Free Deoxycholic Acid Exacerbates Vascular Calcification in CKD through ER Stress-Mediated ATF4 Activation

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Key Points
- CKD increases levels of DCA, which induces endoplasmic reticulum stress in vascular smooth muscle cells and vascular calcification.
- Inhibition of FXR selectively increases DCA, resulting in severe vascular calcification.
- Inhibition of ATF4 in vascular smooth muscle cells blocks DCA-induced vascular calcification in vitro and in vivo.

Abstract
Background Our metabolome approach found that levels of circulating, free deoxycholic acid (DCA) is associated with the severity of vascular calcification in patients with CKD. However, it is not known whether DCA directly causes vascular calcification in CKD.

Methods Using various chemicals and animal and cell culture models, we investigated whether the modulation of DCA levels influences vascular calcification in CKD.

Results CKD increased levels of DCA in mice and humans by decreasing urinary DCA excretion. Treatment of cultured VSMCs with DCA but no other bile acids (BAs) induced vascular calcification and osteogenic differentiation through endoplasmic reticulum (ER) stress–mediated activating transcription factor-4 (ATF4) activation. Treatment of mice with Farnesoid X receptor (FXR)–specific agonists selectively reduced levels of circulating cholic acid–derived BAs, such as DCA, protecting from CKD-dependent medial calcification and atherosclerotic calcification. Reciprocal FXR deficiency and DCA treatment induced vascular calcification by increasing levels of circulating DCA and activating the ER stress response.

Conclusions This study demonstrates that DCA plays a causative role in regulating CKD-dependent vascular diseases through ER stress–mediated ATF4 activation.

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Introduction
Cardiovascular disease (CVD) is a major cause of mortality in individuals with CKD (1). Patients with CKD have a >30-fold higher risk of developing CVD. More than 80% of men and 50% of women with CKD develop vascular calcification, leading to death (2–4). There are two types of vascular calcification: (1) intimal calcification, in which calcified lesions are observed in atherosclerotic lesions; and (2) medial calcification, in which calcified lesions are observed in the aortic media. Intimal calcification progresses with the CKD stage, whereas medial calcification appears from CKD stage 3 and progresses at CKD stages 4–5 and further on dialysis (5–7). However, the cellular and molecular mechanisms by which CKD accelerates vascular calcification are still poorly understood. In addition, no effective therapy for this deleterious vascular disease is currently available.

Bile acids (BAs) are known to facilitate absorption of lipids in the small intestine and to regulate cholesterol homeostasis (8–10). However, it has become clear that BAs are not simply digestive detergents and the primary route governing cholesterol catabolism. BAs are now recognized as signaling molecules contributing to the regulation of various metabolic processes and diseases (8,9). Cholic acid (CA) and chenodeoxycholic acid (CDCA) are the primary BAs formed in humans (10). CA is synthesized in hepatocytes through the reaction of a branch point enzyme, CYP8B1 (11,12). The synthesis of CDCA requires the reaction of CYP27A1 (13,14). CA and deoxycholic acid (DCA) are converted to the secondary BAs DCA and lithocholic acid (LCA), respectively, in the intestine by gut microbacteria (11,12,15). Farnesoid X receptor (FXR) is a nuclear receptor that is activated by BAs

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and regulates BA and lipid homeostasis. FXR is highly expressed in the liver and intestine, where BAs are generated and modified (8,15,16). CDCA was identified as the most potent FXR ligand, whereas secondary BAs (DCA and LCA) are preferential ligands for the G-protein BA receptor 1 (17,18). Increased levels of BAs are a risk factor for a number of chronic diseases, such as cancers, diabetes, steatohepatitis, and atherosclerosis. Our previous study showed that FXR activation inhibited intimal calcification induced by CKD in ApoE knockout (KO) mice (19). Activation of FXR preferentially reduces CA-derived BAs, such as DCA, more than other BAs, such as CDCA and LCA (20–22). Recently, our lipidomic analysis identified that levels of DCA were associated with coronary calcification volume in patients with stage 3 and 4 CKD (23). These data suggest DCA may play a causative role in the pathogenesis of vascular calcification in CKD.

In this study, we examine (1) whether CKD affects levels of circulating DCA, (2) whether DCA has a direct role in the pathogenesis of vascular calcification in CKD, (3) whether FXR could be a therapeutic target for medial calcification in CKD by reducing DCA, and (4) the molecular mechanism by which DCA induces vascular calcification.

Materials and Methods

Animals
SM-ATF4 KO, FXR KO, FXR KO; LDLR KO double KO, and CKD mice were generated as we previously described (22,24,25). Five-week-old animals were subjected to 5/6 nephrectomy to induce CKD-dependent vascular calcification, as previously described (19,24–27). CKD mice were fed a special diet (TD10364) containing INT-747 (10 mg/kg body wt) and P20606 (5 mg/kg body wt); CKD animals consumed 3 g of diet per day (19,21,22). One week after surgery, SMC-ATF4 KO mice were injected intraperitoneally with tamoxifen (1 mg/kg body wt) in vegetable oil. Animals were maintained on a special diet (TD10364) until euthanasia (28). Urine was collected in a metabolic cage 24 hours after 3H-DCA (30 mg/kg body wt; Cambridge Isotope Laboratories) was intraperitoneally injected into the mice. All mouse strains were backcrossed at least ten times with appropriate genetic backgrounds, as described in the figure legends. The genetic backgrounds were checked using a short tandem repeats–based screening at the BioResources Core Facility Molecular Biology Unit on the University of Colorado–Anschutz Medical Campus (AMC) campus. Animal experiments were approved by the University of Colorado-AMC Institutional Animal Care and Research Advisory Committee.

Human Subjects
Serum samples were collected from ten patients with stage 3 and 4 CKD (mean±SD eGFR of 34±7 ml/min per 1.73 m²) and a mean±SD age of 57±7 years, and ten age-matched patients with normal kidney function (mean±SD eGFR of 80±12 ml/min per 1.73 m²) and a mean±SD age of 59±14 years after a 4 hour–minimum fast. Trained personnel at the University of Colorado Clinical and Translational Research Center collected all serum samples. All patients gave informed consent for their blood specimens 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. GFR was estimated using the four-variable Modification of Diet in Renal Disease prediction equation. All participants were enrolled in a separate clinical research study and the procedures were approved by the Colorado Multiple Institutional Review Board or the University of Colorado Boulder Institutional Review Board. The nature, benefits, and risks of the study were explained to the patients, and their written informed consent was obtained before participation.

Histologic Analysis
Calculated lesions in the aortic sinus were analyzed, as previously described, using Alizarin-Red staining (24–27). We performed en face analysis of the aortas using Sudan IV, as we previously described (19,22). Apoptotic cells in the aortas were detected using an In Situ Cell Death Detection Kit (Roche). At least five sections from each sample were analyzed (25,27,29).

Cell Cultures
Human vascular smooth muscle cells (VSMCs) and mouse VSMCs were purchased from Applied Biologic Materials (T0515; Richmond, Canada) and American Type Culture Collection (CRL2797; Manassas, VA), respectively. ATF4 stable knockdown cells were previously generated using a lentiviral short hairpin RNA, ATF4 short hairpin RNA (TRCN0000071723; Thermo Fisher) (28). VSMCs were maintained in DMEM containing 10% FBS with 100 U/ml penicillin and 100 μg/ml streptomycin. VSMCs were treated with complete media (with FBS and amino acids) and 12.5–200 μM BAs (24,25,28).

Calcium Content in Cultured Cells and Aortas
For evaluation of DCA-induced vascular calcification, VSMCs were plated at 1.0×10^5 cells per well in a 12-well plate and grown overnight. VSMCs were treated with BSA (vehicle) and 10–200 μM BAs every 2 days for 6 days. VSMCs were incubated with 0.6 N hydrochloric acid (HCl) overnight at 4°C. After incubation, 0.6 N HCl was collected to measure calcium content, and then VSMCs were lysed with 0.1 N sodium hydroxide/0.1% SDS to measure protein concentration using a bicinchoninic acid assay. Aortas were collected from mice and stored at −20°C. Dried aorta was defatted with chloroform and methanol (2:1) for 48 hours, and dehydrated with acetone for 3 hours. The dried samples were incinerated to ashes at 600°C for 24 hours using an electric muffle furnace (Thermo Scientific), and then extracted with 0.6 N HCl. Calcium content from cultured cells and aortas was quantified using the e-cresolphthalein method. In addition, VSMCs were stained with Alizarin Red to identify calcium deposits 6 days after inorganic phosphate treatment (24,25,28).

RNA Analysis
Total RNA was isolated using a Direct-zol RNA kit (Zymo Research). cDNA was synthesized from 500 ng total RNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biologic Materials Inc). Quantitative RT-PCR was performed using an Applied Biosystems StepOne Plus.
qPCR instrument with SYBR Select Master Mix according to the manufacturer’s instructions (24,25,28).

**Immunoblot Analysis**

Cell and tissue lysates were prepared using radioimmunoprecipitation assay buffer (150 mM sodium chloride, 1% nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0). Cells were disrupted by pipetting ten to 15 times, then centrifuged at 13,800 × g for 10 minutes at 4°C, and the supernatant was collected for total cell lysates. The samples were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted with the following antibodies: PKR-like endoplasmic reticulum kinase (p-PERK, 16F8), ATF4 (D4B8), p-Eukaryotic translation initiation factor 2alpha (p-eIF2alpha, D9G8), and C/EBP homologous protein (CHOP, L63F7) from Cell Signaling Technology; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, V-18) from Santa Cruz Biotechnology; and β-actin (66009) from Proteintech. Samples were visualized using horseradish peroxidase coupled to appropriate secondary antibodies with enhancement by an enhanced chemiluminescence detection kit (Thermo Fisher Scientific) (24,25,28).

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**Figure 1.** CKD increases levels of serum deoxycholic acid (DCA), inducing vascular calcification. Levels of bile acids (BAs) in the serum of (A) age-matched patients with stage 3 CKD and normal kidney function (NKD), (B) LDLR knockout (KO) mice with CKD, and (C) DBA/2J mice with CKD. Eight-week-old mice were subjected to sham operation or 5/6 nephrectomy. CKD mice were maintained for 16 weeks. (D) Urinary DCA excretion. Twenty-week-old CKD DBA/2J mice were intraperitoneally injected with 2H-DCA (30 mg/kg body wt). Urine and feces samples were collected after 24 hours in metabolic cages. Urinary 2H-DCA was analyzed with liquid chromatography–tandem mass spectrometry. (E) Alizarin-Red staining of vascular smooth muscle cells (VSMCs) treated with BAs. (F) Matrix calcium levels and (G) alkaline phosphatase (ALP) activity in human VSMCs. Human VSMCs were treated with 100 μM of various BAs (V, vehicle; 1, cholic acid; 2, DCA; 3, chenodeoxycholic acid; 4, ursodeoxycholic acid; 5, taurocholic acid; 6, taurine-conjugated DCA; 7, taurochenodeoxycholic acid; 8, taurosodeoxycholic acid; 9, β-muricholic acid; 10, lithocholic acid) for 7 days in the presence of 2.6 mM inorganic phosphate. (H) Dose response of DCA in human VSMCs. Human VSMCs were treated with 12.5–100 μM BAs for 7 days. *P<0.05, ***P<0.001.
BA Analysis

Serum BA levels were determined using an Applied Biosystems 3200 qTRAP LC-MS/MS instrument using 3- H-DCA as the internal standard, as previously described (20,22).

Statistical Analyses

Data were collected from more than two independent experiments and reported as the means±SEM. Statistical analysis for two-group comparisons was performed using the t test; for multigroup comparisons, the one-way ANOVA or two-way ANOVA with a Newman–Keuls post hoc test was used. Significance was accepted at P<0.05.

Results

CKD Increases Levels of DCA and Other BAs in Humans and Mice

Our recent lipidomic study identified that levels of serum DCA are associated with coronary artery calcification in patients with CKD (23). In this study, we examine whether CKD increases levels of DCA in humans and mice. As shown in Figure 1A, patients with stage 3 and 4 CKD had significantly higher levels of DCA than patients with normal kidney function. Levels of glycine-conjugated DCA, taurine-conjugated DCA (TDCA), glycine-conjugated CDCA, and taurine-conjugated CDCA were also increased (Supplemental Table 1). Levels of total BAs were also increased by CKD. Similarly to humans, 5/6 nephrectomy–induced CKD significantly increased levels of DCA in both LDLR KO and DBA/2J mice, which are mouse models of intimal calcification and medial calcification, respectively (Figure 1, B and C). CKD significantly and consistently increased levels of CA, DCA, and TDCA in both LDLR KO and DBA/2J mice (Supplemental Tables 2 and 3). To examine whether CKD reduces urinary excretion of BAs, we analyzed urinary DCA excretion using an intraperitoneal injection of 3- H-DCA. Figure 1D shows that CKD significantly reduced urinary 3- H-DCA excretion by 54%, whereas the fecal excretion of DCA was not affected (Supplemental Figure. 1A).

DCA Induces Mineralization and Osteogenic Differentiation of VSMCs through ATF4 Activation

Because serum levels of DCA are associated with the severity of coronary artery calcification in patients with CKD, and CKD increases levels of DCA in human and mouse models, we next examined whether DCA specifically induces vascular calcification using cultured VSMCs. Human VSMCs treated with ten major BAs were analyzed for mineralization and osteogenic differentiation. Alizarin-Red stain, calcium, and alkaline phosphatase (ALP) quantitative analyses showed that DCA but no other BAs strongly and dose dependently induced mineralization (Figure 1, E–G) and osteogenic differentiation (Figure 1H). We previously found that endoplasmic reticulum (ER) stress–mediated ATF4 activation is a major pathway that induces vascular calcification in CKD. Therefore, we examined whether DCA-mediated vascular mineralization and osteogenesis is associated with ER stress–mediated ATF4 activation. Treatment of VSMCs with DCA, but no other BAs, time and dose dependently induced mRNA and protein expression of ER stress markers such as p-PERK, p-eIF2α, ATF4, and CHOP (Figure 2, A–D). To demonstrate that DCA induced mineralization through ATF4 activation, we used ATF4 knockdown VSMCs that we previously generated (25,28). ATF4 inhibition significantly attenuated DCA-induced mineralization, osteogenic differentiation, and ER stress (Figure 2, E–I).

Treatment with FXR Agonists Blocks Intimal and Medial Calcification

We previously reported that a semisynthetic FXR agonist, INT-747, blocks CKD-induced intimal calcification in ApoE KO mice (19). In addition, the FXR agonist selectively reduced CA-derived BAs, such as DCA, over CDCA-derived BAs, such as LCA (20,22). To examine the effect of FXR activation on the prevention of vascular calcification, we examined whether treatment with another nonsteroidal potent FXR agonist, PX20606, prevents CKD-induced athersclerosis and intimal calcification using LDLR KO mice. CKD significantly increased levels of serum creatinine by 4.0-fold as an indicator of CKD, resulting in the induction of atherosclerotic formations (Figure 3, A and B, Supplemental Table 4). CKD-induced atherosclerotic formation was completely blocked by Px20606 treatment (Figure 3, A and B). Px20606 treatment significantly reduced levels of serum cholesterol and triglycerides but not calcium and phosphate (Supplemental Table 4). CKD also induced vascular calcification in LDLR KO mice, which was significantly blocked by Px20606 treatment (Figure 3, C–E). Px20606 completely reduced CYP8B1-derived BAs (such as CA and DCA) and their taurine-conjugated forms, but not CDCA and LCA (Figure 3F, Supplemental Table 5). We next examined whether FXR activation attenuates CKD-dependent medial calcification using DBA/2J mice, which are susceptible to CKD-induced medial calcification, as previously described (24). Treatment of mice with Px20606 and INT-747 strongly blocked medial calcification of CKD DBA/2J mice (Figure 3, G–I), similarly to the atherosclerotic calcification blocked in CKD LDLR KO mice.

These data suggest the anticalcific effect of Px20606 occurs independently of the antiatherosclerotic effect. We analyzed whether FXR activation affects levels of specific serum BAs in LDLR KO and DBA/2J mice under CKD. Treatment with Px20606 and INT-747 significantly reduced levels of CA and CA-derived BAs, such as DCA, whereas CDCA and CDCA metabolites, such as LCA, were increased in DBA/2J mice (Figure 3J, Supplemental Table 6). We next determined whether CKD and FXR activation selectively affect a hepatic CA-DCA synthesis enzyme, CYP8B1. CKD induced CYP8B1 expression, whereas treatment with Px20606 and INT-747 drastically reduced CYP8B1 expression (Supplemental Figure 1B). Whereas other direct FXR targets—such as small heterodimer partner (SHP), ATP-binding cassette sub-family G member 5 (ABCG5) and scavenger receptor class B type 1 (SR-B1)—were reduced in the livers of CKD mice, treatment of CKD mice with FXR agonists normalized expression of those FXR targets. Hepatic expression of CYP27A1, which is a critical enzyme in the synthesis of CDCA-LCA, was not affected by either CKD or FXR activation (Supplemental Figure 1, C–G).
Figure 2. DCA induces mineralization of VSMCs through endoplasmic reticulum (ER) stress–mediated ATF4 activation. (A) CHOP gene expression. VSMCs were treated with 100 μM BA (V, vehicle; 1, cholic acid; 2, DCA; 3, chenodeoxycholic acid; 4, ursodeoxycholic acid; 5, taurocholic acid; 6, taurine-conjugated DCA; 7, taurochenodeoxycholic acid; 8, tauroursodeoxycholic acid; 9, β-muricholic acid; 10, lithocholic acid) for 24 hours. (B) ATF4 and CHOP protein expression. VSMCs were treated with 100 μM BA for 24 hours. (C) Time course of DCA treatment in ER stress marker expression. VSMCs were treated with DCA for 0.5 or 4 hours. (D) Dose response of DCA in ER stress marker expression. VSMCs were treated with various concentrations of DCA for 24 hours. (E) Alizarin-Red stain in ATF4 knockdown VSMCs treated with DCA. Wild-type and ATF4 knockdown VSMCs were treated with 100 μM DCA for 7 days. (F) Matrix calcium content and (G) ALP activity treated with DCA. Wild-type and ATF4 knockdown VSMCs were treated with 100 μM DCA for 7 days. (H) ATF4 protein and (I) CHOP gene expression in ATF4 knockdown VSMCs treated with DCA. VSMCs were treated with 100 μM DCA for 24 hours. *P<0.05. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; shRNA, short hairpin RNA; veh, vehicle.
**Figure 3.** Farnesoid X receptor (FXR) activation reduces DCA, attenuating atherosclerosis and intimal and medial calcification in CKD. (A) Representative pictures of en face analysis using Sudan IV in CKD LDLR KO mice treated with Px20606. (B) Quantitative analysis of en face analysis in CKD LDLR KO mice treated with Px20606 (Px). (C) Representative pictures of aortic sinus stained with Von Kossa in CKD LDLR KO mice treated with Px20606. Arrows (black lesions) indicate calcification. (D) Quantitative analysis of calcified lesions in the aortic sinus in CKD LDLR KO mice treated with Px20606. (E) Aortic calcium content in CKD LDLR KO mice treated with Px20606. (F) Serum DCA levels of CKD LDLR−/− mice treated with Px20606. Eight-week-old male LDLR KO mice were subjected to sham operation or 5/6 nephrectomy. CKD mice were maintained on a Western diet containing Px20606 (5 mg/kg body wt) for 16 weeks. (G) Representative pictures of aortic sinus stained with Alizarin Red in CKD DBA/2J wild-type mice treated with Px20606. (H) Quantitative analysis of calcified lesions in the aortic sinus of CKD DBA/2J wild-type mice treated with Px20606. (I) Aortic calcium content in CKD DBA/2J mice treated with Px20606. (J) Serum DCA levels of CKD DBA/2J wild-type mice treated with Px20606. Eight-week-old male DBA/2J wild-type mice were subjected to sham operation or 5/6 nephrectomy. CKD mice were maintained on a Western diet containing Px20606 (5 mg/kg body wt) or INT-747 (INT; 10 mg/kg body wt) for 16 weeks. **P<0.01, ***P<0.001. NKD, normal kidney function; veh, vehicle.
FXR Deficiency Accelerates CKD-Dependent Atherosclerotic Calcification

To examine whether FXR deficiency alleviates CKD-dependent atherosclerotic vascular calcification in contrast to FXR activation, FXR; LDLR double KO (FXR KO; LDLR KO) mice were subjected to 5/6 nephrectomy. Under CKD, FXR deficiency increased levels of serum cholesterol and triglycerides but not phosphate or calcium (Supplemental Table 7). Under CKD, FXR deficiency significantly induced atherosclerotic formation in LDLR KO mice (Figure 4, A and B). The atherosclerotic, calcified lesions were significantly increased in FXR KO; LDLR KO mice than in LDLR single KO mice (Figure 4, C and D). Aortic calcium content was four-fold higher in FXR KO; LDLR KO mice (Figure 4E). Under CKD, FXR deficiency significantly increased levels of CA and DCA and their taurine-conjugated forms, but not CDCA and LCA in LDLR single KO mice (Figure 4F, Supplemental Table 8).

FXR Deficiency Induces Medial Calcification in FVB Mice

Because FXR affects both atherogenesis and vascular calcification of LDLR KO mice under CKD, the anticalcific effect of FXR occurs secondary to the antiatherogenesis effect. To examine whether FXR directly induces medial calcification under normal kidney function, we characterized FXR KO mice on two genetic backgrounds, C57BL/6 (B6) and FVB, which are resistant to vascular calcification (24,30). Although the phenotypes of FXR deficiency are similar between B6 and FVB backgrounds, the effects of

Figure 4. | FXR deficiency exacerbates atherosclerosis and vascular calcification in LDLR KO (LDLRKO) mice with CKD. (A) Representative pictures of en face analysis with Sudan IV stain in CKD FXR KO; LDLR KO mice. (B) Quantitative analysis of en face analysis in CKD FXR KO; LDLR KO mice. (C) Representative pictures of aortic sinus with Von Kossa stain in CKD FXR KO; LDLR KO mice. Arrows (black lesions) indicate calcification. (D) Quantitative analysis of calcified lesions in the aortic sinus of CKD FXR KO; LDLR KO mice. (E) Aortic calcium content in CKD FXR KO; LDLR KO mice. (F) Serum DCA levels of CKD FXR KO; LDLR KO mice. Eight-week-old male mice were subjected to sham operation or 5/6 nephrectomy. CKD mice were maintained on a Western diet for 16 weeks. **P<0.01, ***P<0.001.
FXR deficiency were more drastically enhanced in the FVB background than the B6 background (Figures 5 and 6, Supplemental Figures 2–4). FVB FXR KO mice had a significantly shorter life span than B6 FXR KO mice and wild-type mice (Figure 5A). When compared with B6 FXR KO mice, FVB FXR KO mice displayed more severe kidney and spleen enlargements, liver steatosis, and hyperlipidemia (Supplemental Figures 2 and 3). FXR deficiency did not affect levels of serum creatinine, glucose, calcium, or phosphate on either genetic background (Supplemental Figure 3). FVB FXR deficiency selectively increased levels of DCA compared with B6 FXR deficiency (Figure 5B, Supplemental Table 9). Levels of other BAs were comparable between B6 and FVB FXR KO mice (Supplemental Table 9). FXR deficiency drastically induced hepatic CYP8B1 but not CYP27A1, especially on the FVB background (Supplemental Figure 4). Alizarin-Red staining of the aortic sinus revealed that FVB FXR KO mice developed more severe medial calcification (Figure 5, C and D) than other groups. The ash assay and terminal deoxynucleotidyl transferase-mediated digoxigenin-deoxyuridine nick-end labeling assay also confirmed that FVB FXR KO mice had significantly higher aortic calcium content and levels of aortic cell death than other groups (Figure 5, E and F).

**DCA Treatment Induces Vascular Calcification through an ATF4-Dependent Pathway**

We next examined whether FVB FXR deficiency induces ER stress in vivo in aortic VSMCs by increasing DCA. Quantitative PCR and immunoblot analyses showed that FXR deficiency significantly increased levels of ER stress markers such as p-eIF2α, binding immunoglobulin protein

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**Figure 5.** FVB FXR KO mice display a shorter life span and more severe medial calcification. (A) Survival rate of FXR KO mice. Four-week-old animals (N=20, ten males and ten females) were maintained on a Western diet. (B) Levels of serum DCA in FXR KO mice. (C) Alizarin-Red stain of the aortic sinus of FXR KO mice. (D) Quantitative analysis of calcified lesions in the aortic sinus in FXR KO mice. (E) Aortic calcium content in FXR KO mice. (F) Aortic cell death in FXR KO mice. For biologic analyses, 8-week-old wild-type (WT) and FXR KO female mice were maintained on a Western diet for 16 weeks. *P<0.05, **P<0.01, ***P<0.001. B6, C57BL/6.
BiP, growth arrest and DNA damage gene-34 (GADD34), ATF4, sodium-dependent phosphate transporter-1 (PiT-1), spliced form of X-box binding protein (sXBP-1), and CHOP (Figure 6, A and B). To demonstrate that DCA-induced vascular calcification occurs through ATF4 activation, SMC-specific ATF4 KO (SM-ATF4KO) mice, which we previously generated, were backcrossed with FVB (25) and treated with a diet containing 0.3% DCA. DCA feeding significantly increased DCA levels by six-fold in both control and SM-ATF4KO mice (Figure 6C). However, DCA-induced vascular calcification was observed in control mice but not in SM-ATF4KO mice (Figure 6, D and E). DCA induced mRNA expression of the ATF4 targets CHOP and PiT1 in the aortic media of control mice, but not SMC-ATF4KO mice (Figure 6, F–H).

**Figure 6.** | ATF4 activation mediates DCA-induced vascular calcification. (A) mRNA and (B) protein expression of ER stress markers in the aortic media of FXR KO mice. Eight-week-old FVB wild-type (WT) and FVB FXR KO mice were maintained on a Western diet for 16 weeks. Medial VSMCs were isolated using a cell sorter. (C) Levels of serum DCA in wild-type and SM-ATF4 KO mice treated with DCA. (D) Alizarin-Red stain of the aortic sinus in wild-type and SM-ATF4KO mice treated with DCA. Arrows (red lesions) indicate calcification. (E) Quantitative analysis of calcified lesions in the aortic sinus of SM-ATF4 KO mice treated with DCA. (F) CHOP, (G) ALP, and (H) PiT-1 mRNA expression in the aortic media of SM-ATF4 KO mice treated with DCA. (C–H) For the DCA study, wild-type and SM-ATF4 KO mice were maintained on a Western diet containing 0.3% DCA for 8 months. *P<0.05, **P<0.01, ***P<0.001.
Discussion

Levels of circulating, free DCA are associated with the severity of vascular calcification in patients with CKD. In this study, we found that (1) CKD increases levels of serum DCA and other BAs in human and mice due to the reduction of urinary excretion; (2) DCA, but no other BAs, induces osteogenic differentiation and mineralization of VSMCs through ER stress–mediated ATF4 activation; (3) FXR activation attenuates atherosclerotic calcification and medial calcification in CKD by reducing circulating DCA; (4) FXR deficiency causes vascular calcification by increasing circulating DCA; and (5) DCA treatment in vivo induces vascular calcification through ER stress–mediated ATF4 activation. These data elucidate that DCA plays a causative role in the regulation of vascular calcification in CKD, and that FXR is a therapeutic target for treating CKD-dependent CVDs.

CKD increases levels of DCA in humans and mice. We believe the major mechanism underlying increased circulating DCA in CKD is a significant reduction in renal BA excretion. Interestingly, however, patients and mouse models with CKD have more significantly increased levels of CA-DCA over CDCA-LCA. We found that CKD clearly represses hepatic FXR pathways to induce the expression of hepatic CYP8B1, which is a bottleneck enzyme of the CA-DCA synthesis pathway. On the other hand, CYP27A1—an enzyme critical for CDCA synthesis but not an FXR target—was not affected by CKD. Mechanistically, it is well known that FXR induces the expression of SHP in the liver, which, in turn, inhibits liver homolog-1– or hepatocyte NF 4–induced gene transcription of enzymes for CA-DCA synthesis, such as CYP8B1 and CYP7A1 (11,31,32). In fact, CKD represses major hepatic FXR targets, such as SHP (33), ABCB4 (34), and SR-BI (35), which are induced by FXR activation. Accordingly, it is known that CKD represses the FXR pathway in the kidney. These data suggest that the selective increase in DCA by CKD occurs through a significant reduction of renal BA excretion and the induction of CYP8B1-mediated CA-DCA synthesis via hepatic FXR repression. Future studies will be required for elucidating the major tissues that contribute to the anticalcific effect of FXR agonists using FXR conditional KO mice.

This study elucidated the mechanism by which DCA induces vascular calcification through ER stress–mediated ATF4 activation. We previously reported that ATF4 mediates vascular calcification through increases in (1) phosphate uptake into VSMCs via PIT-1 induction; and (2) vascular apoptosis via a proapoptotic transcription factor, CHOP (25,27). In addition, other groups have reported that ALP is an ATF4 target that regulates vascular calcification (36). DCA, in fact, induces vascular ALP, PIT-1, and CHOP in an ATF4-dependent manner both in vivo and in cell cultures. In addition, in support of our conclusion, FXR KO mice had significantly higher ER stress levels, accompanied by higher levels of DCA and severe vascular calcification. Although free DCA strongly induces ER stress in VSMCs, TDC did not induce ER stress or mineralization of VSMCs. In addition, taurine-conjugated BAs, such as tauroursodeoxycholic acid, are known to work as a chemical chaperone to block ER stress (37). These data suggest that induction of taurine conjugation of DCA could be a target for blocking CKD-induced vascular ER stress and calcification.

Another interesting finding of this study is that mouse genetic backgrounds strikingly influence the effects of FXR and levels of DCA. FVB FXR KO mice had significantly shorter life spans and more severe vascular calcification, accompanied by a selective increase in DCA. More importantly, even in wild-type mice, FVB mice had higher levels of DCA, but no other BAs, than B6 mice. These data suggest that another genetic modifier regulates DCA metabolism in the FVB genetic background and a future study is required to identify the genetic modifier. In addition to vascular calcification, FVB FXR KO mice developed severe tissue enlargement, hyperlipidemia, and liver steatosis. It has been reported that FVB mice have a higher rate of intestinal lipid absorption than B6 mice (38). In addition, an FXR agonist prevented CKD-dependent and -independent atherosclerosis (21). These phenotypes caused by FVB and FXR modification could be explained by alteration of DCA levels.

A recent study showed that another secondary BA, LCA, indirectly induces vascular calcification by increasing intestinal phosphate and calcium absorption through the activation of vitamin D receptors (39). However, we believe the contribution of LCA in vascular calcification in our models is minor because we did not observe any changes in levels of serum calcium or phosphate through FXR modifications. Taken together, a strategy to reduce secondary BAs, such as DCA, could have major beneficial effects for treating vascular calcification in CKD and other conditions.

Disclosures

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Author Contributions

M. Chonchol, C. Kremoser, and M. Miyazaki were responsible for resources; A.L. Keenan, M. Miyazaki, S. Miyazaki-Anzai, and Y. Shiozaki were responsible for investigation; A.L. Keenan, M. Miyazaki, and Y. Shiozaki reviewed and edited the manuscript; M. Masuda and M. Miyazaki were responsible for formal analysis; M. Masuda, M. Miyazaki, and S. Miyazaki-Anzai were responsible for methodology; M. Miyazaki was responsible for conceptualizing the study, data curation, funding acquisition, project administration, software, validation, visualization, writing the original draft, and providing supervision.

Supplemental Material

This article contains the following supplemental material online at http://kidney360.asnjournals.org/lookup/suppl doi:10.34067/KID.0017502020/-/DCSupplemental.

Supplemental Table 1. CKD increases levels of serum bile acids in humans.
Supplemental Table 2. CKD increases levels of serum bile acids in LDLR KO mice.
Supplemental Table 3. CKD increases levels of serum bile acids in DBA/2J mice.
Supplemental Table 4. Biochemical parameters in CKD LDLRKO mice treated with FXR agonists.
Supplemental Table 5. CKD increases levels of serum bile acids in LDLR KO mice.
Supplemental Table 6. CKD increases levels of serum bile acids in DBA/J mice.
Supplemental Table 7. Serum biochemical parameters in CKD LDLRKO and FXRKO; LDLRKO mice.
Supplemental Table 8. CKD increases levels of serum bile acids in LDLR KO mice.
Supplemental Table 9. Serum bile acid levels in C57BL/6 and FVB FXRKO mice.

Supplemental Figure 1. Fecal DCA excretion in CKD mice and expression of bile acid synthesis enzymes in the livers of CKD mice treated with FXR agonists.
Supplemental Figure 2. FVB FXR KO deficiency elicits severe tissue enlargement and liver steatosis.
Supplemental Figure 3. FVB FXR KO mice develop hyperlipidemia.
Supplemental Figure 4. Hepatic expression of FXR targets and enzymes involved in bile acid synthesis in FVB FXR KO mice.

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