Anti-Inflammatory and Antiatherogenic Role of BMP Receptor II in Endothelial Cells

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Objective—Atherosclerosis is an inflammatory disease with multiple underlying metabolic and physical risk factors. Bone morphogenetic protein 4 (BMP4) expression is increased in endothelium in atherosclerosis-prone regions and is known to induce endothelial inflammation, endothelial dysfunction, and hypertension. BMP actions are mediated by 2 different types of BMP receptors (BMPRI and BMPRII). Here, we show a surprising finding that loss of BMPRII expression causes endothelial inflammation and atherosclerosis.

Approach and Results—Using BMPRII siRNA and BMPRII+/− mice, we found that specific knockdown of BMPRII, but not other BMP receptors (Alk1, Alk2, Alk3, Alk6, ActRIIa, and ActRIIb), induced endothelial inflammation in a ligand-independent manner by mechanisms mediated by reactive oxygen species, nuclear factor-KappaB, and reduced nicotinamide adenine dinucleotide phosphate oxidases. Further, BMPRII+/− ApoE−/− mice developed accelerated atherosclerosis compared with BMPRII+/− ApoE−/− mice. Interestingly, we found that multiple proatherogenic stimuli, such as hypercholesterolemia, disturbed flow, prohypertensive angiotensin II, and the proinflammatory cytokine (tumor necrosis factor-α), downregulated BMPRII expression in endothelium, whereas antiatherogenic stimuli, such as stable flow and statin treatment, upregulated its expression in vivo and in vitro. Moreover, BMPRII expression was significantly diminished in human coronary advanced atherosclerotic lesions. Also, we were able to rescue the endothelial inflammation induced by BMPRII knockdown by overexpressing BMPRII in vivo.

Conclusions—These results suggest that BMPRII is a critical, anti-inflammatory, and antiatherogenic protein that is commonly targeted by multiple pro- and antiatherogenic factors. BMPRII may be used as a novel diagnostic and therapeutic target in atherosclerosis. (Arterioscler Thromb Vasc Biol. 2013;33:1350-1359.)

Key Words: atherosclerosis  blood flow  BMPRII  endothelial cell  inflammation  signal transduction

Atherosclerosis is an inflammatory disease,1,2 associated with multiple systemic risk factors, including hypercholesterolemia, hypertension, diabetes mellitus, smoking, and physical inactivity.3,5 Despite the presence of these systemic risk factors, atherosclerosis preferentially occurs in specific focal regions of branched or curved arteries exposed to disturbed flow (d-flow), suggesting a potential interaction between hemodynamic conditions and systemic risk factors in atherogenesis. More specifically, d-flow induces endothelial inflammation, which may prime these arterial regions for atherogenesis in the presence of the aforementioned systemic risk factors.1,5 Indeed, we have recently demonstrated that exposure of pristine, straight common carotid artery to d-flow by partial carotid ligation leads to rapid and robust atherosclerosis development in apolipoprotein E (ApoE)−/− mice fed a high-fat diet.6,7 However, the underlying mechanisms by which these synergistic effects lead to initiation and progression of atherosclerosis are still unclear.

Previously, we showed that bone morphogenetic protein 4 (BMP4) is induced by d-flow in endothelial cells causing endothelial inflammation and dysfunction in an NfκB and NADPH oxidase–dependent manner.5,6 In addition, BMP2 and BMP4 expression is increased in human atherosclerotic plaques and human coronary artery endothelium overlying early atherosclerotic lesions.8,9 Furthermore, we showed that chronic infusion of BMP4 causes systemic hypertension by stimulating activation of arterial NADPH oxidases and endothelial dysfunction subsequently in mice.10 Moreover, overexpression of a BMP antagonist, matrix gla protein, or treatment with a pharmacological BMP inhibitor protects...
against atherosclerosis. These results clearly suggest the role of BMPs in vascular disease, but specific BMP receptors that mediate this effect are unknown.

BMP actions are mediated by 2 different types of BMP receptors (BMPR): type I receptors (Alk1, Alk2, Alk3, and Alk6) and type II receptors (BMPRII, ActRIIa, and ActRIIb). On binding to BMPs, type I BMPRs are activated by BMPRII and subsequently activate the canonical SMAD-1/5/8–dependent and –independent signaling pathways. Interestingly, it has been shown that loss-of-function mutations of BMPRII are linked to primary pulmonary hypertension in humans, demonstrating its importance in pulmonary artery biology and pathophysiology. However, it is unknown whether the genetic mutations or changes in BMPRII expression have any association with conduit arterial diseases, such as atherosclerosis.

We initially hypothesized that BMP4-induced endothelial inflammation would be mediated by BMPRII, and that its knockdown would prevent the BMP4 effect. However, we found a surprising, paradoxical result that loss of BMPRII in endothelial cells caused robust inflammation and atherosclerosis. Further studies showed that the proinflammatory effect of BMPRII knockdown was unique to this receptor member because knockdown of other BMP receptors did not induce endothelial inflammation. Moreover, we found that multiple proatherogenic factors, either individually or in combination, resulted in a common response, loss of BMPRII in endothelial cells in vitro and in vivo. We also found loss of BMPRII expression in human coronary arteries with advanced atherosclerotic lesions.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

Loss of BMPRII Induces Endothelial Inflammation In Vitro and In Vivo

To examine whether BMPRII mediates BMP4-dependent endothelial inflammation, endothelial cells were treated with either BMPRII siRNA (BRII.si) or control siRNA (Non.si) in the presence or absence of BMP4, and monocyte adhesion assays were performed. As expected, BMP4 increased monocyte adhesion by 2-fold over siRNA control in human umbilical vein endothelial cells (HUVECs) (Figure 1A). To our surprise, however, knockdown of BMPRII markedly elevated basal monocyte adhesion by almost 2-fold over controls, independent of BMP4 (Figure 1A). To determine the underlying mechanisms for this unexpected result, expression of cell adhesion molecules intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), BMP4, and BMPRII was examined in HUVECs. BMPRII siRNA reduced BMPRII expression by ≈90%, whereas ICAM-1 and VCAM-1 expression was increased by 6- and 3-fold, respectively, compared with nonsilencing control, but basal BMP4 expression was unaffected (Figure 1B; Figure 1A in the online-only Data Supplement). We next investigated the effect of BMPRII knockdown on activation of the canonical BMP pathway by examining SMAD1/5/8 phosphorylation in a time-dependent manner, which was blocked by BMPRII siRNA (Figure 1C), consistent with a previous report. This suggests that BMPRII siRNA effectively inhibits the canonical signaling pathway in response to BMP4, while paradoxically inducing endothelial inflammation.

We next tested whether this proinflammatory effect is specific to BMPRII knockdown. To compare the specificity of each BMP receptor, endothelial cells were treated with siRNAs specific for other BMP receptors (type I: Alk1, Alk2, Alk3, and Alk6 and type II: ActRIIa, ActRIIb, and BMPRII). Only BMPRII siRNA was able to induce ICAM-1 and VCAM-1 expression markedly above control siRNA levels, whereas all other BMP receptor siRNAs tested did not (Figure 1D). Interestingly, Alk1 knockdown decreased endothelial inflammation, which suggests that basal expression of ICAM-1 and VCAM-1 is mediated by ligands, such as BMPs or transforming growth factor-β, existing in the culture media. Also, Alk3 and Alk6, like Alk1 showed a moderate downregulation of ICAM-1 compared with the Non.si controls indicating that these receptors might mediate the proinflammatory effects attributable to these ligands, which may be present in the culture media or produced by the cells themselves. These findings suggest that specific loss of BMPRII, but not other BMP receptors, triggers an endothelial inflammatory response (Figure 1D; Figure ID–IF in the online-only Data Supplement).

To validate whether this unexpected in vitro finding was also relevant in vivo, we examined expression of ICAM-1 and VCAM-1 in arterial walls of BMPRII-deficient ApoE knockout mice (BMPRII+/−ApoE−/−) in comparison with homozygotic knockout of BMPRII causes embryonic lethal phenotype, heterozygotic BMPRII+− mice were used. To rule out the effect of high-fat diet on our experimental model, the mice used in the study were fed a standard chow diet. Expression of both ICAM-1 and VCAM-1 was significantly increased in the arterial endothelium of BMPRII+/−ApoE−/− compared with BMPRII+/−ApoE−/− littermate control mice. Because homozygotic knockout of BMPRII causes embryonic lethal phenotype, heterozygotic BMPRII+− mice were used. To rule out the effect of high-fat diet on our experimental model, the mice used in the study were fed a standard chow diet. Expression of both ICAM-1 and VCAM-1 was significantly increased in the arterial endothelium of BMPRII+/−ApoE−/− compared with BMPRII+/−ApoE−/− littermate control mice (Figure 1E; Figure IH in the online-only Data Supplement). These results suggest that specific BMPRII knockdown induces endothelial inflammation in a BMP4-independent manner both in vitro and in vivo.

Loss of BMPRII Induces Endothelial Inflammation by Mechanisms Mediated by Reactive Oxygen Species, NADPH Oxidase, and NFκB Activity

Next we studied the mechanisms by which BMPRII knockdown induces endothelial inflammation. Because reactive oxygen species (ROS) play an important role in endothelial inflammation and atherosclerosis, we tested whether the proinflammatory effect of BMPRII was mediated by ROS. Knockdown of BMPRII increased ROS production in HUVECs as measured by Amplex-Red. This increased ROS production was significantly blunted by the cell-permeable ROS scavenger polyethylene glycol catalase (Figure IIA in the online-only Data Supplement), implicating a potential role for ROS in BMPRII-mediated endothelial inflammation.

Because Nox1 is a reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase member producing ROS involved in endothelial inflammation, we tested the effect of apocynin (a NADPH oxidase inhibitor and ROS scavenger) on BMPRII phosphorylation in a time-dependent manner, which was blocked by BMPRII siRNA (Figure 1C), consistent with a previous report. This suggests that BMPRII siRNA effectively inhibits the canonical signaling pathway in response to BMP4, while paradoxically inducing endothelial inflammation.
knockdown–induced inflammation in HUVECs. Treatment with apocynin significantly inhibited increased monocyte adhesion and ICAM-1 expression after BMPRII siRNA (Figure 2A and 2B; Figure IIB and IIC in the online-only Data Supplement). To validate these in vitro findings, we measured in vivo ROS production in the aortic walls of BMPRII+/−ApoE−/− and BMPRII+/+ApoE−/− mice by dihydroethidium staining. Increased ROS production was observed in the aortic wall, including endothelium (arrows in the inset) and smooth muscle cells of BMPRII+/−ApoE−/− compared with BMPRII+/+ApoE−/− mice (Figure 2C). ROS production in the aortic endothelium coincided with Nox1 expression in BMPRII+/−ApoE−/− and BMPRII+/+ApoE−/− mice by dihydroethidium staining. Increased ROS production was observed in the arterial wall, including endothelium (arrows in the inset) and smooth muscle cells of BMPRII+/−ApoE−/− compared with BMPRII+/+ApoE−/− mice (Figure 2C). ROS production in the aortic endothelium coincided with Nox1 expression in BMPRII+/−ApoE−/− and BMPRII+/+ApoE−/− mice (Figure 2D), indicating a potential link among BMPRII reduction, Nox1 induction, and ROS production in the arterial wall. This was further demonstrated in HUVECs, which showed that BMPRII knockdown induced Nox1 expression (Figure IID in the online-only Data Supplement; Figure 2E). Moreover, simultaneous knockdown of Nox1 and BMPRII significantly prevented VCAM-1 expression, but not ICAM-1, (Figure 2E; Figure IIE in the online-only Data Supplement), showing a specific effect of the BMPRII knockdown on VCAM-1 by a Nox1-dependent mechanism.

Because induction of ICAM-1 and VCAM-1 is known to be regulated by nuclear factor-KappaB (NFκB), we tested the role of NFκB signaling pathway in BMPRII knockdown–induced endothelial inflammation. Treatment of HUVECs with the NFκB inhibitor BAY 11–7082 significantly inhibited monocyte adhesion, as well as ICAM-1 and VCAM-1 mRNA and protein expression induced by BMPRII knockdown (Figure 2F–2H; Figure IIF in the online-only Data Supplement). Collectively, these findings suggest that knockdown of BMPRII in endothelial cells stimulates NADPH oxidase and ROS production, which, in turn, mediates endothelial inflammatory responses, including ICAM-1 and VCAM-1 expression, and monocyte adhesion by a NFκB-dependent pathway.

**BMPRII Deficiency Exacerbates Atherosclerosis Development in ApoE−/− mice**

We then tested the hypothesis that BMPRII deficiency exacerbates atherosclerosis using acute and chronic mouse models. We compared atherosclerosis development in the carotid arteries of ApoE−/− mice using an acute partial ligation model of atherosclerosis as we recently described.6,7 As expected, partial ligation of left carotid artery (LCA) rapidly induced

![Figure 1. Loss of bone morphogenic protein receptors II (BMPRII) induces endothelial inflammation in vitro and in vivo.](http://atvb.ahajournals.org/)
atherosclerosis in BMPRII+/−ApoE−/− by 2 weeks post ligation on a high-fat diet, whereas the contralateral right carotid artery remained lesion-free (Figure 3A). In comparison, BMPRII deficiency in BMPRII+/−ApoE−/− mice increased LCA lesion area by >5-fold compared with BMPRII+/+ApoE−/− littermate controls (Figure 3A and 3B). Increased atherosclerosis in the BMPRII-deficient mice showed correlation with CD45+ leukocyte infiltration (Figure III in the online-only Data Supplement), but was not attributable to exacerbated hypercholesterolemia, or abnormal serum lipid profiles compared with BMPRII+/+ApoE−/− littermate controls (Table I in the online-only Data Supplement). Next, chronic atherosclerosis development was examined in the aortic arches of BMPRII+/−ApoE−/− and BMPRII+/+ApoE−/− mice fed a high-fat diet. Treatment with high-fat diet for 2 months induced atherosclerosis in the aortic arch of BMPRII+/+ApoE−/−, as expected. In comparison, BMPRII deficiency increased plaque burden by 2-fold in BMPRII+/−ApoE−/− mice (Figure 3C and 3D). These results obtained from the 2 different models of atherosclerosis strongly indicate that genetic haplodeficiency of BMPRII is sufficient to exacerbate atherosclerosis development.

BMPRII Expression Is Downregulated by Proatherogenic Risk Factors, While Upregulated by Antiatherogenic Conditions

We next asked whether BMPRII expression in arterial endothelium is regulated by conditions or stimuli relevant to pathophysiology of human atherosclerosis. Because atherosclerosis is caused by multiple risk factors, we hypothesized that various proatherogenic conditions, such as hypercholesterolemia, d-flow, hypertension, and proinflammatory cytokines, either individually or in combination, downregulate BMPRII expression, which, in turn, drives endothelial inflammation and atherosclerosis. We first tested whether BMPRII expression could be decreased by d-flow and hypercholesterolemia using our carotid partial ligation model of d-flow–induced atherosclerosis in ApoE−/− mice fed a high-fat diet (Figure 4A).6,26 Although we did not notice any remarkable change in the BMPRII expression at an early time point (2 days post ligation), BMPRII expression became undetectable in flow-disturbed LCA endothelium and was markedly reduced in smooth muscle cells at 1 week post ligation. In contrast, BMPRII expression in the contralateral right carotid artery remained

Figure 2. Loss of bone morphogenic protein receptors II (BMPRII) induces endothelial inflammation by mechanisms dependent on ROS, reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, and nuclear factor-KappaB (NFκB) activity. A and B, HUVECs transfected with BMPRII siRNA or Non.si were also treated with apocynin (60 μmol/L; 2 days) or vehicle, and monocyte adhesion assay (A) or Western blot analysis (B) of cell lysates using intercellular cell adhesion molecule-1 (ICAM-1) antibody was performed (means±SEM; n=4; *P<0.05). C and D, Thoracic aorta sections of BMPRII+/ApoE−/− and BMPRII+/−ApoE−/− mice were stained with dihydroethidium for ROS detection (C) or Nox1 antibody (D). Shown are representative confocal microscopy images (n=6 each). The insets show magnified views of endothelial regions. Nuclei (blue) and elastic laminas (green) are shown. L indicates lumen. Also shown are en face confocal images of Nox1 antibody staining (bottom, D). E, HUVECs were transfected with Non.si, BMPRII siRNA, BMPRII siRNA+Nox1 siRNA, or Nox1 siRNA for 2 days and analyzed by Western blots using ICAM-1, vascular cell adhesion molecule-1 (VCAM-1), BMPRII, Nox1, and β-actin antibodies. F–H, Human umbilical vein endothelial cells transfected with BMPRII siRNA or Non.si were treated with Bay11-7082 (10 μmol/L; 24 hours) before monocyte adhesion assay (F), quantitative polymerase chain reaction analysis for BMPRII, ICAM-1, and VCAM-1 (G), and Western blots with BMPRII, ICAM-1, and VCAM-1 antibodies (H; n=4; *P<0.05).
relatively unchanged at the 1-week time point (Figure IV in the online-only Data Supplement). These findings suggest that d-flow and hypercholesterolemia in combination downregulated BMPRII expression, whereas 1 week of hypercholesterolemia alone was not sufficient to decrease BMPRII level. Interestingly, we did not observe any BMPRII staining in the intima at 1 week post ligation (Figure IV in the online-only Data Supplement), suggesting that the leukocytes infiltrating the intima at this time point, as we recently demonstrated, do not express detectable level of BMPRII expression.

To determine whether flow itself could downregulate BMPRII expression in the arterial endothelium, endothelial-enriched RNA was collected from the LCA and right carotid artery of C57Bl6 mice fed a chow diet at 48 hours post ligation as we previously described. BMPRII mRNA expression was decreased by >2-fold in the LCA endothelium compared with right carotid artery (Figure 4B). This was further demonstrated in HUVECs in vitro, which we exposed to unidirectional, laminar shear and oscillatory shear stress using a cone-and-plate shear device to mimic stable flow and d-flow in vivo, as we described previously. BMPRII expression was increased in HUVECs exposed to laminar shear compared with oscillatory shear stress and static control (Figure 4C). These findings further substantiate that proatherogenic d-flow downregulates, whereas antiatherogenic stable flow upregulates BMPRII expression in endothelial cells both in vivo and in vitro.

Next we tested whether angiotensin II (AngII), a key regulator of hypertension, and proinflammatory and proatherogenic agent, downregulates BMPRII expression in aortic endothelium. AngII infusion in C57Bl6 mice decreased BMPRII mRNA expression in a time-dependent manner, measured using endothelial-enriched RNA isolated from thoracic aortas at 36 to 72 hours post infusion (Figure 4D). Finally, we examined the role of proinflammatory cytokines using tumor necrosis factor α (TNFα) as a well-known prototypical cytokine that induces acute endothelial cell inflammation, on BMPRII expression in HUVECs. TNFα significantly inhibited BMPRII expression in a concentration- and time-dependent manner (Figure 4E; Figure VA in the online-only Data Supplement). Conversely, we tested whether BMPRII expression could be upregulated or maintained by antiatherogenic stimuli. For this purpose, we chose statins as they are known to provide anti-inflammatory and antiatherogenic benefits in addition to their well-known lipid-lowering effect. Recently, simvastatin has been shown to upregulate the expression of BMPRII in different cell types in vitro, including lung microvascular endothelial cells. Treatment of HUVECs with 3 different statins (simvastatin, rosuvastatin, and mevasstatin) significantly increased BMPRII expression both at the mRNA and at the protein levels (Figure 4F; Figure VB–VD in the online-only Data Supplement). These results show that antiatherogenic conditions, such as stable flow and statin treatment, prevent the loss of BMPRII expression, whereas multiple proatherogenic conditions, including hypercholesterolemia, d-flow, AngII, and TNFα, downregulate BMPRII expression.

**Loss of BMPRII in Human Coronary Arteries With Advanced Atherosclerotic Plaques**

To examine whether our findings in in vitro settings and mouse models are relevant to human atherosclerosis, BMPRII expression was determined in human coronary arteries with atherosclerosis. Immunohistochemical staining showed that BMPRII expression was readily detected in nondiseased human coronary artery wall, including luminal endothelium and smooth muscle cells, but not in adventitia (Figure 5A and 5B). However, BMPRII expression began to decrease (Figure 3. Bone morphogenic protein receptors II (BMPRII) deficiency exacerbates atherosclerosis development in ApoE−/− mice. A, BMPRII+/−ApoE−/− (n=12) and littermate control BMPRII+/+ApoE−/− mice (n=14) were partially ligated and fed a high-fat diet for 2 weeks. Frozen sections from the ligated left carotid artery (LCA; disturbed flow) and contralateral right carotid artery (RCA; stable flow) were stained with Oil-Red-O as shown by representative microscopy images. B, Atherosclerotic lesion area was quantified (*P<0.001). C, BMPRII+/−ApoE−/− (n=8) and BMPRII+/+ApoE−/− mice (n=10) were fed the high-fat diet for 2 months. Aortic arch regions were stained with Oil-Red-O as shown, and (D) atherosclerotic lesion areas were determined (*P<0.05).
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5Ai, 5Av, 5Bi, and 5Bv) in the coronary artery wall in early and intermediate lesions (type II–III) and was nearly undetectable (Figure 5Aiii, 5Avi, 5Biii, and 5Bvi) in advanced lesions (type V–VI).32,33 BMPRII staining was also not detected in intimal regions known to contain leukocyte accumulations, consistent with the above finding in mouse (Figure 4A). Overall, BMPRII expression in coronary artery endothelium showed a significant negative correlation ($R^2=0.68$; $n=14$ patients) with atheroma intensity (Figure 5C), suggesting that a similar relationship exists between BMPRII loss and atherosclerosis development in humans as demonstrated in our mouse models.

Overexpression of BMPRII Rescues Endothelial Inflammation Caused by BMPRII Knockdown

Next we tested whether we could reverse the BMPRII knockdown–induced endothelial inflammation by overexpressing BMPRII. To this end, we overexpressed BMPRII wild type or short form, lacking the C-terminal tail domain using adenoviral constructs in endothelial cells treated with BMPRII siRNA. Overexpression of BMPRII wild type, but not the short form, was able to blunt the endothelial expression of ICAM-1 and VCAM-1 induced by BMPRII knockdown (Figure 6A and 6B). This result suggests that the C-terminal tail region of BMPRII plays a critical role in regulating ICAM-1 and VCAM-1 expression in response to BMPRII knockdown.

Discussion

Our serendipitous finding that the loss of BMPRII induces endothelial inflammation and atherosclerosis came as a surprise at first because we initially hypothesized that knockdown of the receptor mediating BMP4 action would prevent endothelial inflammation. To the contrary, we found that knockdown of BMPRII in endothelial cells induced monocyte adhesion through the expression of ICAM-1 and VCAM-1 (Figure 1). This increase in the adhesion molecule expression and monocyte adhesion was independent of the BMP4 treatment indicating that BMPRII plays a critical role in endothelial inflammation, perhaps in a BMP ligand–independent manner. Further in vivo studies showed that loss of BMPRII induced endothelial inflammation (Figures 1 and 2) and atherosclerosis in BMPRII+/−ApoE−/− mice (Figure 3). Our data also showed that BMPRII expression was progressively lost as advanced atherosclerotic plaques formed in human coronary arteries (Figure 5). The progressive loss of BMPRII was also found in our mouse model of atherosclerosis (Figure 4A). Together, these results suggest that loss of BMPRII may be a common event occurring both in human atherosclerosis and in mouse models of atherosclerosis.

Although our data on loss of BMPRII expression in human coronary arteries with advanced lesions need to be interpreted with caution because of the small number of tissue samples (n=14 patients) used in the study, it does raise the possibility that loss of BMPRII in atherosclerosis may be an important
pathophysiological molecular event that has not been appreciated thus far. Although genomic mutations or downregulation of BMPRII has been linked to human primary pulmonary hypertension, human secondary pulmonary hypertension, and rat hypoxic pulmonary hypertension, they have never been implicated in atherosclerosis. Primary pulmonary hypertension predominantly occurs in young women with a median age at diagnosis of 36.4 years. The rarity of the disease and short survival duration (mean survival of 2.8 years) of these patients may explain why BMPRII has not been previously implicated in more common vascular diseases, such as atherosclerosis.

Our data indicate that seemingly disparate, multiple proatherogenic factors downregulate BMPRII expression through a potentially common pathway. For example, hypercholesterolemia, d-flow, the prohypertensive AngII, and the proinflammatory cytokine TNFα, all downregulated BMPRII expression in endothelial cells in vitro and in vivo (Figure 4). One possibility is that these multiple proatherogenic risk factors converge on increased production of proinflammatory cytokines, such as TNFα, acting as common effectors. These proinflammatory cytokines produced by vascular wall cells and infiltrating leukocytes under various proatherogenic conditions may downregulate BMPRII expression. In contrast, anti-inflammatory conditions, such as treatment with statins or stable flow, increase or maintain BMPRII expression thereby preventing endothelial inflammation and atherosclerosis. BMPRII thus may serve as a common target of these pro- and antiatherogenic risk factors (Figure 6C).

We found that loss of BMPRII induced endothelial inflammation and atherosclerosis by the mechanisms mediated by NADPH oxidases, ROS production, and NFkB activation. How the loss of BMPRII triggers these signaling pathways needs to be further defined, but it may depend on cell types and biological context. Interestingly, we were able to rescue endothelial inflammation caused by BMPRII knockdown by overexpressing BMPRII wild type, but not by the BMPRII short form. This suggests that the C-terminal tail domain of BMPRII plays a constitutive anti-inflammatory role and that its loss or reduction induces proinflammatory response. One potential mechanistic explanation for this is the unique “molecular docking” nature of the long cytoplasmic tail domain of BMPRII, which is not found in other members of transforming growth factor-β/BMP receptor superfamily. The long cytoplasmic tail domain of BMPRII is known to bind various signaling proteins, including NFkB-p50 protein and cSrc.

Loss of the BMPRII, especially the C-terminal domain, may induce abnormal activation of these pathways, leading to NFkB activation and endothelial inflammation. Consistent with this idea, the proinflammatory effect of BMPRII knockdown was unique only to this receptor because knockdown of all other type I and type II BMP receptors examined in our study did not induce endothelial inflammation.

Other possibilities include that the loss of BMPRII may result in altered activation of non-BMP2/4 ligands, such as BMP6/7 and BMP9/10 families as well as Transforming growth factor-β, while inhibiting BMP2/4 pathway. In pulmonary artery smooth muscle cells, BMPRII deletion was shown to inhibit BMP2/4 signaling, while augmenting BMP6/7 pathway via activation of ActRIIa receptor. However, knockdown of ActRIIa, which is the primary receptor for BMP6/7, did not affect endothelial inflammation in our system, suggesting that endothelial inflammation caused by BMPRII knockdown is unlikely to be mediated by BMP6/7. BMP9 is known to be a vascular quiescence factor and binds to Alk1 and BMPRII along with a coreceptor. However, our results showed that
We propose that multiple proatherogenic risk factors, such as hypercholesterolemia, hypertension, d-flow, and inflammatory conditions, either independently or in combination, downregulate BMPRII expression through a common mechanism (Figure 6C). Endothelial cells exposed to d-flow in athero-prone regions produce BMP4, which, in turn, induces an early endothelial inflammation resulting in intimal leukocytes infiltration. In the presence of additional risk factors, such as hypercholesterolemic conditions, these leukocytes, including macrophages and dendritic cells, become foam cells and produce proinflammatory cytokines. These cytokines, such as TNFα, and additional risk factors, including hypertensive AngII and d-flow, would then downregulate BMPRII, which triggers vicious cycle of inflammatory responses leading to atherosclerosis by the ROS and NFκB-dependent mechanisms (Figure 6C).

In conclusion, we show that BMPRII is a common target of multiple proatherogenic factors and that its loss in arterial wall induces endothelial inflammation and atherosclerosis. BMPRII may be a critical, anti-inflammatory, and antiatherogenic protein and may be used as a novel diagnostic and therapeutic target in atherosclerosis.

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Disclosures

None.

References


Atherosclerosis is a multifactorial disease that usually develops many years before any clinical symptoms are manifest. The role of bone morphogenic protein receptor (BMPRII) in atherosclerosis is not known. Here, using BMPRII siRNA and BMPRII+/− mice, we show a surprising finding that loss of BMPRII expression causes endothelial inflammation and atherosclerosis by mechanisms mediated by reactive oxygen species, NFκB, and NADPH oxidases. Interestingly, we found that multiple proatherogenic stimuli, such as hypercholesterolemia, disturbed flow, prohypertensive angiotensin II, and the proinflammatory cytokine (tumor necrosis factor-α), downregulated BMPRII expression in endothelium, whereas antiatherogenic stimuli, such as stable flow and statin treatment, upregulated its expression in vivo and in vitro. Endothelial inflammation induced by BMPRII knockdown was alleviated by overexpressing the BMPRII wild type, but not by the BMPRII short form lacking the carboxyl-terminal tail region. Therefore, BMPRII may be used as a novel diagnostic and therapeutic target in atherosclerosis.

**Significance**

Atherosclerosis is a multifactorial disease that usually develops many years before any clinical symptoms are manifest. The role of bone morphogenic protein receptor (BMPRII) in atherosclerosis is not known. Here, using BMPRII siRNA and BMPRII+/− mice, we show a surprising finding that loss of BMPRII expression causes endothelial inflammation and atherosclerosis by mechanisms mediated by reactive oxygen species, NFκB, and NADPH oxidases. Interestingly, we found that multiple proatherogenic stimuli, such as hypercholesterolemia, disturbed flow, prohypertensive angiotensin II, and the proinflammatory cytokine (tumor necrosis factor-α), downregulated BMPRII expression in endothelium, whereas antiatherogenic stimuli, such as stable flow and statin treatment, upregulated its expression in vivo and in vitro. Endothelial inflammation induced by BMPRII knockdown was alleviated by overexpressing the BMPRII wild type, but not by the BMPRII short form lacking the carboxyl-terminal tail region. Therefore, BMPRII may be used as a novel diagnostic and therapeutic target in atherosclerosis.
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Supplemental Figure I.

(A) Quantification of BMPRII, ICAM-1, VCAM-1 and BMP4 protein expression by Western blot. Cell lysates obtained from HUVECs transfected with BMPRII siRNA (BRII.si) or non-silencing control siRNA (Non.si) were analyzed by Western blot using antibodies to BMPRII, ICAM-1, VCAM-1 and BMP4. The representative Western blot is shown in main Figure 1B. (B) Western blot showing BMPRII expression in various endothelial cell sources. BAEC- bovine arterial endothelial cells; HAEC- human aortic endothelial cells HMEC-human microvascular endothelial cells; iMAEC-immortalized mouse aortic endothelial cells. Some HAECs were treated with BRII.si to determine specificity of Western blot analysis. (C, D) HAECs were transfected with siRNAs specific for BMPRII, Alk2, Alk3, Alk6, ActRIIa, ActRIIb or control (Non.si). Transfection efficiency was quantified by qPCR (n=3, *p<0.05). (C). Note that Alk6 was nearly undetectable in ECs even without the Alk6 siRNA treatment. Western blot analyses using antibodies for BMPRII, VCAM-1, ICAM-1 and b-actin antibodies were carried out and graph in (D) shows quantification of Western blot results (n=3, *p<0.05). The representative Western blot is shown in main Figure 1D. (E) Knockdown efficiency of Alk1 siRNA in HUVECs is shown by qPCR (n=3, *p<0.05). (F) Effect of Alk1 siRNA treatment on ICAM1 and VCAM1 in HUVECs is shown by qPCR (n=3, *p<0.05). (G) Graph shows BMPRII mRNA expression from the endothelial-enriched RNA isolated from thoracic aortas of BMPRII+/− ApoE−/− and BMPRII+/+ApoE−/− mice, respectively, (n=4 each). (H) Quantification of immunofluorescence staining using ICAM-1 and VCAM-1 antibodies on the cross-sections of thoracic aortas obtained from BMPRII+/−ApoE−/− and BMPRII+/+ApoE−/− mice, respectively, (n=6).
Supplemental Figure II

(A) Quantification of Amplex-Red assay. HUVECs were treated with 100 nM BMPRII siRNA (BRII.si) or control (Non.si) siRNA. PEG-catalase was used to scavenge ROS generation. (*, p<0.05, n=4) (B and C) BRII.si and non.si transfected ± apocynin-treated cells were lysed for immunoblot with antibodies to BMPRII, and ICAM-1, using β-actin as loading control. (C) Graph shows quantification of Western blot data shown in B; mean ± SEM (n=4, *p< 0.05). (D) Western blot shows expression of nox1 and BMPRII on cell lysates from HUVECs transfected with BRII.si or Non.si. (E) Graph shows quantification of Western blot analysis shown in main Fig. 2E. Data are expressed as mean ± SEM (n=4, *p< 0.05). (F) Western blot show the expression of BMPRII, ICAM1 and VCAM1 on HUVECs transfected with BRII siRNA or non.si and treated with or without NFkB inhibitor, BAY11-7082 (10μM, 24 h).
Supplementary Figure III. BMPRII deficiency exacerbates CD45+ leukocyte infiltration to atherosclerotic plaques in ApoE−/− mice.

BMPRII+/−ApoE−/− and littermate control BMPRII+/+ApoE−/− mice were partially ligated and fed a high-fat diet for 2 weeks. Frozen sections from the ligated LCA (d-flow) and contralateral RCA (s-flow) were stained with CD45 antibody as shown by representative (n=4) microscopy images. Blue= DAPI nuclear staining; Green= elastic laminas; Red= CD45 staining.
Supplemental Figure IV

ApoE<sup>−/−</sup> mice were partially ligated and fed a high-fat diet for 2 or 7 days. Frozen sections of ligated LCA and RCA exposed to d-flow were stained with BMPRII antibody. Representative confocal microscopy images of mouse carotids show loss of BMPRII expression. DAPI staining for nuclei (blue); BMPRII antibody (red) and auto-fluorescence signal for elastic lamina (green). Arrows point to BMPRII expression in the endothelial layer. Images shown on the right are magnified regions indicated by broken boxes (low-magnification).
Supplemental Figure V

(A) Representative immunoblots show the expression of BMPRII in HUVECs treated with 20ng/ml TNF-α for 1 h, 4 h, and 24 h. (B) Immunoblot shows expression of BMPRII in HUVECs treated with simvastatin and Rosuvastatin (μM). (C) qPCR and (D) immunoblot showing the expression of BMPRII in HUVECs treated with mevastatin (10μM). Graph shows quantification of the immunoblot data (*, p<0.05, n=6).
Supplemental Figure VI

(A) HUVECs were treated with BMP4 (50 ng/ml), BMP9 (10 ng/ml), BMP10 (50 ng/ml), BMP4 neutralizing antibody (1 μg/ml), BMP9 neutralizing antibody (10 μg/ml), BMP10 neutralizing antibody (10 μg/ml), or each IgG control. Cells were lysed and analyzed by Western blot with antibodies specific to phospho-SMAD1/5/8 and total SMAD1/5/8. (B, C) HUVECs were treated with BRII siRNA or Non.si. One day later, cells were incubated in complete medium containing neutralizing antibodies specific to BMP9 or BMP10 (10 μg/ml each) or IgG controls for 1 day and analyzed by Western blot using antibodies to BMPRII, ICAM1, VCAM1 or actin. Representative immunoblots (n=3) show that increased expression of ICAM1 and VCAM1 by BRII.si was not affected by either antibodies used. (D, E) HEK293 cells were infected with adenoviral BMPRII WT or BMPRII Short-form for 24 h, Western blots show expression of FLAG and BMPRII. Note that the BMPRII antibody used here was raised against the C-terminal domain and could not detect the short-form.
Supplemental Table I

Serum lipid profile of partially ligated BMPRII+/ApoE−/+ and BMPRII+/ApoE−/− mice fed with a high-fat diet (two weeks).

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Cholesterol (mg/dL)</th>
<th>Triglyceride (mg/dL)</th>
<th>HDL (mg/dL)</th>
<th>LDL (mg/dL)</th>
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<tbody>
<tr>
<td>BMPRII+/ApoE−/− (n=12)</td>
<td>1384.56±229.62</td>
<td>66.56±47.54</td>
<td>13.53±5.20</td>
<td>786.67±130.24</td>
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<td>BMPRII+/ApoE−/− (n=14)</td>
<td>1501.11±232.72</td>
<td>48.79±12.45</td>
<td>17.12±20.66</td>
<td>842.89±187.44</td>
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</table>
### Supplemental Table II. List of q-PCR primers

<table>
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<th>Target Gene</th>
<th>Species</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMPRII</td>
<td>Human</td>
<td>5'-TCTTTTCAGCCACAAATGTCCCT-3'</td>
<td>5'-TGCCATCTTGTGTTGACTCAC-3'</td>
</tr>
<tr>
<td>BMPRII</td>
<td>Mouse</td>
<td>5'-GAGCCCTCCCTTGACCTG-3'</td>
<td>5'-GTATCGACCCCGTCCAATC-3'</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Human</td>
<td>5'-CTTCCTCACCCTGTACTGG-3'</td>
<td>5'-AGCGTGAGGTAAGGTTCTTGC-3'</td>
</tr>
<tr>
<td>ICAM-1</td>
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<td>5'-AGGTGGTTCTTCTGACCGGC-3'</td>
<td>5'-AAACAGGAACTTTTTCCGCCA-3'</td>
</tr>
<tr>
<td>VCAM-1</td>
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<td>5'-TGGACATAAGAAACTGCGAAAGG-3'</td>
<td>5'-CCACTCATCTGATTTCTGGA-3'</td>
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<tr>
<td>VCAM-1</td>
<td>Mouse</td>
<td>5'-TCTTGGGAGCCTAACGGTA-3'</td>
<td>5'-CAAGTGAGGGCCATGGAGTC-3'</td>
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</table>
Materials and Methods

Cell culture and treatment with siRNAs

Human umbilical vein endothelial cells (HUVECs), purchased from Lonza, were cultured and maintained as described previously\textsuperscript{1}. BMPRII siRNA (5'-UGA ACG CAA CCU GUC ACA UAA UAG GCG-3'), ActRIIa siRNA (5'-GGA CUG AUU GUG UAG AAA ATT-3') and ActRIIb siRNA (5'-GGU GUA CUU CUG CUGCUG UTT-3') were custom synthesized from MWG Biotech. Nox1 siRNA (Santa Cruz), Alk1 and Alk2 siRNA (Dharmacon), Alk6 and Alk3 siRNAs (Ambion) and non-silencing control (Invitrogen) were used at 100 nM (unless indicated otherwise) to transfect HUVECs using Oligofectamine (Invitrogen) in serum-free media for 5h as described previously\textsuperscript{1}. Knockdown efficiency of siRNAs was confirmed by qPCR and Western blot analysis.

Neutralizing BMP9 and 10 antibodies

HUVECs transfected with BMPRII siRNA (BRII.si) or non-silencing siRNA (Non.si) for 5h were incubated in complete medium containing 10% fetal bovine serum and neutralizing antibodies for BMP9 (10 \( \mu \)g/ml), BMP10 (10 \( \mu \)g/ml) (R&D Biosystems) or control IgG as described previously\textsuperscript{2}.

BMPRII overexpression

HUVECs were treated with or without BMPRII siRNA for 5h. Cells were further infected with adenoviral constructs encoding for BMPRII wild-type (WT) or BMPRII short form (short) lacking the c-terminal tail region (provided by Dr. Akiko Hata), for 1 day in complete medium as previously described\textsuperscript{3}. Expression of BMPRII and endothelial inflammation was determined by Western blot analysis using antibodies to BMPRII, intercellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule (VCAM-1).

Human Coronary Arterial Samples

Coronary arteries were obtained from patients undergoing heart transplant in an Institutional Review Board approved study at Emory University with written informed consent. Frozen sections were prepared and were processed from immunohistochemical staining. Atherosclerotic lesions were classified according to the American Heart Association classification\textsuperscript{4,5}.

BMPRII heterozygotic null mice

BMPRII\textsuperscript{+/-} mice on C57BL/6 background\textsuperscript{6}, provided by Dr. Hideyuki Beppu, were crossed with ApoE\textsuperscript{-/-} mice to generate BMPRII\textsuperscript{+/-}ApoE\textsuperscript{-/-} mice. All experiments were performed with littermate BMPRII\textsuperscript{+/-}ApoE\textsuperscript{-/-} as wild-type controls.

Acute atherosclerosis model (Partial carotid ligation)

All animal studies were performed using 8-week-old male BMPRII\textsuperscript{+/-}ApoE\textsuperscript{-/-} and littermate BMPRII\textsuperscript{+/-}ApoE\textsuperscript{-/-} mice as control. All procedures were carried out according to the approved Institutional Animal Care and Use Committee protocol for this study by Emory University. Partial ligation of LCA was carried out under anesthesia as previously described\textsuperscript{7} to induce low and disturbed flow in the LCA. Following carotid ligation, \( d \)-flow in LCA and \( s \)-flow in RCA was confirmed at one day post-ligation by ultrasonography using the Vevo770 system (Visualsonics, Canada)\textsuperscript{7,8}. RCA and LCA were isolated at indicated experimental end-points.
**Chronic atherosclerosis model (High-fat diet fed model)**

BMPRII<sup>+/−</sup>ApoE<sup>−/−</sup> mice and BMPRII<sup>+/+</sup>ApoE<sup>−/−</sup> littermate controls were used as indicated and maintained for 2 months on the Paigen’s high-fat diet (HFD; Science Diets) containing 1.25% cholesterol, 15% fat, and 0.5% cholic acid<sup>9,10</sup>. Mice were provided water ad libitum. Explanted aortic tree were processed for subsequent plaque analysis and immunohistochemical analysis.

**Quantitative real-time PCR (qPCR)**

Total RNA was polyadenylated and reverse transcribed for use in a two-step qPCR setup using High-capacity cDNA synthesis kit (ABI) and using Brilliant II SYBR Green QPCR Master Mix (Stratagene) with custom designed primers on a Real-Time PCR System (StepOne Plus, Applied Biosystems)<sup>8</sup>. Fold changes between LCA and RCA were determined for all targets using the ∆∆Ct method<sup>11</sup>. Sequences for primers used for mRNA expression studies have been listed in Supplementary Table 2.

**Western blotting**

Following treatments, cell lysates were prepared and analyzed by Western blot analysis as described previously<sup>12,13</sup>. The membranes were probed with antibodies to Alk2 (1:1000, R&D), Alk3 (1:500, Santa Cruz), Alk5 (1:1000, Santa Cruz), BMPRII (1:500, BD), ICAM-1 (1:1000, Santa Cruz), VCAM-1 (1:1000, Santa Cruz), Nox-1 (1:2000), FLAG (1:3000) or β-actin (1:1000, Sigma), total SMAD and phospho-SMAD antibodies (1:1000) (Cell Signaling) and appropriate secondary antibodies conjugated to alkaline phosphatase, which were detected by a chemiluminescence method.

**Immunofluorescence staining**

Frozen sections of human coronary arteries were fixed in ice-cold acetone for 5 min, blocked for 1 hour with 10% donkey or goat serum, and incubated with primary antibodies overnight at 4°C, followed by Rhodamine-conjugated secondary antibodies for 2 h at room temperature as described<sup>12</sup>. Nuclei were counter-stained with DAPI (Sigma). Primary antibodies used were specific for Alk2 (1:50, R&D systems), BMPRII (1:50, Santa Cruz), PECAM-1 (1:50, BD), CD45 (1:100, eBioscience), ICAM-1 (1:100, Santa Cruz) and VCAM-1 (1:100, BD). Twenty different human coronary artery sections, containing various stages of atherosclerosis from minimally diseased to advanced atheroma stages, from nine different patients, were examined. Images were taken using a Zeiss epi-fluorescence microscope. The semi-quantification method was used based on the blinded grading for atheroma intensity and BMPRII staining intensity in endothelium using one to five scale (1: minimally diseased, low staining intensity, 5: advanced atheroma, high staining intensity). Similar methodology was used for mouse thoracic aorta as described above in this section was used for ICAM-1 and VCAM-1 staining.

**Monocyte binding assay**

Following BMPRII siRNA transfection for two days and BMP4 treatment for 4h in HUVECs, monocyte binding assay was performed as described previously<sup>12</sup>. In some studies of HUVECs, apocynin (Calbiochem) or an NFκB inhibitor, BAY 11-7082 (Sigma) were treated for two days after 5h of transfection of BMPRII or non-silencing siRNA.

**Amplex-Red assay for measuring hydrogen peroxide**

Extracellular H<sub>2</sub>O<sub>2</sub> was measured using a horseradish peroxidase-linked Amplex Red fluorescence assay. Briefly, after cells were transfected with non-silencing or BMPRII siRNA,
media were washed twice with Krebs Ringer Phosphate (KRP) buffer and incubated with 5 µM Amplex UltraRed (Molecular Probes) and 0.1 U/ml horseradish peroxidase type II (Sigma-Aldrich) in KRP for 40 minutes. Triplicate reading were taken in a 96-well plate using 100 µl samples of media, and fluorescence was detected via plate reader at excitation and emission of 530 nm and 580 nm, respectively. Hydrogen peroxide levels were normalized to cellular protein as measured by the Bio-Rad DC assay.

**Serum Lipid Analysis**

Serum lipid analysis from the mouse samples was performed by Cardiovascular Specialty Laboratories, (Atlanta, GA). All lipid determinations were performed using a Beckman CX7 biochemical analyzer and reagents from Beckman Diagnostics (Fullerton, CA) for total cholesterol, triglycerides, HDL and LDL.

**Atherosclerotic lesion analysis**

To examine atherosclerosis development in the carotid artery, aorta and carotid arteries were isolated en bloc as described above. Aortic trees, RCA and LCA were photographed using a CCD camera (Moticam 2500, Motic) attached to a dissection microscope at 3x magnification and the opaque area covered by plaque and total artery area of LCA were measured using NIH ImageJ software.

**Oil-red-O staining**

Oil-red-O staining was carried out using frozen sections as described.

**Dihydroethidine (DHE) staining for measuring superoxide generation**

The evaluation of basal ROS production in BMPRII−/−ApoE−/− and BMPRII+/−ApoE−/− were performed as described before. DHE hydrochloride (5 µM, Molecular Probes) was applied to the freshly cut frozen aortic sections (10 µm) for 30 min at 37°C to reveal the presence of ROS as red fluorescence (585 nm) by Zeiss (Jena, Germany) fluorescence microscope.

**Statistical Analysis**

Values are expressed as means±SEM unless otherwise indicated. Pairwise comparisons were performed using one-way or two-way Student T-tests. Multiple comparisons of means were performed using 1-way ANOVA followed by Tukey’s Multiple Comparison tests. Differences between groups were considered significant at P values below 0.05.

**References:**

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10. Ishida BY, Blanche PJ, Nichols AV, Yashar M, Paigen B. Effects of atherogenic diet consumption on lipoproteins in mouse strains c57bl/6 and c3h. *Journal of lipid research*. 1991;32:559-568


