 C674 in ECs evoked ER stress and inflammatory response, promoted macrophage adhesion to ECs by interfering with intracellular calcium homeostasis, which may account for the aggravated atherosclerosis in SKI mice.

3.7. 4-PBA oral administration attenuates Western diet-induced atherosclerosis in SKI LDLR $^{-/-}$ mice

Multiple studies have verified ER stress in macrophages and ECs attribute to the development of atherosclerosis and 4-PBA administration can significantly reduce atheromatous plaque deposition *in vivo* (Geng et al., 2019), therefore we didn't focus on 4-PBA treatment in WT mice. To confirm if sustained activation of ER stress accounts for the aggravated atherosclerosis, 4-PBA oral administration was employed in SKI mice. We found 4-PBA administration had no effect on body weight (28.4±0.3g vs 27.8±0.4g; SKI mice vs that treated with 4-PBA) as well as the plasma amount of cholesterol (28.8±0.7 mmol/L vs 28.6±0.6 mmol/L; SKI mice vs that treated with 4-PBA) or triglyceride (3.2±0.12 mmol/L vs 3.6±0.2 mmol/L; SKI mice vs that treated with 4-PBA). However, as shown in Figure 8a, 4-PBA treatment resulted in an obvious decrease (45.1%) in the development of atherosclerotic lesions in *en face* aorta and a 48.4% reduction in aortic root (Figure 8b). However, the percentage of the lesion necrotic core showed no significant difference despite the fact that there was a significant reduction in macrophage infiltration in the lesion in 4-PBA treated SKI mice (Figure 8b).

4. Discussion

C674 is identified as the main cysteine of SERCA2, and it can be S-glutathiolated to increase SERCA2 activity, which is easily blocked by irreversible oxidation of C674 thiols under pathological circumstances (Adachi *et a*l., 2004a), However, the specific regulation mechanism of

SERCA2 C674 oxidation in different vascular cell types during atherosclerosis is currently not known.

In this work, the 50% substitution of SERCA2 C674 with S674 in a heterozygous knock in mouse allows for the assessment of the effect of prolonged removal of the reactive thiol which occurs pathophysiologically in the context of atherosclerosis due to its irreversible oxidation. We revealed the crucial role of the SERCA2 C674 thiol in both macrophages and ECs, the two major cell types in atherogenesis, implying that redox regulation of the thiol is very important in atherogenesis. In BMDMs, C674S substitution causes prolonged ER stress, increases the secretion of pro-inflammatory cytokines and promotes macrophage adhesion. In addition, the overexpression of SERCA2 S674 in ECs leads to ER stress and triggers endothelial dysfunction by causing elevated adhesion molecule abundance and macrophage adhesion, which in turn augments inflammatory response and macrophage infiltration. Since significant increase in intracellular Ca^{2+} was observed in both SKI BMDMs and ECs overexpressing SERCA2 S674, we suppose the vital role redox regulation of the SERCA2 C674 thiol on ER stress and inflammatory response is due to the dysregulation of ER Ca^{2+} handling.

Cellular and ER Ca^{2+} levels and prolonged disturbance of ER function have emerged as a crucial factor for the initiation and progression of atherosclerosis (Ron, 2002; Fe*ng et a*l., 2003; Tab*as et a*l., 2010). ER stress occurs in many cell types that are involved in atherosclerosis, including macrophages and ECs (Lusis, 2000; Lib*by et a*l., 2013), however, the specific role of the SERCA2 C674 thiol on ER stress in atherosclerosis has not been elucidated. Our data demonstrated the formation of atherosclerotic plaque is increased in SKI mice, and is prevented by 4-PBA oral administration *in vivo*, suggests that the SERCA2 C674 thiol regulates atherosclerosis progression by inducing ER stress. Surprisingly, no obvious changes of the plasma amount of cholesterol and triglyceride explain the changes in the SKI mouse or after 4-PBA intervention. To further clarify this issue, our group is working on the role of the SERCA2 C674 thiol in cholesterol metabolism in macrophages in mice of C57BL/6J background, and the preliminary results reveal a key regulation by the SERCA2 C674 thiol redox status on lipid metabolism and homeostasis during atherogenesis. Considering the regulating effect of macrophages and ECs during atherosclerosis, we asked whether ER stress was responsible for increased atherosclerosis

in SKI mice with a focus on BMDMs and ECs. Similar to our previous work, we found that without affecting SERCA2 expression, C674S substitution leads to prolonged activation of ER stress both in BMDMs and ECs, which was prevented by 4-PBA pretreatment. This suggests the key role of redox status of SERCA 2 C674 on ER stress by increasing intracellular Ca^{2+} level, which inevitably disrupts the intracellular microenvironment and eventually promotes inflammation-driven atherogenesis.

NF-κB controls various cellular processes, especially the inflammatory response. The activation of the NF-κB pathway is strongly related to the induction of pro-inflammatory chemokines and cytokines involved in atherogenesis in several cell types. The contribution of the NF-κB driven inflammatory cascade in individual ECs or macrophages has long been appreciated both in animals and patients (Pana*ro et a*l., 2012; Pater*as et a*l., 2014). In our study, we demonstrated elevated expression of VCAM-1 and ICAM-1 in aortic endothelium in SKI mice compared with WT mice, suggested the endothelial inflammatory and the important role of ECs in the progression of atherosclerosis. In cell culture studies, NF-κB phosphorylation and the expression of its downstream chemokines MCP-1 and adhesion molecule VCAM-1 were significantly increased in SKI BMDMs, and increased macrophages adhesion to ECs was observed. Further research showed that the overexpression of SERCA2 S674 in ECs activated NF-κB, increased the target gene VCAM-1, and thus elevated macrophages infiltration. These data indicated the dual role of redox modifiable SERCA2 C674 on macrophage recruitment and inflammatory response through the NF-κB pathway. What's more, inflammation evoked by substitution of the SERCA2 C674 thiol was ameliorated both in macrophages and ECs with 4-PBA pretreatment, indicating sustained ER stress is of importance in regulating the inflammatory response.

In conclusion, the genetic SKI mouse provides us with a model for the first time to study the important role of the redox reactive thiol of SERCA2 C674 on atherosclerosis and a potential mechanism by which it occurs focusing on the cross talk between macrophages and ECs. Our work firstly highlights the important contribution of the SERCA2 C674 thiol as well as its modes of action during atherogenesis. We demonstrated here the substitution of SERCA2 C674 thiol directly accelerates the development of atherosclerosis by inducing ER stress and inflammatory response, which opens up a promising avenue to combat atherosclerosis.

Reference

Figure legends

Figure 1. (a) Body weight, plasma cholesterol and triglyceride in WT LDLR$^{-/-}$ mice and SKI LDLR$^{-/-}$ mice fed for 8-weeks with Western diet. WT vs. SKI, n=12. (b) GTT in WT LDLR$^{-/-}$ mice and SKI LDLR$^{-/-}$ mice with 8-week of Western diet. WT vs. SKI, n=6. All data are presented as mean ± SEM, unpaired Student's t test. ns, no significant difference.

Figure 2. Substitution of SERCA2 C674 thiol aggravates the formation of atherosclerotic plaques in SKI LDLR$^{-/-}$ mice fed with a Western diet

(a) Representative images of the whole aorta stained with oil red O (left panel) and the quantification of plaques (right panel), scale bar: 3 mm. (b) Representative images of the aortic root stained with oil red O for atherosclerotic lesion, hematoxylin and eosin (H&E) for necrotic core (necrotic core was marked with green line) and immunohistochemical staining against CD68 (left panel), and the quantitative analysis in the graph (right panel). *P<0.05 WT vs. SKI, n =12. Scale bar: 200 μm. All data are presented as mean ± SEM, unpaired Student's t test.

Figure 3. of SERCA2 C674 provoked ER stress and inflammatory response in macrophages by increasing intracellular calcium accumulation

(a) Representative Western blots of SERCA2, ER stress and the inflammatory markers from WT and SKI macrophages (left panel) and the analysis of bands density in the graph (right panel). The results for SKI are normalized to the results of the control in the same experiment. all data used a non-parametric statistical test. *P<0.05. WT vs. SKI. SERCA2, p-PERK, ATF-6, BIP and VCAM-1, n=6; CHOP, p-P65 and MCP-1, n=5. (b) Representative images of intracellular calcium levels detected by Fluo-4 AM and macrophages adhesion to ECs (left panel) and the quantification in the graph (right panel). All data are presented as mean ± SEM, unpaired Student t-test. ns, no significant difference. *P<0.05. WT vs. SKI, n=6.

Figure 4. 4-PBA treatment ameliorates ER stress and inflammatory response in SKI macrophages

(a) Representative Western blots of ER stress and inflammatory markers in SKI macrophages

with or without 4-PBA treatment (left panel) and the quantification of bands density in the graph (right panel). Each 4-PBA value was normalized to each matched control value, all data used a non-parametric statistical test. ns, no significant difference. *P<0.05. WT vs. SKI macrophages treated with 4-PBA vs. that without 4-PBA. p-PERK, ATF-6, BIP and VCAM-1, n=6; CHOP, p-P65 and MCP-1, n=5. (b) Representative images of WT macrophages adhesion to ECs that treated with or without 4-PBA (left panel) and the summary plots depicted the average macrophages adhered to ECs in the graph (right panel). All data are presented as mean ± SEM, unpaired Student t-test. *P<0.05. SKI macrophages treated with 4-PBA vs. that without 4-PBA. n=6. Scale bar: 200 μm.

Figure 5. Chronic partial substitution of the SERCA2 C674 thiol upregulated the expression of ICAM-1 and VCAM-1 in aorta endothelium of mice fed normal diet

(a) Representative immunofluorescence of ICAM-1 (green), DAPI (nucleus, blue) in aorta endothelium (left panel) and the analysis of MFI in the graph (right panel). (b) Representative immunofluorescence of VCAM-1 (green), DAPI (nucleus, blue) in aorta endothelium (left panel) and the analysis of MFI in the graph (right panel). *P<0.05 WT vs. SKI. n=12. Scale bar: 500 μm. All data are presented as mean ± SEM, unpaired Student's t test.

Figure 6. Substitution of SERCA2 C674 thiol in ECs activated ER stress and promoted inflammation by increasing intracellular calcium concentration

(a) Representative of Western blots of SERCA2, ER stress and inflammatory markers in ECs transfected with SERCA2 empty adenovirus (Adv) or adenovirus SERCA2 S674 (S674) (left panel) and the analysis of band density in the graph (right panel). *P<0.05. ECs transfected with empty adenovirus vs. that transfected with adenoviral SERCA2 S674. SERCA2, n=9; p-PERK and p-P65, n=7; ATF-6, BIP, VCAM-1 and CHOP, n=5. (b) Representative images of intracellular calcium detected by Fluo-4 AM and macrophages adhesion to ECs (left panel) and the quantification in the graph (right panel). *P<0.05. ECs transfected with SERCA2 empty adenovirus vs. that transfected with adenoviral SERCA2 S674, n=6. All data are presented as mean ± SEM, unpaired Student's t test.

Figure 7. 4-PBA administration ameliorated ER stress and inflammatory response in ECs transfected with adenovirus SERCA2 S674

(a) Representative Western blots of ER stress and inflammatory markers in adenovirus SERCA2 S674 transfected ECs with or without 4-PBA treatment (left panel) and the analysis of bands density in the graph (right panel). SERCA2, n=9; p-PERK and p-P65, n=7; BIP, CHOP and VCAM-1, n=5. ns, no significant difference. *P<0.05. Adenovirus SERCA2 S674 transfected ECs treated with 4-PBA vs. that without 4-PBA (b) Representative images of macrophages adhered to SERCA2 S674 transfected ECs with or without 4-PBA treatment (left panel) and the summary plots of the average number of macrophages adhered to ECs (right panel). *P<0.05. Adenovirus SERCA2 S674 transfected ECs treated with 4-PBA vs. that without 4-PBA. n=5. Scale bar: 200 μm. All data are presented as mean ± SEM, unpaired Student's t test.

Figure 8. 4-PBA oral administration attenuated the atherosclerotic lesion progression in SKI LDLR $^{-/-}$ mice

(a) Representative images of the atherosclerotic lesions in the whole aorta (left panel) and the relative quantification of the plaques (right panel), scale bar: 3 mm. (b) Representative images of the aortic root stained with oil red O for atherosclerotic lesion, hematoxylin and eosin (H&E) for necrotic core (necrotic core was marked with green line) and immunohistochemical staining against CD68 (left panel), and the quantitative analysis in the graph (right panel). ns, no significant difference. *P<0.05 SKI treated with 4-PBA mice vs. that without 4-PBA. n=12. Scale bar: 200 μm. All data are presented as mean ± SEM, unpaired Student's t test.