Inhibition of Bone Morphogenetic Protein Signaling Reduces Vascular Calcification and Atherosclerosis

Matthias Derwall, Rajeev Malhotra, Carol S. Lai, Yuko Beppu, Elena Aikawa, Jasbir S. Seehra, Warren M. Zapol, Kenneth D. Bloch, Paul B. Yu

Objective—The expression of bone morphogenetic proteins (BMPs) is enhanced in human atherosclerotic and calcific vascular lesions. Although genetic gain- and loss-of-function experiments in mice have supported a causal role of BMP signaling in atherosclerosis and vascular calcification, it remains uncertain whether BMP signaling might be targeted pharmacologically to ameliorate both of these processes.

Methods and Results—We tested the impact of pharmacological BMP inhibition on atherosclerosis and calcification in LDL receptor-deficient (LDLR−/−) mice. LDLR−/− mice fed a high-fat diet developed abundant vascular calcification within 20 weeks. Prolonged treatment of LDLR−/− mice with the small molecule BMP inhibitor LDN-193189 was well-tolerated and potently inhibited development of atheroma, as well as associated vascular inflammation, osteogenic activity, and calcification. Administration of recombinant BMP antagonist ALK3-Fc replicated the antiatherosclerotic and anti-inflammatory effects of LDN-193189. Treatment of human aortic endothelial cells with LDN-193189 or ALK3-Fc abrogated the production of reactive oxygen species induced by oxidized LDL, a known early event in atherogenesis. Unexpectedly, treatment of mice with LDN-193189 lowered LDL serum cholesterol by 35% and markedly decreased hepatosteatosis without inhibiting HMG-CoA reductase activity. Treatment with BMP2 increased, whereas LDN-193189 or ALK3-Fc inhibited apolipoprotein B100 secretion in HepG2 cells, suggesting that BMP signaling contributes to the regulation of cholesterol biosynthesis.

Conclusion—These results definitively implicate BMP signaling in atherosclerosis and calcification, while uncovering a previously unidentified role for BMP signaling in LDL cholesterol metabolism. BMP inhibition may be helpful in the treatment of atherosclerosis and associated vascular calcification. (Arterioscler Thromb Vasc Biol. 2012;32:613-622.)

Key Words: atherosclerosis ■ calcification ■ lipids ■ reactive oxygen species ■ signal transduction

Bone morphogenetic protein (BMP) ligands provide critical signals for determining cell fate and embryonic patterning in development and contribute to the postnatal remodeling of diverse tissues.1 More than 20 known BMP ligands, which form a subset of the transforming growth factor-β family, are recognized by heteromeric complexes of BMP type I and type II serine-threonine kinase receptors on the membrane surface.2 Ligand binding induces constitutively active BMP type II receptors to transphosphorylate BMP type I receptors, which in turn phosphorylate the intracellular BMP effector proteins, Smads 1, 5, and 8 (Smad1/5/8). “Canonical” signaling via the activation of Smad1/5/8 and its associated transcriptional coregulators appear to mediate the principal effects of BMPs, although activation of additional pathways including mitogen-activated protein kinases may further refine cellular effects.3 BMP signaling is also modulated by extracellular ligand antagonists, such as noggin, and by membrane-associated BMP coreceptors, including the repulsive guidance molecules and endoglin.4,5

Vascular calcific lesions associated with atherosclerosis, diabetes, and chronic kidney disease are known to be enriched in BMP ligands, a host of bone-specific matrix regulatory proteins, and cells with the phenotypic profile of osteoblasts and chondroblasts, whose differentiation is known to be coordinated by BMPs.6–11 The concept that BMPs regulate vascular calcification is supported by the finding that smooth muscle-targeted overexpression of BMP2 accelerates vascular calcification in athogenic (apolipoprotein (apo)E−/−) mice.12 Similarly, arterial calcification is observed in mice lacking matrix Gla Protein (MGP), a
vitamin K-dependent calcium-binding extracellular matrix protein that inhibits osteogenic differentiation, and which is proposed to function as an endogenous BMP inhibitor. In fact, MGP overexpression in apoE−/− mice reduces vascular calcification, as well as antecedent vascular inflammation and atherosclerosis, suggesting a role for BMP signaling in early vascular injury as well as calcification. However, MGP-deficient apoE−/− mice are also protected from atherosclerosis, despite developing extensive vascular calcification. Moreover, atherosclerosis is not accelerated in apoE−/− mice overexpressing BMP2 in smooth muscle. Although these studies support a causal role of BMP signaling in vascular calcification, there remains some uncertainty about the precise contribution of BMP signaling in atherosclerosis, as well as the causal or mechanistic linkage of atherosclerosis and vascular calcification.

To further delineate the role of BMP signaling in atherosclerosis and vascular calcification, while avoiding the confounding developmental effects of gene disruption or overexpression, we studied the effects of inhibiting BMP signaling postnatally in LDLR−/− mice, using a small molecule inhibitor of BMP type I receptor kinases, LDN-193189. We found that pharmacological blockade of BMP signaling with LDN-193189 is well-tolerated in adult mice, even for prolonged periods of time, as measured by a variety of gross, hematologic, and bone metabolic parameters. Whereas hyperlipidemic vascular injury led to the activation of SMAD1/5/8 in vascular cells and lesions, BMP inhibition using LDN-193189 effectively attenuated the activation of SMAD1/5/8 in these tissues, as well as subsequent inflammation, atherosclerosis, and vascular calcification. BMP signaling inhibition with LDN-193189 inhibited oxidative stress associated with hyperlipidemia and also exerted potent effects on lipoprotein biosynthesis, reducing serum levels of total cholesterol and LDL. Importantly, inhibition of BMP ligand activity using a recombinant BMP type I receptor extracellular domain fusion protein, ALK3-Fc, also attenuated SMAD1/5/8 activation, inflammation, and atherosclerosis in vivo. Although LDN-193189 and ALK3-Fc both inhibited the synthesis of apolipoprotein B100 (apoB) by HepG2 cells in vitro, ALK3-Fc did not reduce serum LDL in hyperlipidemic mice, despite potently inhibiting atherosclerosis. These results, obtained using 2 distinct pharmacologic strategies, demonstrate an unequivocal role of BMP signaling in atherosclerosis and vascular calcification that is independent of modifying serum lipids, while highlighting an additional and previously unknown role of BMP signaling in lipoprotein homeostasis. Taken together, these data suggest that treatments targeting this pathway may be useful for modifying atherosclerotic and vascular calcific disease.

Methods

Chemicals and Reagents
(4-6-(4-piperazin-1-ylphenyl)pyrazolo[1,5-a]pyrimidin-3-yl)quinoline (LDN)-193189, was synthesized, as previously described. ALK3-Fc was provided by Acceleron Pharma (Cambridge, MA). OsteoSense 680 and ProSense 750 were obtained from PerkinElmer (Waltham, MA). Recombinant human BMP2 and noggin were purchased from R&D Systems (Minneapolis, MN). Human oxidized LDL (oxLDL) was purchased from Intracell (Fredrick, MD). Chloromethyl 2′,7′-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) was purchased from Invitrogen (Eugene, OR), and lucigenin was purchased from Sigma (St. Louis, MO).

Animals
Eight-week-old female wild-type and LDLR−/− mice on a C57BL/6 background were obtained from Jackson Laboratories (Bar Harbor, ME). Animals were fed a western-style diet formulated to match Paigen’s Atherogenic Rodent Diet (42% fat, 0.15% cholesterol, and 19.5% casein; Research Diets, New Brunswick, NJ).

Near-Infrared Imaging and Quantitation of Vascular Calcific and Atherosclerotic Lesions
Animals were injected with OsteoSense 680 and ProSense 750 (150 μL each) via the tail vein 24 hours before euthanasia, as described previously. OsteoSense 680 (Osteosense) is a bisphosphonate-derivatized near-infrared fluorescent imaging probe which, being incorporated into hydroxyapatite, marks early osteogenic activity in the vasculature and predicts the development of calcified lesions. Prosense 750 (Prosense) is a cathepsin-activated near-infrared imaging agent that has been previously demonstrated to mark the activity of vascular macrophages and reflect atherosclerotic burden. Aortae were dissected and separated from adventitial and myocardial tissue and analyzed ex vivo by near-infrared fluorescence reflectance imaging using an Odyssey Imaging System (LI-COR Biotechnology, software version 3.0.16, Lincoln, NE) with signal intensities and volumes determined for regions of interest.

Bone Mineral Density
Bone mineral density was measured in femurs from euthanized mice using a dual energy X-ray absorptiometry Scanner from Lunar/GE Medical Systems (Piximus2, Faxitron X-Ray Corporation, Wheeling, IL) and analyzed using the Piximus2 software.

Cell Culture
HepG2 cells were purchased from the American Type Culture Collection (Manassas, VA) and maintained in Eagle’s Minimum Essential Medium supplemented with 10% FBS, 100 U/mL of penicillin, and 0.1 mg/mL of streptomycin and glutamine. For protein secretion and gene expression experiments, HepG2 cells were grown to 70% confluence before incubation in Eagle’s Minimum Essential Medium with 0.1% FBS. ApoB levels were measured in supernatants from HepG2 cells incubated in Eagle’s Minimum Essential Medium containing 0.5% bovine serum albumin using a human apoB ELISA kit (Mbtech AB, Nacka Strand, Sweden).

Human aortic endothelial cells (HAECs), EB-2, and EG-2 medium were purchased from Lonza, (Basel, Switzerland). During protein secretion and gene expression experiments, HAECs were maintained in EB-2 with 0.1% FBS without additional growth factors. BMP2 protein levels were measured in supernatants from HAECs incubated in EB-2 containing 0.1% FBS using a BMP2 ELISA kit (R&D Systems, Minneapolis, MN). For measurements of reactive oxygen species production, HAECs were incubated in serum-free media for 6 hours prior to the experiment.

Quantitative RT-PCR
Total cellular RNA from cultured cells was extracted by the phenol/guanidine method. Reverse transcription was performed using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA). A Mastercycler ep Realplex (Eppendorf, Hamburg, Germany) was used for real-time amplification and quantification of transcripts. Relative expression and changes in the expression of target transcripts were normalized to levels of 18S ribosomal RNA, determined using the relative Cq method. Quantitative PCR was performed using primer sequences as provided in Table I in the online-only Data Supplement.
Measurement of Reactive Oxygen Species Production

HAECs were plated overnight in a 96-well format. Following starvation in serum-free media for 6 hours, cells were pretreated with and without LDN-193189, ALK3-Fc, or noggin for 30 minutes followed by incubation with vehicle, oxLDL, or BMP2 for 20 hours. H$_2$O$_2$ and O$_2^-$ production were measured with CM-H$_2$DCFDA and lucigenin, respectively, as described previously.$^{24-26}$

Histology and Immunohistochemistry

For histology, aortae were embedded and cryopreserved in optimal cutting-temperature medium (Sakura Tissue-Tek, Zoeterwoude, Netherlands) before sectioning into 6-μm sections. Paraformaldehyde-fixed aortic tissue samples were used to prepare en face specimens, and stained with Oil Red O to detect lipid. Calcification was detected in cryosections by Alizarin Red or von Kossa staining. To quantify the extent of calcification, equivalent longitudinal sections of the aortic arch including the minor curvature were obtained from mice subjected to various treatments, and the surface areas stained by von Kossa or Alizarin Red were quantified (ImageJ software, NIH, Bethesda, Maryland). To quantify atheroma, whole-mount aortae were subjected to Oil Red O staining and areas of involvement for given regions of interest (root, arch, carotid bifurcations, and thoracic aorta) quantified by a similar approach. For immunofluorescence, frozen tissue sections were postfixed in cold methanol and incubated with polyclonal antibodies specific for p-SMAD1/5/8 (1:100 dilution; Cell Signaling, Danvers, MA) or MAC2 (1:100 dilution; Cedarlane, Burlington, ON) followed by reaction with FITC-labeled goat anti-rabbit IgG (for p-SMAD 1/5/8, Jackson Labs, West Grove, PA) or rhodamine-labeled goat anti-rat IgG (for MAC2, Jackson Labs, respectively). Nuclei were identified using 4',6-diamidino-2-phenylindole (DAPI). Liver tissues were fixed with paraformaldehyde, embedded in paraffin, and cut in 6-μm thick sections. Liver sections were stained with hematoxylin and eosin (H+E).

Serum Analysis

Total cholesterol, triglycerides, and hemoglobin, were analyzed using a HemaTrue™ Hematology Analyzer (Heska AG, Switzerland). Blood urea nitrogen, glucose, alkaline phosphatase, total protein, alanine transaminase, and creatinine were determined using a HemaTrue™ Hematology Analyzer (Heska AG, Switzerland) before sectioning into 6-μm sections. Paraformaldehyde-fixed aortic tissue samples were used to prepare en face specimens, and stained with Oil Red O to detect lipid. Calcification was detected in cryosections by Alizarin Red or von Kossa staining. To quantify the extent of calcification, equivalent longitudinal sections of the aortic arch including the minor curvature were obtained from mice subjected to various treatments, and the surface areas stained by von Kossa or Alizarin Red were quantified (ImageJ software, NIH, Bethesda, Maryland). To quantify atheroma, whole-mount aortae were subjected to Oil Red O staining and areas of involvement for given regions of interest (root, arch, carotid bifurcations, and thoracic aorta) quantified by a similar approach. For immunofluorescence, frozen tissue sections were postfixed in cold methanol and incubated with polyclonal antibodies specific for p-SMAD1/5/8 (1:100 dilution; Cell Signaling, Danvers, MA) or MAC2 (1:100 dilution; Cedarlane, Burlington, ON) followed by reaction with FITC-labeled goat anti-rabbit IgG (for p-SMAD 1/5/8, Jackson Labs, West Grove, PA) or rhodamine-labeled goat anti-rat IgG (for MAC2, Jackson Labs, respectively). Nuclei were identified using 4',6-diamidino-2-phenylindole (DAPI). Liver tissues were fixed with paraformaldehyde, embedded in paraffin, and cut in 6-μm thick sections. Liver sections were stained with hematoxylin and eosin (H+E).

HMG-CoA Reductase Activity

HMG-CoA reductase activity was measured and quantified using the HMG-CoA Reductase Activity Kit (Sigma-Aldrich, St. Louis, MO).

Statistical Analysis

Statistical analysis was performed using SPSS 14.0 Data package for Windows (SPSS, Chicago, IL) and GraphPad Prism 5.02 (GraphPad Software, La Jolla, CA). Data are reported as mean±SEM, unless otherwise indicated. Normal distribution of the data were confirmed using the Shapiro-Wilk-Test. For group comparisons of continuous variables, analysis of variance (ANOVA) with posthoc Bonferroni-adjusting testing was used. To determine the relationship between 2 variables in dose-response experiments, Pearson’s rank correlation coefficient was calculated. In all cases, a P≤0.05 was considered to indicate statistical significance.

Results

Activation and Inhibition of the BMP Pathway in the Vasculature of LDLR$^{-/-}$ Mice

Atheroma formation was evident in LDLR$^{-/-}$ mice within 3 to 6 weeks of beginning a high-fat diet (HFD), followed by development of intimal and medial calcification at 16 to 20 weeks (Figure 1a). Also within 3 to 6 weeks of beginning HFD, phosphorylated BMP-responsive SMADs 1, 5, and 8 (p-SMAD1/5/8) were detected in the nuclei of endothelial, intimal macrophages, and medial cells underlying atheromatous lesions (Figure 1b and Figure I in the online-only Data Supplement). SMAD1/5/8 activation was most intense at the aortic root and lesser curvature of the aorta. Activation of BMP signaling in vascular lesions persisted for at least 20 weeks (Figure 1). To confirm its bioavailability and impact on BMP signaling, LDN-193189 was administered for 5 days (2.5 mg/kg IP daily) to LDLR$^{-/-}$ mice that had received a HFD for 6 weeks. Short-term treatment with LDN-193189 markedly diminished nuclear p-SMAD1/5/8 immunoreactivity within atheromatous lesions (Figure 1c).

Impact of Long-Term BMP Inhibition on Vascular Calcification and Atherosclerosis

To determine if modifying BMP signaling in the vasculature might prevent the development of vascular calcification in an atherogenic milieu, adult LDLR$^{-/-}$ mice were fed a HFD and simultaneously treated with LDN-193189 (2.5 mg/kg IP daily) or vehicle for 20 weeks. Treatment with LDN-193189 appeared to inhibit osteogenic activity in the aortae of LDLR$^{-/-}$ mice, based on marked reduction of Osteosense labeling (Figure 2a). A similar reduction in vascular calcification was confirmed by diminished Alizarin red staining (Figure 2b) or von Kossa silver stain (Figure IIa in the online-only Data Supplement). The reduction of vascular calcification by treatment with LDN-193189 was accompanied by a marked reduction in vascular inflammation, as determined by Prosense labeling (Figure 3a). The localization of Osteosense and Prosense labeling were distinct and partially overlapping in merged images (Figure III in the online-only Data Supplement), as observed previously in other atherosclerotic models.$^{21}$ Consistent with an effect of reducing vascular inflammation and atheroma formation, LDN-193189 treatment reduced the aortic Oil Red O staining, which marks lipid-rich atherosclerotic plaque (Figure 3b and Figure IIb in the online-only Data Supplement). Importantly, the impact of LDN-193189 on atherosclerosis and vascular calcification was not associated with a reduction in body weight or food intake (Figure IVa and IVb in the online-only Data Supplement). Sustained treatment with LDN-193189 had no significant effect on bone mineral density in LDLR$^{-/-}$ or wild-type animals (Figure V in the online-only Data Supplement).

To confirm that the effects of LDN-193189 on atherogenesis were mediated by its impact on BMP signaling, a recombinant BMP inhibitor, ALK3-Fc, was administered to HFD-fed LDLR$^{-/-}$ mice for 6 weeks (2 mg/kg IP every other day). Treatment with ALK3-Fc markedly reduced vascular p-SMAD1/5/8 immunoreactivity and intimal macrophage accumulation (Figure 4a and 4b), and significantly reduced cathepsin (Prosense) activity throughout the aorta (Figure 4c). In certain regions such as the aortic arch and carotid bifurcations, the impact of LDN-193189 on cathepsin activity was significantly greater than that observed with ALK3-Fc, as administered under this protocol.
Effect of BMP Inhibition on Endothelial Reactive Oxygen Species Production

A variety of mechanisms have been implicated in the pathogenesis of atherosclerosis. Among these is the induction of reactive oxygen species (ROS) synthesis in endothelial cells exposed to oxLDL, thought to be a critical event contributing to vascular injury in atherogenesis. To gain insight into how BMP inhibition might impact endothelial ROS generation, we measured ROS production in HAECs exposed to oxLDL after pretreatment with vehicle, LDN-193189, ALK3-Fc, or recombinant noggin. Exposure of HAECs to oxLDL increased H$_2$O$_2$ and O$_2^-$ production, as reflected by an increase in dichlorofluorescein and lucigenin fluorescence, respectively (Figure 5a and 5b). LDN-193189 and ALK3-Fc (Figure 5b and 5c), as well as noggin but not control protein (bovine serum albumin, data not shown), attenuated oxLDL-induced ROS production. These results suggested the possibility that oxLDL increases endothelial ROS production via a mechanism requiring BMP ligand expression. In fact, exposure of HAECs to oxLDL for 8 hours increased BMP2 mRNA levels and BMP2 protein expression (Figure VI in the online-only Data Supplement), similar to observations in other endothelial cell types, without significantly altering levels of mRNAs encoding BMP4, BMP6, BMP7, or BMP9.
Moreover, incubation of HAECs with BMP2 increased ROS generation in a manner that could also be attenuated by treatment with LDN-193189 or ALK3-Fc (Figure 5c).

BMP Inhibition Lowers Hepatic Cholesterol Biosynthesis

Serum lipoprotein levels are known to be an important risk factor for atherosclerosis, and total cholesterol and LDL levels are markedly elevated in HFD-fed LDLR\(^{-/-}\) mice (Figure 6a and Table II in the online-only Data Supplement). We observed that treatment with LDN-193189 reduced total cholesterol and LDL levels, but not HDL or triglyceride levels, in LDLR\(^{-/-}\) mice fed a HFD for 20 weeks. Similarly, LDN-193189 reduced total serum cholesterol in wild-type animals fed a HFD (Table III in the online-only Data Supplement).
The ability of LDN-193189 to reduce LDL levels did not appear to be mediated by a direct effect on HMG CoA reductase (HMGCR) activity (Figure 6b) or hepatic HMGCR gene expression (data not shown). To further investigate the role of BMP signaling in the regulation of LDL synthesis, we studied production of apoB, the primary LDL apolipoprotein, in a human hepatoma cell line, HepG2. We observed that incubation of HepG2 cells with BMP2 increased apoB production (Figure 6c) in a time- and dose-dependent manner (Figure VIIa and VIIb in the online-only Data Supplement). Incubation with LDN-193189 (Figure 6c) and ALK3-Fc (Figure VIII in the online-only Data Supplement) inhibited apoB production by HepG2 cells in the absence of exogenous BMP2 and prevented the BMP2-induced apoB secretion. In contrast, the HMGCR inhibitor, atorvastatin, reduced apoB production in the absence of BMP2 but did not prevent the induction of apoB synthesis by BMP2, providing further support for the independence of HMGCR-mediated and BMP-regulated cholesterol biosynthesis.

BMP Inhibition With LDN-193189 Prevents Hepatic Steatosis in LDLR<sup>−/−</sup> Mice

Fatty infiltration of the liver or steatosis is known to be an independent risk factor for coronary heart disease and atherosclerosis and is a prominent feature in LDLR<sup>−/−</sup> mice receiving a HFD. Recent reports have shown that reduction of serum lipoprotein levels can prevent steatosis in LDLR<sup>−/−</sup> mice and may help to prevent the development of hepatic dysfunction in humans with nonalcoholic steatohepatitis. To ascertain the impact of LDN-193189 on steatosis and associated hepatic function, we performed liver histology...
and biochemical liver function tests in these animals. LDLR<sup>−/−</sup> mice fed a HFD for 20 weeks and treated with vehicle exhibited severe steatosis in hepatic tissues, which was markedly reduced in LDN-193189-treated animals (Figure 6d). Consistent with the reduction in steatosis, treatment with LDN-193189 reduced blood alanine transaminase (ALT) and alkaline phosphatase (ALP) levels in LDLR<sup>−/−</sup> mice (Tables II and IV in the online-only Data Supplement).

**Discussion**

We report that pharmacological inhibition of BMP signaling reduced vascular calcification in atherogenic animals, likely by limiting antecedent atherogenesis and vascular inflammation. We confirm in LDLR<sup>−/−</sup> mice, as has been recently shown in apoE<sup>−/−</sup> mice, that early atherosclerotic lesions are marked by the activation of the BMP signaling pathway in the vascular endothelium, smooth muscle, and subintimal macrophages. A small molecule BMP inhibitor attenuated the activation of SMAD1/5/8 and subsequent vascular inflammation and atheroma—results that were replicated using a complementary BMP ligand trap strategy. These pharmacological approaches overcome some of the limitations of genetic overexpression and targeted-disruption strategies to implicate BMP signaling definitively in atherogenesis and associated vascular calcification. Moreover, some of the evidence implicating BMP signaling in the pathogenesis of vascular calcification has been based on the impact of disrupting MGP, and the notion that MGP functions primarily as a BMP inhibitor. However, some recent data suggest that MGP may inhibit vascular calcification via mechanisms that
are independent of its ability to inhibit BMP signaling, such as the direct inhibition of hydroxyapatite formation.\[40\] Although the overexpression and deficiency of MGP in mice on an apoE\(^{-/-}\) background result in decreased and increased vascular calcification, respectively, both strains exhibit decreased atherosclerosis, suggesting that the mechanisms by which MGP modulates vascular calcification and atherosclerosis may be distinct.\[16\] In contrast to previous models, the present data suggest that atherosclerosis and associated vascular calcification are closely coupled and are both enhanced by BMP signaling in the vasculature.

BMP signaling was found to be required for the induction of ROS in endothelial cells by oxLDL, a critical process in atherogenesis.\[25,41,42\] BMP signaling and ROS both enhance the osteogenic differentiation of smooth muscle cells and may, thereby, contribute to subsequent vascular calcification.\[33-45\] BMP ligands have been previously reported to enhance ROS production in endothelial cells via the activation of NADPH oxidase 1, resulting in the induction of monocyte adhesion factor ICAM-1, COX-2, and a proinflammatory transcriptional program.\[25,46,47\] Thus, our observation that oxLDL stimulates HAECs to generate ROS in a BMP-dependent manner is consistent with the notion that BMP signaling mediates vascular inflammation via proinflammatory effects of lipid-mediated endothelial injury, and provides a potential explanation of the marked decrease in macrophage recruitment observed in LDN-193189-treated animals. Given recent evidence that BMP signaling may mediate macrophage function and cytokine expression,\[48-49\] a direct impact of BMP inhibition on macrophages cannot be excluded and would be an important subject of future investigation.

The observation that inhibition of the BMP type I receptor activity with LDN-193189 reduced lipoprotein metabolism and hepatic steatosis in LDLR\(^{-/-}\) mice, as well as BMP2-induced lipoprotein synthesis in vitro, suggested the possibility that inhibition of BMP signaling reduced atherosclerosis exclusively by reducing LDL levels. However, treatment of hyperlipidemic mice with ALK3-Fc nearly replicated the effects of LDN-193189 on atherosclerosis in a short-term (6-week) experiment, and, in contrast to LDN-193189, without impacting cholesterol levels. This latter result supports the notion that BMP inhibition may attenuate atherosclerosis by direct effects on the vasculature, and that the impact of BMP inhibition on atherosclerosis does not require a reduction in LDL levels. However, it is possible the increased potency of LDN-193189 in reducing vascular inflammation (Figure 4c) may be attributable to the reduction of LDL levels seen with LDN-193189 treatment combined with direct vascular effects. There are a number of potential explanations for the differences in the ability of LDN-193189 and ALK3-Fc to reduce LDL levels. We considered the possibility that the reduction in LDL levels seen in mice treated with LDN-193189 was attributable to an “off-target” effect of the small molecule. However, the likelihood of this explanation is reduced by the observation that LDN-193189, ALK3-Fc, and a third BMP signaling inhibitor, noggin, all reduced apoB synthesis by HepG2 cells in culture. It is possible that the BMP ligand(s) or receptors responsible for regulating lipoprotein synthesis in vivo may be more sensitive to LDN-193189, which acts broadly against the known BMP ligands, vs ALK3-Fc, which targets primarily BMP2 and BMP4.\[20,50\] Alternatively, the bioavailability, pharmacokinetics, pharmacodynamics, or potency of ALK3-Fc may make it a less effective inhibitor of hepatic lipoprotein synthesis than LDN-193189, as administered in this study.

The striking impact of LDN-193189 on hepatic steatosis was likely due to its ability to lower serum cholesterol levels, given the established link between serum cholesterol levels and steatosis.\[34\] With a prevalence of 20% to 30% in Western adults and 70% to 90% among obese or diabetic patients, nonalcoholic fatty liver disease constitutes a novel landmark feature of the metabolic syndrome.\[51\] Because nonalcoholic fatty liver disease appears to be an independent risk factor for cardiovascular disease,\[52\] therapeutic interventions for asymptomatic nonalcoholic fatty liver disease may yield benefits beyond protecting hepatic function. Whether the reduction in steatosis seen in our model was mediated by effects on lipid metabolism or other hepatic effects of LDN-193189 remains to be determined.

Given the pleiotropic roles of BMP signaling in regulating cell growth and differentiation, necessary caution about pharmacological BMP inhibition is warranted. Although previous studies have examined the effects of LDN-193189 at similar doses in neonatal and adult mice for up to 60 days,\[20,50\] the current study extends these findings to mice treated for 140 days in LDLR\(^{-/-}\) and 210 days in WT animals. Surprisingly, treatment with LDN-193189 did not have significant effects on bone mineral density, nor did it elicit evidence of renal, hepatic, or hematopoietic toxicity, despite having measurable effects on tissue SMAD1/5/8 activation and serum alkaline phosphatase as administered. The limited impact of LDN-193189 on postnatal skeletogenesis might potentially be explained by studies by Tsuji et al demonstrating that prototypic BMP ligands BMP2 and BMP4 are dispensable for skeletogenesis when ablated from the limb bud during embryogenesis,\[52,53\] indicating that signals other than BMP may predominate in this process. These investigators also found that BMP4 was dispensable for fracture healing, whereas BMP2 was necessary. These data suggest that prolonged suppression of BMP signaling in adult animals may be well-tolerated, but more comprehensive toxicology in multiple species and perhaps in fracture healing models would be necessary to confirm the safety of BMP inhibition as a long-term therapy.

This investigation highlights the BMP signaling pathway as a therapeutic target in the treatment of vascular calcification and atherosclerosis, confirming the role of BMP signaling in regulating vascular oxidative stress and inflammation, while identifying a novel role in lipid metabolism. The HMGCR-independent impact of BMP inhibition on LDL biosynthesis suggests a novel therapeutic strategy for patients who do not tolerate statin medications (ie, due to statin-induced myopathy, drug sensitivity, chronic hepatic disease, or interactions with other medications) or in whom standard regimens fail to achieve lipid-lowering targets. Given the large unmet need for novel strategies for achieving lipid and cardiovascular risk reduction, the potent effects of BMP signaling modulation on lipoprotein metabolism is a subject...
that strongly merits further clinical and mechanistic investigation.

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Disclosures
Massachusetts General Hospital and Partners Healthcare have applied for patents related to small molecule inhibitors of BMP type I receptors and the application of ALK3-Fc to treat atherosclerosis and vascular calcification, and Matthias Derwall, Rajevaj Malhotra, Kenneth D. Bloch, and Paul B. Yu may be entitled to royalties. Jasbir S. Seehra is a former employee of Acceleron Pharma, Inc, the manufacturer of ALK3-Fc.

References
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Supplemental material

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(J.S.S.), Cambridge, MA.
# Supplemental Tables

## Supplementary Table I

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<td>AGGTGACCACACCCCACAGAT</td>
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<tr>
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<tr>
<td>BMP9</td>
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<td>GTCACAATGTGGGACGCTG</td>
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</table>

**Supplementary Table I.** List of primers used for quantitative RT-PCR.
Supplementary Table II

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>LDN-193189</th>
<th>p=</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol [mg/dl]</td>
<td>1957 ± 159</td>
<td>1401 ± 87</td>
<td>0.01</td>
</tr>
<tr>
<td>Triglycerides [mg/dl]</td>
<td>125 ± 23</td>
<td>135 ± 16</td>
<td>0.73</td>
</tr>
<tr>
<td>Hemoglobin [g/dl]</td>
<td>11.6 ± 1.0</td>
<td>12.9 ± 0.8</td>
<td>0.34</td>
</tr>
<tr>
<td>Blood urea nitrogen [mg/dl]</td>
<td>25 ± 1</td>
<td>25 ± 2</td>
<td>0.84</td>
</tr>
<tr>
<td>Glucose [mg/dl]</td>
<td>216 ± 21</td>
<td>230 ± 19</td>
<td>0.65</td>
</tr>
<tr>
<td>Alkaline phosphatase [IU/L]</td>
<td>158 ± 15</td>
<td>84 ± 11</td>
<td>0.00</td>
</tr>
<tr>
<td>Total protein [g/dl]</td>
<td>4.9 ± 0.1</td>
<td>4.3 ± 0.4</td>
<td>0.12</td>
</tr>
<tr>
<td>Alanine transaminase [IU/L]</td>
<td>257 ± 44</td>
<td>130 ± 21</td>
<td>0.03</td>
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<tr>
<td>Creatinine [mg/dl]</td>
<td>0.5 ± 0.0</td>
<td>0.4 ± 0.0</td>
<td>0.61</td>
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</tbody>
</table>

n=10                  n=8

**Supplementary Table II.** Blood biochemical analysis in LDLR<sup>−/−</sup> mice fed a high fat diet. LDLR<sup>−/−</sup> mice were started on a HFD at eight weeks of age that was continued for 20 weeks during which mice received daily injections of either vehicle or LDN-193189 (2.5 mg/kg ip, data are presented as mean ± SEM).
Supplementary Table III

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>LDN-193189</th>
<th>p=</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol [mg/dl]</td>
<td>218 ± 5</td>
<td>183 ± 6</td>
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</tr>
<tr>
<td>Triglycerides [mg/dl]</td>
<td>144 ± 26</td>
<td>82 ± 21</td>
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<tr>
<td>Hemoglobin [g/dl]</td>
<td>13.7 ± 0.9</td>
<td>13.5 ± 1.0</td>
<td>0.89</td>
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<tr>
<td>Blood urea nitrogen [mg/dl]</td>
<td>32 ± 4</td>
<td>25 ± 1</td>
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<tr>
<td>Glucose [mg/dl]</td>
<td>283 ± 16</td>
<td>311 ± 12</td>
<td>0.17</td>
</tr>
<tr>
<td>Alkaline phosphatase [IU/L]</td>
<td>177 ± 12</td>
<td>109 ± 8</td>
<td>0.00</td>
</tr>
<tr>
<td>Total protein [g/dl]</td>
<td>4.8 ± 0.1</td>
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<tr>
<td>Alanine transaminase [IU/L]</td>
<td>275 ± 21</td>
<td>159 ± 19</td>
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<tr>
<td>Creatinine [mg/dl]</td>
<td>0.6 ± 0.0</td>
<td>0.5 ± 0.0</td>
<td>0.06</td>
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</table>

n=8  n=9

Supplementary Table III. Blood biochemical analysis in WT mice fed a HFD for 30 weeks. C57BL/6 mice were started on a HFD at eight weeks of age that was continued for 30 weeks during which mice received daily injections of either vehicle or LDN-193189 (2.5 mg/kg ip, data are presented as mean ± SEM).
## Supplementary Table IV

<table>
<thead>
<tr>
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<th>Vehicle</th>
<th>ALK3-Fc</th>
<th>LDN-193189</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol [mg/dl]</td>
<td>1953 ± 102</td>
<td>2206 ± 139</td>
<td>1553 ± 77*:$</td>
</tr>
<tr>
<td>Triglycerides [mg/dl]</td>
<td>122 ± 10</td>
<td>108 ± 11</td>
<td>112 ± 15</td>
</tr>
<tr>
<td>Hemoglobin [g/dl]</td>
<td>13.7 ± 1.3</td>
<td>14.2 ± 1.1</td>
<td>12.9 ± 1.5</td>
</tr>
<tr>
<td>Blood urea nitrogen [mg/dl]</td>
<td>28 ± 1</td>
<td>25 ± 1</td>
<td>24 ± 2</td>
</tr>
<tr>
<td>Glucose [mg/dl]</td>
<td>253 ± 19</td>
<td>243 ± 16</td>
<td>259 ± 20</td>
</tr>
<tr>
<td>Alkaline phosphatase [IU/L]</td>
<td>141 ± 14</td>
<td>207 ± 22</td>
<td>112 ± 20$</td>
</tr>
<tr>
<td>Total protein [g/dl]</td>
<td>4.9 ± 0.1</td>
<td>5.2 ± 0.1</td>
<td>5.1 ± 0.1</td>
</tr>
<tr>
<td>Alanine transaminase [IU/L]</td>
<td>408 ± 74</td>
<td>310 ± 72</td>
<td>233 ± 56</td>
</tr>
<tr>
<td>Creatinine [mg/dl]</td>
<td>0.5 ± 0.1</td>
<td>0.6 ± 0.0</td>
<td>0.5 ± 0.1</td>
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</tbody>
</table>

Supplementary Table IV. Blood biochemical analysis in LDLR<sup>−/−</sup> mice fed a high fat diet. LDLR<sup>−/−</sup> mice were started on a HFD at eight weeks of age that was continued for 6 weeks during which mice received daily injections of vehicle or LDN-193189 (2.5 mg/kg ip) or received ALK3-Fc (2 mg/kg ip) every other day. Data are presented as mean±SEM. *p≤0.05 LDN-193189 vs. vehicle. §p≤0.05 LDN-193189 vs. ALK3-Fc.
Supplementary Figure I. LDLR−/− mice on HFD develop atherosclerotic lesions demonstrating marked activation of SMAD1/5/8. Aortic sections from LDLR−/− mice fed HFD for 3, 6, 7, 9, and 20 weeks showed evidence of phospho-(p-)SMAD immunoreactivity in evolving atheromatous plaques (left panels), as compared to serial sections from the same aortae reacted with FITC-labeled secondary Ab alone (right panels). Nuclear staining for p-SMAD1/5/8 was observed in the intimal, subintimal, and medial areas of involvement in atheromatous lesions (images representative of ≥3 aortae at each interval, bar= 500 μm).
**Supplementary Figure II.** Reduction of vascular calcification and atheroma formation in conventional histology. (a) Calcified surface area (left panel) determined by von Kossa-staining of longitudinal sections from the aortic minor curvature from LDLR<sup>−/−</sup> mice fed HFD for 20 weeks was markedly reduced with LDN-193189 treatment (n=5, 2.5 mg/kg ip) vs. vehicle (n=5, p<0.05). Similarly, Alizarin Red staining of serial sections from the same aortae revealed decreased areas calcification in LDN-193189-treated vs. vehicle-treated mice (n=4 each group, p<0.05) (b) Lipid plaque surface area, determined by areas of Oil Red O staining in whole-mount aortae from LDLR<sup>−/−</sup> mice fed HFD for 20 weeks, was significantly reduced in animals receiving LDN-193189 (n=3, 2.5 mg/kg ip) vs. vehicle (n=3, mean±SEM, *p <0.05).
Supplementary Fig. III. Vascular calcification and inflammation in aortae from LDLR−/− mice are detected by ex vivo molecular imaging with Osteosense and Prosense in overlapping but distinct areas of the aorta, and are both inhibited by treatment with a BMP antagonist. Aortae were harvested from HFD-fed LDLR−/− mice treated with vehicle (left panel) or LDN-193189 (right panel) for 20 weeks, dissected and imaged by near-infrared fluorescence reflectance imaging 24h after iv injection with OsteoSense 680 (a near-infrared fluorescent bisphosphonate probe). Brightfield images (outside) correspond to colorized intensity maps (inside) demonstrating localization and degree of osteogenic activity (red) and inflammation (green). Treatment with LDN-193189 diminished aortic osteogenic activity, and markedly diminished vascular inflammation.
Supplementary Figure IV

(a) Mean weight (g) in LDLR^{-/-} mice fed a HFD for 20 weeks while receiving daily injections of vehicle (n=20) or LDN-193189 (n=20, 2.5 mg/kg ip). (b) Food intake per g weight over 6 weeks of HFD administration while receiving daily injections of vehicle (n=10) or LDN-193189 (n=10, 2.5 mg/kg ip). Data are presented as mean ± SEM.
Supplementary Figure V. Bone mineral density did not differ between LDLR<sup>−/−</sup> mice treated with vehicle or LDN-193189. Bone mineral density (BMD) was measured in femurs from sacrificed LDLR<sup>−/−</sup> mice fed a HFD for 20 weeks while receiving daily injections of vehicle (n=8) or LDN-193189 (n=10, 2.5 mg/kg ip) using dual energy X-ray absorptiometry in the distal femur (Distal), the femur shaft (Shaft) or in the whole bone (Total, mean ± SEM).
Supplementary Figure VI

(a) BMP2 mRNA levels were measured by quantitative RT-PCR. Data presented as mean±SEM, n=4 measurements. (b) BMP2 protein levels in the culture medium were measured using the BMP-2 Quantikine ELISA Kit (DBP200, R&D Systems, Minneapolis, MN). BMP2 mRNA and protein levels increased over time in response to incubation with oxLDL (80 µg/mL). Data presented as mean ± SEM, n=4 measurements. *p≤0.05 versus HAEC not exposed to oxLDL (0 h).
Supplementary Figure VII. BMP2 induces Apolipoprotein B 100 production in a time- and dose-dependent manner in HepG2 cells. (a) After starvation in EMEM culture media containing 0.1% fetal bovine serum for 24 h, HepG2 cells were incubated with BMP2 (100 ng/mL) for varying periods of time. Apolipoprotein B 100 (ApoB) levels, measured in culture medium by ELISA, were increased after 24 h of BMP2 stimulation (mean ± SEM, n=4, *p ≤ 0.05 vs. control). (b) After starvation in EMEM culture media containing 0.1% fetal bovine serum for 24 h, cells were incubated with varying concentrations of BMP2 for 24 h. Apo B levels increased with BMP2 stimulation in a dose-dependent fashion (mean ± SEM, n=4, *p≤0.05 vs. control, Pearson's correlation, p<0.001).
Supplementary Fig. VIII. Recombinant or small molecule inhibition of BMP signaling inhibits BMP2-induced ApoB production. After serum deprivation for 24 h, HepG2 cells stimulated with BMP2 (100 ng/mL) for 24 h secreted ApoB 100 into secretion in a manner that was inhibited by ALK3-Fc (400 ng/mL) or LDN-193189 (LDN, 100 nM, mean ± SEM, n=4, *p≤0.05 vs. control, #p≤0.05 vs. BMP2).