Vascular Calcification and Aortic Fibrosis
A Bifunctional Role for Osteopontin in Diabetic Arteriosclerosis

Jian-Su Shao, Oscar L. Sierra, Richard Cohen, Robert P. Mecham, Attila Kovacs, James Wang, Kathryn Distelhorst, Abraham Behmann, Linda R. Halstead, Dwight A. Towler

Objective—Calcification and fibrosis reduce vascular compliance in arteriosclerosis. To better understand the role of osteopontin (OPN), a multifunctional protein upregulated in diabetic arteries, we evaluated contributions of OPN in male low-density lipoprotein receptor (LDLR)/−/− mice fed a high-fat diet.

Methods and Results—OPN had no impact on high-fat diet–induced hyperglycemia, dyslipidemia, or body composition. However, OPN−/−:LDLR−/− mice exhibited an altered time-course of aortic calcium accrual—reduced during initiation but increased with progression—versus OPN+/+;LDLR−/− controls. Collagen accumulation, chondroid metaplasia, and mural thickness were increased in aortas of OPN−/−:LDLR−/− mice. Aortic compliance was concomitantly reduced. Vascular reexpression of OPN (SM22-OPN transgene) reduced aortic Col2A1 and medial chondroid metaplasia but did not affect atherosclerotic calcification, Col1A1 expression, collagen accumulation, or arterial stiffness. Dosing with the proinflammatory OPN fragment SVVYGLR upregulated aortic Wnt and osteogenic gene expression, increased aortic β-catenin, and restored early-phase aortic calcification in OPN−/−:LDLR−/− mice.

Conclusion—OPN exerts stage-specific roles in arteriosclerosis in LDLR−/− mice. Actions phenocopied by the OPN metabolite SVVYGLR promote osteogenic calcification processes with disease initiation. OPN limits vascular chondroid metaplasia, endochondral mineralization, and collagen accumulation with progression. Complete deficiency yields a net increase in arteriosclerotic disease, reducing aortic compliance and conduit vessel function in LDLR−/− mice. (Arterioscler Thromb Vasc Biol. 2011;31:1821-1833.)

Key Words: calcification ■ matrix ■ peripheral arterial disease ■ transgenic models ■ fibrosis

atherosclerosis, calcification, mural fibrosis, endothelial dysfunction, and elastin matrix senescence cause arteriosclerosis, the age-associated conduit vessel stiffening that impairs Windkessel physiology necessary for efficient distal tissue perfusion.1,2 Vascular remodeling processes also increase wall thickness and reduce conduit artery lumen area, thus worsening arterial compliance and its impact.2 In the musculoskeletal system, lower extremities experience the brunt of arteriosclerotic disease. Claudication and amputation are salient manifestations that diminish mobility and increase morbidity, but hip fracture is also increased.3 Importantly, the presence and extent of arterial calcification conveys risk for progression of arteriosclerosis to critical limb ischemia requiring amputation.4,5 Osteochondrocytic gene programs are elaborated by calcifying arteries of patients with arteriosclerosis, indicating that active osteogenic processes contribute to arterial calcium accrual.6 The increasing prevalence of type II diabetes—a risk factor for arterial calcification7,8—will increase arteriosclerotic disease burden in the future. A fundamental understanding of procalcific and profibrotic arterial remodeling is required to develop new strategies to better address this burgeoning clinical need.9,10

In our studies of arteriosclerotic calcification, we have emphasized the low-density lipoprotein receptor (LDLR)−/− mouse model. When male LDLR−/− mice are fed high-fat diets (HFD) characteristic of Westernized caloric composition, animals develop obesity, insulin-resistant diabetes, hypertriglyceridemia, and hypercholesterolemia with progressively severe arterial calcification.11 Although patchy arterial medial calcification predominates in the model at early stages,11 arteriosclerotic calcification with or without ectopic ossification (endochondral bone formation) increasingly accounts for vascular calcium load over time.11,12 At very early stages of HFD-induced disease, active osteogenic gene regulatory programs are elaborated in the artery wall. Within a few weeks of HFD challenge and the initiation of “diabetes,” the osteoblast transcription factor Msx2 is upregulated along with osteopontin (OPN),13 a polyphosphorylated matrix cytokine and integrin ligand first identified in bone.14 We have shown that mesenchymal Msx2 expression promotes cardiovascular calcification by activating osteogenic Wnt/β-catenin signals.13 However, the contributions of OPN to vascular biology are more complex.14 OPN is a small
integrin binding ligand N-glycosylated (SIBLING) family member. Like all SIBLINGs, OPN is a highly processed multifunctional protein. Full-length phosphorylated OPN is relatively protease resistant, promotes cell attachment and migration, and is an inhibitor of calcium deposition. Scatena et al first noted that OPN is expressed in calcifying vascular segments and that intact phosphorylated OPN limits the extent of atherosclerotic calcium deposition. Thrombin-processed OPN — namely, the N-terminal fragment that contain the C-terminal epitope SVVYGLR (SLAYGLR in mouse) — is proinflammatory, proangiogenic, and proosteogenic, and it supports aortic matrix metalloproteinase 9 (MMP9) activation. Indeed, in calcifying human aortic valves, the SVVYGLR proinflammatory OPN “N-half” epitope is enriched at sites of calcification, correlating with activation of thrombin. OPN is pivotal in MMP-dependent vascular remodeling during aortic aneurysm formation and arteriogenic collateralization responses to limb ischemia.

To better understand the role of OPN in the arteriosclerosis of type II diabetes, we have examined the effects of OPN deficiency in the LDLR−/− model of HFD-induced disease. We find that OPN SVVYGLR signals promote calcification and osteogenic signaling with disease initiation, whereas other OPN actions limit arterial fibrosis, vascular chondroid metaplasia seen in advanced human disease, and vascular calcium accrual with disease progression. Complete OPN deficiency yields a net increase in arteriosclerotic disease and reduces aortic compliance in diabetic LDLR−/− mice.

Methods

Reagents and Animals
Biochemicals, histochemical stains, and molecular biology reagents were obtained from Fisher, Sigma, or Invitrogen. TaqMan Gene Expression Assays for analysis of aortic mRNAs by quantitative reverse transcription–polymerase chain reaction were purchased from Applied Biosystems. The custom synthetic OPN heptapeptide SVVYGLR was obtained from Invitrogen. All procedures for maintaining and handling mice were approved by the Washington University Animal Studies Committee. All other reagents and protocols are detailed in the supplemental materials, available online at http://atvb.ahajournals.org.

Statistics
Statistical analyses were performed using GraphPad Instat Software (version 3.06), implementing standard parametric or nonparametric (Mann–Whitney U test for histological image analysis scores) methods as indicated. Post hoc analysis for significance between groups in 1-way ANOVA was carried out using Student-Neuman–Keuls multiple comparison testing. Differences were deemed significant at \( P < 0.05 \). Graphic data are presented as the mean ± SEM.

Results

OPN Differentially Regulates Initiation and Progression Phases of Aortic Calcification in Diabetic LDLR−/− Mice
In the MGP- and male apolipoprotein E (apoE)-deficient models of arterial calcification, concomitant OPN deficiency yields a net increase in arteriosclerotic disease and reduces aortic compliance in diabetic LDLR−/− mice.
clearly increases arterial calcium accrual.\textsuperscript{26,27} However, \textit{apoE}\textsuperscript{-28} and \textit{MGP}\textsuperscript{-29} deficient mice do not develop the diet-induced obesity and diabetes that commonly afflicts patients with vasculopathy.\textsuperscript{8} Because OPN is a multifunctional modulator of migration and inflammation,\textsuperscript{14} we hypothesized that OPN may exert distinct contributions to the initiation and progression of diabetic arteriosclerosis. To test this notion and better understand the role of OPN, we studied the impact of OPN deficiency on arterial calcification in the male \textit{LDLR}\textsuperscript{-}/\textsuperscript{+};\textit{LDLR}\textsuperscript{-}/\textsuperscript{-} mouse fed diabetogenic HFD. HFD increases aortic calcium accrual 3-fold by 3 months in male \textit{LDLR}\textsuperscript{-}/\textsuperscript{-}/\textsuperscript{-} mice (Figure 1A). Thus, beginning at 1.5 to 2 months of age, cohorts of male OPN\textsuperscript{-}/\textsuperscript{-}/\textsuperscript{-};\textit{LDLR}\textsuperscript{-}/\textsuperscript{-}/\textsuperscript{-} and OPN\textsuperscript{-}/\textsuperscript{-}/\textsuperscript{-};\textit{LDLR}\textsuperscript{-}/\textsuperscript{-}/\textsuperscript{-} mice were challenged with HFD feeding for 1, 2, and 3 months’ duration (Supplemental Figure I) to assess the impact of OPN on vascular calcium load during disease initiation and progression. After 1 month of HFD, OPN\textsuperscript{-}/\textsuperscript{-}/\textsuperscript{-};\textit{LDLR}\textsuperscript{-}/\textsuperscript{-}/\textsuperscript{-} mice exhibited significantly reduced aortic calcium content (\mu g/mg aorta) as compared with OPN\textsuperscript{-}/\textsuperscript{-}/\textsuperscript{-};\textit{LDLR}\textsuperscript{-}/\textsuperscript{-}/\textsuperscript{-} mice (Figure 1A). This reduction was not related to increased aortic weight in OPN\textsuperscript{-}/\textsuperscript{-}/\textsuperscript{-};\textit{LDLR}\textsuperscript{-}/\textsuperscript{-}/\textsuperscript{-} mice (trended lighter, $P=\text{not significant}$). Of note, at this early disease stage, OPN\textsuperscript{-}/\textsuperscript{-}/\textsuperscript{-};\textit{LDLR}\textsuperscript{-}/\textsuperscript{-}/\textsuperscript{-} mice exhibit significantly diminished oxidative stress\textsuperscript{20} and reduced aortic expression of \textit{Msx2} and \textit{Sox2} (Supplemental Figure II)—transcriptional regulators of self-renewing osteoprogenitors.\textsuperscript{30,31} Alizarin red calcium staining localized the early reduction in aortic calcium content in OPN\textsuperscript{-}/\textsuperscript{-}/\textsuperscript{-};\textit{LDLR}\textsuperscript{-}/\textsuperscript{-}/\textsuperscript{-} animals to the tunica media (Figure 1B, upper panels). By contrast, by 3 months of HFD challenge—a stage at which atherosclerotic calcification increasingly contributes to aortic calcium accrual\textsuperscript{11}—OPN\textsuperscript{-}/\textsuperscript{-}/\textsuperscript{-};\textit{LDLR}\textsuperscript{-}/\textsuperscript{-}/\textsuperscript{-} mice exhibited significantly increased aortic calcification versus OPN\textsuperscript{-}/\textsuperscript{-}/\textsuperscript{-};\textit{LDLR}\textsuperscript{-}/\textsuperscript{-}/\textsuperscript{-} cohorts (Figure 1A). This was confirmed by Alizarin red staining (Figure 1B, lower panels) and reflected in the aortic expression of osteochondrocytic transcription factors, including \textit{Msx2} (Supplemental Figure II, lower panel). At the intermediate stage of 2 months on the HFD, no difference was noted in aortic calcium load between genotypes (Figure 1A). Importantly, OPN deficiency did not significantly affect HFD-induced changes in body composition (Figure 1C) or in fasting serum glucose, triglycerides, or total cholesterol (Figure 1D). Thus, \textit{OPN} supports the early phase of aortic calcification in diabetic...
LDLR−/− mice but limits the extent of aortic calcium accrual with disease progression.

Aortic Chondroid Metaplasia, Elastin Fragmentation, Mural Thickness, and Fibrosis Are Increased in OPN−/−;LDLR−/− Mice

Alizarin and picrosirius red stains suggested that arterial chondroid metaplasia might be increased in OPN−/−;LDLR−/− mice as compared with OPN+/+;LDLR−/− cohorts (Supplemental Figure IIIA). Metaplasia was histologically confirmed by Alcian blue staining for cartilage glycosaminoglycans (Figure 2A). Chondroid metaplasia was much more prominent in the tunica media of OPN−/−;LDLR−/− mice and was also more pronounced in the large atheroma elaborated by these animals (Figure 2A). In comparison with calcium (Alizarin red) and inorganic phosphate (von Kossa stain) deposits, chondroid metaplastic changes (Alcian stain) were much more extensive and observed in areas not yet involved with mineral deposition (Supplemental Figure IIIB). Histological scoring of the intensity and extent of Alcian blue staining confirmed increased medial chondroid metaplasia in OPN−/−;LDLR−/− versus OPN+/+;LDLR−/− mice on HFD (Figure 2B; P=0.02, U test) and dependent on HFD challenge (data not shown). A nonsignificant trend for atheroma staining was also observed in OPN-null cohorts (Figure 2B). Differences in metaplasia did not relate to differences in lesion monocyte/macrophage content as quantified by immunohistochemical enumeration (Supplemental Figure IV). Aortic Col2A1—a marker of early chondrocyte differentiation—was dramatically upregulated in OPN−/−;LDLR−/− mice versus OPN+/+;LDLR−/− cohorts after 3 months of HFD (Figure 2C). Immunohistochemistry confirmed the increased intensity and extent of type II collagen protein accumulation in aortas of OPN−/−;LDLR−/− mice (Figure 2D). Aortic endochondral ossification is histologically evident at between 3 and 4 months of HFD (Supplemental Figure IIIB). During this later phase, Col2A1 decreased in OPN−/−;LDLR−/− aortas, whereas Col10A1, a hypertrophic chondrocyte marker upregulated with endochondral bone mineralization, concomitantly increased (Figure 2C), thereby confirming endochondral

Figure 2. OPN deficiency increases aortic chondroid metaplasia in diabetic LDLR−/− mice. A, Representative Alcian blue histochemistry of aortic chondroid metaplasia in OPN+/+;LDLR−/− and OPN−/−;LDLR−/− mice on HFD. B, Histological scoring of the extent and intensity of staining demonstrated a statistically significant increase in OPN−/−;LDLR−/− mice. C, OPN−/−;LDLR−/− mice exhibited augmented aortic expression of Col2A1 vs OPN+/+;LDLR−/− controls. Although Col10A1 was significantly increased, aortic VSMC markers were significantly decreased in OPN−/−;LDLR−/− vs OPN+/+;LDLR−/− between 3 and 4 months of HFD. D, Immunohistochemistry revealed increased medial and atherosclerotic staining for type II collagen in OPN−/−;LDLR−/− mice vs OPN+/+;LDLR−/− cohorts. NS indicates not significant.
ossification with disease progression. Vascular smooth muscle cell (VSMC) markers Myh11, Acta2, and SM22 were significantly reduced by 40% to 45% in aortas of OPN−/−;LDLR−/− mice after 4 months of HFD (Figure 2C and Supplemental Figure V). Thus, OPN deficiency promotes chondroid metaplasia in diabetic LDLR−/−/− mice.

Because vascular OPN supports aortic collagenase activity and is associated with elastin fibers, we examined collagen and elastin matrix characteristics. Elastin morphology was abnormal in diabetic OPN−/−;LDLR−/− animals; histology revealed frequent fragmentation of thickened lamellae (Figure 3A), and aortic wall thickness was increased (Figure 3B). Even before HFD challenge, aortic collagen content was 70% greater in OPN−/−;LDLR−/− animals (P<0.001), but elastin initially did not differ between the 2 genotypes (data not shown). However, with HFD-induced disease, both total aortic collagen and elastin protein content were significantly greater in OPN−/−;LDLR−/−/− mice versus OPN−/−;LDLR−/− cohorts (Figure 3C; 3 to 4 months of diabetic HFD). Picrosirius red staining with dark-field illumination analysis confirmed increased aortic fibrosis in OPN−/−;LDLR−/−/− mice.

Figure 3. Aortic collagen accumulation and elastin morphology are perturbed in diabetic OPN−/−;LDLR−/− mice. A, OPN−/−;LDLR−/− mice exhibited increased lamellar fragmentation and thickness (arrow), as assessed by histomorphometry. B, Total thoracic aortic wall thickness was increased in OPN−/−;LDLR−/−/− mice. C, Diabetic OPN−/−;LDLR−/−/− mice accrued greater aortic collagen and elastin content vs OPN−/−;LDLR−/− cohorts. D, Histochemical staining of aortic sections with picrosirius red and dark-field digital image analysis confirmed increased aortic fibrosis in OPN−/−;LDLR−/−/− mice.
Aortic Compliance Is Reduced in Diabetic OPN−/−;LDLR−/− Mice

The presence of aortic fibrosis with elastin fragmentation and wall thickening strongly suggested that vessel mechanical properties would be altered by OPN deficiency.2,36 We first examined aortic arch pulse wave velocity—an in vivo parameter that increases with reduced arterial compliance—as an assessment of aortic stiffness. As shown in Figure 4A, aortic pulse wave velocity was significantly increased in OPN−/−;LDLR−/− mice versus OPN+/+;LDLR−/− cohorts. We then implemented ex vivo video aortic plethysmography (see supplemental material) to characterize the diameter-pressure relationships of thoracic segments isolated from OPN−/−;LDLR−/− and OPN+/+;LDLR−/− mice. After 4 months on HFD, the change in vessel diameter per mm Hg hydrostatic pressure load was significantly reduced in OPN−/−;LDLR−/− mice versus OPN+/+;LDLR−/− cohorts (Figure 4B), confirming reduced aortic compliance. Similar reductions in pressure-diameter relationships were noted at baseline in OPN−/−;LDLR−/− mice even in the absence of HFD challenge (data not shown). Reduced arterial compliance is predicted to impair conduit vessel Windkessel functions and distal tissue perfusion.39 Indeed, when assessed by fluorescent microsphere analysis, blood flow to the femur was reduced by ≈65% in OPN−/−;LDLR−/− mice compared with OPN+/+;LDLR−/− cohorts (Figure 4C). Thus, OPN deficiency reduces aortic compliance and femur blood flow in LDLR−/− mice.

An SM22 Promoter-Driven OPN Transgene Diminishes Medial Chondroid Metaplasia but Does Not Does Restore Vascular Compliance in Arteriosclerotic OPN−/−;LDLR−/− Mice

In diabetic vessels, both VSMCs and macrophages express OPN,41 and OPN may exert different actions in these 2 cell types. We wished to better understand the cell type–specific mechanisms whereby VSMC OPN regulates the latter phases of arteriosclerotic disease. Thus, we generated SM22-OPN transgenic mice expressing the OPN protein from the VSMC-specific 0.5 kb SM22 promoter (Figure 5A), and examined the impact on osteochondrocytic gene expression, chondroid metaplasia, and collagen accumulation in OPN−/−;LDLR−/− animals. Compared with OPN−/−;LDLR−/− SIBLINGs, SM22-OPN;OPN−/−;LDLR−/− mice exhibited significant aortic OPN message (Figure 5B). Col1A1 mRNA was unaffected, but aortic expression of Col2A1 and Col10A1 was significantly suppressed (Figure 5B). Histological scoring of Alcian blue–stained sections (Figure 5C) confirmed that the medial chondroid metaplasia of OPN
deficiency was partially reversed by the SM22-OPN transgene (Figure 5D). However, no significant change was observed in Alcian staining of adjacent atheroma (Figure 5E). Furthermore, no significant alteration in total aortic collagen content or calcium content was observed in response to the SM22-OPN transgene (Figure 5F), and aortic compliance quantified by ex vivo plethysmography was not improved ($2.0 \pm 0.3$ versus $1.3 \pm 0.5$ mm Hg load, $P = 0.24$, OPN+/+;LDLR−/− versus SM22-OPN+/+;LDLR−/−). Thus, although it partially mitigates medial chondroid metaplasia, the SM22-OPN transgene cannot reverse arteriosclerotic calcification and fibrosis in OPN−/−;LDLR−/− mice.

**OPN SVVYGLR Signaling Promotes Early Phase Osteogenic Mineralization in OPN−/−;LDLR−/− Mice on HFD**

OPN deficiency reduces aortic oxidative stress (reactive oxygen species) and MMP activation in newly diabetic LDLR−/− mice. Dosing with the OPN N-half peptide metabolite SVVYGLR restores early aortic reactive oxygen species production and MMP activation in OPN−/−;LDLR−/− animals. HFD feeding increases urinary N-terminal OPN fragments with the murine epitope in LDLR−/− mice (Supplemental Figure VII). This OPN metabolite is significantly enriched at sites of aortic calcification in humans. Therefore, we hypothesized that this OPN-dependent proinflammatory signal, lacking in OPN-null mice, supports early osteogenic vascular mineralization at the initiation of diabetic arteriosclerosis. To test this notion, we quantified the impact of SVVYGLR OPN peptide dosing on aortic osteogenic gene expression and calcification in OPN−/−;LDLR−/− mice fed HFD. Eight days of daily SVVYGLR administration to OPN−/−;LDLR−/− mice upregulated aortic expression of COL10A1, Wnt3a, and Wnt7 (Figure 6A) and increased aortic $\beta$-catenin protein accumulation 3-fold (Figure 6B)—hallmarks of osteochondrocytic differentiation and Wnt/β-catenin signaling. A smaller but significant 1.8-fold increase in Runx2 was also noted. A shorter, independent experiment indicated that SVVYGLR (thrice daily) increased total aortic $\beta$-catenin within 3 days, with concomitant increases in $\beta$-catenin Ser-675 phosphorylation that further confirmed activation (see Supplemental Figure VIIA to VIIB). The expression of aortic bone morphogenetic protein (BMP)-2 and BMP4—and the BMP targets Msx2, Sox9, and Smad6—were unaltered by SVVYGLR (Figure 6A). Moreover, in OPN−/−;LDLR−/− mice on HFD, levels of aortic phospho-Smad1/5/8 were not regulated by SVVYGLR.

![Aortic Pulse Wave Velocity (m/s)](image1)

**Figure 4.** Aortic compliance is reduced in diabetic OPN−/−;LDLR−/− mice. A, Aortic arch pulse wave velocity (PWV) (m/second) was increased in OPN−/−;LDLR−/− mice vs OPN+/+;LDLR−/− controls. B, Ex vivo aortic video plethysmography also demonstrated reduced aortic compliance. Note reductions in aortic diameter (D; μm) as a function of intravascular pressure (P; mm Hg). C, Femur blood flow as assessed by fluorescent microsphere analysis was significantly reduced in arteriosclerotic OPN−/−;LDLR−/− mice. NS indicates not significant.
dosing (Figure 6C). Finally, the early stage of aortic calcium accumulation deficient in OPN/H11002/LDLR mice (Figure 1A and 1B, upper panel) was substantially restored by SVVYGLR administration (Figure 6D). Alizarin red staining reflected this and localized SVVYGLR-induced aortic calcification to the tunica media (Figure 6E). Thus, the OPN metabolite SVVYGLR preferentially augments aortic Wnt/β-catenin signaling and restores early-stage medial artery calcium deposition in OPN/H11002/LDLR mice, dependent in part on doxycycline-sensitive MMP activity.

MMP Activity Contributes to β-Catenin Signaling and Calcium Accumulation in OPN−/−;LDLR−/− Mice Treated With SVVYGLR

Recent studies have highlighted the crucial role for proteases in vascular mineralization. Moreover, extracellular MMP activities promote activation of β-catenin signaling by cleaving N-cadherin. Because the OPN SVVYGLR peptide upregulates vascular MMP9 activity, we assessed whether MMP activity contributed to SVVYGLR actions in vivo. Coadministration of doxycycline, a prokaryotic protein synthesis antagonist and inhibitor of multiple eukaryotic MMPs, downregulated aortic β-catenin accumulation elicited by SVVYGLR (Supplemental Figure IXA and IXB). A smaller but statistically significant decrease in aortic calcium accumulation was also observed (Supplemental Figure IXC). Thus, at early stages of HFD challenge, OPN SVVYGLR signaling supports aortic Wnt/β-catenin signaling and calcification in OPN−/−;LDLR−/− mice, dependent in part on doxycycline-sensitive MMP activity.

Discussion

OPN plays multiple modulatory roles in postnatal physiology. OPN regulates the hematopoietic niche, controls T-cell programming by dendritic cells, promotes migration and activities of the monocyte/macrophage (M/MΦ) lineage,
helps eradicate certain pathogens, supports tumor metastasis, and facilitates vascular remodeling. Skeletal calcium mobilization is also impaired by OPN deficiency. Scatena et al were the first to identify that OPN participates in vascular disease processes. Implementing elegant murine models of genetically programmed spontaneous arterial chondroid metaplasia, they highlighted the importance of OPN as an inducible inhibitor of vascular mineralization. Our study demonstrates that OPN in fact plays both pathogenic and protective roles in the diet-induced diabetic arteriosclerosis of LDLR−/− mice. Endogenous OPN supports calcium accrual with disease initiation via actions phenocopied by exogenous OPN SVVYGLR peptide dosing. These mechanistic insights are consistent with recent observations of Breyne et al, who demonstrated increased N-half OPN SVVYGLR epitope levels in calcified versus noncalcified zones of human aortic valves, arising from tissue factor-related thrombin activation. The cryptic OPN peptide motif—SVVYGLR in humans, SLAYGLR in mice—is liberated by the thrombin-mediated proteolysis and promotes inflammation, angiogenesis, and calcification. Our data show that the in vivo aortic calcium accrual promoted by SVVYGLR dosing represents, in part, ectopic recapitulation of an osteogenic repair process. The upregulation of β-catenin and vascular calcification by SVVYGLR is antagonized by doxycycline, an inhibitor of both MMP bioactivity and mRNA accumulation. In preclinical models of Marfan aortopathy, doxycycline and angiotensin receptor 1 antagonists synergize to improve aortic structure and function. Furthermore, reductions in MMP activation by doxycycline reduce vascular inflammation, a key contributor to vascular calcification. Thus, a better understanding of the interactions between OPN-linked extracellular proteolytic cascades (thrombin, MMP activation), inflammation, and the intracellular transcription factors (β-catenin; Runx, Sox, Msx family members) that control osteogenic mineralization promises to yield novel strategies for mitigating diabetic arteriosclerosis.

However, OPN clearly plays a protective role in the advanced phase of diabetic arteriosclerosis, limiting adventitial and mural fibrosis in addition to chondroid metaplasia atherosclerotic calcium accrual as first described in nondiabetic models. With respect to fibrosis, the effects of OPN deficiency in the remodeling conduit arteries differ unexpectedly from those observed in myocardial fibrosis. Implementing SM22-OPN transgenic mice, we demonstrate that chondroid metaplasia of mural VSMCs is diminished by OPN in vivo (Figure 5) and is cell autonomous in vitro (not shown). However, the advanced fibrosis and endochondral calcification observed in OPN−/−;LDLR−/− mice was not inhibited by the SM22-OPN transgene. Thus, cell types other than the VSMC primarily mediate OPN-dependent inhibition of vascular collagen and calcium accrual and the tempo of disease progression. The monocyte/macrophage (M/Mφ) lineage plays a critical role in the adventitial, medial, and intimal remodeling necessary for arteriogenesis. Thus, it is tempting to speculate that the changes in time course and severity of arteriosclerosis arising in OPN−/−;LDLR−/−...
mice relates in part to altered vascular M/Mc activities (Supplemental Figure X). Future studies will directly test this notion and the impact of SLAYGLR-mutated OPN transgenes on arteriosclerotic disease kinetics.

The earliest phase of osteochondrogenic mineral deposition occurs in conjunction with acidic phospholipid vesicles and multiple annexin complexes with little inorganic phosphate. This may help explain the frequent disconnect observed between arteriosclerotic vascular Alizarin (calcium) and von Kossa (inorganic phosphate) stains first addressed by Puchtler 4 decades ago. Although type II collagen is unable to support such mineralization, type I collagen, type X collagen, and elastin can. Activation of MMPs and osteogenic β-catenin signals by OPN SVVYGLR rapidly promotes aortic calcium accrual—demonstrated by biochemical assay and Alizarin histochemistry—without initially enhancing calcium phosphate deposition and ossification that are characteristic of von Kossa inorganic phosphate staining (Figure 6 and data not shown). This suggests that the early medi calcalcification processes supported by OPN during disease initiation in LDLR mice on HFD differ from the vascular ossification processes that ensue following chondroid metaplasia with advanced disease. Future studies will examine whether SVVYGLR and Wnt/β-catenin signals regulate vascular annexin-phospholipid or other mineralizing lipoprotein complexes. A combination of sensitive near-infrared fluorescent bisphosphonate probes for calcium with annexin immunofluorescence and von Kossa phosphate staining may help temporally and spatially resolve the forms of vascular calcium biochemically quantified during disease initiation and progression. Once vascular calcium phosphate crystals accumulate, however, autocrine processes involving both BMP2 and OPN signaling are likely to rapidly drive progressive mineralization.

There are, of course, limitations to our study. The mechanisms whereby complete OPN deficiency perturbs elastin morphology and promotes fragmentation in diabetic LDLR mice have not been established—and may differ

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**Figure 6.** The OPN peptide SVVYGLR increases aortic calcification and osteochondrocytic gene expression in diabetic OPN/−/−; LDLR/−/− mice. A, Dosing with SVVYGLR upregulated aortic expression of Runx2, Col10A1, and key osteogenic Wnt ligands in diabetic OPN/−/−; LDLR/−/− mice. B, Aortic β-catenin, a marker of canonical Wnt signaling, was increased by SVVYGLR. C, Aortic phospho-Smad1/5/8 was not regulated by SVVYGLR. D, Administration of OPN SVVYGLR to OPN/−/−; LDLR/−/− mice on HFD increased aortic calcification. E, Alizarin red staining confirmed increased medial calcification in SVVYGLR-treated mice. NS indicates not significant.
dependent on the specific disease model. Furthermore, in the cardiomyocyte, OPN excess increases myocardial fibrosis via T-cell mediated mechanisms. However, we demonstrated that OPN deficiency increased aortic adventitial and mural fibrosis in diabetic LDLR<sup>−/−</sup> mice. Given the cell type–specific contributions of OPN, we speculate that OPN actions in M/M<sub>ϕ</sub> versus striated muscle versus VSMC lineages will differentially affect collagen accumulation. Reductions in OPN-dependent collagenase activity likely play a role in adventitial collagen accumulation, but the precise mechanisms whereby global OPN deficiency increases aortic fibrosis in LDLR<sup>−/−</sup> mice have yet to be delineated. P1NP levels indicate that increased collagen synthesis contributes as well. Nevertheless, our study demonstrates the differential impact of OPN on the initiation and progression phases of arteriosclerosis with diet-induced diabetes in LDLR<sup>−/−</sup> mice. At the initiation of diabetes and vascular disease, OPN promotes calcification via actions recapitulated by the proinflammatory OPN SVVYGLR metabolite but OPN limits mural fibrosis, chondroid metaplasia, and arterial calcium accrual with disease progression. Complete OPN deficiency thus yields a net increase in arteriosclerotic disease severity in the LDLR<sup>−/−</sup> mouse. Strategies that selectively inhibit OPN proinflammatory SVVYGLR actions yet preserve OPN production in cells that limit arterial fibrosis and calcium accrual may improve arterial compliance and distal tissue perfusion in diabetic vascular disease.

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**Disclosures**

None.

**References**


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**General Scheme**

- **OPN+/+;LDLR-/-**
  - 1.5 – 2 months of age
  - Male mice
    - HFD, 1 month
    - HFD, 2 months
    - HFD, 3 months

- **OPN-/--;LDLR-/-**
  - 1.5 – 2 months of age
  - Male mice
    - HFD, 1 month
    - HFD, 2 months
    - HFD, 3 months

- **OPN+/+;LDLR-/-**
  - 1.5 – 2 months of age
  - Male mice
    - CHOW, 3 months
    - HFD, 3 months

- **OPN+/+;LDLR-/-**
  - OPN-/--;LDLR-/-
  - 1.5 – 2 months of age
  - Male mice
    - Chow, 3 months
    - Chow, 3 months

**Analyses at 1, 2 and 3 months:** Aortic calcium content, body composition by DXA, fasting serum biochemistries.

**Analyses at baseline and 3 months:** Alizarin red histochemistry, Alcian blue metaplasia scoring, osteogenic transcription factor expression by RT-qPCR.

**Analyses at 3 months:** Biochemical assessment of aortic collagen and elastin content. Picosiris red staining for aortic collagen deposition. Immunohistochemistry for type II collagen, CD68 and F4/80. Aortic compliance by ex vivo video microplethysmography. Cartilage and myogenic gene expression by RT-qPCR.
Supplement Figure S2

Relative Aortic mRNA Accumulation, Chow Diet
% OPN+/+;LDLR−/− Control

- Msx2: p = 0.06
- Runx2: p = 0.76
- Sox2: p = 0.02
- Sox9: p = 0.30

n = 5 – 7 per group

Relative Aortic mRNA Accumulation, HFD x 3 months
% OPN+/+;LDLR−/− Control

- Msx2: p = 0.03
- Runx2: p = 0.08
- Sox2: p = 0.89
- Sox9: p = 0.41
Supplement Figure S3A

OPN-/-;LDLR-/-
HFD 3 months

Alizarin  Picrosirius Red

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Supplement Figure S4A

Aortic Sinus CD68+ Cell Number (Percent of Total Cells)

p = NS

Aortic Lesion F4/80+ Cell Number (Percent of Total Cells)

p = NS
Supplement Figure S4B

Brightfield
(CD68+ cells)

CD68 + DAPI

Fluorescence
(DAPI stained total nuclei)

No primary Aby + DAPI
Aortic mRNA Accumulation at 4 months HFD
Fold Relative to OPN+/+;LDLR-/- at 3 months
(GAPD Normalized)

ANOVA p < 0.01
ANOVA p < 0.0001,
n = 8 – 16 per group

Supplement Figure S5

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Serum P1NP, A Marker of Type I Collagen Biosynthesis, Is Increased in OPN-/-;LDLR-/- Mice

Chow Diet, 2 month old cohorts, n = 13-16 per group
Urinary N-Terminal OPN Concentration (mol/L)

Chow

HFD

p = 0.042 (two tail t-test)

OPN+/+;LDLR-/-, HFD x 1 month, n = 5 per group
Aortic β-Catenin and phospho Ser-675 β-Catenin Protein Levels (Lamin B Normalized, % Vehicle Control)

- Total β-catenin
- phospho Ser-675 β-catenin

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n = 3–5 per group
Supplement Figure S8B

VEHICLE

SVVYGLR

β-catenin
phospho Ser-675

Total β-catenin

Lamin B

VEHICLE

SVVYGLR

75 kDa

75 kDa

75 kDa

n = 3– 5 per group

OPN-/-;LDLR-/-, Dosed 3 times daily for 3 days

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Supplement Figure S9A

Aortic β-Catenin Protein Level
(Lamin B Normalized)

SVVYGLR + VEHICLE

SVVYGLR + DOXYCYCLINE

\( p = 0.029 \) (two-tailed Mann-Whitney U test)

\( n = 4-5 \) per group

OPN-/-;LDLR-/-, HFD x 0.75 months (3 weeks)
Dosed once daily
Supplement Figure S9B

Representative Western Blots

SVVYGLR + VEHICLE

SVVYGLR + DOXYCYCLINE

β-catenin

Lamin B

OPN−/−;LDLR−/−, HFD x 0.75 months (3 weeks)
Dosed once daily

n = 4–5 per group

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Aortic Calcium

(ug calcium / mg aortic dry weight)

\[ p = 0.045 \text{ (2 tail t test)} \]

OPN-/-;LDLR-/-, HFD x 0.75 months (3 weeks)
Dosed once daily
**Diabetes, Obesity, Dyslipidemia**

- Inflammation

**Proteolyzed OPN SVVYGLR liberation**

**ROS**

- Osteogenic Wnt signals
- Vascular remodeling
- Collagen accumulation
- Arterial lamellar calcification
- Fragmentation of elastin lamellae

**β-catenin activation**

- Pre-osteogenic chondroid matrix metaplasia
- Progressive endochondral mineralization

**Intact Osteopontin**

- **Structural and material impairment of vessel compliance**
  - Windkessel function of conduit arteries
  - Distal tissue perfusion

**Arterial stiffening**