

Endoplasmic Reticulum Stress Effector CCAAT/Enhancer-binding Protein Homologous Protein (CHOP) Regulates Chronic Kidney Disease–Induced Vascular Calcification

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Background—Cardiovascular diseases such as atherosclerosis and vascular calcification are a major cause of death in patients with chronic kidney disease (CKD). Recently, the long-awaited results of the Study of Heart and Renal Protection trial were reported. This large randomized clinical trial found that an extensive cholesterol-lowering therapy through the combination of simvastatin and ezetimibe significantly reduced cardiovascular diseases in a wide range of patients with CKD. However, the mechanism by which this cholesterol-lowering therapy reduces CKD-dependent vascular diseases remains elusive. The objective of the present study was to determine the contribution of the oxysterol-induced pro-apoptotic transcription factor CCAAT/enhancer-binding protein homologous protein (CHOP) on the pathogenesis of CKD-dependent cardiovascular diseases through endoplasmic reticulum stress signaling.

Methods and Results—CKD increased levels of serum oxysterols such as 7-ketocholesterol in human patients and ApoE^{−/−} mice. Treatment with simvastatin plus ezetimibe strongly reduced levels of serum oxysterols and attenuated CKD-dependent atherosclerosis, vascular cell death, vascular calcification, and cardiac dysfunction. This therapy also reduced aortic endoplasmic reticulum stress induced by CKD. The short hairpin RNA-mediated knockdown of CHOP and activating transcription factor-4 in vascular smooth muscle cells attenuated oxysterol-induced mineralization, osteogenic differentiation, and endoplasmic reticulum stress. In addition, CHOP deficiency protected ApoE^{−/−} mice from CKD-dependent vascular calcification, cardiac dysfunction, and vascular cell death.

Conclusions—These data reveal that the cholesterol-lowering therapy of simvastatin plus ezetimibe attenuates CKD-dependent vascular diseases through a reduction of oxysterol-mediated endoplasmic reticulum stress. CHOP plays a crucial role in the pathogenesis of CKD-dependent vascular calcification. (*J Am Heart Assoc.* 2014;3:e000949 doi: 10.1161/JAHA.114.000949)

Key Words: CHOP • chronic kidney disease • endoplasmic reticulum stress • oxysterol • vascular calcification

Patients with chronic kidney disease (CKD) continue to experience higher mortality rates caused by an extremely high burden of cardiovascular diseases such as atherosclerosis, vascular calcification, and heart failure. Abnormalities in lipid metabolism are known to be a major risk factor for cardiovascular diseases in patients with CKD. Recently, the Study of Heart and Renal Protection confirmed that an extensive cholesterol-lowering therapy through treatment with a combination of simvastatin and ezetimibe reduced the risk of cardiovascular diseases in patients with CKD.^{1,2} However, (1) whether the combination of simvastatin and ezetimibe is more effective than simvastatin alone and (2) the molecular mechanism by which this cholesterol-lowering therapy reduces cardiovascular risk in CKD have not been examined.

Oxidized low-density lipoprotein (LDL) is present in atherosclerotic and calcified plaques and is thought to play an important role in the development of atherosclerosis and vascular calcification.^{3–6} Serum oxidized sterols (oxysterols) such as 7-ketocholesterol and 25-hydroxycholesterol are a major component in oxidized LDL and are thought to mediate the lipotoxic effects of oxidized LDL.⁷ Oxysterols induce

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apoptosis of vascular cells including macrophages,^{6,8,9} but they also induce mineralization and osteoblastic differentiation of vascular smooth-muscle cells (VSMCs). Oxysterols are endogenously generated through either the enzyme reaction of cholesterol hydroxylases or oxidative stress.^{10–12} In addition, oxysterols such as 7-ketocholesterol are detected at high levels in advanced atherosclerotic plaques and in the plasma of patients at high risk for cardiovascular diseases.¹³ However, the role of oxysterols in CKD-dependent atherosclerosis and vascular calcification has not yet been investigated in detail.

The endoplasmic reticulum (ER) is a multifunctional organelle that regulates a wide range of cellular processes including calcium homeostasis, protein synthesis and modification, and lipid synthesis. Structural and functional disruptions of the ER lead to the accumulation of unfolded and misfolded proteins in the ER lumen, thereby provoking ER stress signaling. ER stress signaling is composed of three signaling axes that are initiated by three corresponding ER resident proteins: inositol-requiring protein-1, double-stranded RNA-dependent protein kinase-like ER kinase (PERK), and activating transcription factor 6 (ATF6).^{6,8,9,14,15} Among these three pathways of the ER stress signal, the PERK-mediated axis is considered to play a predominant role in the development of atherosclerosis.^{6,9,16,17} Activation of the PERK pathway during ER stress leads to the phosphorylation of an α subunit of the eukaryotic initiation factor 2, which in turn promotes the translation of the activating transcription factor 4 (ATF4) protein. ATF4 subsequently induces the CCAAT/enhancer-binding protein homology protein (CHOP), a pro-apoptotic transcription factor, leading to vascular apoptosis and advanced atherosclerosis.^{6,16,18,19} CHOP expression is detected at high levels in atherosclerotic lesions.^{4,17,20} Inhibition of CHOP attenuates atherosclerosis, vascular apoptosis, and vascular necrosis in ApoE^{-/-} and LDLR^{-/-} mice.^{16,18} In addition to atherosclerosis, our group previously reported that vascular calcification induced through ER stress signaling contributes to vascular calcification and osteoblastic differentiation of VSMCs.^{21–23} The CKD-induced aortic PERK-eukaryotic initiation factor 2-ATF4-CHOP axis of ER stress signaling is believed to accelerate atherosclerosis and vascular calcification in ApoE^{-/-} mice.²¹ Furthermore, treatment with ER chemical chaperones such as sodium tauroursodeoxycholate and sodium 4-phenylbutyrate reduced CKD-induced aortic ER stress, leading to a significant attenuation of vascular calcification in several animal models including ApoE^{-/-} mice with CKD.^{21,24,25} In addition to inflammatory cytokines, several lipotoxic mediators including oxysterols induce ER stress signaling in vascular cells including VSMCs and macrophages.^{6,8,9} We therefore hypothesized that the oxysterol-mediated induction of CHOP through the PERK-mediated pathway of the ER stress

signal directly contributes to CKD-dependent vascular calcification and atherosclerosis, and is blocked by the extensive cholesterol-lowering therapy of simvastatin and ezetimibe.

To test our hypothesis, we examined whether (1) levels of a specific oxysterol are elevated in the serum of humans and ApoE^{-/-} mice with CKD using Liquid chromatography mass spectrometry (LC-MS/MS); and (2) treatment with the combination of simvastatin and ezetimibe reduces levels of serum oxysterols increased by CKD, resulting in the attenuation of CKD-dependent atherosclerosis, vascular cell death, cardiac dysfunction, and vascular calcification. Using cell culture models and the short hairpin RNA (shRNA)-mediated gene knockdown technique, we also examined whether ER stress contributes to oxysterol-induced mineralization and osteoblastic differentiation of VSMCs. Furthermore, we examined whether CHOP deficiency can attenuate atherosclerosis, vascular cell death, and vascular calcification induced by CKD.

Methods

Animal Studies

Eight-week-old male ApoE^{-/-} and CHOP^{-/-} mice (C57Bl6/J background) were purchased from the Jackson Laboratory. CHOP^{-/-};ApoE^{-/-} double mutant mice were generated by brother-sister mating of CHOP^{+/-};ApoE^{-/-} mice. CHOP^{+/-}; ApoE^{-/-} littermates were used as controls. Eight-week-old animals were subjected to either 5/6 nephrectomy (nx) or sham operations.²⁶ Ten-week-old ApoE^{-/-} mice were fed a Western diet (TD10364) containing simvastatin (20 mg/kg body weight) and ezetimibe (10 mg/kg body weight) for 12 weeks. All experiments were conducted with 8 animals per group. Simvastatin and ezetimibe were kindly provided by Merck (Rahway, NJ). All animals were euthanized by isoflurane overdose after 4 hours of fasting. Animal experiments were approved by the Institutional Animal Care and Research Advisory Committee of the University of Colorado at Denver.

Human Subjects

Serum samples were collected from 10 patients with stages 3 and 4 CKD (mean estimated glomerular filtration rate 34±7 mL/min per 1.73 m²) and mean age (57±7 years) and 10 age-matched patients with normal kidney function (mean estimated glomerular filtration rate 80±12 mL/min per 1.73 m²) and mean age (59±14 years) after a minimum 4-hour fast. Trained personnel at the University of Colorado Clinical and Translational Research Center collected the serum samples. All patients gave informed consent for their blood specimen to be used for research purposes. Basic metabolic panels and cholesterol levels were performed at the University of Colorado Clinical Laboratory using standard techniques.

Creatinine was measured using the Jaffe rate method with colorimetry via the Synchron Systems AQUA CAL 1 and 2. Glomerular filtration rate was estimated using the 4-variable Modification of Diet in Renal Disease prediction equation.²⁷ All participants were enrolled in a separate clinical research study and the procedures were approved by the Colorado Multiple Institutional Review Board or the Institutional Review Board of the University of Colorado Boulder. The nature, benefits, and risks of the study were explained to the volunteers, and their written informed consent was obtained prior to participation.

Cell Culture Studies

CHOP-knockdown and ATF4-knockdown VSMCs were previously generated using appropriate shRNAs (Thermo Fisher).^{21,22} These cells were cultured in DMEM containing 10% fetal bovine serum and 1 to 25 $\mu\text{g}/\text{mL}$ oxysterols. The medium was changed every 2 to 3 days. All cell culture experiments were performed independently at least 2 times.

Histological Analysis

For en face analysis, mouse aortas were obtained, opened longitudinally from the heart to the iliac arteries, and stored at 4°C as previously described.^{21,26,28} The dissected aortas were stained with Sudan IV. Images were obtained using a Zeiss AxioCam-CCD video camera and analyzed with Axio-Vision image-analysis software. Atherosclerotic and calcified lesions of the aortic sinus were analyzed as previously described.^{21,26,28} The upper portions of the heart and proximal aorta were obtained, embedded in optimal cutting temperature compound, and stored at -80°C . Sections were stained with Oil Red O and von Kossa. The atherosclerotic and calcified areas on 6 sections were determined in a blinded fashion by light microscopy. To confirm the presence of macrophage and smooth-muscle cells within the arterial wall, 5- μm -thick sections of the aortic arch from mice were processed, cut, and prepared for immunohistochemical analysis. As previously described,^{29,30} anti-CD68 and anti- α -smooth muscle cell actin were used for detecting macrophage and smooth-muscle cells, respectively. Apoptotic cells in aortic sinuses were detected using an In Situ Cell Death Detection Kit (Roche) as previously described.¹⁶

Oxysterol Analysis

We used the analysis method as reported by MacDonald et al.³¹ Briefly, serum lipids were extracted with dichloromethane:methanol (1:1) containing butylated hydroxytoluene (50 $\mu\text{g}/\text{mL}$). The lipid fractions were hydrolyzed with 0.5 N potassium hydroxide for 1.5 hours at 35°C and subjected to an aminopropyl solid-phase extraction. The eluted lipid samples

were analyzed with LC-MS/MS (Applied Biosystems 3200 Qtrap with Shimadzu LC20AD) with a reverse-phase column (Kinetex C18 HPLC column; Phenomenex) using an acetonitrile/2-propanol/5 mmol/L ammonium acetate gradient.

Biochemical Analysis

Serum FGF23 was determined using ELISA kits (Immutopics). Serum creatinine was measured using an LC-MS/MS method as we previously described. Serum levels of total cholesterol, LDL-cholesterol, triglyceride, phosphorus, and calcium were analyzed using commercially available kits according to the manufacturer's protocols (Wako Chemicals, Stanbio Laboratory, and Pointe Scientific).

Calcium Content in Cultured Cells

Calcium deposition in the plates was quantified as previously described.^{21–23,26} Cells were decalcified using a 0.6 N hydrochloric acid solution. After collecting the supernatant, the cells were washed with PBS and solubilized with a 0.1 N sodium hydroxide/0.1% SDS solution for protein quantification. Calcium content was quantified calorimetrically using the o-cresolphthalein method. Protein content was measured using a BCA protein assay kit.^{21–23,26}

RNA Analysis

Total RNA was isolated using Tri reagent in conjunction with an RNAeasy kit. Real-time quantitative polymerase chain reaction assays were performed using an Applied Biosystems StepOne qPCR instrument as previously described.^{21,22} Using the $\Delta\Delta\text{Ct}$ method, quantification of gene expression was calculated as the mRNA level normalized to a ribosomal housekeeping gene (18S or 36B4). Primer sequences are available upon request.

Western Blotting

Cell and tissue lysates were prepared using RIPA buffer (Cell Signaling). The samples were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted with the following antibodies: p-PERK (Cell Signaling), PiT-1, ATF4, CHOP, and GAPDH (Santa Cruz Biotechnology). Samples were visualized using horseradish peroxidase coupled to an anti-mouse secondary antibody, with enhancement by an Electrochemiluminescence detection kit.

Echocardiography

Using a Vevo-770 high-resolution imaging system from Visual Sonics and a 30-MHz mechanical transducer (707B),

echocardiography on mice was performed after 12 weeks of the cholesterol-lowering drug treatment. Mice were anesthetized with 2% isoflurane and maintained at a body temperature of 37°C for the duration of the noninvasive imaging procedure. Mouse hearts were imaged in a parasternal short-axis view. M-mode images of the left ventricle (LV) were recorded at the level of the papillary muscles to measure LV wall dimensions and internal diameter in diastole and systole.³²

Statistical Analysis

Data were collected from more than 2 independent experiments and reported as the means±SEM. Statistical analysis for 2-group comparison was performed using the Student's *t* test, or one-way ANOVA with a Newman–Keuls post-hoc test for multi-group comparison. Significance was accepted at $P<0.05$.

Results

Although oxysterols play a causative role in the development of atherosclerosis and vascular calcification,^{3,6} whether CKD increases levels of oxysterols in human and animal models has not been analyzed. We therefore analyzed and compared levels of oxysterols in the serum of human subjects with stage 3 to 4 CKD to those of age-matched human subjects with normal kidney function using a recently developed LC-MS/MS method that is capable of analyzing over 30 oxysterol molecules. As shown in Table 1, human subjects with CKD had significantly higher levels of serum oxysterols including 5β,6β-epoxycholesterol, 7-ketocholesterol, 24-hydroxycholesterol, and 27-hydroxycholesterol (Table 1), despite no significant difference of total and LDL-cholesterol levels (Table S1). As expected, hypertriglyceridemia and hyperphosphatemia

were observed in human subjects with CKD (Table S1). Similarly, in ApoE^{-/-} mice, CKD induced by 5/6 nx increased levels of serum oxysterols including 7-ketocholesterol, 25-hydroxycholesterol, and 27-hydroxycholesterol (Table 1). Consistent with our previous reports, CKD significantly worsened atherosclerosis and vascular calcification in ApoE^{-/-} mice (data not shown).

To examine whether increased oxysterol levels caused by CKD contribute to atherosclerosis and vascular calcification, we treated 5/6 nx ApoE^{-/-} mice with either simvastatin alone or a combination of simvastatin and ezetimibe. The serum oxysterols 7-ketocholesterol, 25-hydroxycholesterol, and 27-hydroxycholesterol induced by CKD were reduced with a combination of simvastatin plus ezetimibe, but not simvastatin alone. 7α-hydroxycholesterol levels were also significantly reduced in 5/6 nx ApoE^{-/-} mice treated with simvastatin plus ezetimibe (Figure 1). Consistent with previous reports from our group and other groups,^{1,26} CKD also increased levels of total cholesterol and LDL-cholesterol in serum. These levels were significantly reduced by treatment with a combination of simvastatin and ezetimibe and only slightly reduced by treatment with simvastatin alone (Table S1). CKD also induced hypertriglyceridemia in ApoE^{-/-} mice, whereas both cholesterol-lowering therapies prevented CKD-induced hypertriglyceridemia (Table S1). CKD increased serum phosphorus and serum FGF23 levels, which were not affected by a combination of simvastatin plus ezetimibe (Table S1).

Next, we examined whether the cholesterol-lowering therapy attenuated CKD-induced cardiovascular dysfunctions including atherosclerosis, vascular calcification, and cardiac systolic dysfunction. Aortic en face analysis showed that treatment with a combination of simvastatin and ezetimibe drastically reduced atherosclerotic lesions induced by CKD (Figure 2A). Treatment with simvastatin alone slightly reduced

Table 1. Serum Oxysterol Levels in Human Patients and ApoE^{-/-} Mice With Chronic Kidney Disease

ng/mL	4β-OH	5β,6β-EP	7α-OH	7-keto	24-OH	25-OH	27-OH
<i>Human*</i>							
Normal	11.1±2.3	178.1±21.9	208.1±28.3	35.7±5.3	19.7±2.9	14.6±3.1	164.8±2.4
CKD1	4.9±3.2	464.1±51.1	181.8±15.21	21.6±15.7	34.8±4.0	52.5±22.3	286.4±3.7
<i>P</i> value [†]	0.12	0.001**	0.69	0.001**	0.01**	0.09	0.04**
<i>ApoE^{-/-} mice[‡]</i>							
Sham	285.5±44.5	2742.8±301.8	8618.9±1565.2	429.4±55.1	307.5±52.7	141.4±32.4	819.8±102.9
5/6nx	212.0±22.3	3006.8±343.0	10 283.6±1052.4	1037.6±133.5	384.4±30.6	260.7±27.5	1469.5±249.2
<i>P</i> value	0.17	0.58	0.40	0.002**	0.24	0.008**	0.04**

CKD indicates chronic kidney disease; EP, epoxy; OH, hydroxy.

*Serum samples (N=10) were collected from human subjects with stage 3 and 4 chronic kidney disease and age-matched subjects with normal kidney function after fasting overnight.

[†]Statistical significance ($P<0.05$) was analyzed using a 2-tailed Student's *t* test.

[‡]Serum samples (N=6) were collected from 22-week-old ApoE^{-/-} mice with normal kidney function or CKD after 4 h of fasting.

**Level of statistical significance.

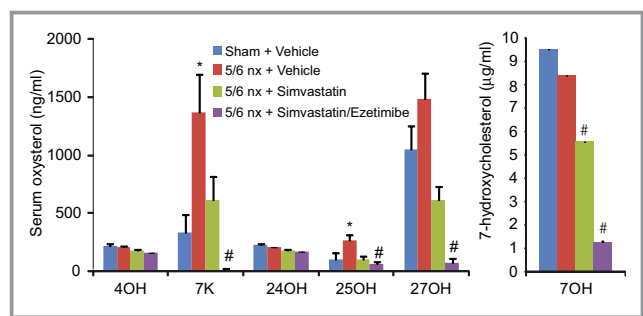


Figure 1. Treatment with simvastatin plus ezetimibe profoundly reduces levels of oxysterols increased by CKD. Serum oxysterol levels. 5/6 nx ApoE^{-/-} mice were treated with either 20 mg/kg simvastatin alone or 20 mg/kg simvastatin plus 10 mg/kg ezetimibe for 12 weeks. Blood was drawn after 4-hour fasting. N=6, **P*<0.05 vs sham-operated mice with vehicle. #*P*<0.05 vs 5/6 nx ApoE^{-/-} mice treated with vehicle. CKD indicates chronic kidney disease.

CKD-induced atherosclerosis (Figure 2B). Treatment with a combination of simvastatin and ezetimibe, but not simvastatin alone, significantly attenuated both CKD-induced medial and intimal vascular calcification (Figures 2C, 2D, and S1A). These data suggest that a combination of simvastatin and ezetimibe is more effective than simvastatin alone in the treatment of CKD-dependent cardiovascular diseases. We therefore eliminated the group treated with simvastatin alone from further analyses. We next analyzed whether (1) simvastatin/ezetimibe therapy reduces macrophage and smooth muscle cell content; (2) CKD deregulates cardiac function of ApoE^{-/-} mice; and (3) simvastatin/ezetimibe therapy improves CKD-dependent cardiac dysfunctions. Immunofluorescence analysis revealed that treatment with a combination of simvastatin and ezetimibe reduced macrophage content but not smooth-muscle cell content (Figure 2E, 2F and 2G). ApoE^{-/-} mice with CKD were noted to have significantly impaired LV systolic function, as determined by echocardiographic assessment of ejection fraction (EF). Treatment with the combination of simvastatin and ezetimibe significantly improved LV systolic function in these animals, and also reduced pathological LV dilation (Figure 3).

Since we previously reported that the aortic PERK-eukaryotic initiation factor 2 α -ATF4-CHOP axis of the ER stress signal contributes to CKD-dependent cardiovascular diseases,²¹ we next analyzed whether the treatment with simvastatin plus ezetimibe affects ER signaling in the aortas of ApoE^{-/-} mice with CKD. In agreement with our previous report, CKD significantly induced the mRNA expression of ER stress makers such as ATF4, CHOP, and Binding immunoglobulin protein, osteogenic markers such as alkaline phosphatase and osteopontin, and inflammatory markers such as TNF α (Figure 4A). Treatment with simvastatin plus ezetimibe

significantly reduced mRNA levels of these ER stress, osteogenic, and inflammatory markers. In addition, CKD increased levels of aortic p-PERK, p-ATF4, total ATF4, and CHOP proteins, which were significantly reduced by simvastatin plus ezetimibe treatment (Figures 4B and S1). Immunohistological analysis confirmed that aortic CHOP expression was significantly reduced by this treatment (Figure 4C and 4D). Since CHOP is a pro-apoptotic effector of ER stress, we performed terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining of the aortas of ApoE^{-/-} mice with CKD to examine whether: (1) CKD induces cell death in the aorta and (2) this cholesterol-lowering therapy reduces CKD-induced aortic cell death. As hypothesized, CKD significantly increased aortic cell death (Figure 4E and 4F), whereas simvastatin/ezetimibe treatment attenuated CKD-induced cell death (Figure 4E and 4F).

To examine whether oxysterol-induced CHOP and ATF4 through ER stress signaling is involved in mineralization and osteogenic differentiation of VSMCs, mouse vascular smooth muscle cell line (MOVAS-1) cells were treated with 7-ketocholesterol. CKD significantly increases 7-ketocholesterol in both humans and mice, as shown in Table 1. 7-ketocholesterol dose-dependently increased calcium content and alkaline phosphatase activity (Figure 5A and 5B). ATF4 and CHOP protein levels were significantly increased by 7-ketocholesterol with dose-dependent and time-dependent manners (Figure 5C and 5D). We next treated ATF4 knockdown and CHOP knockdown MOVAS-1 cells with 7-ketocholesterol. ATF4 knockdown and CHOP knockdown significantly attenuated oxysterol-induced mineralization (Figure 5E). ATF4 knockdown decreased ATF4 protein expression induced by 7-ketocholesterol treatment, resulting in the reduction of oxysterol-induced CHOP expression (Figure 5F). As expected, CHOP knockdown reduced oxysterol-induced CHOP expression, but not ATF4 expression (Figure 5G). Treatment with 25-hydroxycholesterol also induced mineralization and expression of ATF4 and CHOP. Similar to 7-ketocholesterol, ATF4 and CHOP knockdown both attenuated mineralization and ER stress induced by 25-hydroxycholesterol treatment (Figure S2).

To obtain direct evidence that the ER stress-mediated induction of CHOP plays a causative role in CKD-dependent cardiovascular diseases in vivo, we compared the development of CKD-dependent cardiovascular diseases in CHOP^{-/-}; ApoE^{-/-} mice to CHOP^{+/+}; ApoE^{-/-} mice. 5/6 nx increased serum levels of total cholesterol, phosphorus, blood urea nitrogen, and calcium, which were not affected by CHOP deficiency (Table S3). Atherosclerotic lesions in CHOP^{-/-}; ApoE^{-/-} mice were quantified by aortic en face analysis. Quantification of the en face analysis showed that atherosclerosis developed in ApoE^{-/-} mice with sham operation, and was accelerated by 5/6 nx. Consistent with previous

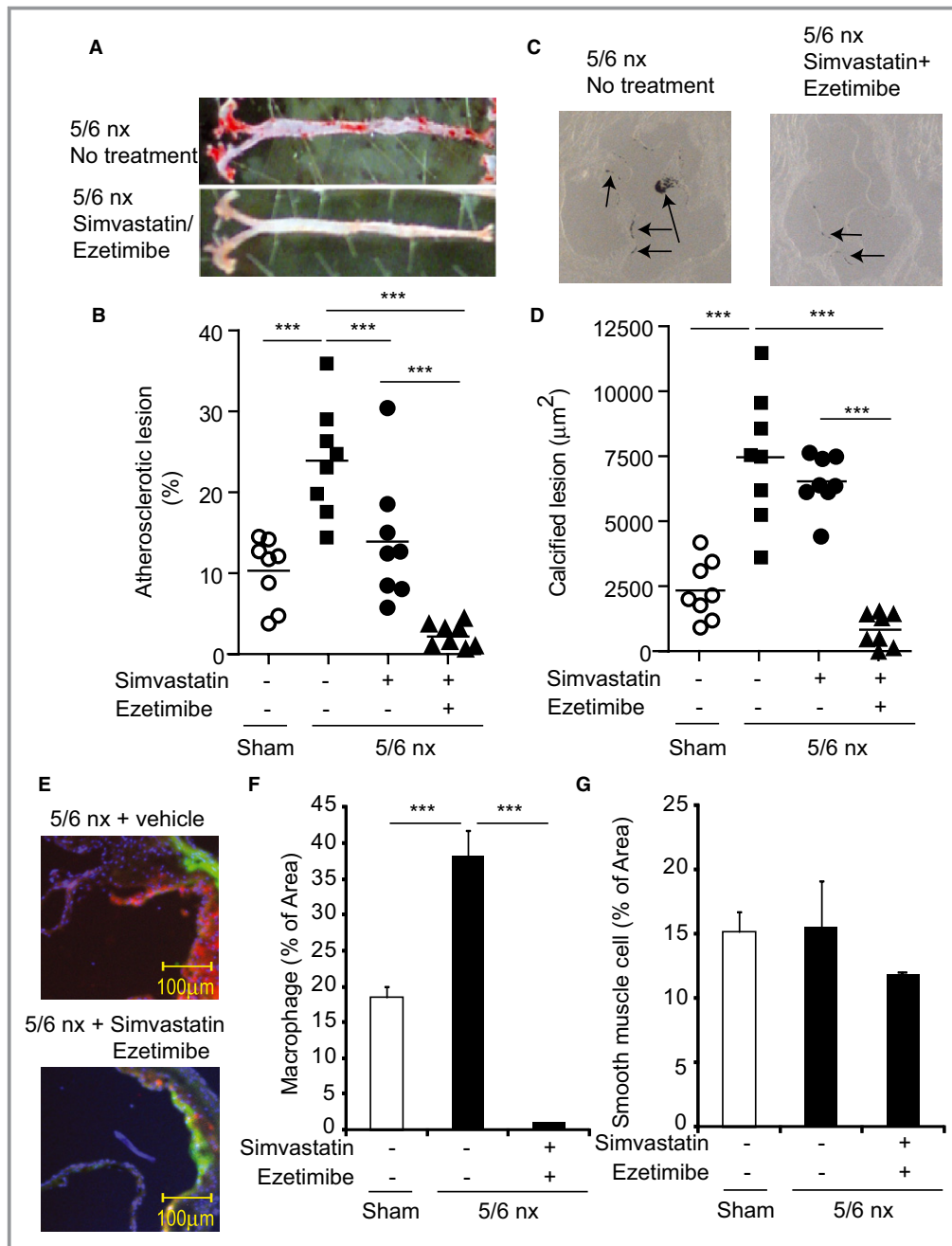


Figure 2. Treatment with simvastatin plus ezetimibe profoundly attenuates CKD-dependent atherosclerosis and vascular calcification. **A**, Representative pictures of aortic en face analysis with Sudan IV staining in 5/6 nx ApoE^{-/-} mice treated with either vehicle or simvastatin plus ezetimibe. **B**, Quantitative analysis of atherosclerotic lesions. **C**, Representative pictures of histological analysis with von Kossa staining in the aortic sinuses of 5/6 nx ApoE^{-/-} mice treated with either vehicle or simvastatin plus ezetimibe. Black arrows indicate calcified lesions. **D**, Quantitative analysis of calcified lesions in the aortic sinus. Sham-operated ApoE^{-/-} mice treated with vehicle (open circle), 5/6 nx ApoE^{-/-} mice treated with vehicle (closed square), 5/6 nx ApoE^{-/-} mice treated with simvastatin (closed circle) and 5/6 nx ApoE^{-/-} mice treated with simvastatin plus ezetimibe (closed triangle). **E**, Representative pictures of immunofluorescence analysis in the aortic sinuses of 5/6 nx ApoE^{-/-} mice treated with either vehicle or simvastatin plus ezetimibe. CD68-positive macrophages (red), α -smooth muscle cell actin (α SMA)-positive smooth muscle cells (SMC, green) and 4',6-diamidino-2-phenylindole (DAPI, blue). **F**, Quantification of macrophage content in the aortic sinus. **G**, Quantification of SMC content in the aortic sinus. 5/6 nx ApoE^{-/-} mice were treated with either 20 mg/kg simvastatin alone or 20 mg/kg simvastatin plus 10 mg/kg ezetimibe for 12 weeks. N=8, ***P<0.001. CKD indicates chronic kidney disease.

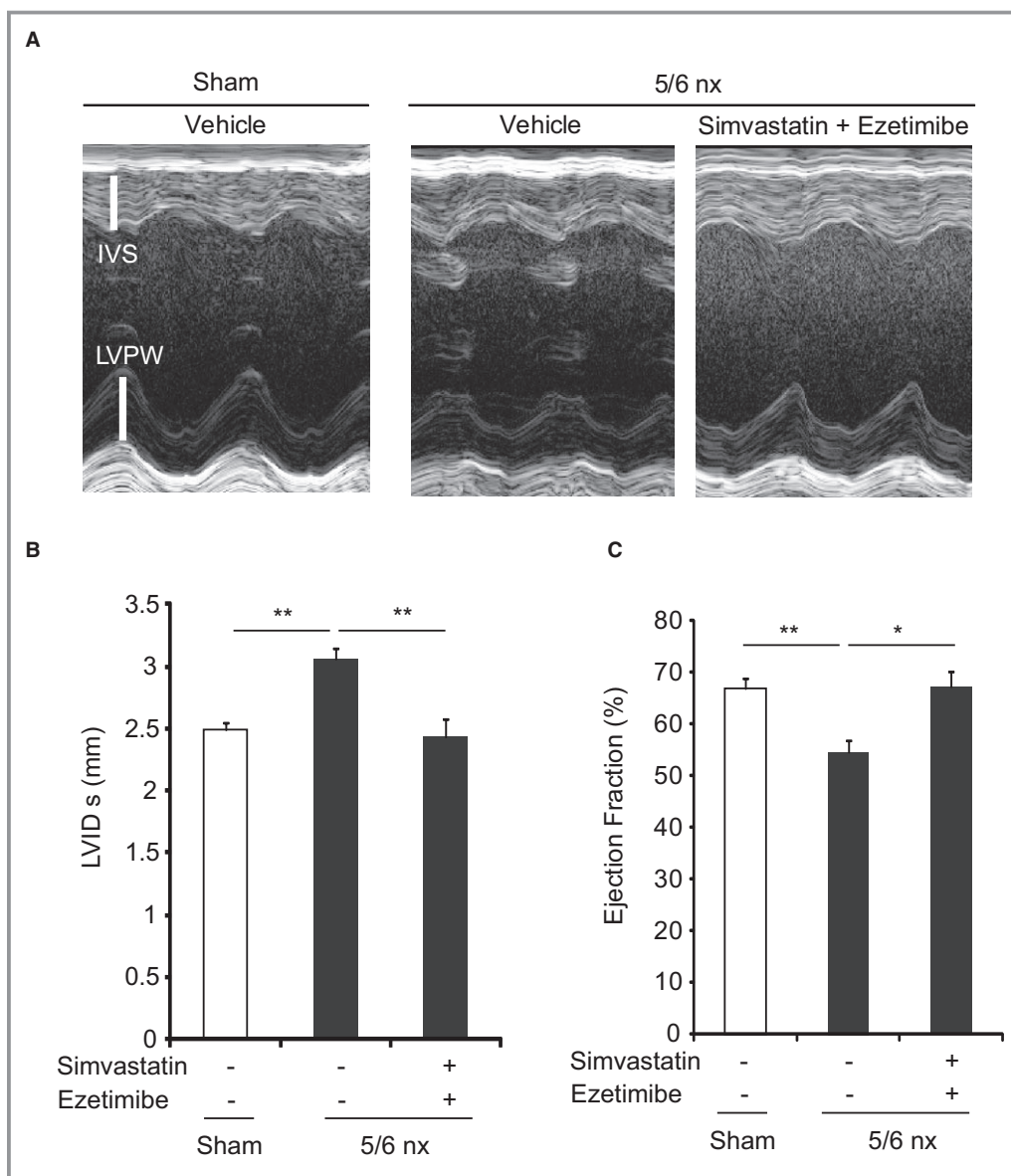


Figure 3. Treatment with simvastatin plus ezetimibe rescues CKD-dependent cardiac dysfunctions. A, Representative pictures of echocardiographic analysis in 5/6 nx ApoE^{-/-} mice treated with simvastatin plus ezetimibe. IVS, interventricular septum; LVPW, LV posterior wall. B, Left ventricular internal dimension at end systole (LVIDs), and C, Ejection fraction (EF) rate. Echocardiograms were performed at 10 weeks of the treatment with simvastatin plus ezetimibe. N=8, *P<0.05 and **P<0.01. CKD indicates chronic kidney disease.

reports, CHOP deficiency attenuated atherosclerosis in ApoE^{-/-} mice with normal kidney function (data not shown). Unexpectedly, however, CHOP deficiency had no effect on atherosclerotic plaque formation in ApoE^{-/-} mice in the presence of CKD (Figure 6A). In order to examine the effectiveness of CHOP deficiency on aortic calcification, aortic sinus lesions from 5/6 nx CHOP^{-/-};ApoE^{-/-} mice were analyzed with von Kossa staining. Quantification of calcified lesions revealed a significant reduction in calcified lesion areas in both the intimal and medial lesions of 5/6 nx

CHOP^{-/-};ApoE^{-/-} mice compared with 5/6 nx CHOP^{+/+}; ApoE^{-/-} littermates (Figures 6B and S4A). Immunofluorescence analysis showed that CHOP deficiency did not affect macrophage and SMC content in the aortic sinus (Figure S4B through S4E). TUNEL staining showed that CHOP deficiency drastically reduced aortic cell death induced by CKD (Figure 6C). In addition, we examined whether CHOP deficiency improves CKD-dependent cardiac dysfunction using echocardiography. CHOP deficiency improved LV systolic function of ApoE^{-/-} mice with 5/6 nx, and also reduced

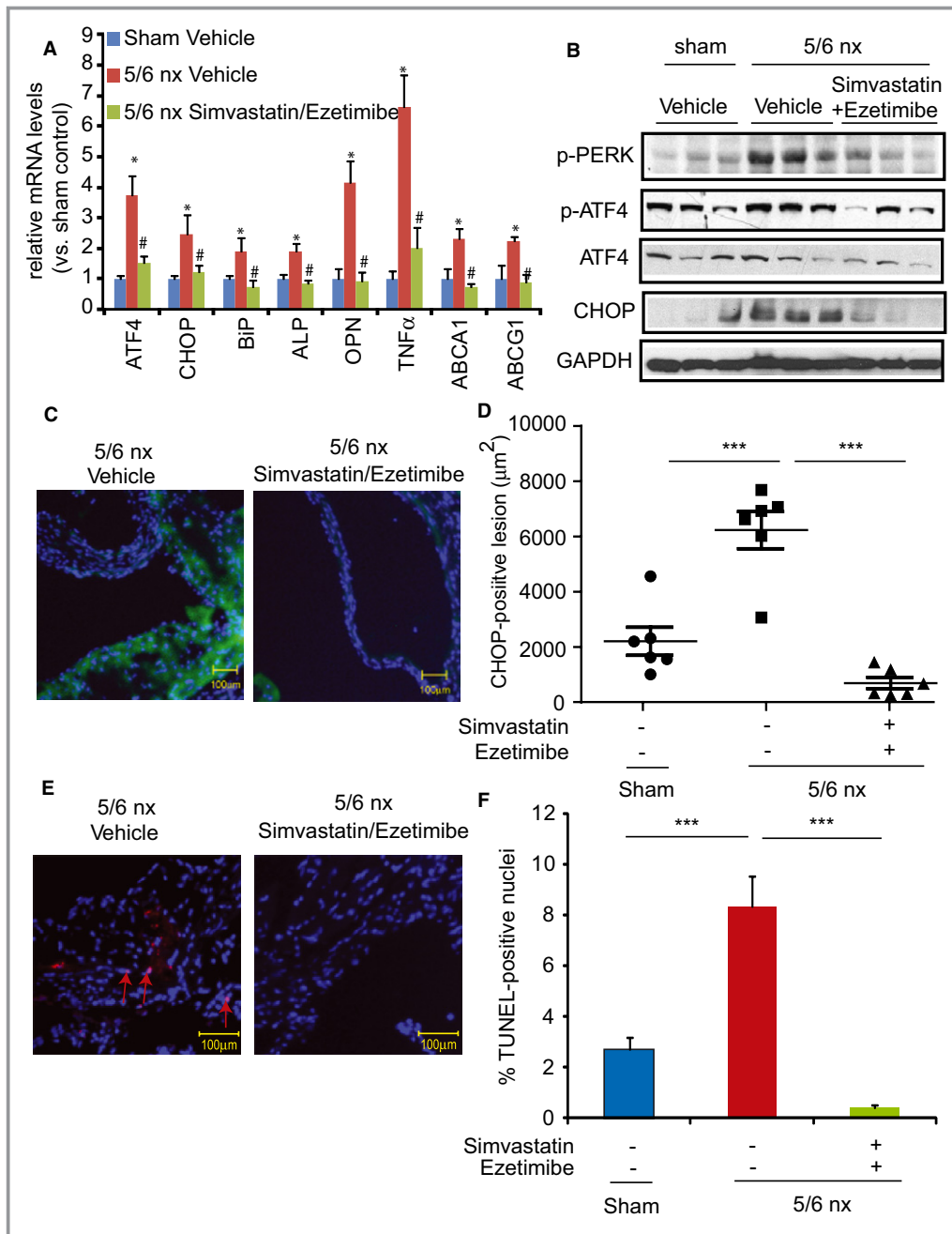


Figure 4. Treatment with simvastatin plus ezetimibe reduces endoplasmic reticulum stress and cell death induced by CKD. A, Aortic mRNA levels and B, aortic, p-PERK, ATF4, and CHOP protein expression in 5/6 nx ApoE $^{-/-}$ mice treated with simvastatin plus ezetimibe. C, Representative micrographs show less CHOP-positive signal (green) in nuclei (blue) of aortic sinus lesions from 5/6 nx ApoE $^{-/-}$ mice treated with simvastatin plus ezetimibe. D, Immunofluorescence detection of CHOP in the aortic sinuses of 5/6 nx ApoE $^{-/-}$ mice treated with simvastatin plus ezetimibe. E, Representative micrographs show less TUNEL-positive signal (red, arrows) in nuclei (blue) of aortic sinus lesions from 5/6 nx ApoE $^{-/-}$ mice treated with simvastatin plus ezetimibe. F, Quantitative analysis of TUNEL-positive nuclei conducted on lesions from 5/6 nx ApoE $^{-/-}$ mice treated with either vehicle or simvastatin plus ezetimibe. 5/6 nx ApoE $^{-/-}$ mice were treated with either vehicle or 20 mg/kg simvastatin plus 10 mg/kg ezetimibe for 12 weeks. N=6, * $P < 0.05$ vs sham-operated mice with vehicle. # $P < 0.05$ vs 5/6 nx ApoE $^{-/-}$ mice treated with vehicle. ALP indicates alkaline phosphatase; ATF4, activating transcription factor 4; BiP, binding immunoglobulin protein; CKD, chronic kidney disease; CHOP, C/EBP homology protein; OPN, osteopontin; p-PERK, phospho protein kinase-like endoplasmic reticulum kinase; TNF, tumor necrosis factor; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling. *** $P < 0.001$.

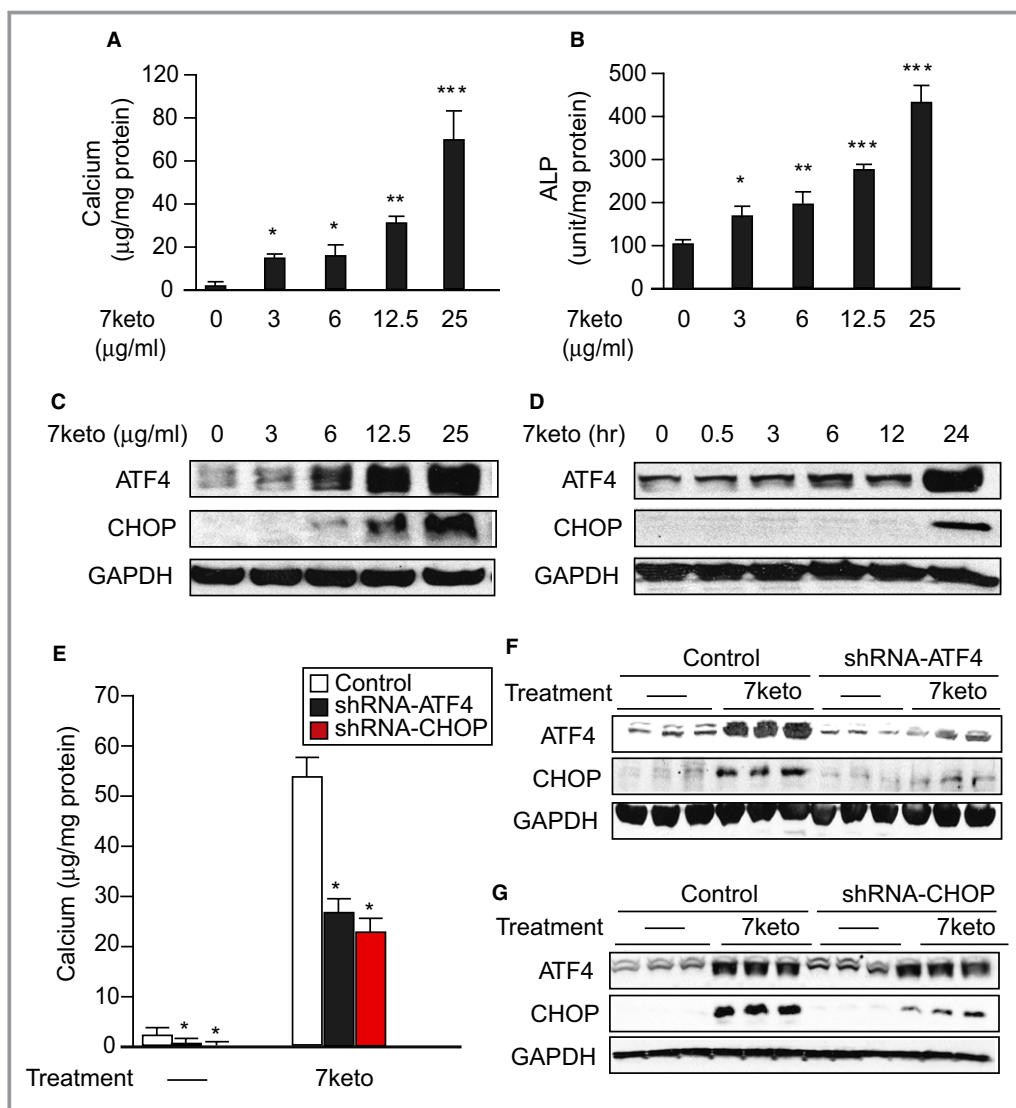


Figure 5. 7-Ketocholesterol induces mineralization through ER stress response in MOVAS-1 cells. A, Mineralization and (B) ALP activity of MOVAS-1 cells treated with different concentrations of 7-ketocholesterol (0, 3, 6, 12.5, and 25 µg/mL) for 7 days. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs no treatment. C, Dose-dependent effect of 7-ketocholesterol on ATF4 and CHOP expression. MOVAS-1 cells were treated with different concentrations of 7-ketocholesterol (0, 3, 6, 12.5, and 25 µg/mL) for 24 hours. GAPDH was used as a loading control. D, Time-dependent effect of 7-ketocholesterol on ATF4 and CHOP expression. MOVAS-1 cells were treated with 2.5 µg/mL 7-ketocholesterol for the indicated time periods (0, 0.5, 3, 6, 12, and 24 hours). E, Mineralization of ATF4- or CHOP-knockdown MOVAS-1 cells treated with 7-ketocholesterol (2.5 µg/mL) for 7 days. F and G, ATF4 and CHOP protein expression in ATF4- and CHOP-knockdown MOVAS-1 cells treated with 7-ketocholesterol (2.5 µg/mL) for 24 hours. * $P < 0.05$ vs control MOVAS-1 cells. ALP indicates alkaline phosphatase; ATF4, activating transcription factor 4; CHOP, C/EBP homology protein; ER, endoplasmic reticulum; MOVAS, mouse vascular smooth-muscle cell line.

pathological LV dilation (Figure 6D and 6E). Quantitative polymerase chain reaction and immunoblotting analyses showed that Pit-1, but not Pit-2, was significantly reduced in the aortas of 5/6 nx CHOP^{-/-};ApoE^{-/-} mice (Figures 6F, 6G and S3). In addition, CHOP deficiency reduced the expression of inflammatory markers including tumor necrosis factor- α (TNF α) and osteopontin (Figure S3).

Discussion

In agreement with the results of the Study of Heart and Renal Protection,¹ extensive cholesterol-lowering therapy through the combination of simvastatin and ezetimibe significantly alleviated CKD-dependent cardiovascular dysfunctions including atherosclerosis, vascular calcification, and systolic cardiac

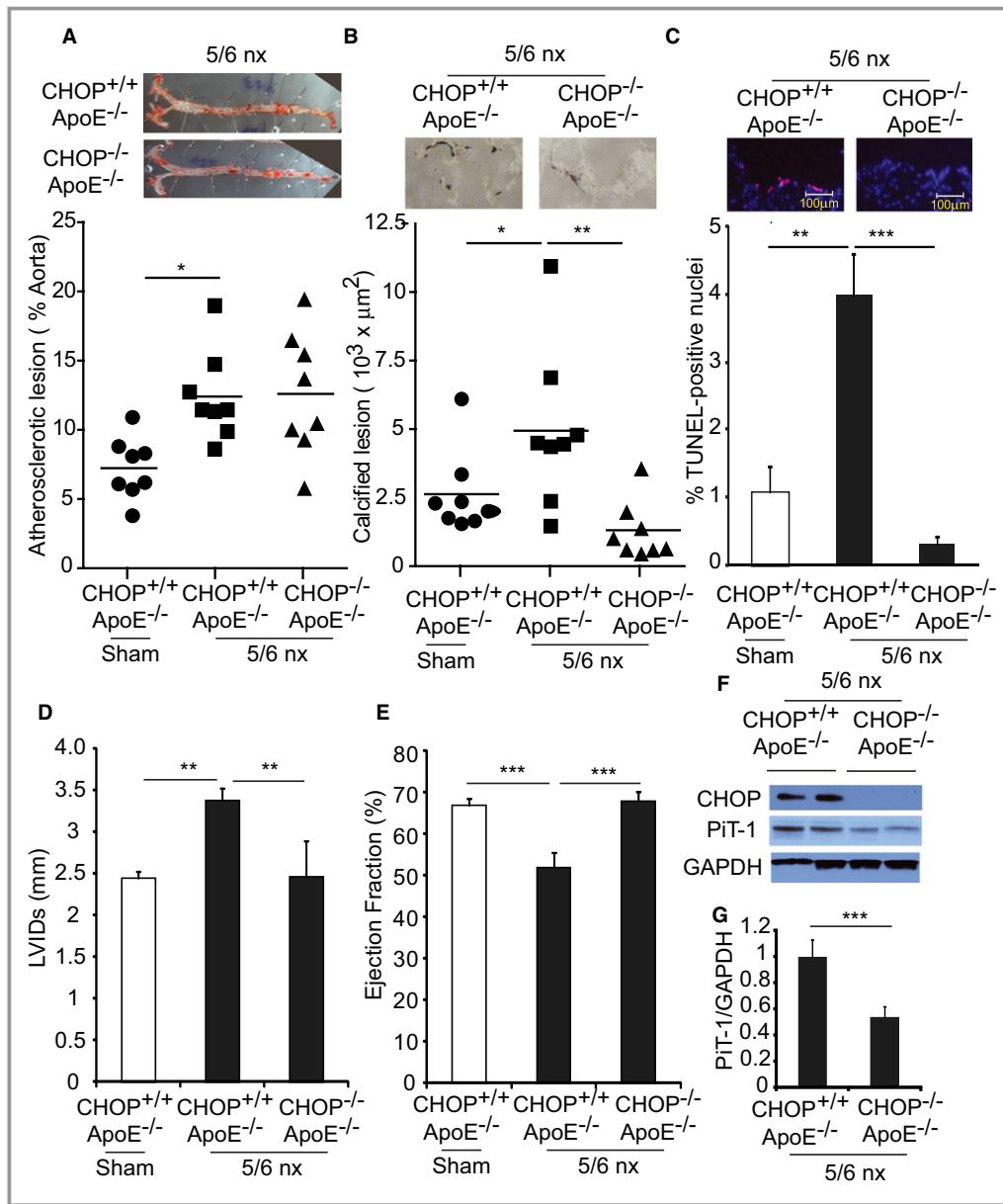


Figure 6. CHOP deficiency attenuates CKD-dependent cell death and vascular calcification but not atherosclerosis. A, Atherosclerotic lesions determined by aortic en face analysis. B, Vascular calcification in the aortic sinuses of 5/6 nx CHOP^{-/-};ApoE^{-/-} mice determined by von Kossa staining. C, Cell death in the aortic sinuses determined by immunofluorescence-based TUNEL assay. D, Left ventricular internal dimension at end systole (LVIDs) and E, ejection fraction rate. F, The representative pictures and G, the densitometry analysis of immunoblotting in the aortas of 5/6 nx CHOP^{-/-};ApoE^{-/-} mice. CHOP^{+/+}; ApoE^{-/-} and CHOP^{-/-};ApoE^{-/-} littermates were fed a Western diet for 12 weeks after the nx or sham operations. Echocardiograms were performed at 10 weeks of the feeding. N=8, *P<0.05, **P<0.01 and ***P<0.001. CHOP indicates C/EBP homology protein; CKD, chronic kidney disease; PiT, sodium-dependent phosphate co-transporter; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling.

function in ApoE^{-/-} mice with CKD. In addition, we found that CKD in humans and mice leads to a significant increase in levels of circulating oxysterols such as 7-ketocholesterol. Increased oxysterols in CKD were significantly reduced by treatment with a combination of simvastatin and ezetimibe. The reduction of oxysterols was highly associated with the

attenuation of CKD-dependent vascular diseases. Furthermore, treatment with the combination of simvastatin and ezetimibe reduced aortic ER stress. Several recent studies suggest that prolonged activation of aortic ER stress plays a significant role in the development of atherosclerosis and vascular cell death.¹⁶⁻¹⁸ In particular, CHOP-mediated cell

death is most widely characterized in atherosclerosis and is a crucial contributor to the development of advanced atherosclerotic lesions.¹⁶ In addition, recent evidence from our laboratory and others indicates that an enhanced ER stress signal also contributes to vascular calcification.^{21–25} Increased levels of free sterols such as 7-ketocholesterol and 25-hydroxycholesterol are considered to be a major cause of ER stress in advanced atherosclerotic lesions. However, whether oxysterol-mediated ER stress is involved in CKD-dependent cardiovascular diseases has not been studied. In this study, we demonstrated for the first time that ER stress-mediated CHOP induction contributes to CKD-dependent vascular calcification. Among the three branches of ER stress signaling, we focused on the PERK-eukaryotic initiation factor 2 α -ATF4-CHOP branch because both ATF4 and CHOP are crucial regulators of osteoblast differentiation.^{33–35} We previously showed that ER stress-induced ATF4 and CHOP mediate mineralization and differentiation of VSMCs induced by other factors such as TNF α , inorganic phosphate, and stearate.^{21,22} Similar to previous reports, the treatment of VSMCs with 7-ketocholesterol and 25-hydroxycholesterol induced ER stress, mineralization, and osteogenic differentiation. In this study, we found that shRNA-mediated knockdowns of ATF4 and CHOP significantly attenuate oxysterol-induced mineralization of VSMCs. In addition, CHOP deficiency attenuated CKD-dependent vascular calcification in ApoE^{-/-} mice. These data demonstrate that ER stress-mediated induction of CHOP plays a causative role in vascular calcification in CKD.

Type III sodium-dependent phosphate transports (PiT-1 and PiT-2) are major phosphate transporters of VSMCs and play causative roles in the pathogenesis of CKD-dependent vascular calcification.^{36–39} We previously reported that CHOP induces PiT-1 expression, resulting in a significant increase of inorganic phosphate uptake of VSMCs.^{21,22} Consistently, PiT-1 expression was reduced in the aortas of CHOP^{-/-};ApoE^{-/-} mice. Our results suggest that increased phosphate uptake of VSMCs by the induction of PiT-1 contributes to the mechanism by which CHOP deficiency attenuates CKD-dependent vascular calcification. There are several other potential mechanisms by which CHOP contributes to CKD-dependent vascular calcification. CHOP deficiency reduced levels of osteogenic markers such as alkaline phosphatase and osteopontin as well as the inflammatory marker TNF α . Treatment with a TNF α monoclonal antibody attenuated CKD-dependent vascular calcification.²¹ In addition, CHOP deficiency significantly reduced vascular apoptosis, which is considered to be a key event in CKD-dependent vascular calcification.^{39,40} Although it is not known whether CHOP directly alters expression of genes in these pathways, increased expression of genes in osteogenesis, inflammation, and apoptosis may also contribute to the procalcific effect of CHOP.

Consistent with previous reports,^{21,22} we confirmed that CHOP deficiency attenuates atherosclerosis in ApoE^{-/-} mice with normal kidney function. However, CHOP deficiency did not affect atherosclerosis in ApoE^{-/-} mice with CKD. These data suggest that CHOP inhibition alone is not enough to inhibit CKD-dependent atherosclerosis. Other deleterious ER stress components such as ATF4 and the IRE1 α -NF κ B axis of the ER stress signal may be more important in the development of CKD-dependent atherosclerosis.

In our mouse model, the combination of simvastatin and ezetimibe improved CKD-dependent cardiovascular diseases more effectively than simvastatin alone. Similar to our current study, a previous report from Massy's group showed that simvastatin treatment alone had a minimal effect on CKD-dependent vascular diseases in a similar uremic ApoE^{-/-} mouse model.¹ However, Massy's study found that treatment of uremic ApoE^{-/-} mice with simvastatin significantly reduced vascular calcification but not atherosclerosis or serum cholesterol levels.¹ Our current study found that simvastatin treatment significantly reduced CKD-induced atherosclerosis and serum cholesterol levels, but not vascular calcification. The differences between the two studies are possibly explained as follows: (1) the dose of simvastatin (100 to 200 mg/kg versus 20 mg/kg); (2) the method of the treatment (oral gavage versus dietary); and (3) experimental diet (standard chow versus Western diet). Nevertheless, the combination of simvastatin plus ezetimibe is more effective in the attenuation of CKD-dependent vascular diseases than simvastatin alone. This observation regarding vascular calcification agrees with our previous *in vitro* study using LDLR-deficient VSMCs treated with another statin drug, mevastatin.⁴¹ Mevastatin treatment alone did not inhibit mineralization and osteogenic differentiation of VSMCs induced by a protein kinase A activator, whereas mevastatin treatment coupled with cholesterol-uptake blockage in VSMCs significantly reduced vascular mineralization.⁴¹ These results suggest that a simultaneous inhibition of endogenous cholesterologenesis and cholesterol uptake of VSMCs is required for the inhibition of vascular calcification. The combination of simvastatin and ezetimibe, but not simvastatin alone, creates a similar condition *in vivo*. This treatment causes a profound reduction of LDL-cholesterol and oxysterol levels as well as the inhibition of hydroxymethyl glutaryl coenzyme A reductase in VSMCs, leading to a significant inhibition of vascular calcification. In addition, we previously reported that the activation of liver X receptor (LXR) induced mineralization and osteogenic differentiation of VSMCs.^{23,41} Since oxysterols are endogenous ligands of LXR, another possible mechanism of the anticalcific effect of the cholesterol-lowering therapy is a significant reduction of LXR activity by reducing levels of oxysterols. mRNA levels of ABCA1 and ABCG1, major targets of LXR, were consistently reduced in

the aortas of 5/6 nx ApoE^{-/-} mice treated with the combination of simvastatin and ezetimibe (Figure 4).

These findings provide a mechanistic insight into how this extensive cholesterol-lowering therapy attenuates CKD-dependent vascular diseases. The combination of simvastatin and ezetimibe reduces levels of oxysterols as well as other lipids including LDL-cholesterol and triglycerides, resulting in a significant reduction of aortic ER stress, which leads to the attenuation of CKD-dependent vascular diseases. In addition, the results of this study, combined with our previous recent findings that show the beneficial effect of ER chemical chaperones such as 4-phenylbutyric acid and tauroursodeoxycholic acid²¹ on vascular diseases, suggest that the ER stress-mediated CHOP may be a potential therapeutic target for CKD-dependent vascular calcification.

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Disclosures

None.

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