CARDIOVASCULAR, PULMONARY, AND RENAL PATHOLOGY

Lack of Endothelial Nitric Oxide Synthase Accelerates Ectopic Calcification in Uremic Mice Fed an Adenine and High Phosphorus Diet

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Ectopic calcification is a risk of cardiovascular disease in chronic kidney disease (CKD) patients, and impaired endothelial nitric oxide synthase (eNOS) is involved in the CKD complications. However, whether eNOS dysfunction is a cause of ectopic calcification in CKD remains to be elucidated. To address this issue, we investigated the role of eNOS in ectopic calcification in mice with renal injury caused by an adenine and high-phosphorus (Ade + HP) diet. DBA/2J mice, a calcification-sensitive strain, were fed Ade + HP for 3 weeks. Expression levels of eNOS-related genes were reduced significantly in their calcified aorta. C57BL/6J is a calcification-resistant strain, and wild-type mice showed mild calcified lesions in the aorta and kidney when given an Ade + HP diet for 4 weeks. In contrast, a lack of eNOS led to the development of severe aortic calcification accompanied by an increase in runt-related transcription factor 2, an osteochondrogenic marker. Increased renal calcium deposition and the tubular injury score were remarkable in mice lacking eNOS-fed Ade + HP. Exacerbation of ectopic calcification by a lack of eNOS is associated with increased oxidative stress markers such as nicotinamide adenine dinucleotide phosphate oxidases. In conclusion, eNOS is critically important in preventing ectopic calcification. Therefore, the maintenance of eNOS is useful to reduce cardiovascular disease events and to improve prognosis in CKD patients. (*Am J Pathol* 2021, 191: 283–293; <https://doi.org/10.1016/j.ajpath.2020.10.012>)

Abnormal mineral homeostasis is common in advanced stages of chronic kidney disease (CKD), and is referred to as CKD mineral and bone disorder (MBD). CKD-MBD encompasses ectopic calcification such as vascular calcification (VC).^{1,2} Uremia-related VC is characterized by medial artery calcification and contributes to left ventricular hypertrophy, ischemic cardiac events, and cardiovascular disease-related mortality.³ Because cardiovascular disease is a strong prognostic factor in CKD,⁴ understanding its pathogenesis and novel therapeutic options are urgent needs.

The underlying mechanisms of ectopic calcification in uremia have been actively studied. Most importantly, high phosphate levels induce vascular smooth muscle cell

calcification accompanied by osteochondrocytic differentiation; thus, decreasing plasma phosphate is an important strategy to prevent VC.^{5,6} In addition, oxidative stress, inflammatory cytokines, and uremic toxins, such as indoxyl sulfate and p-cresyl sulfate, are known to promote VC.^{7–9} On the other hand, protective roles of osteopontin, fetuin-A, and magnesium against VC have been shown in human and animal studies.^{10–12}

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Endothelial nitric oxide synthase (eNOS) is a NOS isoform, and NO derived from eNOS plays a major role in vascular relaxation, inhibiting both leukocyte infiltration and platelet activation.¹³ A large number of studies have shown that impaired eNOS is important in the CKD pathogenesis. In humans, eNOS (*NOS3*) variants, such as 894G>T (Glu298Asp) and intron 4 variable number of tandem repeat, are polymorphisms that predict the progression of CKD risk.^{14–16} Furthermore, 894G>T (Glu298Asp) has been shown to be associated with left ventricular function in CKD patients.¹⁷ In animal models, a lack of eNOS exacerbates kidney injury, including diabetic kidney disease, unilateral ureter obstruction, glomerulonephritis, and thrombotic microangiopathy.^{18–23}

Some studies have shown the role of eNOS in the pathogenesis of VC. Indeed, eNOS overexpression in vascular smooth muscle cells (VSMCs) has been shown to reduce high-phosphate-induced calcium deposition *in vitro*.²⁴ Moreover, a lack of eNOS is associated with the development of valve calcification in aged mice.²⁵

However, the causal link between eNOS and VC under uremic conditions remains unclear. Herein, we showed the role of a lack of eNOS in both kidney and VC using uremic renal injury mice fed an adenine and high-phosphorus diet.

Materials and Methods

Animals

All experiments were conducted at Tohoku University in compliance with regulations on animal experiments and related activities. DBA/2J mice were purchased from CLEA Japan (Tokyo, Japan). eNOS deficiency (*eNOS*^{−/−}) in C57BL/6J mice was established previously²⁶ and have been maintained at Tohoku University. Control wild-type mice on a C57BL/6J background were purchased from CLEA Japan. To develop uremic calcification, AIN93M-based diets containing 0.2% to 0.25% adenine (Sigma, St. Louis, MO) and 1.8% high phosphorus (referred to as Ade + HP)

Figure 1 Vascular calcification in DBA/2J mice fed an adenine and high-phosphorus (Ade + HP) diet. **A:** An experimental protocol. **B–G:** Plasma blood urea nitrogen (BUN) (**B**), plasma creatinine (**C**), plasma phosphate (**D**), plasma calcium (**E**), plasma calcium and phosphate product (Ca × P) (**F**), and aortic calcium content (**G**). **H:** Representative photomicrographs of Von Kossa and Elastica-Masson (EM) stains in aorta. Arrowheads indicate a disorganized elastic layer. **I:** Expression of endothelial nitric oxide synthase-related genes in the aorta. Data are expressed as means ± SEM or box-and-whisker plots. $n = 6$ to 8 (**B–G**); $n = 6$ (**I**). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Scale bars = $100\ \mu\text{m}$. Pi, inorganic phosphate.

were provided by Oriental Yeast (Tokyo, Japan).^{9,27} In the first experiment, female DBA/2J mice (age, 8 to 10 weeks; $n = 6$ to 8) were used to examine the expression of eNOS-related molecules in the calcified aorta. Mice were fed a diet containing 0.25% adenine and 1.8% phosphorus for 3 weeks. The control group was fed an AIN93M diet containing 0.3% phosphorus (Figure 1A). In the second experiment, male $eNOS^{-/-}$ (age, 12 to 14 weeks; $n = 12$) and wild-type mice ($n = 6$) on a C57BL/6J background were used. They were fed a 0.25% adenine and 1.8% phosphorus diet for a week, and 0.2% adenine and 1.8% phosphorus for 3 weeks. The control $eNOS^{-/-}$ ($n = 5$) and wild-type ($n = 6$) mice were fed a 0.3% phosphorus diet for 4 weeks (Figure 2A). Although females are more susceptible to ectopic calcification and often used in previous studies,^{5,9} the diet containing both adenine and high

phosphorus was too toxic and weight loss was evident for female $eNOS^{-/-}$ mice. As a result, male $eNOS^{-/-}$ mice were used in this study.

Plasma Analysis

Plasma blood urea nitrogen was determined by a colorimetric kit (K024-H1; Arbor Assays, Ann Arbor, MI). Plasma calcium also was determined by a colorimetric kit (CA01M; Metallogenics, Chiba, Japan), and plasma phosphate was measured using a phosphate colorimetric assay (MAK030; Sigma). $Ca \times P$ product is defined as the plasma calcium level multiplied by phosphate level. Plasma fibroblast growth factor 23 (FGF23) was measured using an enzyme-linked immunosorbent assay (DY2629-05; R&D Systems, Minneapolis, MN), and plasma creatinine was

Figure 2 Renal function and plasma levels of calcium, phosphate, and fibroblast growth factor 23 (FGF23) in $eNOS^{-/-}$ fed an adenine and high-phosphorus (Ade + HP) diet. **A:** An experimental protocol. **B:** Plasma blood urea nitrogen (BUN). **C:** Plasma creatinine. **D:** Plasma phosphate. **E:** Plasma calcium. **F:** Plasma calcium and phosphate product ($Ca \times P$). **G:** The level of plasma FGF23. Ade + HP indicates a group fed an adenine and high-phosphorus diet. Data are expressed as means $\pm$ SEM. $n = 5$ to 9. * $P < 0.05$, *** $P < 0.001$. eNOS, endothelial nitric oxide synthase; KO, knockout; NC, normal chow; N.D., not detectable; Pi, inorganic phosphate.

determined using liquid chromatography-tandem mass spectrometry as previously described.²⁸

Histologic Evaluation

The abdominal aorta and kidney samples were fixed in 2% paraformaldehyde. The paraffin-embedded tissues were cut into 3-μm-thick sections and stained with hematoxylin and eosin, Elastica-Masson, and periodic acid-Schiff stains. Calcified lesions were visualized by Von Kossa staining. For the evaluation of renal calcified, tubular, or fibrosis lesions, five high-power fields were selected randomly in an individual renal cortex and quantified using ImageJ software version 1.49 (NIH, Bethesda, MD; <https://imagej.nih.gov/ij>).

Immunohistochemistry

The samples were embedded in paraffin and cut into 3-μm-thick sections for immunohistochemistry. A rabbit antinitrotyrosine antibody (06-284, 1:1000; Merck Millipore, Burlington, MA), rat anti-human runt-related transcription factor 2 antibody (MAB2006, 1:300; R&D Systems), rabbit anti-human nicotinamide adenine dinucleotide phosphate oxidase 4 (NOX4) antibody (14347-1-AP, 1:400; Proteintech, Rosemont, IL), rabbit anti-human cleaved caspase-3 antibody (9661, 1:300; Cell Signaling Technology, Danvers, MA), and rabbit anti-human plasminogen activator inhibitor-1 (PAI-1) antibody (ab66705, 1:200; Abcam, Cambridge, UK) were used. Heat-induced antigen retrieval was performed using sodium citrate buffer. The slides were incubated with primary antibodies overnight at 4°C, and N-Histofine simple stain kits (Nichirei Biosciences, Tokyo, Japan) were used as secondary antibodies. Sections were incubated without primary antibody as a negative control. The ratio of the targeted protein-positive area to the aorta or renal area was assessed using ImageJ software.

Aortic Calcium Deposition

Harvested thoracic aorta was dried overnight at 37°C and incubated further overnight in 0.6 mol/L of hydrochloric acid at 37°C. The calcium content in the supernatant was measured using a colorimetric method (CA01M; Metallogenics). Calcium content was normalized to the weight of the dried tissue.

Real-Time Quantitative PCR

The thoracic aorta homogenates were prepared using a bead homogenizer (TOMY, Tokyo, Japan). Total RNA was extracted using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH). RT-PCR and real-time PCR were performed using the iScript Advanced cDNA Synthesis kit (Bio-Rad, Hercules, CA) and KAPA SYBR Fast qPCR kit (Kapa Biosystems, Wilmington, MA) according to the

Table 1 Primers Used in This Study

Gene	Catalog no.*
Mouse <i>Nos3</i>	MA128570
Mouse <i>Ass1</i>	MA107464
Mouse <i>Asl</i>	MA115339
Mouse <i>Tnfa</i>	MA165780
Mouse <i>Il6</i>	MA152279
Mouse <i>p47phox</i>	MA163291
Mouse <i>Rps18</i>	MA050364

*Takara Bio (Shiga, Japan).

manufacturer’s protocol. The reaction protocols were as follows: initial denaturation at 98°C for 3 minutes, reaction was 45 cycles, and each cycle comprised denaturation at 95°C for 5 seconds and annealing and extension at 60°C for 10 seconds *Rps18* was used as a reference gene. Primer sequences were designed and provided by Takara Bio (Shiga, Japan). Their catalog numbers are shown in Table 1.

Statistical Analysis

All data are presented as means ± SEM. Data were analyzed using JMP Pro 14 (SAS Institute, Cary, NC). After checking normality with the Shapiro-Wilk test, the groups were compared using a two-tailed *t*-test with Welch correction or one-way analysis of variance followed by the Tukey–Kramer test. For non-normally distributed variables, log transformation was applied or the *U*-test or the Kruskal–Wallis test with the Dunn test was used. Differences were considered statistically significant when *P* < 0.05.

Results

Characteristics of VC in DBA/2J Mice Fed an Ade + HP Diet

The aortic expression of eNOS-related markers was first characterized using calcification-prone DBA/2J mice.²⁹ Mice were fed an Ade + HP diet for 3 weeks (Figure 1A). The results showed that the mice had increased levels of plasma blood urea nitrogen, creatinine, and phosphate compared with mice fed normal chow (Figure 1, B–F). The Ade + HP diet significantly increased calcium deposition in the aorta (Figure 1G). Furthermore, VC lesions and a disorganized elastic layer in the abdominal aorta were detected easily by Von Kossa and Elastica-Masson stains (Figure 1H).

eNOS converts arginine to citrulline and produces NO in endothelial cells (ECs). Furthermore, citrulline is recycled to arginine by the action of arginosuccinate synthase and arginosuccinate lyase, which together provide the substrate arginine for eNOS and further NO production.³⁰ We therefore evaluated the expression of the eNOS–NO–pathway–related genes (*Nos3*, *Ass1*, and *Asl* mRNA) in the aorta. These results show that *Nos3* and *Asl* mRNA

Figure 3 A lack of endothelial nitric oxide synthase (eNOS) exacerbates aortic calcium deposition in mice fed an adenine and high-phosphorus (Ade + HP) diet. **A:** Representative photomicrographs of Von Kossa, hematoxylin and eosin (HE), and Elastica-Masson (EM) stains. **Arrowheads** indicate the disorganized elastic layer. **B:** Quantification of the aortic calcium content in each group. **C:** Comparison of the Runx2-positive area in the aorta. **D:** Representative photomicrographs of immunohistochemistry against runt-related transcription factor 2 (Runx2). Data are expressed as means $\pm$ SEM. $n = 5$ to 9 (**B**); $n = 5$ to 8 (**C**). * $P < 0.05$, ** $P < 0.01$. Scale bars = 100 μ m (**A** and **D**).

were reduced in the mice fed Ade + HP compared with the mice in the control group (Figure 1I).

Renal Function and Plasma Levels of Calcium, Phosphate, and FGF23 in Mice Lacking eNOS and Fed an Ade + HP Diet

To test the effect of a lack of eNOS on uremic VC, wild-type and *eNOS*^{-/-} C57BL/6J mice were fed Ade + HP for 4 weeks (Figure 2A). A lack of eNOS drastically increased plasma blood urea nitrogen in mice fed Ade + HP compared with other groups. An Ade + HP diet significantly increased plasma creatinine in both wild-type and *eNOS*^{-/-} groups compared with normal chow control groups. Plasma phosphate increased in wild-type mice fed an Ade + HP diet, while a lack of eNOS significantly reduced plasma phosphate, although the reason for this is unknown. The Ca $\times$ P product predicts cardiovascular disease and mortality in CKD,³¹ and the Ca $\times$ P product of *eNOS*^{+/+} and *eNOS*^{-/-} mice fed

Ade + HP was increased significantly compared with that of normal chow control groups. FGF23, a phosphaturic hormone, regulates phosphate homeostasis,³² and an Ade + HP diet drastically increased the level of plasma FGF23, while a lack of eNOS had no significant effect on the level of FGF23 (Figure 2, B–G). The survival rates of the mice are shown in Supplemental Figure S1. Collectively, eNOS deficiency exacerbates a parameter of renal dysfunction such as blood urea nitrogen, although it only minimally affects the balance of plasma calcium and phosphate in mice with CKD.

Lack of eNOS Increases Calcium Deposition in the Aorta of Mice Fed an Ade + HP Diet

Next, the calcium content in the thoracic aorta was quantified. Because C57BL/6J mice are resistant to VC,²⁹ the level of aortic calcium in wild-type mice fed Ade + HP was low and similar to those of normal chow control groups, whereas *eNOS*^{-/-} mice with uremia had significantly increased

calcium deposition in the aorta (Figure 3, A and B). Furthermore, VC lesions in the aortic media and disorganized elastic layer were observed only in *eNOS*^{-/-} mice fed an Ade + HP diet (Figure 3A).

Runt-related transcription factor 2 is an established marker of VSMCs reprogramming into osteochondrogenic progenitors.⁵ Evaluating the expression of aortic runt-related transcription factor 2 using immunohistochemistry showed that the area of vascular runt-related transcription factor 2

was increased significantly in the aorta from *eNOS*^{-/-} mice fed an Ade + HP diet compared with the groups fed normal chow (Figure 3, C and D).

Increased Expression of Oxidative Stress Markers in Mice Lacking eNOS Fed an Ade + HP Diet

Because an increase of inflammation and oxidative stress is important in the pathogenesis of VC,^{7,9} we quantified their

Figure 4 A lack of endothelial nitric oxide synthase (eNOS) increases aortic oxidative stress in mice fed an adenine and high-phosphorus (Ade + HP) diet. **A:** Expression levels of *Tnfa*, *Il6*, and *p47phox* mRNA in the aorta. **B:** Representative photomicrographs of immunohistochemistry against nicotinamide adenine dinucleotide phosphate oxidase 4 (NOX4) and nitrotyrosine. **C** and **D:** Comparison of NOX4 and nitrotyrosine-positive area in the aorta. Data are expressed as means ± SEM or box-and-whisker plots. *n* = 5 to 9 (**A**); *n* = 5 to 8 (**C** and **D**). **P* < 0.05. Scale bars = 100 μm. *eNOS*^{+/+} NC, wild-type mice fed normal chow; *eNOS*^{-/-} NC, *eNOS* deficiency mice fed normal chow.

Figure 5 A lack of endothelial nitric oxide synthase (eNOS) exacerbates both nephrocalcinosis and kidney injury in mice fed an adenine and high-phosphorus (Ade + HP) diet. **A:** Representative photomicrographs of Von Kossa stain. **B:** Comparison of calcified area in the kidney. **C:** Representative photomicrographs of Elastica-Masson (EM) stain and immunohistochemistry against cleaved caspase-3. **Arrowheads** indicate cleaved caspase-3-positive tubular cells in the kidney. **D** and **E:** Comparison of renal tubular and fibrosis area in the kidney. Data are expressed as means $\pm$ SEM. $n = 5$ to 6 (**B**); $n = 5$ to 6 (**D** and **E**). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Scale bars: 500 μ m (**A**, and **C**, top row); 200 μ m (**C**, bottom row).

markers in the aorta. Of *Tnfa*, *Il6*, and *p47phox* mRNA expression, the level of *p47phox* mRNA was increased significantly in the aorta from *eNOS*^{-/-}-fed Ade + HP compared with those of normal chow groups (Figure 4A). In immunohistochemistry, NOX4 and nitrotyrosine were strongly positive in aorta from *eNOS*^{-/-}-fed Ade + HP. The positive area of Nox4 was increased significantly in *eNOS*^{-/-}-fed Ade + HP compared with wild-type mice fed Ade + HP or normal chow groups. Nitrotyrosine was increased in *eNOS*^{-/-} mice fed Ade + HP without reaching significance (Figure 4, B–D). While the importance of pro-fibrotic PAI-1 in VC has been shown,^{24,33} it was found that

PAI-1 protein was also increased in the aorta from *eNOS*^{-/-} mice fed an Ade + HP diet (Supplemental Figure S2).

Exacerbated Nephrocalcinosis in *eNOS*^{-/-} Fed an Ade + HP Diet

Calcium deposition in the kidney was evaluated next. The results showed that mice lacking eNOS fed an Ade + HP diet had a drastically increased area of calcium deposition compared with other groups, as determined by Von Kossa staining (Figure 5, A and B). Furthermore, the increase in kidney calcification was accompanied by exacerbation of

Figure 6 Increase of oxidative stress markers in the kidney from endothelial nitric oxide synthase (eNOS) deficiency mice fed an adenine and high-phosphorus (Ade + HP) diet. **A:** Representative photomicrographs of nicotinamide adenine dinucleotide phosphate oxidase 4 (NOX4) and nitrotyrosine in immunohistochemistry. **B:** Comparison of NOX4-positive area in the kidney. **C:** Comparison of nitrotyrosine-positive area in the kidney. Data are expressed as means $\pm$ SEM. $n = 5$ to 6. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Scale bars = 200 μ m.

histologic injury as identified by a decrease in the tubular area and an increase in the fibrotic area. Cleaved caspase-3-positive tubular cells also clearly were observed in the kidney of *eNOS*^{-/-} mice fed an Ade + HP diet (Figure 5, C–E).

Expression of NOX4 and Nitrotyrosine in the Uremic Kidney of *eNOS*^{-/-} Mice

To determine whether the increase in renal calcification was associated with exacerbation of oxidative stress in *eNOS*^{-/-} mice, NOX4 and nitrotyrosine expression in the kidneys was evaluated by immunohistochemistry. Both NOX4 and nitrotyrosine were strongly positive in kidneys from *eNOS*^{-/-} mice fed an Ade + HP diet; the NOX4-positive area was increased significantly in *eNOS*^{-/-} mice fed an Ade + HP diet compared with that of wild-type mice fed normal chow. The nitrotyrosine-positive area was increased significantly in *eNOS*^{-/-} mice fed an Ade + HP diet compared with that of wild-type mice fed Ade + HP or normal chow (Figure 6).

Calcification in the Heart

The possibility that a lack of eNOS increased calcification in the heart was also examined. Arterial calcification in the heart was observed in *eNOS*^{-/-} mice fed Ade + HP, but no calcified lesions were observed in the other groups (Figure 7).

Discussion

The current study showed the following: i) Calcification-prone DBA/2J mice showed reduced expression of eNOS-related genes in the aorta under uremic conditions; ii) a lack of eNOS exacerbated both vascular and kidney calcification; and iii) ectopic calcification was accompanied by an increase in oxidative stress such as NOX4 and nitrotyrosine.

Dysfunctional eNOS is a hallmark of vascular dysfunction in CKD. A previous report showed that uremic toxins

Figure 7 Calcified lesion in the heart. Representative photomicrographs of Von Kossa stain. Scale bars = 200 μ m. Ade + HP, adenine and high-phosphorus diet.

affect endothelial function, and, most strikingly, uremic sera and indoxyl sulfate reduces eNOS expression via micro-RNA 92a and oxidative stress—dependent mechanisms in human umbilical vein endothelial cells.³⁴ In a dog nephrectomy model, eNOS mRNA and protein levels were reduced in the coronary arteries, and were restored by treatment with all-trans retinoic acid.³⁵ Furthermore, the bone morphologic protein type 1 receptor has been shown to reduce aortic eNOS phosphorylation, resulting in vascular dysfunction in CKD mice.³⁶ The current study further characterized reduced eNOS expression using an established VC model.

Previous reports have suggested the importance of eNOS in VC pathogenesis.^{24,33,37} Metformin has been shown to prevent β -glycerophosphate-induced calcium deposition via up-regulation of the AMP-activated protein kinase-eNOS-NO pathway in cultured rat aortic smooth muscle cells.³⁷ Moreover, NO donors reduce osteogenic/chondrogenic transformation of VSMC *in vitro*, and vitamin D—overloaded VC in a murine model.³³ Furthermore, overexpression of eNOS has been shown to inhibit osteoblastic differentiation in murine VSMCs.²⁴ However, *in vivo* evidence of a causal link between ectopic calcification and eNOS deficiency was unclear, and was newly identified in our study.

eNOS-derived NO is an antioxidant,³⁸ and reduced NO bioavailability increases oxidative stress in CKD patients.³⁹ Several studies have shown that reduced eNOS causes vascular dysfunction accompanied by an increase in oxidative stress,^{40,41} which consequently contributes to VC. Tumor necrosis factor α induces nicotinamide adenine dinucleotide phosphate oxidase and procalcific responses in the aorta and are inhibited by nicotinamide adenine dinucleotide phosphate oxidase inhibitors.⁴² Moreover, the antioxidant tempol ameliorates arterial medial calcification in uremic rats.⁷ Therefore, we focused on the changes in oxidative stress markers in the uremic aorta, and showed that increases in nicotinamide adenine dinucleotide phosphate oxidase—related molecules and nitrotyrosine were associated with the severity of VC. Furthermore, eNOS/NO likely reduces VC via suppression of the profibrotic response (transforming growth factor β and PAI-1).²⁴ Consistent with this observation, the level of aortic PAI-1 was increased in *eNOS*^{−/−} mice with uremia.

Progressive nephrocalcinosis is associated with kidney injury and progression of CKD.⁴³ Although a high-phosphorus diet induces nephrocalcinosis in rodent models,⁴⁴ the mediator of the endothelium-derived factor is unknown. It is a noteworthy finding that eNOS deficiency drastically exacerbated renal calcium deposition and kidney injury. Similar to VC pathogenesis, the importance of inflammation and oxidative stress in the pathogenesis of nephrocalcinosis was addressed, and inhibition of inflammasomes or tumor necrosis factor—receptor signaling and antioxidant medications was shown to reduce nephrocalcinosis in experimental oxalate nephropathy.^{45,46} Consistent

with previous reports,^{45,46} exacerbation of nephrocalcinosis by eNOS deficiency is associated with an increase in oxidative stress markers in the kidneys.

Although eNOS is considered to be rich in ECs, VSMCs and red blood cells are also known to express eNOS.^{24,47} Recent work has shown that red blood cell—derived eNOS is harmful in the development of VC⁴⁷; in light of this research, we were limited to using systemic deletion of eNOS in the current study. In the future, we plan to use conditional knockout mice to elucidate the cell-specific role of eNOS, and we believe that this experimental design will prove to be more informative.

In conclusion, this research identified a protective role of eNOS in ectopic calcification under uremic conditions. These findings suggest that preventing endothelial dysfunction and preserving eNOS is important to reduce both the risk of cardiovascular disease and CKD progression (Supplemental Figure S3).

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Author Contributions

Y.O., S.M., E.S., N.S., K.K., and A.S. performed the experiments; Y.O. and E.S. analyzed data; Y.O. and N.T. co-wrote the manuscript; M.M., H.S., and S.I. interpreted data and edited the manuscript; and Y.O. and N.T. contributed to the research conception.

Supplemental Data

Supplemental material for this article can be found at <http://doi.org/10.1016/j.ajpath.2020.10.012>.

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