Activation of Vascular Smooth Muscle Parathyroid Hormone Receptor Inhibits Wnt/β-Catenin Signaling and Aortic Fibrosis in Diabetic Arteriosclerosis

Su-Li Cheng, Jian-Su Shao, Linda R. Halstead, Kathryn Distelhorst, Oscar Sierra, Dwight A. Towler

Rationale: Vascular fibrosis and calcification contribute to diabetic arteriosclerosis, impairing Windkessel physiology necessary for distal tissue perfusion. Wnt family members, upregulated in arteries by the low-grade inflammation of “diabesity,” stimulate type I collagen expression and osteogenic mineralization of mesenchymal progenitors via β-catenin. Conversely, parathyroid hormone (PTH) inhibits aortic calcification in low-density lipoprotein receptor (LDLR)-deficient mice fed high-fat diabetogenic diets (HFD).

Objective: We sought to determine the impact of vascular PTH receptor (PTH1R) activity on arteriosclerotic Wnt/β-catenin signaling in vitro and in vivo. We generated SM-caPTH1R transgenic mice, a model in which the constitutively active PTH1R variant H223R (caPTH1R) is expressed only in the vasculature.

Methods and Results: The caPTH1R inhibited Wnt/β-catenin signaling, collagen production, and vascular smooth muscle cell proliferation and calcification in vitro. Transgenic SM-caPTH1R;LDLR−/− mice fed HFD develop diabetes, with no improvements in fasting serum glucose, cholesterol, weight, body composition, or bone mass versus LDLR−/− siblings. SM-caPTH1R downregulated aortic Col1a1, Runx2, and Nox1 expression without altering TNF, Msx2, Wnt7a/b, or Nox4. The SM-caPTH1R transgene decreased aortic β-catenin protein accumulation and signaling in diabetic LDLR−/− mice. Levels of aortic superoxide (a precursor of peroxide that activates pro–matrix metalloproteinase 9 and osteogenic signaling in vascular smooth muscle cells) were suppressed by the SM-caPTH1R transgene. Aortic calcification, collagen accumulation, and wall thickness were concomitantly reduced, enhancing vessel distensibility.


Key Words: arteriosclerosis ■ β-catenin ■ diabetes ■ parathyroid hormone ■ Wnt

Atherosclerosis and medial artery calcification are 2 contributors to arteriosclerosis: the vascular stiffening that impairs Windkessel physiology necessary for smooth distal tissue perfusion and efficient cardiac performance. Arteriosclerotic changes in mural biochemical and geometric properties are responsible for functional ageing of conduit arteries. In the musculoskeletal system, the lower extremities bear the brunt of disease burden arising from arteriosclerosis. Claudication, critical limb ischemia, and amputation are salient manifestations that reduce mobility and increase morbidity in our patients. In either the presence or absence of type 2 diabetes (T2DM), the presence and extent of vascular calcification portends lower extremity amputation risk. Demer, Shanahan, and colleagues demonstrated the elaboration of osteochondrogenic regulatory programs in calcifying segments of patients with vascular disease, indicating that active biomineralization programs contribute to arterial calcium accrual. Rajamannan, Miller, Heistad, and colleagues have made similar observations in calcific aortic stenosis, a lethal sclerotic vasculopathy also increased by T2DM and advancing age. A fundamental understanding of the procalcific and profibrotic mechanisms of diabetic arteriosclerosis is necessary to develop new strategies to address the burgeoning vascular disease burden afflicting our population.

In our preclinical studies of arterial calcification, we have implemented the low-density lipoprotein receptor (LDLR)-deficient mouse model. When LDLR−/− mice are fed high-fat Western diets (HFDs), male animals develop obesity, diabetes, hyperinsulinemia, hypertriglyceridemia, and hypercholesterolemia, with progressively severe arterial calcification. Whereas medial calcification predominates at early
disease stages, at later stages, atherosclerotic intimal calcification increasingly accounts for vascular calcium load.\textsuperscript{11,12} Without HFD-induced “diabesity,” vascular disease phenotypes are markedly attenuated. However, in the presence of selective apolipoprotein (Apo)B\textsubscript{100} expression, LDLR-deficient mice develop hemodynamically significant calcific aortic stenosis, even when aged on standard rodent chow diets.\textsuperscript{13} In LDLR\textsuperscript{−/−};ApoB\textsubscript{100/100} mice, cholesterol-dependent initiation and reversal of age-associated aortic valve calcification is heralded by vascular changes in osteogenic transcription factors, including Msx2 (muscle segment homeobox protein homolog 2).\textsuperscript{14} Similarly, at the very earliest stages of HFD-induced diabesity and dyslipidemia, osteogenic gene regulatory programs are upregulated in the aortas of LDLR-deficient mice.\textsuperscript{15,16} Within 2 weeks of HFD challenge, the osteogenic transcription factor Msx2 is ectopically induced in aortic valve fibrosa and aortic adventitia.\textsuperscript{15,16} Msx2 enhances cardiovascular calcification by activating paracrine Wnt (wingless-type integration site family member) signals that drive osteogenic\textsuperscript{17} and myofibroblast\textsuperscript{17,18} differentiation programs in adjacent mesenchymal progenitors.\textsuperscript{16–18} Inflammatory cytokines, matrix metalloproteinases (MMPs), and reactive oxygen species (ROS) initiate and propagate these processes, activating mural \(\beta\)-catenin signals.\textsuperscript{11,19}

\(\beta\)-Catenin is a transcriptional coadaptor indispensable for osteogenic tissue mineralization\textsuperscript{20} and participates in osteochondrogenic differentiation of mural mesenchymal progenitors.\textsuperscript{21} In the vasculature, \(\beta\)-catenin activity is not only entrained to tumor necrosis factor (TNF) and Msx2-Wnt activity;\textsuperscript{11} N-cadherin– and MMP-regulated cell–cell interactions reciprocally control \(\beta\)-catenin signals that drive vascular smooth muscle cell (VSMC) proliferation and neointima formation.\textsuperscript{19,22} Moreover, ROS activate pro-MMP9,\textsuperscript{23} which, in turn, cleaves cell surface N-cadherin and liberates the cadherin-sequestered pool of \(\beta\)-catenin to promote signaling.\textsuperscript{19} Thus, data from multiple laboratories have converged on \(\beta\)-catenin activation as a key component of arteriosclerotic physiology.\textsuperscript{8,16,19,21,24}

Previously, we demonstrated that dosing with the bioactive human parathyroid hormone (PTH) fragment PTH\textsubscript{(1–34)} reduced vascular mineralization, downregulated aortic Msx2-Wnt expression, and inhibited Wnt-activated \(\beta\)-catenin signaling.\textsuperscript{15,25} However, in vivo PTH\textsubscript{(1–34)} administration may exert both direct and indirect actions on vascular physiology. Whereas the PTH/PTHrP receptor (PTH1R) is expressed and biologically active in VSMCs,\textsuperscript{26,27} PTH1R activation by PTH\textsubscript{(1–34)} in kidney, bone, and hematopoietic niche may also influence arteriosclerotic processes.\textsuperscript{28} Thus, to better understand the role of VSMC PTH1R signaling in arteriosclerosis, we generated SM-caPTH1R transgenic mice. In this model, the constitutively active Jansen PTH1R (caPTH1R) variant PTH1R(H223R)\textsuperscript{29} is expressed in VSMCs using the 0.5-kb SM22 (transgelin) promoter.\textsuperscript{11,30} We find that VSMC-autonomous PTH1R signaling decreases aortic activation of procalcific and profibrotic \(\beta\)-catenin signals diminishes vascular oxidative stress and reduces aortic collagen and calcium accrual the LDLR-deficient mouse model of diabetic vascular disease.

\section*{Methods}

An expanded Methods section detailing the materials, methods, and statistics implemented in this study is available in the Online Data Supplement at http://circres.ahajournals.org.

Procedures for generating and evaluating mice transgenic for SM-caPTH1R were approved by the Washington University Animal Studies Committee. LDLR\textsuperscript{−/−} mice (stock no. 002207) and TOPGal mice (stock no. 004625) were obtained from The Jackson Laboratories. Diet-induced diabetes was initiated in male sibling cohorts at 5 to 8 weeks of age by ad libitum feeding with the “Western” HFD (Harlan TD.88137; 42% of calories from fat). Following 3 to 4 months of dietary challenge, body composition, serum biochemistries, aortic pathology, and aortic gene expression were analyzed.\textsuperscript{11} Data are presented as the means±SE of independent replicates (n=3 to 22, dependent on the assay).
Results

**Wnt/β-Catenin Signaling Elicits Profibrotic and Procalcific Responses in C3H10T1/2 Multipotent Mural Mesenchymal Progenitors**

In male LDLR−/− mice, diabetogenic HFD upregulates TNF-dependent aortic Msx2-Wnt signaling cascades that promote vascular calcium deposition.11,25 Wnt7a and Wnt7b are vascular Wnts11,16,31,32 that participate in Msx2 proosteogenic actions.16,33 Like Wnt3a, Wnt7a, and Wnt7b also stimulate canonical β-catenin signaling in C3H10T1/2 cells, as evident in TCF/LEF optimal promoter (TOP), luciferase reporter (LUC) activation (Figure 1A). To better understand the contributions of the Wnt7 family to vascular fibrosis and calcification, we examined Wnt7 effects on C3H10T1/2, a multipotent mural mesenchymal progenitor34 that elaborates both osteogenic17 and vascular smooth muscle18,34 phenotypes. We generated SFG-Wnt7a and SFG-Wnt7b (vesicular stomatitis virus G protein pseudotyped retroviral vectors [SFG35] for Wnt7 expression17) and assessed the impact of SFG-Wnt7 transduction on collagen gene expression, collagen protein production, and mineral deposition. As compared to cultures transduced with SFG-LacZ (β-galactosidase; control), cultures transduced with SFG-Wnt7a or SFG-Wnt7b exhibited increased expression of type I collagen (Col1A1 and Col1A2; Figure 1B) and increased collagen protein content (Figure 1C). Bone alkaline phosphatase enzyme activity (Figure 1D) and mRNA (data not shown) were also induced by SFG-Wnt7. Moreover, when cultured under conditions permissive for matrix mineralization, SFG-Wnt7 significantly increased calcium deposition, as revealed by Alizarin red staining (n=2 per condition, replicated >5 times).

![Figure 1.](image-url)
PTH(1–34) (also known as teriparatide) is a clinically useful agonist of the PTH1R that simultaneously stimulates bone formation and inhibits vascular calcification in diabetic25 and uremic37 rodent models. Because Wnt/β-catenin cascades24 participate in diabetic arteriosclerosis,11,16 we examined the impact of PTH/PTH1R activation on Wnt/β-catenin–regulated transcription, including type I collagen expression. For these experiments, we implemented primary mouse aorta VSMCs and A7r5 cells, a SM22α-expressing rat aorta VSMC line that faithfully recapitulates key features of VSMC-specific gene transcription.38 PTH(1–34) inhibits TOPLUC activation by either Wnt3a (Figure 2A) or by Wnt7a/b (data not shown), indicating that Wnt7a, Wnt7b, and Wnt3a elaborate similar PTH-sensitive canonical signals in VSMCs. Likewise, PTH(1–34) significantly reduces Wnt7a- and Wnt7b-stimulated Col1A1 and Col1A2 expression in primary aortic VSMCs (Figure 2B). Moreover, using the 0.5-kb SM22-promoter30 to drive expression, coexpression of SM-caPTH1R (a constitutively active form of the PTH1R [caPTH1R] that does not require ligand for activation29) inhibited Wnt7-activated TOPLUC transcription in A7r5 VSMCs (Figure 2C) and primary aortic VSMC cultures (data not shown). Suppression of transcription by caPTH1R was promoter-specific, because SM-caPTH1R stimulated pCRE-LUC (n=6 per condition, replicated twice.) E, SM-caPTH1R inhibits basal and pcDNA3-Wnt7a–induced Col1A1 promoter activity (n=6 per condition, replicated 4 times). F, Primary aortic VSMCs transduced with SFG-caPTH1R exhibit decreased collagen protein accumulation vs SFG-LacZ controls (n=3 per condition).

![Figure 2. PTH and the constitutively active receptor caPTH1R inhibit canonical Wnt/β-catenin signaling, Col1A1 transcription, and collagen accumulation.](http://circres.ahajournals.org/)

**A**, A7r5 aortic VSMCs were transfected with the TOPLUC reporter and treated with 10 ng/mL Wnt3a and 100 nmol/L PTH either alone or in combination. PTH(1–34) abrogated Wnt3a-mediated activation (n=6 per condition, replicated twice). B, PTH(1–34) inhibits SFG-Wnt7a and SFG-Wnt7b induction of Col1A1 and Col1A2 expression in transduced primary aortic VSMCs (n=3 per condition, replicated twice). C, Cotransfection of SM-caPTH1R, a VSMC expression plasmid for constitutively active caPTH1R, also inhibits TOPLUC activation by pcDNA3-Wnt7a or pcDNA-Wnt7b (n=6 per condition, replicated twice). D, TOPLUC inhibition by SM-caPTH1R was promoter-specific, because SM-caPTH1R stimulates pCRE-LUC (n=6 per condition, replicated twice). E, SM-caPTH1R inhibits basal and pcDNA3-Wnt7a–induced Col1A1 promoter activity (n=6 per condition, replicated 4 times). F, Primary aortic VSMCs transduced with SFG-caPTH1R exhibit decreased collagen protein accumulation vs SFG-LacZ controls (n=3 per condition).
tion assays (Figure 2D). The caPTH1R also inhibited basal (Figure 2E), Wnt3a (data not shown), and Wnt7a-stimulated (Figure 2E) Col1A1 promoter activity, as reflected by 3.6ColA1-LUC (3.6-kb Col1A1 promoter) activity in aortic VSMCs. Similar responses were observed with the 2.3-kb Col1A1 promoter fragment (data not shown).1 Finally, retroviral transduction of primary aortic VSMC with SFG-caPTH1R reduced basal collagen protein accumulation versus cultures transduced with SFG-LacZ (Figure 2F). Thus, PTH/PTH1R activation inhibits Wnt/β-catenin signaling, type I collagen expression, and collagen protein accumulation in aortic VSMC cultures.

**SM-caPTH1R Transgene Expression**

**Downregulates Aortic β-Catenin Signaling and Aortic β-Catenin Protein Accumulation In Vivo**

PTH(1–34) administration reciprocally regulates aortic versus skeletal osteogenic programs.16,25 Specifically, PTH(1–34) suppresses aortic osteopontin (OPN), Msx2, and Wnt7a but stimulates bone formation, skeletal OPN expression, and circulating OPN levels in LDLR-deficient mice on HFD.25 PTH(1–34) downregulates aortic calcium accumulation in diabetic LDLR-deficient mice (Online Figure I and elsewhere25). However, PTH(1–34) dosing also exerts global effects (eg, changes in skeletal hematopoietic niche size,28 circulating OPN,25,39 and ROS-generated oxylipid signals23; Online Figure II) that control vascular disease processes.4,39 We sought to assess whether VSMC-autonomous PTH1R signaling could convey a subset of PTH(1–34) responses; therefore, we generated mice transgenic for a constitutively active PTH1R (caPTH1R),29 using the 0.5-kb SM22 promoter as a delivery module (SM-caPTH1R; Figure 3A). The SM-caPTH1R expression vector we validated by A7r5 transient transfection (Figure 2D) was digested with AclI and NdeI to liberate the SM-caPTH1R-globin untranslated region fragment (Figure 3A), and transgenic mice were generated (see Methods). Subsequently, we bred the SM-caPTH1R transgene onto the heterozygous LDLR+/−/− background, a LDLR-deficient background of intermediate vascular disease severity.40 As in LDLR+/−/− mice, HFD upregulates aortic expression of Msx2 in LDLR+/−/− mice (Online Figure III); however, 3 to 4 months of HFD feeding in LDLR+/−/− mice was required to achieve significant diabetes, and cholesterol levels were much lower in LDLR+/−/− versus LDLR+/−/− mice (Online Figure IV). Analysis of aortic extracts from SM-caPTH1R;LDLR+/−/− transgenic mice demonstrated a 2.5-fold increase in PTH1R protein levels versus nontransgenic siblings (Figure 3B; \( P < 0.01 \)). We then challenged male SM-caPTH1R;LDLR+/−/− and LDLR+/−/− sibling cohorts with 4 months of HFD, assessing the impact on body composition and metabolic profiles. As shown in Figure 3C, SM-caPTH1R;LDLR+/−/− mice exhibited no significant differences in bone mineral content, lean body mass, or fat mass, as assessed by dual-electron x-ray absorptiometry.25 Serum glucose and cholesterol levels were not reduced by the transgene and were, in fact, slightly higher in SM-caPTH1R animals (Figure 3D). No significant differences were observed in serum triglycerides and free fatty acids. Similarly, no significant differences were observed in fasting serum calcium, PTH, 8-F-isoprostane, or markers of bone turnover (Online Table I).

![Figure 3. LDLR+/−/− mice transgenic for SM-caPTH1R exhibit increased levels of aortic PTH1R protein without improvements in diabesity phenotype or alterations in bone turnover. A, Schematic of the SM-caPTH1R expression construct and AclI-NdeI fragment used to generate transgenic mice. B, Immunoreactive PTH1R protein was significantly increased in aortic extracts from SM-caPTH1R;LDLR+/−/− mice vs nontransgenic LDLR+/−/− littermates (n=4). C, Body composition, as assessed by dual-electron x-ray absorptiometry, was unaffected by the SM-caPTH1R transgene (n=14 to 16/group; 4 months HFD). D, Likewise, serum fasting glucose, cholesterol, triglycerides, and free fatty acids were not improved by the SM-caPTH1R transgene.](http://circres.ahajournals.org/lookup/suppl/doi:10.1161/CIRCRESAHA.117.312664/-/DC1/fig-3)
Because SM-caPTH1R inhibited Wnt activation of TCF/LEF-LUC in A7r5 VSMCs, we hypothesized that SM-caPTH1R may inhibit β-catenin activation signaling in VSMCs of the aortic tunica media.11,16 To test this notion, we generated SM-caPTH1R;TOPGAL⁺;LDLR⁻/⁻ mice and scored the aortic extent of LacZ (β-galactosidase) staining as an index of mural β-catenin activation registered by the TOPGAL reporter.41 As shown in Figure 4A and 4B, SM-caPTH1R;TOPGAL⁺;LDLR⁻/⁻ mice exhibited significantly fewer aortic LacZ⁺ cells versus nontransgenic TOPGAL⁺;LDLR⁻/⁻ siblings fed HFD. Furthermore, by Western blot analysis,16 aortic β-catenin protein levels were significantly reduced in SM-caPTH1R transgenics (Figure 4C), confirming the inhibition of β-catenin signaling. We next examined Dkk1 regulation. Dkk1 is highly expressed in aorta42 and is a direct endogenous target of β-catenin signaling43 that participates in a negative feedback loop to curtail excessive Wnt receptor activation.24 In vitro, Wnt5a upregulates Dkk1 promoter (LUC reporter) activity in aortic VSMCs, and caPTH1R inhibits Wnt5a-dependent induction (Figure 4D). In vivo, the SM-caPTH1R transgene also significantly reduced aortic Dkk1 mRNA accumulation (Figure 4E) and circulating levels of Dkk1 protein (data not shown). Thus, caPTH1R signaling downregulates aortic VSMC β-catenin signaling in vivo.

SM-caPTH1R Transgene Reduces Diabetic Arteriosclerotic Gene Expression and Fibrosis Without Altering Expression of Vascular TNF, Msx2, BMP2, or Wnt7

Diabetes-induced TNF upregulates vascular BMP2 and Msx2-Wnt expression and activates Wnt/β-catenin signals in vivo.11,16 Thus, reductions in vascular β-catenin signaling in response to the SM-caPTH1R transgene could be mediated via reductions in aortic TNF, Msx2, BMP2, or Wnt7. To test this, we extracted whole aorta RNA from SM-caPTH1R;LDLR⁺/⁻ transgenic and nontransgenic LDLR⁻/⁻ siblings fed HFD and evaluated gene expression by reverse transcription–quantitative PCR. Unlike results obtained with PTH(1–34)16,25 or infliximab11 administration, aortic expression of TNF, Msx2, BMP2, and Wnt7a were not significantly altered by the SM-caPTH1R transgene (Figure 5A). The expression of other vascular Wnt ligands such as Wnt5a and Wnt7b were also unaltered (data not shown). By contrast, as compared with nontransgenic sibling cohorts, SM-caPTH1R mice on HFD exhibited reduced aortic OPN and COL1A1 gene expression (Figure 5A). MMP9, a target of OPN-dependent ROS signaling in diabetic arteriosclerosis,23,44 was also diminished by caPTH1R (Figure 5A), whereas MMP2 and Nox4, the most abundant NADPH oxidase of VSMCs, were unaffected. However, Nox1, a NADPH oxidase that selectively conveys VSMC activation by advanced glycosylation products in diabetes,45 was decreased. Moreover, expression of Runx2, an osteochondrogenic transcription factor targeted by ROS signaling in VSMCs,46 was significantly diminished (confirmed by Western blot; data not shown). The reductions in aortic β-catenin signaling and Runx2 expression without changes in upstream TNF, Msx2, BMP2, or Wnt7 arteriosclerotic stimuli suggested that mural PTH1R activation might reduce aortic collagen accumulation and calcium accrual in vivo as in vitro. To test this notion, SM-caPTH1R;LDLR⁺/⁻ mice and nontransgenic LDLR⁺/⁻ siblings were placed on diabetogenic HFD for 12 weeks, and aortas were extracted for calcium and collagen content. As shown in Figure 5B, no difference in body weight was observed between cohorts. However, aortic calcium content (P=0.03) and collagen content (P=0.04) were significantly reduced in SM-caPTH1R;LDLR⁺/⁻ transgenic mice versus nontransgenic siblings. Histological staining for calcium deposition with Alizarin red demonstrated that transgene-regulated calcium deposition occurred primarily within the aortic tunica media (Figure 5C). Furthermore, cultures of transgenic aortic VSMCs exhibited reduced mineralization in vitro as compared with nontransgenic LDLR⁺/⁻ “WT” controls (Figure 5D). Twenty-four-hour fasting urinary calcium/creatinine ratios were not altered, indicating that the transgene did not enhance urinary calcium clearance (Online Figure V). Serum P1NP, a marker of type I collagen biosynthesis,36 was reduced in SM-caPTH1R;LDLR⁺/⁻ mice on HFD (Figure 5E). Histological staining of collagen with Picrosirius red confirmed reductions in aortic fibrosis by the SM-caPTH1R transgene (Figure 5F). Diabetic SM-caPTH1R;LDLR⁻/⁻ mice also exhibited reduced aortic collagen content as compared to nontransgenic LDLR⁻/⁻ siblings (Online Figure VI). Histomorphometric analysis revealed reduced aortic wall thickness in SM-caPTH1R;LDLR⁺/⁻ mice (Figure 5G), reflecting transgene-induced reductions in VSMC proliferation (Online Figure VII). Consistent with reductions in collagen, calcification, and wall thickness, aortas from SM-caPTH1R transgenic mice exhibited increased distensibility by ex vivo plethysmography (Figure 5H). Thus, VSMC PTH1R activity directly inhibits procalcific and profibrotic β-catenin signaling in aortas of diabetic LDLR-deficient mice, downregulating Nox1 and Runx2 without altering TNF, Msx2, BMP2, or Wnt7 expression. VSMC-autonomous PTH1R signaling recapitulates many, but not all, effects of systemic PTH(1–34) on diabetic arteriosclerosis.35

SM-caPTH1R Transgene Reduces Aortic Superoxide Accumulation

Oxidative stress plays a key role in diabetic arteriosclerosis,6,44,45 and systemic PTH(1–34) administration globally reduced oxidative stress reflected in serum 8-F-isopropanol in LDLR⁻/⁻ mice (Online Figure II).23 SM-caPTH1R transgene did not globally reduce oxidative stress (Table I); however, the SM-caPTH1R transgene did reduce aortic Nox1 (a key VSMC source of superoxide) and aortic OPN (a stimulus for Nox activation)23,44. Therefore, we assessed the impact of SM-caPTH1R transgene on aortic superoxide, a direct measure of local tissue oxidative stress. As shown in Figure 6A, the SM-PHT1R transgene significantly reduced aortic superoxide levels in LDLR⁻/⁻ mice as measured by lucigenin assay45; similar inhibition was observed in LDLR⁻/⁻ mice (Online Figure VIII). Moreover, VSMC pro-MMP9 activation (a target and index of Nox/ROS signaling)19 was also downregulated by the caPTH1R transgene (Figure 6B). Dihydroethidium staining23 confirmed reductions in
superoxide in VSMC expressing caPTH1R (data not shown). Thus, VSMC PTH1R signaling reduces aortic VSMC superoxide levels in LDLR-deficient mice fed diabetogenic diets.

Discussion

Once considered a passive process of dead and dying cells, research from laboratories worldwide has identified that arteriosclerotic matrix calcification is an actively regulated form of tissue mineralization.4 As in the developing skeleton,36 molecular and histoanatomic heterogeneity exists in vascular calcium deposition.15 In advanced atherosclerosis, such as that recapitulated by the ApoE-null mouse47 and LDLR-null mice on fatty diets for 4 months or more, 12 osteochondrogenic regulatory programs drive endochondral-type vascular mineralization, with contributions of dystrophic calcification also observed within cholesterol-laden lipid deposits.11 With time, advanced lesions can remodel to form woven bone with active hematopoiesis (ie, vascular osteogenesis).4,48 Elegant data from Giachelli and colleagues demonstrate that osteochondrogenic transdifferentiation of VSMCs drives atherosclerotic calcification process in ApoE-deficient mice.47 In the setting of chronic renal insufficiency, the hyperphosphatemic milieu of uremia further enhances osteochondrogenic transdifferentiation,49 with VSMC apoptosis contributing to medial and intimal calcium load.50 In T2DM, medial calcification predominates, driven by low-grade systemic inflammation of obesity and diabetes.4,15 These clinically relevant stimuli are recapitulated in male LDLR-deficient mice fed HFD15,25,51 but not in ApoE-deficient mice.15,51 When fed the HFD, male LDLR−/− mice become increasingly obese, hyperglycemic, hyperinsulinemic, and dyslipidemic with concomitant aortic calcification.11 During early disease stages, medial artery calcification predominates, as revealed by Alizarin red staining, without significant endochondral contributions.11,15,16 At later stages, aortic medial calcification is progressively accompanied by atherosclerotic calcification, including mineralization of cholesterol-laden lipid deposits.11 Thus, in response to clinically relevant stimuli (eg, diet-induced diabetes, obesity, and dyslipidemia), initiation and progression phases of arterial calcification in T2DM can be studied in detail.15 In both diabetic LDLR-deficient and atherosclerotic ApoE-deficient models, inflammation, oxidative stress, and MMP proteolytic remodeling have emerged as contributors to vascular calcium deposition.4,15

Figure 4. SM-caPTH1R transgene reduces aortic β-catenin signaling and β-catenin protein accumulation in diabetic LDLR−/− mice. SM-caPTH1R;LDLR−/− transgenic mice were bred with TOPGAL−/−;LDLR−/− reporter mice, and the extent of aortic LacZ staining was assessed in SM-caPTH1R;LDLR−/−;TOPGAL−/− mice vs LDLR−/−;TOPGAL−/− siblings following 1.5 months of HFD challenge. A, Representative aortic LacZ reporter visualization by staining frozen sections from these cohorts. B, Extent of LacZ staining was reduced in aortas of LDLR−/−;TOPGAL−/− mice possessing the SM-caPTH1R transgene (n=12). C, Accumulation of aortic β-catenin was reduced in LDLR−/−;TOPGAL−/− mice possessing the SM-caPTH1R transgene (n=4). D, Wnt-induced Dkk1 promoter activity is inhibited by SM-caPTH1R in aortic VSMCs in vitro. E, Expression of aortic Dkk1, an endogenous target of Wnt/β-catenin signaling, was reduced in vivo in mice possessing the SM-caPTH1R transgene (n=12).
Figure 5. SM-caPTH1R transgene downregulates aortic fibrosis and calcification without inhibiting TNF, BMP2, Wnt, and Msx2 gene expression in diabetic LDLR−/− mice. **A**, Aortic Col1A1, OPN, MMP2, Nox1, and Runx2 were reduced in LDLR−/− mice possessing the SM-caPTH1R transgene. Nox4 and upstream osteogenic signals were not altered (n=10; 4 months HFD). **B**, Aortic calcium and collagen content were decreased in aortic extracts from diabetic LDLR−/− mice possessing the SM-caPTH1R transgene (n=6 to 12 per group; 3 months HFD). **C**, Aortic calcium deposition occurred primarily within the tunica media. **D**, SM-caPTH1R transgene suppressed VSMC calcification in vitro. **E**, Serum P1NP, a marker of collagen biosynthesis, was also decreased (n=19 to 22 per group). **F**, Aortic collagen content, as assessed by Picrosirius histochemistry and digital image analysis, confirmed that SM-caPTH1R transgene decreased fibrosis (n=4). **G**, Thickness of the aortic tunica media was reduced by the SM-caPTH1R transgene. **H**, Aortic distensibility was increased by the SM-caPTH1R transgene.
The endocrine mechanisms controlling arteriosclerotic mineralization are only beginning to be understood. In this study, we focused on VSMC-autonomous PTH1R signaling and its impact on diabetic vascular disease. Daily PTH(1–34) administration, a potent stimulus for PTH1R activation and bone formation, reduces aortic OPN expression, downregulates aortic osteogenic Msx2-Wnt signaling, and decreases aortic calcification in LDLR−/− mice. Because the PTH1R is expressed in VSMCs, as well as in osteoblasts, reductions in vascular mineralization could arise from direct vascular actions of PTH(1–34), as well as indirect actions via the hematopoietic bone marrow–vascular axis. Implementing the VSMC-specific 0.5-kb SM22 promoter30 to express constitutively active Jansen receptor PTH1R(H223R),29 we now demonstrate that the PTH1R exerts VSMC-autonomous actions that inhibit proosteogenic and profibroblast Wnt/β-catenin signaling. Mural oxidative stress was concomitantly reduced by the SM-caPTH1R transgene. Because ROS inactivate the autoinhibitory MMP9 propeptide, oxidative stress enhances matrix turnover. Guzman and colleagues demonstrated the critical role for MMPs in vascular calcification, using a vitamin D toxicity model. Elastinolysis is certainly one contributor; however, seminal studies by George and colleagues have demonstrated that MMP9 and MMP12 stimulate β-catenin signaling by proteolytically liberating the N-cadherin-associated VSMC β-catenin pool. Thus, in addition to direct inhibition of β-catenin signaling, PTH1R-mediated downregulation of vascular ROS and MMP9 may also help preserve N-cadherin–dependent restraint of β-catenin in diabetic arteriosclerosis (Figure 7).

The precise intracellular signaling cascade whereby the PTH1R inhibits VSMC β-catenin actions has yet to be delineated. Because β-catenin protein levels are reduced by PTH1R activity in VSMCs, ubiquitin-dependent β-catenin proteolysis is likely involved. However, if so, this mechanism must be cell type–specific because protein kinase A, a key PTH1R mediator that inhibits Wnt/β-catenin signaling in VSMCs, augments β-catenin accumulation in other cell types. Cell type–specific protein–protein interactions between PTH1R and LRP6 (LDLR-related protein 6), the Wnt coreceptor important in paracrine Msx2-Wnt signaling, may also participate. Recently, however, peroxide has been shown to upregulate β-catenin levels and signaling via a ROS-sensitive nucleoredoxin-disheveled relay. Of note, protein kinase A phosphorylates Nox1 and thereby reduces Nox1 activity, a key source of ROS in diabetic VSMCs. Thus, it is tempting to speculate that inhibition of Nox1-dependent ROS signaling mediates PTH1R downregulation of aortic β-catenin and pro-MMP9. Alternatively, PTH1R...
activity may upregulate antioxidant defenses in VSMCs.6 These potential mechanisms remain to be evaluated.

There are limitations to our study. We have emphasized the impact of VSMC PTH1R signaling on diabetic arterial calcification in the absence of significant renal insufficiency. Chronic renal insufficiency is a common consequence of T2DM and profoundly accelerates vascular calcium accrual.57 The phosphate retention that characterizes uremia is a powerful stimulus not only for osteochondrogenic signaling but also for VSMC apoptosis.50 Thus, it remains to be determined whether VSMC-autonomous PTH1R signaling has any impact on vascular calcification with uremia. Anatomically distinct vascular beds may be differentially impacted by VSMC PTH1R activation.15 Although MMP9, BMP, and Wnt7 family members are prominent in diseased vessels,8,11,16,23,32,58 the relative contributions of individual components to the biology of diabetic arteriosclerosis have yet to be established. Moreover, although increased aortic distensibility was observed ex vivo, improvements in vascular compliance in response to VSMC PTH1R actions have yet to be demonstrated in vivo, and reductions in vascular ROS may potentially improve endothelium-dependent vasodilatation. Because systemic PTH(1–34) administration reduces adventitial Msx2 and Wnt7 expression,16 as well as regulates cell products released from the skeletal hematopoietic niche,28 PTH1R signaling beyond medial VSMCs may play even more important roles in the regulation of vascular calcification. Nevertheless, our study newly identifies the important contributions of VSMC-autonomous PTH1R signaling in limiting diabetic arteriosclerosis. This adds to accumulating data indicating that modulation of vascular PTH1R signaling may be useful as pharmacotherapy for treating cardiovascular disease.16,25,27,28

Sources of Funding
This work was supported by NIH grants HL069229 and HL081138 (to D.A.T.) and the Barnes-Jewish Hospital Foundation.

Disclosures
D.A.T. has served as a paid consultant to Merck, Pfizer, and the Institute of Medicine’s Committee to Review Daily Recommended Intake for Calcium and Vitamin D.

References
11. Al-Aly Z, Shao JS, Lai CF, Huang E, Cai J, Behrmann A, Cheng SL, Fowler DA. Aortic Msx2-Wnt calcification cascade is regulated by TNF-

Figure 7. VSMC PTH1R and β-catenin signaling in diabetic arteriosclerosis: a working model. Low-grade vascular inflammation and ROS that accompany T2DM promote mural elaboration of TNF, BMP2/4, and Msx2-Wnt signaling cascades that direct osteogenic differentiation of arterial mesenchymal progenitors.16 VSMC-autonomous PTH1R activation downregulates ROS accumulation, MMP9 activation (degrades N-cadherin19), β-catenin protein accumulation and signaling, and Runx2 expression. By mechanisms yet to be identified, systemic PTH(1–34) administration additionally reduces adventitial Msx2 and Wnt7 mRNA accumulation.16,25 The impact of PTH/PTH1R signaling on elastinolysis and matrix crosslinking has yet to be evaluated. Not shown is the important role of Sox9 in specifying the common osteo-/chondroprogenitor, or the Smad signals that convey vascular BMP actions.4,58


---

**Novelty and Significance**

**What Is Known?**

- Arteriosclerotic fibrosis and calcification of conduit vessels compromises smooth distal tissue perfusion, increasing the risk of lower extremity amputation.
- Arteriosclerosis is an actively regulated process potentiated in part via oxidative stress and osteogenic Wnt/beta-catenin signaling.
- The PTH receptor (PTH1R) is expressed in bone and vascular smooth muscle, and intermittent PTH administration stimulates bone formation but suppresses arterial calcification.

**What New Information Does This Article Contribute?**

- Vascular smooth muscle cell–autonomous PTH1R signaling reduces arterial oxidative stress.
- Inhibition of vascular Wnt/beta-catenin and ROS signaling with PTH1R agonists may exert beneficial actions in diabetic arteriosclerosis.

Arteriosclerotic calcification and fibrosis reduce conduit vessel compliance and distal tissue perfusion, thereby increasing the risk for amputation. Osteogenic gene regulatory programs control matrix deposition and calcification in both bone and vasculature and are reciprocally regulated by the osteotropic hormone PTH. We demonstrate that procalcific and proosteogenic Wnt/beta-catenin signaling cascades are inhibited in vascular smooth muscle cells by cell-autonomous PTH receptor actions that reduce beta-catenin signaling and vascular oxidative stress.
Activation of Vascular Smooth Muscle Parathyroid Hormone Receptor Inhibits Wnt/β-Catenin Signaling and Aortic Fibrosis in Diabetic Arteriosclerosis
Su-Li Cheng, Jian-Su Shao, Linda R. Halstead, Kathryn Distelhorst, Oscar Sierra and Dwight A. Towler

Circ Res. 2010;107:271-282; originally published online May 20, 2010;
doi: 10.1161/CIRCRESAHA.110.219899

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/107/2/271

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2010/05/20/CIRCRESAHA.110.219899.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at: http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at: http://circres.ahajournals.org//subscriptions/
Reagents, antibodies, growth factors, and tissue culture supplies – All biochemicals were purchased from Fisher, Sigma, or Pierce. Real-time fluorescent PCR and Dye Terminator Sequencing reagents were purchased from Applied Biosystems. RNAlater Tissue Collection:RNA Stabilization Solution (#AM7020) and RNase-free DNAse (DNA Free Kit; cat #AM1906) were purchased from Ambion. Total RNA purification kits were purchased from Qiagen (RNeasy MiniKit, #74104). Other molecular biology reagents and restriction enzymes were purchased from Invitrogen or Promega. Protease inhibitor cocktails and buffer constituents for protein analyses were purchased from Sigma. Reagents and standards for Bradford protein assays were purchased from Biorad (#500-0002). Sircol Collagen Assay kits from Biocolor Life Sciences were purchased via Accurate Chemical and Scientific, Westbury, NY (cat # CLR S1000). Slides, fixatives, and stains for histological assessment were purchased from Fisher Scientific. Tissue-Tek O.C.T. compound was purchased from Sakura Finetek U.S.A. Custom synthetic oligodeoxynucleotides, pre-cast SDS polyacrylamide gels, and Zymogram pre-cast zymography gels were purchased from Invitrogen. Reagents for protein preparation were obtained from Pierce, Sigma, and Fisher. Fetal bovine serum, tissue cultures media, media supplements, and antibiotics were purchased from Sigma and Invitrogen. Corning disposable tissue culture plasticware was purchased from Fisher. Human PTH(1-34) was obtained from Bachem (#H-4835). Recombinant mouse Wnt3a protein (#1324-WN) was purchased from R&D Systems. Mouse anti-PTH1R (sc-12722; 3D1.1; lot E0903) tubulin (sc-8035; Tu-02; lot D231), β-catenin (sc-7199; H-102; lot D038), and eIF2α (sc-133227;G-12; lot B1209) were purchased from Santa Cruz. Immobilon-P membranes for western blot were purchased from Millipore. I-block, alkaline phosphatase conjugated secondary antibodies (goat anti-mouse IgG+IgM #T2192; goat anti-rabbit #AC31RL) and chemiluminescent alkaline phosphatase substrate (CSPD) were purchased from Applied Biosystems / Tropix. Lucigenin (#B49203) and Krebs-Ringer bicarbonate buffer (#K4002) was purchased from Sigma-Aldrich. Dihydroethidium (DHE) was obtained from Molecular Probes. The colorimetric BrdU uptake and DNA incorporation ELISA for assaying cellular DNA synthesis was purchased from Roche Diagnostics (cat # 11647229001). Urinary calcium and creatinine concentrations were determined using the BioAssay Systems Quantichrom kit DICA-500 (Hayward, CA) and the Cayman Chemical Company creatinine assay kit 500701 (Ann Arbor, MI), respectively.

Tissue culture -- All cells were cultured at 37 °C with humidified air supplemented with 5% CO2. A7r5 rat aortic VSMCs were obtained from American Type Culture Collection (ATCC CRL-1444), and were maintained in Dulbecco's Modified Eagle's Media (DMEM) with 4.5 g/liter glucose, 4 mM glutamine, and 10% fetal bovine serum (FBS) with penicillin-streptomycin supplementation. C3H10T1/2 cells were obtained from the American Type Cell Culture and cultured in Basal Eagle's medium containing 10% FBS. Primary mouse aortic VSMCs were obtained using a modification of our previously published method for obtaining adventitial myofibroblasts, passed in the same media composition described above for A7r5 cells. Briefly, 6 to 8 male C57BL/6 mice, aged 2 to 3 months, were euthanized by exsanguination under ketamine-xylazine general anesthesia following protocols reviewed and approved by the institutional Animal Studies Committee. Subsequently, under sterile conditions, aortic segments from ascending aorta to the diaphragm were resected en bloc, rinsed twice with DMEM supplemented with antibiotics (200 IU/ml penicillin and 200 ug/ml of streptomycin) at room temperature, and placed into two 10 cm Corning tissue culture dishes containing fresh sterile
DMEM/penicillin/streptomycin. The adventitial cell layer was exhaustively dissected away, and the remaining aortic tissue sectioned into rings of ca. 2 mm in length using a sterile forceps and No. 11 scalpel. Aortic rings were then processed by transient digested with 2 mg/ml collagenase (Worthington, 243 U/mg) in serum – free DMEM for 4 hours at 37 °C, followed by brief centrifugation with aspiration of the supernatant. The processed aortic rings were then placed into two separate sterile 10 cm tissue culture dishes containing 3 mL of growth media (10% fetal calf serum in Dulbecco’s Modified Eagle’s Medium, high glucose, supplemented with 100 IU / ml of penicillin and 100 ug/ml of streptomycin), and cultured at 37 °C in a humidified incubator under 5% CO2. This amount of media is just sufficient to provide nutrition and hydration, and permits adherence of the aortic rings to the underlying tissue culture plastic. Two days later, 3 ml of additional growth media was added and changed every three days with daily monitoring. Two weeks after the initial plating, a sterile Pasteur pipette was used to aspirate the residual elastin-rich aortic rings, the outgrowth of adherent smooth muscle cells released by trypsinization, and re-plated at a 1:3 split onto 10 cm Corning tissue culture plates. Cells were amplified over the next 2 passages, and then used for transduction, gene expression, and collagen protein assays as indicated.

Expression plasmids, reporter plasmids, transient transfections, and luciferase assays –

The vascular smooth muscle cell- specific murine SM22 promoter fragment - 441 to + 44 was generated by PCR from mouse genomic DNA, introducing 5’-Xhol and 3’-KpnI restriction sites. As we’ve previously detailed, the SM22 promoter fragment was ligated into the eukaryotic expression plasmid pTRE2 (Clontech; Cat. #631008), following removal of the vector’s tetracycline response element and CMV promoter via Xhol-KpnI digestion. The resulting plasmid, pSM22/DT58.11, contains the SM22 promoter placed upstream of the poly linker, rabbit β-globin 3’-UTR, and polyA sequences of pTRE2. The human Jansen receptor PTH1R(H223R) cDNA (kind gift of H. Juppner) was used as a template and amplified by PCR to introduce (a) a unique 5’- BamHI restriction site just upstream of the initiator Met in good Kozak context; and (b) a unique 3’ KpnI restriction site just downstream of the stop codon of PTH1R(H223R). After digestion, this 1.8 kb PCR fragment was vectorially subcloned into the BamHI – KpnI of DT58.11, downstream of the 0.5 kb SM22 promoter, to create the SM-caPTH1R expression vector. The cDNA insert in SM-caPTH1R was re- sequenced to ensure fidelity and integrity of the open reading frame. The TOP (#21-204) and FOP (#21-205; control) luciferase reporter plasmids for activated Wnt/β-catenin signaling were purchased from Millipore. The cyclic AMP – responsive reporter pCRE-LUC (#219076) was purchased from Stratagene. The CMV-promoter driven eukaryotic expression vector pcDNA3 was purchased from Invitrogen. Mouse Wnt7a and Wnt7b cDNAs were obtained by RT-PCR amplification from C3H10T1/2 cells and subcloned into the KpnI-BamHI sites of pcDNA3 (Invitrogen, Carlsbad, CA) using techniques previously detailed. The 3.6 Col1A1 promoter – luciferase reporter construct 3.6Col1A1LUC was generated by PCR to amplify the 3.6 kb mouse Col1A1 promoter fragment using mouse genomic DNA as template, followed by vectorial 2-step assembly of the full 3.6 kb fragment upstream of the luciferase reporter gene in the Xhol / Bgl II sites of pGL2 (Promega). The promoter insert in 3.6Col1A1LUC was sequenced to ensure fidelity following amplification and ligation. Empty expression vectors (pcDNA3 or pSM2/DT58.11) were used to maintain constant DNA concentrations in all transient transfections. Transient transfections and luciferase assays were carried out precisely as we’ve previously detailed.

Generation of SFG retroviruses expressing Wnt7a, Wnt7b, and caPTH1R – Recombinant retrovirus pseudotyped with vesicular stomatitis virus G glycoprotein (VSV-G) was utilized to introduce human PTH1R(H223R), mouse Wnt7a, or mouse Wnt7b cDNAs to aortic smooth muscle cells or C3H10T1/2 cell line using methods we’ve previously detailed. The SFG
retroviral vector, SFG-LacZ control, and 293GPG packaging cell line were kindly provided by Dr. Dan Orly (Washington University in St. Louis). PCR was used to introduce 5' Nco I and 3'-BamHI sites onto the PTH1R(H223R) cDNA, and was vectorially cloned into the Ncol/BamHI site of SFG to create SFG-caPTH1R. However, because of the presence of 2 Ncol sites near the 3' end of the open reading frame, a single internal Kpnl site upstream of this region was used to divide PTH1R(H223R) cDNA into 2 fragments by PCR to facilitate cloning. Following 3-way ligation, the cDNA insert was re-sequenced to ensure fidelity of the open reading frame. SFG-caPTH1R plasmid and pMDHygro were co-transfected using LipofectAMINE Plus reagent (Invitrogen) into the 293GPG packaging cell line, which stably expresses MuLV gag-pol and tetracycline-suppressed vesicular stomatitis virus G glycoprotein. After hygromycin selection, the stable packaging cells expressing SFG-caPTH1R were expanded. To generate the pseudotyped retrovirus, packaging cells were incubated in regular growth medium without tetracycline and hygromycin to upregulate expression of VSV-G protein. The conditioned medium was harvested daily and the media harvested from day 3 to day 7 containing the highest titer of viral particles (≥ 5 x 10^6 colony-forming units/ml) were combined, centrifuged to remove cell debris, and used for transduction. For negative control, the SFG-LacZ pseudotyped retrovirus was also prepared. The SFG-Wnt7b plasmid was kindly provided by Dr. F. Long (Washington University in St. Louis). Due to the presence of internal Ncol sites in the Wnt7a cDNA, we generated the SFG-Wnt7a plasmid from our pcDNA3-Wnt7a construct using a strategy similar to that for generation of SFG-caPTH1R. A unique internal SacI site was used to divide Wnt7a cDNA into 2 fragments which were then cloned into the Ncol and BamHI sites of SFG vector. Of note, the introduction of Ncol primer to the 5' end of Wnt7a resulted in a non-synonymous Thr → Ala change at codon 2. Thus, site-directed mutagenesis (Stratagene) was employed to revert to the wild-type codon, and the Wnt7a cDNA insert was re-sequenced to ensure fidelity of the open reading frame (ABI Prism Dye Terminator kit, Foster City, CA).

**Transduction of cultured cells with pseudotyped retroviruses** – Retrovirus preparation and transduction was carried out using methods we’ve previously detailed. Briefly, the day before transduction, cells (either primary aortic myofibroblasts or C3H10T1/2 cells) were seeded in 10 cm culture dishes at a density of 0.5 x 10^5/dish. The next day, 10 mL of 50% conditioned medium containing pseudotyped retrovirus and 8 μg/mL polybrene was added to each dish. Virus infection was allowed to proceed for 24 h. At that time point, the virus medium was removed, and fresh medium was added. Cells were grown to confluence in Dulbecco’s Modified Eagle’s Medium supplemented with 10% FBS. Cells were passages and cultured for 7 – 14 days as outlined below. Media was changed every 2 – 3 days. For mineralization, cells were maintained in 10% FBS in DMEM supplemented with 10 mM β-glycerol phosphate and 50 ug/ml ascorbic acid.

**Analysis of transduced cell cultures for collagen gene expression and protein accumulation, alkaline phosphatase enzyme activity, and matrix calcification** – Assays of gene expression (RT-qPCR) and collagen protein accumulation (Sircol Assay) in transduced cell cultures closely followed the protocols as detailed for analysis of aortic tissue (vide infra). Alizarin red staining for calcium was carried out essentially as described. Briefly, the cell monolayers grown as above in 6 well cluster dishes were rinsed with Tris-buffered saline (TBS; 50 mM Tris pH 7.4 / 0.15 M NaCl) and fixed in ice cold 70% ethanol for 1 hour. Following 3 washes in water, 1 mL of filtered 0.4% Alizarin red S in distilled deionized H2O was added per well, and plates incubated at room temperature for 10 minutes with gentle shaking. After aspiration, monolayers were then washed five times x 5 minutes in 3 - 4 mL of water, followed by one wash in PBS for 5 minutes. Following aspiration, stained monolayers were imaged by digital photography as previously detailed. For alkaline phosphatase assays, cell monolayers...
In 24 well cluster dishes were washed three times with TBS, then harvested by scraping into 500 uL of 10 mM Tris pH / 0.5 mM MgCl2 / 0.1% Triton X-100, and cells further disrupted by probe sonication (3 serial 20 second bursts at 40% maximum). Aliquots were then assayed for cellular protein (10 uL; Bradford protein assay) and alkaline phosphatase enzyme activity (40 uL aliquots). Assays for alkaline phosphatase activity implemented 3.3 mM para-nitrophenyl phosphate as substrate in 6.7 mM MgCl2 buffered with 0.07 M 2- amino-2-methyl-1-propanol at pH 10.3. Enzyme activity was monitored by measuring absorbance at 405 nm with a BioTek microQuant plate spectrophotometer, and specific activity expressed as nmol of para-nitrophenol produced per min / mg cellular protein.

**Generation of SM-caPTH1R;LDLR+/— and SM-caPTH1R;LDLR+/-;TOPGAL transgenic mice**

Colonies were maintained following procedures reviewed and approved by the Washington University Animal Studies Committee. SM-caPTH1R transgenic mice (C57Bl/6 background) were generated using methods described5,11. Briefly, the vascular smooth muscle cell- specific murine SM22 promoter fragment - 441 to + 44 (relative to start site of transcription) was generated by PCR from mouse genomic DNA, introducing 5'-Xhol and 3'-KpnI restriction sites. The 0.5 kb SM22 promoter fragment was ligated into the eukaryotic expression plasmid pTRE2 (Clontech; Cat. #631008), following removal of the vector’s tetracycline response element and CMV promoter via Xhol-KpnI digestion. The resulting plasmid, pSM22/DT58.115, contains the SM22 promoter placed upstream of the polylinker, rabbit β-globin 3'-UTR, and polyA sequences following the protocol of Stratman5,12. After sequencing the insert to validate integrity of the open reading frame, the plasmid was digested with Acl I and Nde I to release the 3.6 kb fragment containing the SM22 promoter, human PTH1R(H223R) cDNA, rabbit β-globin 3'-UTR, and the polyA signal. This fragment was gel purified, bound and eluted from Qiagen silica resin, acetate-ethanol precipitated, re-solubilized in transgene buffer (10 mM Tris pH 7.4 / 0.1 mM EDTA), and transgenic (Tg) mice made via male C57BL/6 pronuclear injection (Washington University Mouse Genetics Core, Mia Wallace, Director). PCR genotyping for the SM-caPTH1R transgene was directed toward uniquely juxtaposed human PTH1R cDNA and rabbit β-hemoglobin 3'-UTR sequences following the protocol of Stratman12. Briefly, 5 mm tail segments were extracted for DNA using methods previously described7,13. SM-caPTH1R Tg genotyping amplimers were 5'-CAC TAC ATT GTC TTC ATG GCC ACA CCA TAC-3' and 5'-GAG GAG ACA ATG GTT GTC AAC AGA GTA G-3'. The PCR cycling conditions were as follows: initiation / polymerase activation at 95 ºC for 5 min, followed by 30 cycles of denaturation at 94 ºC for 30 seconds, annealing at 65 ºC for 30 seconds, and extension at 72 ºC for 1 min. The LDLR+/- mice were purchased from the Jackson Laboratory (stock #002207; C57BL/6 background), and stocks maintained using amplimer pairs directed toward the neomycin cassette (5'-AGG ATC TCG TCG TGA CCC ATG GCG A-3' and 5'-GAG CGG CCA TAC CGT AAA GCA CGA GG-3') and the genomic site of insertion in the LDLR gene (5'-CGC AGT GCT CCT CAT CTG ACT TGT C-3' and 5'-ACC CCA AGA CTT GCT CCC AGG ATG-3') to ensure genotype fidelity. SM-caPTH1R transgenic mice (LDLR+/—, C57BL/6) were crossed with LDLR+/- mice14 (C57BL/6 background) to generate SM-caPTH1R;LDLR+/- experimental animals and LDLR+/- sibling controls. Further backcross onto the LDLR+/- parental background generated SM-caPTH1R;LDLR+/- mice. SM-caPTH1R;LDLR+/- mice were bred with TOPGAL reporter mice15 to generate SM-caPTH1R;LDLR+/-;TOPGAL+ and LDLR+/-;TOPGAL+ littermates on a mixed C57BL/6:CD1 background. The TOPGAL reporter mice (TCF/LEF
Optimal Promoter / galactosidase reporter transgenic mice (stock #004623; CD1 background) were purchased from Jackson Labs. The TOPGAL genotyping amplimers were: 5'-GAG TGA CGG CAG TTA TCT GGA AGA TCA GGA-3' and 5'-GGA AAC CGA CAT CGC AGG CTT CTG CTT CAA TCA-3'.

Body composition and serum biochemical analyses — After overnight fasting, mice were anesthetized and body composition was determined by dual energy x-ray absorptiometry (DEXA) with a GE Lunar PixiMus using techniques previously detailed10,16. Subsequently, blood was withdrawn from the inferior vena cava in heparinized syringes. Blood samples were layered on top of Microtainer Serum Separator tubes (BD Biosciences #365956) and incubated overnight at 4 °C. Sera were obtained after centrifugation at 2,000 × g for 20 min and processed for analysis as previously described5,7,10. Serum concentration of osteoclast-derived tartrate-resistant acid phosphatase form 5b was determined by using MouseTRAP assay kit (Code SB-TR103; IDS Inc., Fountain Hills, AZ). Commercial EIAs and ELISAs for CTX (RatLaps, 1RTL4000, Nordic Bioscience Diagnostics via IDS Inc.) collagen N-terminal telopeptide (Rat/Mouse P1NP Assay; AC-33F1, IDS Inc.), and Dkk1 (DuoSet DY1765;R&D Systems) were carried out per the manufacturer’s instructions. The mouse intact PTH ELISA kit (#60-2300) was purchased from Immutopics International. The rodent osteopontin RIA (#900-090A) was purchased from Assay Designs. Biochemical assays for serum cholesterol (Thermo Scientific #2350-400H), glucose (Thermo Scientific #1524-400H), triglycerides (Sigma #TR0100) and free fatty acids (Wako #994-75409) were performed using commercially available assay kit, following the manufacturer’s instructions.

Western blot analysis of aortic PTH1R and β-catenin protein accumulation – Western blot analysis of extracted aortic protein was carried out using methods we’ve previously detailed11. Mouse aortas were rinsed twice with ice cold PBS, then extracted at 4°C with a hand-held Tissue-Tearor rotary homogenizer (BioSpec Products Inc.) in 5 volumes of 10 mM Hepes, pH 7.4, 0.5% Triton X-100, 0.15 M NaCl, 2 mM EDTA, 2 mM EGTA, 0.5 mM DTT, and 0.02% sodium azide supplemented with comprehensive protease (P8340) and phosphatase (P2850 and P5726) inhibitor cocktails from Sigma. After centrifugation, 30 µg of tissue protein extract (Bradford) was resolved by SDS-PAGE then electotransferred to Immobilon-P membranes (Millipore Corp.); proteins were immunovisualized by Western blot as previously detailed, using anti-PTH1R (sc-12722; 3D1.1; lot E0903) and β-catenin (sc-7199; H-102; lot D038), tubulin (sc-8035; Tu-02; lot D231), and eIF2α (sc-133227;G-12; lot B1209) antibodies at 1:1000. Alkaline phosphatase conjugated secondary antibodies (1:5000; goat anti-mouse IgG+IgM #T2192; goat anti-rabbit #AC31RL) and chemiluminescent alkaline phosphatase substrate (CSPD) were used to visualize immune complexes by exposure to x-ray film in autoradiography cassettes as previously detailed7. After digital capture of developed film images using a Hewlett-Packard ScanJet 5370C bed scanner, image analysis was performed by importing scanned jpeg images into Kodak 1D image analysis software. Absolute net pixel intensity of the CSPD-visualized bands with uniform rectangular region of interest were used to quantify western blot signals7. Data are expressed as net pixel intensity of the band, normalized to housekeeping protein control.

Quantitative assessment of gene expression by fluorescence RT-PCR — Messenger RNA accumulation was quantified using techniques we’ve previously detailed3,5,11. Fluorescence RT-PCR was performed to quantify relative mRNA levels of aortic osteogenic signaling molecules; RNA extraction techniques, methods, and validated amplifier pairs for these genes have been previously detailed7. Briefly, a 2 step reverse transcription – PCR was carried with use of TaqMan FAM dye fluorescence to quantify specific messages in cDNA aliquots arising from
reverse transcription of 75 ng of total RNA. Following the overnight fast, experimental animals are euthanized by exsanguination under ketamine-xylazine general anesthesia following protocols approved by the institutional Animal Studies Committee. Subsequently, aortic segments are resected en bloc from ascending aorta to the diaphragm, rinsed twice with sterile PBS (phosphate buffered normal saline), and each aorta then placed in 1.5 mL of RNAlater Tissue Collection:RNA Stabilization Solution (Ambion Cat. # AM7020), minced with a scalpel, and stored at 4 °C. The next day, aortic tissue is processed with an Omni International TH tissue homogenizer using a 5 mm x 95 mm generator (Fisher cat.# 15-338-203) in 600 uL of Qiagen RNAeasy MiniKit (Qiagen cat. #74104), to which 10 uL / mL fresh β-mercaptoethanol is added just prior to use. Total RNA is then isolated by spin column chromatography as per the manufacturer's instructions, eluted in 35 uL of nuclease-free sterile water. Total RNA is then treated with RNAse-free DNase to remove any small amount of contaminating genomic DNA (Ambion DNA Free Kit; cat #1906), and quantified by spectrophotometry. Subsequently, reverse-transcription (RT) reaction (20 uL) were carried out with Superscript II Reverse Transcriptase (Invitrogen) as per the manufacturer's protocol, using oligo dT15 + random hexamer (1.25 uM final of each) primed total RNA (1.5 ug – 2 ug). After heat inactivation of RT (95 °C for 15 minutes then snap cooled on ice), cDNA arising from 75 ng or100 ng (per assay) reverse transcribed total RNA was analyzed by biophasic fluorescence PCR (total volume 20 uL; 2 minutes at 50 °C, 10 minutes at 95 °C, then 40 cycles, 15 sec at 95 °C, 1 min at 60 °C). Expression of the indicated collagen, metalloproteinase, and osteogenic signaling genes was quantified by FAM dye-labeled fluorescence with an ABI 7300 Sequence Detection System in duplicate, implementing validated TaqMan probes (cat. #4331182) purchased from Applied Biosystems. Expression was normalized to the signal arising from 18S rRNA assayed in parallel duplicate aliquots, to control for efficiency in all RT-PCR studies. Relative mRNA accumulation was then expressed as the percent of control values. All analyses were repeated at least twice for each individual aortic specimen, with ≥ 5 animals per group per genotype.

Quantitative assessment of aortic collagen content by Sircol assay 18 -- Following the overnight fast, experimental animals were euthanized by exsanguination under ketamine-xylazine general anesthesia following protocols approved by the institutional Animal Studies Committee. Subsequently, aortic segments were resected en bloc from ascending aorta to just below the diaphragm (level of renal arteries), very quickly rinsed twice with sterile deionized water then 5 times with fresh water and blotted x 5 on a Kimwipe tissue wipe to remove adherent moisture, and stored in a sterile microfuge tube on ice until weighed in a tared, small polystyrene weighing dish (Fisher Cat. #02-294A) on a Mettler Toledo XS105 microbalance. Total aortic collagen was then extracted from murine aortas and quantified using the Sirius red dye binding assay 18 (Sircol Collagen Assay, Biocolor Life Sciences, US supplier Accurate Chemical and Scientific, Westbury, NY, cat # CLR S1000). Pre-weighed samples of insoluble cross-linked collagen processed identically in parallel were used to generate the standard curve for this assay. Briefly, aortic specimens were minced into 3 pieces and total collagen extracted by adding 500 uL of extraction reagent (10 mg of pepsin / ml in 0.5M acetic acid). Collagen was solubilized for 7 days at 25 °C. After equilibration of all reagents to 25 °C, duplicate aliquots or standards (50 uL / sample) were treated with 1 mL of Sircol dye reagent in 1.5 ml microcentrifuge tubes, mixed thrice by gentle inversion, then further treated by gentle agitation at room temperature for 60 minutes. The insoluble collagen-dye pellet was then collected by centrifugation (12,500 g x 10 minutes), the supernatant decanted, and collagen-bound Sirius red solubilized by addition of 1 mL of Sircol alkali reagent and vigorous vortexing for 5 minutes. After a brief centrifugation (1 minute x 12,500g), 200 uL of sample or standard was transferred to 96 microwell plates, and OD measured at 540 nm with a BioTek microQuant plate spectrophotometer, with correction for path length monitored at 900 nm.
**LacZ / β-galactosidase histochemistry and quantitative assessment of aortic sections**

Aortic β-galactosidase staining was carried using methods we’ve previously detailed.\(^3,5,11,19\) Comparisons of LacZ activity elaborated by \textit{SM-caPTH1R;LDLR\(^{+/−}\);TOPGAL} mice vs. \textit{LDLR\(^{+/−}\);TOPGAL} littermate controls were made in aortic frozen sections. A Microm HM 550 cryostat was used to prepare 16 micron frozen sections of ascending aortas from these TOPGAL reporter cohorts that had been previously imbedded in O.C.T. Compound (Tissue-Tek, Sakura Finetek). After thawing slides to room temperature, sections were immediately fixated in 0.25% glutaraldehyde in PBS for 10 minutes. After washing once with water and once with PBS, approximately 200 uL of X-gal staining solution\(^15\) (1 mg/ml X-gal in 2 mM PBS supplemented with 2 mM MgCl\(_2\), 5 mM K\(_3\)Fe(CN)\(_6\) and 5 mM K\(_4\)Fe(CN)\(_6\)) was layered over each section on the slide, and LacZ enzyme activity revealed by overnight development at 37°C in a sealed humidified chamber. The next morning, slides were rinsed once with PBS, twice with deionized water, and counterstained with nuclear fast blue stain. After 2 x 2 minute washes in deionized water, sections were rapidly dehydrated by 30 second serial incubations in graded alcohol solutions (50%, 70%, 80%, 90% and 100%), immersed in xylene, transiently air dried, then covered in Permount slide mounting solution and sealed with a coverslip. Digital photomicrographs were obtained on either (a) a Nikon CoolPix 5000 camera mounted on a Nikon Eclipse TS100 microscope or (b) Leica DFC 420 Digital Camera mounted on a Leica 4000 DM digital microscope. After importing JPEG files into Adobe Photoshop, digital image analysis was performed by counting the numbers of vessel wall cells and aortic valve leaflet cells that stained blue with X-gal.

**Quantitative assessment of aortic collagen content by picrosirius red histochemistry**

Quantitative histochemical analysis of collagen accumulation by digital image analysis of picrosirius red stained specimens was performed using a modification of previously published dark field imaging protocols.\(^20,21\) Following the overnight fast, experimental animals were euthanized by exsanguination under ketamine-xylazine general anesthesia following protocols approved by the institutional Animal Studies Committee. Subsequently, the heart and ascending aortic segment to the take-off of the innominate artery was resected en bloc, the heart transected with a sharp razor midway between the base and apex, rinsed thoroughly with sterile PBS to remove adherent blood and coagulum. The upper heart and ascending aorta were then fixed in 10% neutral buffered formalin with gentle agitation at room temperature. Two days later, the upper heart and ascending aorta were placed in cassettes with the transected cardiac “flat” surface faced downward, and imbedded in paraffin. Subsequently, 6 micron paraffin sections were prepared cutting from the transected surface towards the base and aortic outflow tract through the aortic sinus into the ascending aorta. Sections were selected for evaluation of collagen by picrosirius red staining at the level of the ascending aortas distal to the aortic sinus and just proximal to the innominate artery. Inner aortic diameters at this level were equivalent for both LDLR\(^{+/−}\) and SM-caPTH1R;LDLR\(^{+/−}\) animals. Sections were de-paraffinized in xylene and rehydrated via graded alcholic solutions to water, nuclei stained with Weigert’s hematoxylin for 5 minutes, then rinsed in water for 10 minutes. Sections were subsequently stained for 1 hour in 0.1% picrosirius red F3B prepared in a saturated aqueous solution of picric acid, and then rinsed twice with 0.5% (v/v) glacial acetic acid in distilled deionized water. After shaking to encourage egress of adherent water, slides were dehydrated by treated three times with 100% ethanol, cleared in xylene, and covered with Permount underneath a cover slip. Digital photomicrographs were obtained on a Leica DFC 420 Digital Camera mounted on a Leica 4000 DM digital microscope using a 10X objective lens with dark field illumination with the following settings: dark field illumination intensity of 2X, exposure time of 1s, gain of 1X and color saturation of 1.5X, with gamma curve settings of gamma = 0.1, black = 8 and white = 21. Image analysis was performed by importing digital JPEG images into Kodak 1D image analysis.
software. Absolute net pixel intensity of the picrosirius – stained collagen visible with darkfield illumination was quantified in each aortic cross-section. For each animal, three adjacent sections were analyzed and averaged to provide the aortic fibrosis net intensity value for that animal. Graphed data are presented as the mean ± S.E. net pixel intensity obtained from analyses of aortas from n = 4 LDLR+/- and n = 5 SM-caPTH1R;LDLR+/- male mice challenged with HFD for 3 months. Digital micrometry was implemented to quantify aortic thickness, measured from the outer elastic lamina to the luminal / endothelial surface in these same animals. Digital images were captured on a Leica 4000 DM digital microscope equipped with a DFC 420 Digital Camera using FW 4000 Software, and mural thickness measured with Leica LAS Image Analysis Software. Wall thickness was measured approximately every 30 degrees on the 360 degree aortic circumference at a level just distal to the aortic sinus. These 11 – 12 measurements were then averaged to provide the mean ascending aorta wall thickness for each individual animal. Data are presented as the mean +/- S.E. wall thickness measured for n = 4 animals of each genotype.

**Analysis of aortic calcium content** – Aortic calcium content was measured using techniques we’ve previously detailed. Following the overnight fast, experimental animals were euthanized by exsanguination under ketamine-xylazine general anesthesia following protocols approved by the institutional Animal Studies Committee. Subsequently, aortic segments were resected en bloc from ascending aorta to just below the diaphragm (level of renal arteries), very quickly rinsed twice with sterile deionized water, blotted x 4 on a Kimwipe tissue wipe to remove adherent moisture, weighed in a tared, small polystyrene weighing dish (Fisher Cat.# 02–294A) on a Mettler Toledo XS105 microbalance, and then transferred to a 2.0 ml polypropylene screw cap tube with o-ring seals. After heating 20 minutes at 70 ºC, samples are speed evaporated under 1000 mTorr vacuum at 70 ºC with centrifugation for 60 minutes, and the tared tubes reweighed with the dried aortic tissue inside. Subsequently, 20 volumes (20 uL per ug dry aortic mass) of 10% formic acid in deionized water was added to each sample, a new screw cap tightly fixed, and aortic calcium extracted overnight at 37 ºC. The following morning, samples are cooled to room temperature for 30 minutes, centrifuged for 5 minutes at 13,200 rpm, and the supernate transferred by pipetting to another tube. Two 10 uL aliquots for each specimen are added to 50 uL of deproteinization buffer (0.3 ml of glacial acetic acid and 3.8 ml of 1 N KOH diluted to 50 mL with deionized water, pH 5.2). After heating for 5 minutes at 95 ºC in a screw cap centrifuge tube, samples are immediately centrifuged 5 minutes at 13,200 rpm, snap cooled on ice at 4 ºC for 2 minutes, and 10 uL aliquots mixed with 100 ul of freshly prepared OCPC color reagent (ortho-cresolphthalein complexone; Sigma phthalein purple cat. #P5631) as described by Connerty and Briggs. The purple OCPC calcium complex is spectrophotometrically determined by absorbance at 570 nm using a uQuant BIO-TEK plate reader. Background was determined in parallel using a sham extracted pair of empty microfuge tubes starting with 200 uL of 10% formic acid. A standard curve performed with each assay from a 1 mg/ml stock calcium solution prepared in deionized water from heat-dried calcium carbonate.

**Urinary calcium and creatinine measurements** – SM-caPTH1R;LDLR+/- and LDLR+/- male sibling cohorts were place on HFD for 3 months, then individually house overnight with ad libitum access to water in metabolic cages to collect feces-free urine during a 24 hour fast (n = 7 SM-caPTH1R+/-;LDLR+/- mice vs. 4 LDLR+/- sibling cohort controls). The next day, urinary calcium and creatinine concentrations were determined using the BioAssay Systems QuantiChrom kit DICA-500 (Hayward, CA) and the Cayman Chemical Company creatinine assay kit 500701 (Ann Arbor, MI), respectively. All samples were measured in duplicate, and
data presented as the mean +/- S.E. for the urinary calcium concentration (mg/dL) normalized to urinary creatinine concentration (mg/dL).

**Quantitative assessment of aortic distensibility by ex vivo aortic video plethysmography** -- Aortic distensibility was measured implementing a modification of the method of Mecham et al.\(^\text{23}\) using a plethysmography system purchased from Living Systems Instrumentation (LSI) (Burlington, VT). Briefly SM-caPTH1R;LDLR+/- (n = 4) and LDLR+/- (n =5) sibling controls were maintained on HFD for 3 months beginning at 5- 8 weeks of age. Mice were euthanized by exanguination under ketamine-xylazine anesthesia using protocols approved by the Washington University Animal Studies Committee. After careful dissection in supine position to reveal the ventral aspect of the thoracic aorta in situ, the native length (15 – 20 mm) of the thoracic aorta from the left subclavian artery to the diaphragm was recorded with a digital Vernier caliper (ILAC-MRA) and landmarked under dissection scope magnification (Olympus SZ-PT). The thoracic aorta was then transected at the landmarks, excised by gentle lifting at the caudal end with careful dissection to separate from the adjacent vertebrae, and rinsed in HEPES-buffered saline solution (HBSS) supplemented with 0.02% sodium azide. In a small puddle of HBSS that maintains hydration and lumen patency, the aorta held firmly gently in place on a glass plate via the cephalad end with a cotton swap. The adherent adventitial was then removed by a repetitive, gentle “rolling and stroking” action with an un-treated cotton swab (cephalad to caudal strokes). Any residual fat was removed by extremely cautious dissection with a scalpel under magnification. The cleaned thoracic aortic specimen is then ligated with 4-0 braided suture to two custom-machined, externally etched 0.3 mm metal cannulae (Washington University School of Medicine Machine Shop, St. Louis, MO) mounted onto the Living Systems Instruments (LSI) CH/1/SH chamber (see **Online Figure IX**). The aorta is then extended to native in situ length by adjusting the screw-gear mounts of the CH/1 chamber that orient the cannula longitudinally, using digital Vernier caliper measurement to guide distraction. The vessel is then gently perfused (pressure <50 mmHg) with HBSS to identify “leaks” at thoracic intercostals arteries. Intercostal arteries were ligated with 0.05 mm diameter microsutures using fine forceps (see **Online Figure IX**) -- within the fluid-filled chamber and visualized under the dissecting scope -- to eliminate the possibility of leakage when pressurized. Once all sites of leakage had been identified and sutured, the chamber was moved to the stage of a Nikon inverted microscope (Nikon Eclipse Model TE2000-S) outfitted with a Sony XC-ST30 CCD digital camera, and the image clearly focused by digital projection onto the screen of a Costar video monitor (Carrollton, TX). Intraluminal pressure was applied using a peristaltic pump (PS/200 servo with pump, PM/4 pre-and post- chamber in-line pressure transducers) with native proximal – distal direction fluid flow through the aorta at rates of 0 to 5 ml/min. Changes in outer vessel diameter were measured using the V94 Video Dimension Analyzer. Manual calibration and loading of the scan line with an over-stage micrometer slide was performed prior to each day’s measurements (maximum of 2 aortas per day) as per the manufacturer’s instructions. The change in thoracic aortic diameter (microns) per mmHg increase in pressure loading over the range of 0 mmHg to 50 mm Hg was measured twice for each aorta, averaged, and recorded. Aortic distensibility data is expressed as the mean +/- S.E. aortic diameter change in microns per mm Hg pressure load.

**Quantitative assessment of aortic superoxide levels** -- The chemiluminescent substrate lucigenin was utilized to quantify aortic oxidative stress – viz., aortic superoxide – following a modification of previously described methods\(^\text{24,25}\). Briefly, following euthanasia by exsanguination under ketamine-xylazine anesthesia, aortic segments were resected en bloc from diaphragm through the aortic arch, rinse 5 times with intermittent blotting on Kimwipe to remove adherent blood and coagulum, and aorta placed in 2 mL of Krebs-Ringer bicarbonate buffer on ice until all animal processed. Each aorta is divided into 2 mm segments which are
Supplement Material: Cheng SL, Shao JS, Halstead LR, Distelhorst K, Sierra O, and Towler DA. Activation of Vascular Smooth Muscle Parathyroid Hormone Receptor Inhibits Wnt/β-Catenin Signaling and Aortic Fibrosis in Diabetic Arteriosclerosis
CIRCRESAHA/2010/219899/R1

individually incubated in 100 μL of Krebs-Ringer buffer within a 96-well white luminometry microplate (Berthold #23300). Subsequently, 100 μL of 10 μM (2X) lucigenin in Krebs-Ringer bicarbonate buffer added with mixing to each well, and the plate allow to equilibrate in the dark within the luminometer at room temperature for 10 minutes. Subsequently, photons are counted in the Centro XS3 LB 960 Berthold luminometer, integrating light emitted from all aortic segments over a period of 60 seconds. Readings are repeated 3 to 4 times to ensure stability of signal, and the penultimate measurement is used to quantify ROS. Background signal is determined by integrating readings from blank wells containing 200 μL of 5 μM lucigenin in Krebs-Ringer buffer.

**Quantitative gelatin zymography of cultured primary cells**

Quantitative zymography was used to assess collagenase activity using techniques we’ve previously detailed. Before each analysis, 0.5 million aortic VSMCs obtained from either SM-caPTH1R;LDLR+/- or LDLR+/- siblings were plated on a 15 cm tissue culture dish, grown until confluent, then seeded at high density 1.0 – 1.5 x 10^5 cells per well (12-well cluster dishes). After recovery from plating, cells were maintained in serum-free DMEM (25 mM glucose) for 24 hours or 48 hours as indicated. Twenty microliter aliquots of serum-free conditioned media were analyzed by zymography (n = 3) using precast 10% Zymogram Gelatin Gels (Invitrogen - Novex) per the manufacturer’s instructions (Novex technical bulletin IM-1002, Version B). With constant gentle agitation, gels were renatured for 30 minutes at room temperature, developed overnight at 37 °C, fixed and stained with Colloidal Blue (Novex technical bulletin IM-6025), extensively washed (> 20 hours) to yield uniform background signal, and digital images of stained wet gels captured using a Hewlett-Packard ScanJet 5370C bed scanner. Image analysis was performed by importing scanned JPEG images into Kodak 1D image analysis software, using absolute net pixel intensity of the pro-MMP9 zymogram with uniform rectangular region of interest to quantify metalloproteinase activity. Data are expressed as net pixel intensity of the band, normalized either to mass of extracted tissue protein, or the control treatment condition as indicated. Graphed data are presented as the means ± S.E. of 3 independent replicates.

**Statistical analysis** – All data points are presented as the mean +/- S.E. of independent replicates (n = 3 to 22, dependent upon the assay). Each in vitro experiment was carried out at least twice. For scoring LacZ histochemical scoring, non-parametric Mann-Whitney U test was implemented. All other statistical analyses performed by one-way ANOVA or Student’s t-test analysis as indicated using GraphPad InStat Software (Version 3.06 for Windows). When the ANOVA p was significant (p < 0.05), analysis for intergroup differences was then performed using either Student-Newman-Keuls (for all pairwise comparisons) or Dunnett’s (for comparison vs. control) post-hoc tests for multiple comparisons with GraphPad Instat.
CITATIONS IN THE SUPPLEMENT


Online Table I: Serum Biochemistries of LDLR+/- and SM-caPTH1R;LDLR+/- Transgenic Mice

<table>
<thead>
<tr>
<th>Serum marker</th>
<th>LDLR+/-</th>
<th>SM-caPTH1R;LDLR+/-</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium (mmol/L) *</td>
<td>2.08 +/- 0.03</td>
<td>2.11 +/- 0.04</td>
<td>0.567</td>
</tr>
<tr>
<td>PTH (pg /mL)</td>
<td>54.0 +/- 10.0</td>
<td>59.9 +/- 12.5</td>
<td>0.71</td>
</tr>
<tr>
<td>Osteopontin (nmol / L)</td>
<td>38.0 +/- 0.1</td>
<td>37.0 +/- 0.1</td>
<td>0.74</td>
</tr>
<tr>
<td>TRAP (U/L)</td>
<td>46.2 +/- 1.2</td>
<td>46.2 +/- 1.2</td>
<td>0.10</td>
</tr>
<tr>
<td>Type I collagen CTX C-terminal peptide (ng/ml)</td>
<td>20.1 +/- 3.2</td>
<td>24.5 +/- 4.8</td>
<td>0.22</td>
</tr>
<tr>
<td>8-F-Isoprostane (pg/ml)</td>
<td>41.0 +/- 12.3</td>
<td>46.2 +/- 5.1</td>
<td>0.41</td>
</tr>
</tbody>
</table>

*n = 17 – 23 per group; all other parameters 7 – 13 per group.

SUPPLEMENTARY DATA

**Online Figure I:** PTH(1-34) down-regulates aortic calcification in LDLR-/- mice fed high fat diabetogenic diets (HFD). LDLR-/- mice were fed HFD for 1 month\(^27\), and subcutaneously dosed once daily for 5 days per week with either vehicle or 400 ng/gm PTH(1-34) precisely as detailed\(^10\). Following overnight fast, aortic calcium was extracted and quantified as outlined in the online Methods section above (n = 10 / group).

**Online Figure II:** PTH(1-34) down-regulates serum 8-F-isoprostane, a circulating marker of systemic oxidative stress\(^28\), in LDLR-/- mice fed high fat diabetogenic diets (HFD). LDLR-/- mice were fed HFD for 1 months, and treated once daily 5 days per week with either s.c. vehicle or 400 ng/gm PTH(1-34) precisely as detailed\(^10\). Following overnight fast, serum was obtained and 8-F-isoprostane levels quantified as an index of systemic oxidative stress\(^28\) using a commercially available EIA from Cayman as outlined in the online Methods section above\(^26\) (n = 10 / group).

**Online Figure III:** High fat diabetogenic diet (HFD) upregulates aortic expression of Msx2 in male LDLR+/- mice. LDLR+/- mice were fed HFD for 4 months, and total aortic RNA extracted and analyzed for Msx2 gene expression\(^5,10,11\). Data are presented as the mean +/- S.E.M. relative Msx2 mRNA accumulation observed (n = 5 / group), expressed as % of 18s rRNA signal. As in LDLR-/- mice\(^5,11,27\), HFD upregulates aortic Msx2 expression in LDLR+/- mice.

**Online Figure IV:** High fat diabetogenic diet (HFD) induces diabetes in male LDLR+/- mice. LDLR+/- mice were fed HFD for 4 months, and fast serum glucose and cholesterol levels compared to that observed in LDLR-/- mice fed HFD for 1 month. LDLR+/-, n = 10 / group; LDLR-/-, n = 9 / group. Dashed line, the 14 mmol /L (250 mg/dL) fasting glucose threshold for diabetes in mice\(^29\). As in LDLR-/- mice\(^27\), HFD induces diabetes in LDLR+/- mice, but with significantly less hypercholesterolemia.

**Online Figure V:** The SM-caPTH1R does not increase urinary calcium excretion. LDLR+/- and SM-caPTH1R;LDLR+/- mice were placed on HFD for 3 months, then individually house in metabolic cages with ad libitum access to water for assessment of 24 hour fasting urinary calcium and creatinine excretion. The SM-caPTH1R transgene did not increase urinary calcium excretion.
Online Figure VI: The SM-caPTH1R transgene reduces aortic collagen content. Aortic collagen content was also decreased in LDLR-/- mice possessing the SM-caPTH1R transgene (n = 12-14 / group, three months HFD).

Online Figure VII: The SM-caPTH1R transgene reduces proliferation of primary aortic myofibroblasts. Primary cultures of aortic myofibroblasts were prepared as described in Methods. As a quantitative index of cell proliferation, DNA synthesis was measured using bromodeoxyuridine (BrdU) uptake and incorporation into DNA, implementing a commercially available ELISA. When compared with LDLR+/- sibling control myofibroblasts, myofibroblasts from SM-caPTH1R;LDLR+/- exhibited significantly reduced DNA synthesis.

Online Figure VIII: The SM-caPTH1R transgene reduces aortic superoxide levels. Aortic superoxide levels were reduced in LDLR-/- mice possessing the SM-caPTH1R transgene (n = 7-11 / group).

Online Figure IX: Ex vivo aortic video plethysmography using customized cannulae on a LSI CH/1/SH vessel chamber.
Online Figure I: PTH(1-34) down-regulates aortic calcification in LDLR-/- mice fed high fat diabetogenic diets (HFD).

LDLR-/- mice were fed HFD for 1 month\textsuperscript{26}, and subcutaneously dosed once daily for 5 days per week with either vehicle or 400 ng/gm PTH(1-34) precisely as detailed\textsuperscript{10}. Following overnight fast, aortic calcium was extracted and quantified as outlined in the online Methods section above (n = 10 / group).
Online Figure II: PTH(1-34) down-regulates serum 8-F-isoprostane, a circulating marker of systemic oxidative stress \(^{28}\), in LDLR\(^{-/-}\) mice fed high fat diabetogenic diets (HFD).

LDLR\(^{-/-}\) mice were fed HFD for 1 months, and treated once daily 5 days per week with either s.c. vehicle or 400 ng/gm PTH(1-34) precisely as detailed\(^{10}\). Following overnight fast, serum was obtained and 8-F-isoprostane levels quantified as an index of systemic oxidative stress\(^{28}\) using a commercially available EIA from Cayman as outlined in the online Methods section above\(^{26}\) (n = 10 / group).
Online Figure III: High fat diabetogenic diet (HFD) upregulates aortic expression of Msx2 in male LDLR+/- mice.

LDLR+/- mice were fed HFD for 4 months, and total aortic RNA extracted and analyzed for Msx2 gene expression5,10,11. Data are presented as the mean +/- S.E.M. relative Msx2 mRNA accumulation observed (n = 5 / group), expressed as % of 18S rRNA signal. As in LDLR-/- mice5,11,27, HFD upregulates aortic Msx2 expression in LDLR+/- mice.
Online Figure IV: High fat diabetogenic diet (HFD) induces diabetes in male LDLR+/- mice.

LDLR+/- mice were fed HFD for 4 months, and fast serum glucose and cholesterol levels compared to that observed in LDLR-/- mice fed HFD for 1 month. LDLR+/-, n = 10 / group; LDLR-/-, n = 9 / group. Dashed line, the 14 mmol/L (250 mg/dL) fasting glucose threshold for diabetes in mice. As in LDLR-/- mice, HFD induces diabetes in LDLR+/- mice, but with significantly less hypercholesterolemia.
Online Figure V: The SM-caPTH1R does not increase urinary calcium excretion.

LDLR+/- and SM-caPTH1R;LDLR+/- mice were placed on HFD for 3 months, then individually house in metabolic cages with ad libitum access to water for assessment of overnight fasting urinary calcium and creatinine excretion. The SM-caPTH1R transgene did not increase urinary calcium excretion.
Online Figure VI: The SM-caPTH1R transgene reduces aortic collagen content.

Aortic collagen content was also decreased in LDLR-/- mice possessing the SM-caPTH1R transgene (n = 12-14 / group, three months HFD).
Online Figure VII: The SM-caPTH1R transgene reduces proliferation of primary aortic myofibroblasts

Primary cultures of aortic myofibroblasts were prepared as described in Methods. As a quantitative index of cell proliferation, DNA synthesis was measured using bromodeoxyuridine (BrdU) uptake and incorporation into DNA, implementing a commercially available ELISA. When compared with LDLR+/- sibling control myofibroblasts, myofibroblasts from SM-caPTH1R;LDLR+/- exhibited significantly reduced DNA synthesis.
Aortic superoxide levels were reduced in LDLR-/- mice possessing the SM-caPTH1R transgene (n = 7-11 / group).
Ex vivo aortic video plethysmography

Online Figure IX

- Mouse aorta
- Distal ligation to cannula magnified
- Video microscopy image of mouse thoracic aorta with ligated intercostal artery
- Mouse mid-thoracic aorta subsegment
- Ex vivo aortic video plethysmography
- Video microscopy image of mouse thoracic aorta with ligated intercostal artery

Online Figure IX

- CIRCRESAHA/2010/219899/R1
- Supplement Material
- page S23