Bmper Inhibits Endothelial Expression of Inflammatory Adhesion Molecules and Protects Against Atherosclerosis

Xinchun Pi, Pamela Lockyer, Laura A. Dyer, Jonathan C. Schisler, Brooke Russell, Stephen Carey, Daniel Timothy Sweet, Zhongming Chen, Ellie Tzima, Monte S. Willis, Jonathon W. Homeister, Martin Moser, Cam Patterson

Objective—Bone morphogenetic proteins (Bmps) are important mediators of inflammation and atherosclerosis, though their mechanism of action is not fully understood. To better understand the contribution of the Bmp signaling pathway in vascular inflammation, we investigated the role of Bmper (Bmp endothelial cell precursor–derived regulator), an extracellular Bmp modulator, in an induced in vivo model of inflammation and atherosclerosis.

Methods and Results—We crossed apolipoprotein E–deficient (ApoE−/−) mice with mice missing 1 allele of Bmper (Bmper+/−;ApoE−/− mice used in the place of Bmper−/− mice that die at birth) and measured the development of atherosclerosis in mice fed a high-fat diet. Bmper haploinsufficiency in ApoE−/− mice (Bmper+/−;ApoE−/− mice) led to a more severe phenotype compared with Bmper+/+;ApoE−/− mice. Bmper−/−;ApoE−/− mice also exhibited increased Bmp activity in the endothelial cells in both the greater and lesser curvatures of the aortic arch, suggesting a role for Bmper in regulating Bmp-mediated inflammation associated with laminar and oscillatory shear stress. Small interfering RNA knockdown of Bmper in human umbilical vein endothelial cells caused a dramatic increase in the inflammatory markers intracellular adhesion molecule 1 and vascular cell adhesion molecule 1 at rest and after exposure to oscillatory and laminar shear stress.

Conclusion—we conclude that Bmper is a critical regulator of Bmp-mediated vascular inflammation and that the fine-tuning of Bmp and Bmper levels is essential in the maintenance of normal vascular homeostasis. (Arterioscler Thromb Vasc Biol. 2012;32:2214-2222.)

Key Words: bone morphogenetic protein ■ Bmp endothelial cell precursor–derived regulator ■ atherosclerosis ■ inflammation ■ fluid shear stress

Atherosclerosis is a disease that results from plaque formation within arteries, resulting in arterial hardening and narrowing. It is mediated by a chronic inflammatory process characterized by the accumulation of lipids and inflammatory cells (plaques) along the inner walls of arteries. Although plaque formation is a complex process, endothelial inflammation has been identified as one of the critical initiating factors. Endothelial inflammation can be induced by decreases or disruptions in blood flow, making some regions of the vasculature more prone to plaque formation. For example, arterial regions that are exposed to uniform, unidirectional blood flow with high shear stress are protected from endothelial inflammation and have a lower incidence of atherothrombotic plaque formation. In comparison, atherosclerotic lesions develop predominantly at branches, bends, and bifurcations in arteries, where endothelial cells are exposed to low or disturbed fluid shear stress, resulting in low mean and oscillatory shear stress on the endothelial cells. In these lesion-prone regions, disturbed or oscillatory shear stress increases expression of bone morphogenetic proteins (Bmps) and their antagonists in the vascular endothelium. In turn, Bmps activate an inflammatory response characterized by the expression of adhesion molecules, like intracellular adhesion molecule 1 (ICAM1) and vascular cell adhesion molecule 1 (VCAM1) on the endothelial surface. Despite this, animal studies examining the direct role of bone morphogenetic protein (Bmp) on vascular inflammation have been inconclusive, with some studies reporting a decrease in inflammatory responses and atherosclerotic lesion formation when Bmp activity is increased, whereas others concluding that Bmp plays a proinflammatory role. Although each of these studies support a central role for Bmp-mediated endothelial inflammation in atherosclerosis, some uncertainty remains about the precise contribution of Bmp signaling to endothelial inflammation and atherosclerosis.

Bmps belong to the transforming growth factor-β superfamily and play important roles in cellular processes,
such as bone formation, proliferation, differentiation, motility, vasculogenesis, and angiogenesis (reviewed by Moreno-Miralles). Specifically, Bmp4, together with Bmp2 and Bmp6, demonstrate important roles in endothelial differentiation, migration, and angiogenesis. Previously, we identified a novel extracellular modulator of Bnp, Bmp endothelial cell precursor-derived regulator (Bmrper; also called crossveinless 2 in Drosophila melanogaster), which is required for hematopoietic and vascular development and hypoxia–induced retinal neovascularization. We have also demonstrated that Bmrper regulates Bmp4 activity in a dose-dependent manner. Recent reports show that Bmrper is induced by inflammatory-regulatory stimuli, such as oscillatory shear stress and mevastatin, and inhibits tumor necrosis factor-α–induced endothelial inflammation, suggesting that Bmrper acts in an anti-inflammatory capacity in endothelial cells by inhibiting Bmp activity. This led us to question whether Bmrper may also inhibit the endothelial inflammation and subsequent pathology associated with atherosclerosis.

In this study, we used the apolipoprotein E–deficient (ApoE−/−) mouse atherosclerotic model to study the effects of Bmrper haploinsufficiency on the development of atherosclerosis. We used Bmrper−/− mice instead of Bmrper−/− mice because Bmrper−/− mice die at birth. Bmrper−/−;ApoE−/− mice fed a high-fat (HF) diet displayed an exacerbated inflammatory vascular response compared with ApoE−/− mice with the genetic background. were crossed with ApoE−/− mice to generate aorta and aortic sinuses, which is required for hematopoietic and vascular development and hypoxia–induced retinal neovascularization. We have also demonstrated that Bmrper regulates Bmp4 activity in a dose-dependent manner. Recent reports show that Bmrper is induced by inflammatory-regulatory stimuli, such as oscillatory shear stress and mevastatin, and inhibits tumor necrosis factor-α–induced endothelial inflammation, suggesting that Bmrper acts in an anti-inflammatory capacity in endothelial cells by inhibiting Bmp activity. This led us to question whether Bmrper may also inhibit the endothelial inflammation and subsequent pathology associated with atherosclerosis.

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Materials and Methods

Animals and Diets
Bmrper−/− mice, previously generated in our laboratory on a C57BL/6J genetic background, were crossed with ApoE−/− mice to generate Bmrper−/−;ApoE−/− mice. All adult mice were fed with the standard chow or a HF/high-cholesterol diet (Western diet) (Harlan Laboratories, Indianapolis, IN) for 20 weeks. Body weight of mice was monitored before and after they were fed different diets. Blood samples were drawn from mice after consuming the HF diet or standard chow for 4 weeks. Soluble VCAM and soluble ICAM were measured in plasma in triplicate using an ELISA method (R&D Systems, Minneapolis, MN).

Reagents
Recombinant human Bmp4 and Bmp protein and antibodies recognizing Bmrper and Bmp4 were obtained from R&D Systems. VCAM1 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) for Western blotting and Chemicon for immunofluorescence (Millipore, Billerica, MA). The ICAM1 antibody was purchased from Cell Signaling Technology (Danvers, MA) and was used for Western blotting experiments. An additional ICAM1 antibody (purchased from Chemicon) was used for immunofluorescence experiments. The phosphorylated Smad1, 5, and 8 antibody was purchased from Cell Signaling Technology and was used for both Western blotting and immunofluorescence experiments.

Cell Culture and siRNA Transfection
Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza and cultured in endothelial basal medium (EBM; Lonza, Allendale, NJ) supplemented with hydrocortisone, bovine brain extract, epidermal growth factor, and 2% fetal calf serum. The cells from passages 4–8 were used for experiments. The Stealth small interfering RNA (siRNA) duplexes were obtained from Invitrogen (Grand Island, NY). The siRNAs against mouse Bmrper were a mixture of the duplexes of and . The control siRNA is the Stealth RNAi negative control duplex (Cat. No. 12935-300) and was purchased from Invitrogen. The siRNAs were transfected into HUVECs according to the manufacturer’s recommended protocol. Briefly, for each sample, 2×10⁵ HUVECs were transfected with 300 pmol siRNA. The experiments with Bmp4 or Bmrper siRNA-transfected HUVECs were performed 1 day or 4 days later, respectively. The siRNAs resulted in >70% knockdown of the protein levels of Bmp4 and Bmrper.

Lipid Analysis
Mice were fasted for 18 hours before blood sampling. Less than 200 µL of blood was collected through submandibular bleeding using a lancet. The total cholesterol level was measured enzymatically using a commercially available kit (Infinity kits, Thermo Scientific, Waltham, MA).

Lesion Quantification
The mice were euthanized and perfusion fixed with 10% buffered formalin via the left ventricle for 5 minutes. The lesions were dissected, stained with Oil Red O, cleaned of adventitial tissue, and stained with Oil Red O (Sigma, St. Louis, MO). The surface lesion area was quantified with ImageJ software (Version 1.42q, NIH, Bethesda, MD) and is presented as a percentage of the total surface area of the whole aorta. To measure the lesions in the aortic sinuses, the heart and proximal aorta were excised, and the apex and lower half of the ventricles were removed. The remaining sample was embedded in optimal cutting temperature compound (Tissue-Tek, Fisher Scientific, Pittsburgh, PA) and frozen on dry ice. Starting from the appearance of the aortic valve, serial frozen sections at 5-µm thickness were collected until the aortic valves were completely sectioned after the previously described protocol. Sections were stained with eosin and Oil Red O. The slides were imaged by light microscopy, and the atherosclerotic lesion area located in aortic sinus area was quantified with ImageJ and averaged over a 280-µm region.

Calcification Quantification
Deposited calcium in the aorta was detected by staining with von Kossa. The 5-µm cryosections of aortic sinus were prepared as described above and subjected to the von Kossa staining procedure. The calcification area from each section was quantified as a percentage of the total vessel cross-sectional area using ImageJ software.

ELISA Measurements
Blood samples were drawn from mice after consuming the HF diet or standard chow for 4 weeks. Soluble VCAM and soluble ICAM were measured in plasma in triplicate using an ELISA method (R&D Systems, Minneapolis, MN).

Shear Stress Assays
HUVECs were postconfluent for 48 hours before the performance of fluid shear stress experiment to decrease the background signals.
Laminar shear stress assay was described previously. Briefly, confluent cells in a 10-cm dish were exposed to shear stress using the cone-and-plate-flow chamber system for 8 hours at 20 dyn cm⁻² for laminar shear stress or ±5 dyn cm⁻² for oscillatory shear stress experiments.

**Immunoblotting**

Cells were harvested in lysis buffer (1% Triton X-100, 50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1 mmol/L Na₂VO₄, and 0.1% protease inhibitor mixture; Sigma) and clarified by centrifugation at 16,000g. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes.

**Immunofluorescence**

The aortic arch segments were dissected out and gently cleaned of the adventitia. The aortic fragments located at the greatest curvature (GC) and lesser curvature (LC) were separated and fixed in 3.7% formaldehyde for 10 minutes at room temperature. The aortic fragments were sequentially treated with 70% ethanol for 30 minutes and 5% hydrogen peroxide in methanol. Then, the segments were washed with water for 5 minutes. For the phosphorylated Smad1, 5, and 8 antibody, the samples were soaked in boiling citric acid buffer (10 mmol/L; pH, 6.0) for 9 minutes to expose the antigens. Next, the aortic fragments or 5-µm cryosections of the aortic root were blocked with 5% heat–inactivated goat serum for 1 hour and then incubated overnight with primary antibodies against ICAM1, VCAM1, CD31, or CD68 diluted in the blocking solution. After 3 washes in tris buffered saline, the fragments were incubated in the dark with a second antibody conjugated with Alexa Fluor 488 or 568 (Molecular Probes, Eugene, OR) in blocking solution for 90 minutes at 37°C. After 3 washes in tris buffered saline, cells were incubated in the dark with a second antibody conjugated with Alexa Fluor 488 or 568 (Molecular Probes, Eugene, OR) in blocking solution for 90 minutes at 37°C. After 3 washes in tris buffered saline, cells were incubated in the dark with a second antibody conjugated with Alexa Fluor 488 or 568 (Molecular Probes, Eugene, OR) in blocking solution for 90 minutes at 37°C.

**Statistical Analysis**

Data are shown as the mean±SE for 3 to 4 separate experiments. Differences were analyzed with 2-way ANOVA and post hoc analyses such as Student t test when needed. Values of P≤0.05 were considered statistically significant.

**Results**

**Bmpr Expression Protects Against Atherosclerotic Lesion Formation and Calcification**

Accumulated evidence suggests that Bmpr protects endothelial cells from inflammation by inhibiting Bmp activity. Therefore, we used the ApoE⁻/⁻ mouse model, in which a HF diet leads to accelerated atherosclerotic lesion formation and arterial calcification, to analyze the in vivo effect of reduced Bmpr expression on vascular inflammation. We crossed wild-type or ApoE⁻/⁻ mice with mice that had either 1 or 2 functional Bmpr alleles, resulting in 4 genotypes of experimental mice: Bmpr⁺⁺;ApoE⁻/⁻, Bmpr⁺⁺;ApoE⁻/⁻, Bmpr⁺⁺;ApoE⁻/⁻, Bmpr⁺⁺;ApoE⁻/⁻. These mice were then fed either a standard chow or HF diet for 20 weeks, and the formation of atherosclerotic plaques in the aorta and aortic sinus regions was evaluated by Oil Red O staining. ApoE⁻/⁻ mice that were haploinsufficient for Bmpr expression (Bmpr⁺⁺;ApoE⁻/⁻) responded to the HF diet with enhanced plaque formation compared with Bmpr⁺⁺;ApoE⁻/⁻ mice (28.35±2.23% versus 17.96±3.00%, P=0.016), as measured by en face staining of aortic lesions (including both thoracic and abdominal aorta, Figure 1A and 1B). In addition, cross-sectional analysis of aortic sinus lesions revealed that the plaques formed in the Bmpr⁺⁺;ApoE⁻/⁻ mice were larger compared with lesions in Bmpr⁺⁺;ApoE⁻/⁻ mice (0.38±0.02% versus 0.29±0.03%, P=0.018), demonstrating a protective effect of Bmpr expression on the degree of plaque growth (Figure 1C and 1D). When lesion calcification was compared between genotypes, Bmpr⁺⁺;ApoE⁻/⁻ mice again showed an exacerbated response, with a 120% increase over baseline levels of calcification as determined by von Kossa staining, compared with a 68% increase in the Bmpr⁺⁺;ApoE⁻/⁻ mice. This equated to an overall increase in total calcification of 1.12±0.25% in Bmpr⁺⁺;ApoE⁻/⁻ mice versus 0.37±0.22% in Bmpr⁺⁺;ApoE⁻/⁻ mice. Bmpr⁺⁺;ApoE⁻/⁻ mice fed the HF diet showed no differences in body weight or serum cholesterol levels compared with Bmpr⁺⁺;ApoE⁻/⁻ mice (Figure IA and IB in the online-only Data Supplement), indicating that diet-induced increases in weight and lipid levels are not responsible for the more robust atherosclerotic phenotype in the Bmpr⁺⁺;ApoE⁻/⁻ mice. Together, these results indicate that Bmpr plays a protective role in plaque formation and arterial calcification in an in vivo model of atherosclerosis.

**Bmpr Expression Inhibits Aortic Inflammation**

Several reports have demonstrated a central role for the Bmp signaling pathway in promoting endothelial inflammatory responses. Therefore, we sought to determine whether the protective influence of Bmpr on the development of atherosclerotic lesions is attributable to changes in vascular inflammation. To test this, we measured the degree of macrophage infiltration (a common phenotype observed with the onset of atherosclerotic plaques) and expression of inflammatory markers in atherosclerotic plaques in Bmpr⁺⁺;ApoE⁻/⁻ and Bmpr⁺⁺;ApoE⁻/⁻ mice. Quantitative analysis revealed a more robust degree of macrophage infiltration (determined by CD68 expression) in aortas of Bmpr⁺⁺;ApoE⁻/⁻ mice compared with Bmpr⁺⁺;ApoE⁻/⁻ mice (0.14±0.07 versus 0.05±0.03%, respectively) after 20 weeks of the HF diet (Figure 2A and 2B). Bmpr⁺⁺;ApoE⁻/⁻ mice also exhibited a dramatic increase in the expression of 2 inflammatory markers, ICAM1 and VCAM1, in the intima associated with aortic lesions, compared with Bmpr⁺⁺;ApoE⁻/⁻ mice after 20 weeks of the HF diet (Figure 2C and 2D), supporting the notion that Bmpr functions as an anti-inflammatory mediator. Similar increases in ICAM1 and VCAM1 expression were also observed in serum from Bmpr⁺⁺;ApoE⁻/⁻ mice compared with Bmpr⁺⁺;ApoE⁻/⁻ mice after 4 weeks of the HF diet (Figure II in the online-only Data Supplement). We also performed immunostaining with anti-Bmpr and Bmp4 antibodies to determine whether there is a change in the level of expression of these proteins in response to vascular inflammation induced by HF diet. We observed a robust increase in Bmpr (Figure IIIA in the online-only Data Supplement) and Bmp4 (Figure IIIB in the online-only Data Supplement) levels in mice fed a HF diet compared with those fed a control diet. This increase in Bmpr and Bmp4 correlated with the increased expression of ICAM1/VCAM1 and CD68 signals (Figure 2), further supporting the notion that the modulation
of Bmp signaling by Bmper plays an important role in the inflammatory responses induced by HF diet. Collectively, these data demonstrate that Bmper haploinsufficiency leads to phenotypic changes correlative with an increased chronic, vascular inflammatory response and likely contributes to the aggravated atherosclerotic lesion formation observed in the Bmper+/−;ApoE−/− mice.

**Bmper Inhibits Shear Stress–Dependent Induction of Inflammatory Adhesion Molecules in the Endothelium**

Aortic lesions develop predominantly in regions that are exposed to low or disturbed fluid shear stress, such as the LC of the aorta. Even under normal hemodynamic conditions, previous studies demonstrate higher endothelial inflammation in the LC compared with other regions of the aorta, such as the GC.3,4 possibly because of the increased expression of Bmps in the vascular endothelium of these regions.8 This difference in lesion formation in the GC and LC was also observed in the Bmper atherosclerosis model. Specifically, we observed that there was a very significant increase in lesion area in the LC compared with GC of both Bmper+/+;ApoE−/− and Bmper+/−;ApoE−/− mice (Figure IV in the online-only Data Supplement). Immunostaining of the cross sections of the LC and GC with an antibody specific for Bmper demonstrated a dramatic increase of Bmper protein levels in the intima and media of the LC compared with the GC in both Bmper+/+ and Bmper+/− mice (Figure VA in the online-only Data Supplement). To quantitatively determine the difference of Bmper protein level in the GC and LC, we also performed Western blotting with vessel lysates obtained from the GC and LC. We observed significantly more Bmper protein located in the LC than GC (Figure VB in the online-only Data Supplement), suggesting that Bmper protein level is modulated by different fluid shear stress. Encouraged by these observations, we used this inherent difference in vascular inflammation between the LC and GC to analyze the effect of reduced Bmper expression on downstream mediators and effectors of BMP activation in these regions of mouse aortas. To simplify our experimental setup, we only examined mice on the ApoE +/+ background forwarding subsequent experiments. Endothelial cells located in the LC region of aortas of Bmper+/+ mice displayed abundant Smad1, 5, and 8 activation (as detected by phosphorylated Smad1, 5, and 8 signals) compared with...
endothelial cells in the GC region (Figure 3A), consistent with previous reports.\textsuperscript{11} In contrast, Bmper\textsuperscript{+/−} mice exhibited an enhanced increase in Smad activation in the LC region in comparison with Bmper\textsuperscript{+/+} mice; but in addition, Bmper\textsuperscript{+/−} mice had clearly detectable Smad activation in the GC region of the aortas, consistent with our in vivo data described above indicating that Bmpr functions as an anti-inflammatory mediator (Figure 3B). Expression patterns of both ICAM1 and VCAM1 paralleled that of Smad activation (Figure 3C–3F), demonstrating that Bmper inhibits the endothelial response to oscillatory shear stress–mediated induction of endothelial inflammatory adhesion molecules, and in the context of atherosclerosis, may directly inhibit the endothelial inflammatory response.

Bmper Inhibits Bmp4-Induced Inflammatory Gene Expression in Endothelial Cells and Prevents Fluid Shear Stress–Induced Inflammatory Responses

Our in vivo results clearly support a role for Bmper in suppressing shear stress–mediated inflammation. To determine the effects of Bmper on inflammation in the endothelial cell compartment, we established a cell-based model to determine whether Bmper’s ability to attenuate inflammation is Bmp4 dependent and is in response to fluid shear stress. Treatment of primary HUVECs with Bmp4 increased ICAM1 and VCAM1 expression at both the RNA and protein levels in a time-dependent manner, with peak expression occurring at 8 hours posttreatment (Figure 4A and 4B and data not shown), consistent with the previous reports.\textsuperscript{9,18} This robust Bmp4-mediated increase in ICAM1 and VCAM1 expression was blocked in the presence of exogenous Bmper (Figure 4C), demonstrating that Bmper directly antagonizes Bmp4-mediated inflammatory signaling. Next, we examined the ability of endogenous Bmp4 and Bmper to affect the expression of inflammatory markers. Given the robust inflammation seen in the normally quiescent GC region of the aorta in Bmper\textsuperscript{+/−};ApoE\textsuperscript{−/−} mice, we hypothesized that reducing endogenous Bmp4 or Bmper expression in endothelial cells would affect the inflammatory signature of the cells even in the absence of exogenous mediators. As expected, Bmp4-targeted siRNA reduced expression of ICAM1 and VCAM1 in transfected cells, whereas siRNA-mediated reduction of endogenous Bmper expression resulted in a significant increase in these same inflammatory markers (Figure 4D and 4E). Collectively, our data demonstrate that the anti-inflammatory function of Bmper in endothelial cells is mediated, at least in part, through antagonizing Bmp activity.
Given the effect of Bmper gene dosage on endothelial inflammation in vivo (Figure 3) and the in vitro effects of a reduction of Bmper expression on endothelial inflammation in the absence of any stimuli (Figure 4), we hypothesized that the inflammatory response to shear stress in the aorta may be mediated by changes in the levels of Bmper expression. To test this hypothesis directly, we subjected HUVECs to conditions that mimic the shear stress conditions in the LC and GC aortic regions using either oscillatory stress (±5 dyne cm⁻²) or laminar shear stress (20 dyne cm⁻²) for 8 hours, respectively. Consistent with our in vivo data, endothelial cells subjected to oscillatory shear had a larger inflammatory response (a 2.33-fold and 4.56-fold increase in ICAM1 and VCAM1 expression, respectively) compared with cells subjected to laminar shear, respectively (Figure 5A). Interestingly, we also observed increases in Bmp4 and Bmper expression in oscillatory shear conditions compared with laminar shear conditions (Figure 5A). Moreover, the protein ratio of Bmp4 to Bmper increased more dramatically in oscillatory shear conditions compared with laminar shear conditions (Figure 5B). To determine whether the inflammatory response observed in our shear stress model is mediated by changes in Bmper protein level, we used siRNA-mediated gene silencing of Bmper and...
compared the extent of inflammatory marker expression with control siRNA-treated cells. In control siRNA-treated cells, we detected higher ICAM1 and VCAM1 expression after oscillatory shear was compared with laminar shear (Figure 5C–5E), with similar patterns of Bmp4 and Bmper expression as what was seen in untreated cells exposed to both modes of shear stress (Figure 5A). In Bmper siRNA-treated cells, we observed an increased inflammatory response in oscillatory shear conditions compared with control siRNA–treated cells (Figure 5C–5E), consistent with the increased inflammation we observed in the LC regions of Bmper+/− aortas (Figure 3). Additionally, even in the low inflammatory environment (laminar shear stress), reducing Bmper expression resulted in 34% and 68% higher expression of ICAM1 and VCAM1, respectively, compared with control siRNA–treated cells. In control siRNA–treated endothelial cells (Figure 3). In addition, decreased levels of endothelial inflammatory adhesion molecules in the aorta in regions subjected to oscillatory and laminar shear stress. Similar results were found in cultured cell studies, solidifying the notion that Bmper is a critical regulator of vascular inflammation and broadening our understanding of the role that Bmper plays in the myriad events that result from the Bmp signaling pathway.

Previously, our studies revealed the essential roles of Bmp and Bmper signaling in endothelial cell differentiation, migration, and angiogenesis.13,14,16,17,23,24 With the results from the present study, we can now add vascular inflammation to the growing list of Bmp signaling events that are regulated by Bmper. Bmper+/−;ApoE−/− mice demonstrated increased Smad activation and expression of the inflammatory markers ICAM1 and VCAM1 in the endothelium of the LC of the aorta, a region known to be predisposed to atherogenic activity attributable to the Bmp-mediated vascular inflammation brought about by oscillatory shear stress effects on endothelial cells (Figure 3). In addition, decreased levels of Bmper in Bmper+/−;ApoE−/− mice led to an increase in the number of macrophages that were recruited and that migrated to the inflamed, atherogenic regions of the aortas (Figure 2A and 2B), supporting the notion that Bmper acts as a protective regulator of vascular inflammation. It is worth noting, however, that Bmper may also play a role in maintaining general vascular health in addition to its role in inflammatory responses. Our analysis of aortas taken from ApoE−/− mice that were haploinsufficient for Bmper (Bmper+/−;ApoE−/− mice) revealed a significant increase in the area of atherosclerotic lesions in mouse fed a regular diet (Figure 1A and 1B).
sustains that Bmpr may be important in maintaining vascular health even under basal conditions, a theory that will require further experiments to determine.

As compelling as these in vivo results are, however, it is important to remember that the decrease in Bmpr expression in the Bmpr+/− mouse is not limited to endothelial cells. Because atherosclerosis is a pathological condition that results from the dysfunction of multiple cell types and involves different cellular events, it is not possible, from these in vivo studies, to localize the protective effect of Bmpr to endothelial cells alone. Indeed, published reports demonstrate that Bmps enhance smooth muscle cell migration and induce proinflammatory factors, such as inducible nitric oxide synthase and tumor necrosis factor in macrophages. The inhibition of Bmp activity by specific inhibitors and antagonists decreases vascular calcification, suggesting important roles of Bmp in vascular calcification, as well as early vascular injury. Therefore, it is entirely possible that Bmpr, a secreted extracellular Bmp modulator, may also be able to influence Bmp activity not only in endothelial cells but in additional cell types such as smooth muscle cells and macrophages. Therefore, the contribution of Bmpr to protection against atherogenic processes and vascular inflammation will need further investigation.

As mentioned above, a number of published reports have detailed the role of Bmpr/Bmp signaling in various aspects of endothelial cell function. However, the ability of Bmpr to inhibit endothelial inflammatory responses has not been investigated directly. To examine this aspect of endothelial Bmpr signaling, we cultured HUVECs and subjected them to fluid shear stress. The band intensity of phospho-eNOS (Ser1177) and eNOS antibodies. G. The band intensity of phospho-eNOS (Ser1177) was quantified with ImageJ and is presented as the relative fold change of protein level compared with the control protein level. *P<0.02 compared with the cells at the static condition; **P<0.03 compared with the cells transfected with control siRNA, n=3.
in hypoxia-induced retinal neovascularization.\textsuperscript{13,14} Previously, we reported a gradient effect of Bmpr’s ability to influence Bmp signaling, whereby superstoichiometric concentrations of Bmpr compared with Bmp inhibit Bmp signaling and substoichiometric concentrations of Bmpr compared with Bmp activate Bmp signaling.\textsuperscript{15} In this study, we observed 61% and 15% higher Bmp4 and Bmpr expression in oscillatory shear stress conditions, resulting in a significantly lower Bmpr to Bmp4 ratio compared with laminar shear stress conditions, an expression pattern consistent with an anti-inflammatory role for Bmpr (Figure 5A and 5B). This observation further suggests that the fine-tuning of Bmp activity by Bmpr is essential for modulating Bmp-mediated cellular functions. Collectively, the data presented in this report demonstrate that the regulation of Bmp activity by Bmpr is essential for the maintenance of normal vascular homeostasis and its disruption increases the risk of inflammatory vascular diseases, such as atherosclerosis.

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**Disclosures**

None.

**References**

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SUPPLEMENT MATERIAL

Methods

Animals and diets

ApoE<sup>−/−</sup> mice on C57BL/6J background were kindly provided by Dr. Maeda (UNC, Chapel Hill, NC). Bmper<sup>+/−</sup> mice, previously generated in our laboratory on a C57BL/6J genetic background<sup>1</sup>, were crossed with ApoE<sup>−/−</sup> mice to generate Bmper<sup>+/−</sup>;ApoE<sup>−/−</sup> mice. We used the Bmper<sup>+/−</sup> mice instead of Bmper<sup>−/−</sup> mice because Bmper<sup>−/−</sup> mice die at birth<sup>1</sup>. All adult mice were fed with the standard chow or a high-fat/high-cholesterol diet (Western diet) (Harlan Laboratories, Indianapolis, IN) for 20 weeks. Body weight of mice was monitored before and after they were fed with different diets. Blood serum was obtained every four weeks. All mouse experimental procedures were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Committee for the Use of Animals in Research.

Lipid analysis

Mice were fasted for 18 hours before blood sampling. Less than 200 µl of blood was collected through submandibular bleeding using a lancet. The total cholesterol level was measured enzymatically with a commercially available kit (Infinity kits, Thermo Scientific, Waltham, MA).

Lesion quantification

The mice were euthanized and perfusion fixed with 10% buffered formalin via the left ventricle for 5 minutes. The lesions located in the aorta and aortic sinuses were analyzed using Oil Red O staining. To measure lesions in the aorta, the whole aorta, including the ascending arch, thoracic and abdominal segments, was dissected, gently cleaned of adventitial tissue and stained with Oil Red O following the previously described method<sup>2</sup>. The surface lesion area was quantified with ImageJ software and is presented as a percentage of the total surface area of the whole aorta. To measure the lesions in the aortic sinuses, the
heart and proximal aorta were excised, and the apex and lower half of the ventricles were removed. The remaining sample was embedded in OCT (Tissue-Tek, Fisher Scientific, Pittsburgh, PA) and frozen on dry ice. Starting from the appearance of the aortic valve, serial frozen sections at 5-µm thickness were collected until the aortic valves were completely sectioned following the previously described protocol\(^2\). Sections were stained with eosin and Oil Red O. The slides were imaged by light microscopy, and the atherosclerotic lesion area located in aortic sinus area was quantified with ImageJ and averaged over a 280 µm region.

**Calcification quantification**

Deposited calcium in the aorta was detected by staining with von Kossa. The 5-µm cryosections of aortic sinus were prepared as described above and subjected to the von Kossa staining procedure. The calcification area from each section was quantified as a percentage of the total vessel cross-sectional area using ImageJ software.

**ELISA measurements**

Blood samples were drawn from mice after consuming the high fat diet or standard chow for 4 weeks. Soluble VCAM (sVCAM) and soluble ICAM (sICAM) were measured in plasma in triplicate using an enzyme-linked immunosorbent assay (ELISA) method (R&D Systems, Minneapolis, MN).

**Reagents**

Recombinant human Bmp4 and Bmper protein and antibodies recognizing Bmper and Bmp4 were obtained from R&D Systems. VCAM1 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) for Western blotting and Chemicon for immunofluorescence (Millipore, Billerica, MA). The ICAM antibody was purchased from Cell Signaling Technology (Danvers, MA) and used for western blotting experiments. An additional ICAM1 antibody (purchased from Chemicon) was used for immunofluorescence experiments.
The pSmad1,5,8 antibody was purchased from Cell Signaling Technology and used for both western blotting and immunofluorescence experiments.

**Cell culture and siRNA transfection**

HUVECs (human umbilical vein endothelial cells) were purchased from Lonza and cultured in endothelial basal medium (EBM; Lonza, Allendale, NJ) supplemented with hydrocortisone, bovine brain extract, epidermal growth factor and 2% fetal calf serum. The cells from passages 4-8 were used for experiments. The stealth siRNA duplexes were obtained from Invitrogen (Grand Island, NY). The siRNAs against mouse Bmp4 are a mixture of the duplexes of 5'-GAAUUUCAGCCAGAAGGAAGCAAAU-3' and 5'-GGAGAGAGAUGUGGUCCUAAUCAAUU-3'. The siRNA against mouse Bmper is a duplex of 5'-GCAUGUCAGGAUAGCCGAUCGUUA-3'. The control siRNA is the Stealth™ RNAi negative control duplex (Cat. No. 12935-300) and was purchased from Invitrogen. The siRNAs were transfected into HUVECs according to the manufacturer’s recommended protocol for Nucleofection (Amaxa; the HUVEC protocol). Briefly, for each sample, 2x10^5 HUVECs were transfected with 300 pmol siRNA. The experiments with Bmp4 or Bmper siRNA-transfected HUVECs were performed one day or four days later, respectively. The siRNAs resulted in more than 70% knockdown of the protein levels of Bmp4 and Bmper.

**Shear stress assays**

HUVECs were post-confluent for 48 hours before the performance of fluid shear stress experiment to decrease the background signals. Laminar shear stress assay was described previously. Briefly, confluent cells in 10-cm dish were exposed to shear stress using the cone and plate flow chamber system for eight hours at 20 dyne cm^{-2} for laminar shear stress or ±5 dyn cm^{-2} for oscillatory shear stress experiments.

**Immunoblotting**
Cells were harvested in lysis buffer (1% Triton X-100, 50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1 mmol/L Na₃VO₄ and 0.1% protease inhibitor mixture; Sigma) and clarified by centrifugation at 16,000 g. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes.

**Immunofluorescence**

The aortic arch segments were dissected out and gently cleaned of the adventitia. The aortic fragments located at the greater curvature (GC) and lesser curvature (LC) were separated and fixed in 3.7% formaldehyde for 10 minutes at room temperature. The aortic fragments were sequentially treated with 70% ethanol for 30 minutes and 5% hydrogen peroxide in methanol. Then, the segments were washed with water for 5 minutes. For the phospho-Smad 1, 5, 8 antibody, the samples were soaked in boiling citric acid buffer (10 mmol/L, pH 6.0) for 9 minutes to expose the antigens. Next, the aortic fragments or 5-µm cryosections of the aortic root were blocked with 5% heat-inactivated goat serum for 1 hour and then incubated overnight with primary antibodies against ICAM1, VCAM1, CD31 or CD68 diluted in the blocking solution. After three washes in TBS, cells were incubated in the dark with a second antibody conjugated with Alexa Fluor 488 or 568 (Molecular Probes, Eugene, OR) in blocking solution for 90 minutes at 37 °C. After 3 washes in TBS, the fragments were counterstained with DAPI for phospho-Smad1, 5 and 8 staining. The *en face* images of the endothelial layer and the cross-sectional images of the aortic root were visualized by confocal laser scanning microscopy.

**Immunohistochemistry**

The aortic arch segments were dissected out and gently cleaned of the adventitia. The aortic fragments located at the greater curvature (GC) and lesser curvature (LC) were separated and fixed in 3.7% formaldehyde for 10 minutes at room temperature. After incubating tissue in a sucrose gradient, aortic samples were embedded in OCT compound and submitted for frozen sectioning. For the anti-Bmpr and Bmp4 antibody, the samples were soaked in boiling citric acid buffer (10 mmol/L, pH 6.0) for 10 minutes to
expose the antigens. Next, the 5-µm cryosections of the aortic root or aortic arch were blocked with 5% heat-inactivated rabbit serum for 1 hour and then incubated overnight with primary antibodies against Bmper or Bmp4 antibodies diluted in the blocking solution. After three washes in TBS, samples were incubated in a second antibody. For Bmper staining, we utilized amplification processes including the serial incubation with ABC (Vector Labs, Burlingame, CA, USA) and tyramide signal amplification reagent (Waltham, MA, USA) in blocking solution for 30 minutes for each at 37 °C. After 3 washes in TBS, the sections stained with Bmper and Bmp4 antibodies were developed with DAB. The images were recorded using the bright field microscopy with 10x and 20x objective lens.

**Statistical analysis**

Data are shown as the mean ± SE for 3 to 4 separate experiments. Differences were analyzed with two-way ANOVA and post-hoc analyses such as Student’s t-test when needed. Values of $P \leq 0.05$ were considered statistically significant.

**Figure legends**

Figure S1. The changes in body weight and total cholesterol level of Bmper/ApoE mice after 20 weeks on the standard chow (CH) or high fat diet (HF). A, The change in body weight after 20 weeks was calculated and demonstrated as a fold-change of body weight at Week 20 over Week 0. The numbers listed below the graph are the number of mice used for this experiment. B, The total serum cholesterol level was measured at Week 20. *, $P < 0.05$, compared to mice with the same genotype but fed with the control diet. #, $P < 0.05$, compared to mice fed with the same diet but with the ApoE<sup>−/−</sup> genotype.
Figure S2. sICAM1 and sVCAM1 plasma levels increased in Bmper\(^{+/-}\) mice after consuming the high fat diet for 4 weeks. Blood was collected, and the protein levels of soluble ICAM1 (A) and VCAM1 (B) were measured at Week 4. \(n=3\).

Figure S3. Bmper and Bmp4 expression was increased in mice fed a high fat diet. The cryo-sctions of aortic root were stained with anti-Bmper (A) and Bmp4 (B) antibodies. All the mice were on the ApoE\(^{-/-}\) background. Scale bar: 200 µm.

Figure S4. Bmper haploinsufficiency leads to aggravated atherosclerotic plaque formation in aortic arch area. Mice were fed a high fat diet (HF) or standard chow (CH) for twenty weeks. The aortas were dissected out and stained with Oil Red O. The lesions on the surface of each greater and lesser curvature of aorta arch were quantified as a percentage of the total area of GC and LC. *, \(P<0.002\), compared to that lesions located in GC of the same mouse. #, \(P<0.05\), compared to Bmper\(^{+/-}\) mice fed with the same diet. All the mice were on the ApoE\(^{-/-}\) background.

Figure S5. Bmper protein level was increased in the LC compared to GC. (A) The regions of GC and LC located in aortic arch of mouse aortas were processed for staining with Bmper antibody. Scale bar: 200 µm. The arrows represent the positive staining of Bmper protein. Scale bar: 200 µm. (B) The vessel lysates obtained from the GC and LC region of mice were subjected to the Western blotting with anti-Bmper antibody. Each sample was obtained from two mice. *, \(P<0.05\), compared to the sample of the GC in the same mouse. #, \(P<0.05\), compared to the GC region of Bmper\(^{+/-}\) mice. \(n=3\).
References


A

\[ \text{sICAM1 (\text{\mu g/mL})} \]

\[ \bar{\text{Bmpr}^{+/+}} \text{ ApoE}^{-/-} \]

\[ \bar{\text{Bmpr}^{+/+}} \text{ ApoE}^{-/-} \]

\[ p = 0.02 \]

B

\[ \text{sVCAM1 (\text{\mu g/mL})} \]

\[ \bar{\text{Bmpr}^{+/+}} \text{ ApoE}^{-/-} \]

\[ \bar{\text{Bmpr}^{+/+}} \text{ ApoE}^{-/-} \]

\[ p = 0.05 \]

Pi et al. Supplemental Figure II
Lesion area in GC/LC (%)

HF

CH

GC
LC
GC
LC
GC
LC
GC
LC

Pi et al. Supplemental Figure IV