

Forum Review Article

Hemodynamic Forces, Vascular Oxidative Stress, and Regulation of BMP-2/4 Expression

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Abstract

Changes in the hemodynamic environment (*e.g.*, hypertension, disturbed-flow conditions) are known to promote atherogenesis by inducing proinflammatory phenotypic alterations in endothelial and smooth muscle cells; however, the mechanisms underlying mechanosensitive induction of inflammatory gene expression are not completely understood. Bone morphogenetic protein-2 and -4 (BMP-2/4) are TGF- β superfamily cytokines that are expressed by both endothelial and smooth muscle cells and regulate a number of cellular processes involved in atherogenesis, including vascular calcification and endothelial activation. This review considers how hemodynamic forces regulate BMP-2/4 expression and explores the role of mechanosensitive generation of reactive oxygen species by NAD(P)H oxidases in the control of BMP signaling. *Antioxid. Redox Signal.* 11, 1683–1697.

Introduction

IN THE PAST DECADES, it has been established that vascular inflammation and atherosclerosis develop in hemodynamically well-defined regions. The facilitating role of pressure/wall tension in this context is undeniable: despite uniform levels of circulating proatherogenic factors (LDL, homocysteine, cigarette smoke constituents, *etc.*), atherosclerosis is limited to arteries characterized by high pressure/wall tension, whereas it never develops in low-pressure vascular beds (pulmonary circulation, arterioles, or veins). Moreover, hypertension has been recognized as an independent risk factor for atherosclerosis. In the late 1960s, Caro *et al.* (11) also called attention to the association of low-shear-stress areas with early atherosclerotic lesion localization in human arteries. This led to the hypothesis that low time-averaged wall shear stresses, such as found at atherosclerosis-prone vascular curvatures and branch points, might promote the localized attachment and infiltration of platelets and monocytes as well as atherogenic lipoproteins into the arterial wall. This original working concept invoked rheologic factors *per se* as the critical determinant of vulnerability of the vessel wall.

During the last two decades, major refinements have emerged in our understanding of how hemodynamic forces regulate vascular homeostasis. It has become established that changes in hemodynamic forces acting on both endothelial and smooth muscle cells activate cellular signaling pathways, translating biomechanical stimuli into biologic responses. During normal vascular homeostasis, laminar shear stress maintains an antiinflammatory, antiatherogenic phenotype of endothelial cells. In contrast, adverse changes in the hemodynamic environment, in particular a combination of low shear stress and high pressure, elicit proinflammatory phenotypic changes favoring atherogenesis (including endothelial activation, proinflammatory cytokine expression, smooth muscle hypertrophy, hyperplasia, migration and differentiation, and extracellular matrix reorganization).

The mechanisms by which disturbed flow conditions and high pressure act as potent proatherogenic forces have been the subject of intense studies in this field (74, 75). This review focuses on the emerging evidence that reactive oxygen species (ROS) play a central role in vascular mechanotransduction and discusses the potential link between alterations in hemodynamic environment, expression of bone morphogenetic proteins, and vascular inflammation.

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Activation of NAD(P)H Oxidases in Endothelial and Smooth Muscle Cells by Hemodynamic Forces

Potential vascular sources of ROS include NAD(P)H oxidase, nitric oxide synthase (NOS), xanthine oxidase, cytochrome P450, cyclooxygenase, and mitochondria. In recent years, increasing evidence has accumulated that production of ROS, particularly O_2^- and H_2O_2 , through activation of vascular NAD(P)H oxidases, plays a central role in vascular mechanotransduction. The activities of other oxidases in the vessel wall appear less likely to influence signaling systems under baseline physiologic conditions, although some of them may be also upregulated by adverse hemodynamic conditions (84). Endothelial and smooth muscle cells express different NAD(P)H oxidases that consist of multiple oxidase and regulatory subunits. In phagocytic cells, the gp91^{phox} (Nox-2) oxidase subunit has been reported to be activated by stimulation of the assembly of p47^{phox}, p67^{phox}, and p40^{phox} regulatory subunits and activation of the small G protein rac, which bind to the cell membrane-bound NOX-2-p22^{phox} complex. Many of the NAD(P)H oxidase subunits expressed in neutrophils (p22^{phox}, p47^{phox}, p67^{phox}, NOX-2, and Rac) are expressed in the vascular tissue. However, the vascular and neutrophil NAD(P)H oxidases differ: whereas the neutrophil oxidase releases large amounts of O_2^- outside the cell in bursts, the vascular counterparts continuously produce low levels of O_2^- in the intracellular compartment. In vascular endothelial and smooth muscle cells, both Nox-2 and its homologues Nox-1 and Nox-4 subunits are expressed (37, 40, 127). Furthermore, vascular NAD(P)H oxidase activity is supported by both NADH and NADPH (37, 40, 89).

Several lines of evidence suggest that hemodynamic forces can either directly or indirectly activate vascular NAD(P)H oxidase-derived ROS production (74, 75). Ample indications suggest that hypertension is associated with an increased activity of vascular NAD(P)H oxidases [reviewed in references (9, 68, 130)]. Although earlier studies attributed this largely to circulating neurohumoral factors, such as angiotensin II (35, 91, 103), by now it has become evident that oxidative stress is present in virtually all forms of hypertension (4, 103), including low-renin hypertension (69, 112), despite differences in plasma levels of circulating vasoactive factors [for a detailed discussion of the topic, please see references (68, 127, 128)].

A primary role for high intraluminal pressure itself in the promotion of vascular NAD(P)H oxidase-dependent O_2^- generation is supported by the observations that in rats with aortic banding, increases in vascular O_2^- production are observed exclusively in blood vessels proximal to the coarctation that are exposed to high pressure, but not in distal normotensive vascular beds, despite the presence of the same circulating factors throughout the vascular tree (128). Direct evidence for high pressure-induced, NAD(P)H oxidase-dependent oxidative stress has come from studies showing that exposure of isolated arteries to high pressure in an organoid culture system elicited significant O_2^- and H_2O_2 (Fig. 1) production (76, 127) and endothelial dysfunction (55). Short-term increases in pressure *in vivo* also impair endothelial function (23, 36, 67, 139) by activating ROS-dependent mechanisms (67). It is likely that cellular stretch due to increased wall tension is the primary mechanical stimulus for NAD(P)H oxidase activation, because exposure of isolated

arterial rings to *in vitro* stretching also activates vascular NAD(P)H oxidase-dependent O_2^- generation (97), mimicking the effects of high pressure. Moreover, increased production of ROS has been detected in cultured endothelial and smooth muscle cells subjected to *in vitro* stretching (39, 50, 51). Importantly, aging appears to potentiate high pressure-induced Nox activation and O_2^- production in peripheral arteries (61). In whole vessels, mechanically induced ROS comprise not only O_2^- but also H_2O_2 (21, 96), indicating that a significant portion of NAD(P)H oxidase-derived O_2^- is likely dismutated by superoxide dismutase (SOD) isoforms, which vascular cells express abundantly.

The underlying mechanisms by which high pressure/wall tension-related cell stretch elicits NAD(P)H oxidase activation probably involve increases in $[Ca^{2+}]_i$ and phosphorylation and activation of PKC α (127). PKC-dependent serine phosphorylation of the regulatory p47^{phox} subunit (126) results in its translocation from the cytosol to the membrane oxidase subunits (97), activating NAD(P)H oxidase function. The long-term presence of high pressure may also affect PKC expression in some vascular beds (98). It is of note that the activation of Nox-1 and Nox-4 in response to hemodynamic forces may differ. Nox-4 does not seem to be regulated by PKC phosphorylation of p47^{phox}, and Nox-1 and Nox-4 are likely to be expressed in different cellular compartments (41, 49) [reviewed recently in reference (130)].

In addition to their role in pressure-induced signal transduction, ROS are likely to participate in the mechanotransduction of other modalities of blood flow, such as pulsatility and oscillatory shear stress. Previous studies have shown that pulsatile stretch increases O_2^- production in human coronary artery smooth muscle cells (50) and in cultured rabbit aortas (115). Moreover, in porcine coronary arterioles exposed to pulsatile flow, the bioavailability of NO seems to be decreased as a result of increased O_2^- generation (115). Disturbed flow conditions also were shown to increase endothelial ROS production (53, 72, 120), likely *via* stimulation of NAD(P)H oxidases (13, 58). Oscillatory shear stress is a particularly potent stimulus of O_2^- production in cultured endothelial cells (24, 44, 58, 84).

Many existing hypotheses seek to explain how increased production of ROS affects vascular function and phenotype. According to the free radical theory of aging (43), chronic oxidative stress leads to the accumulation of oxidative damage to cellular constituents, resulting in a progressive decline in cellular function and ultimately to cellular senescence. Other laboratories emphasize the potential vasoactive effects of ROS, including degradation of vasodilator NO by O_2^- (10) or vasomotor effects elicited by H_2O_2 or both (17, 81, 88, 142). In addition to eliciting macromolecular damage and exerting direct vasoactive effects, ROS can activate signaling pathways involved in vascular inflammation and atherogenesis. Importantly, accumulating evidence from experimental and clinical studies indicates that NAD(P)H oxidase activation plays a central role in atherogenesis and other pathophysiologic conditions, in part by regulating cell proliferation and inflammatory gene expression. This review discusses available data on the link between hemodynamic forces, NAD(P)H oxidase activation, and regulation of a novel class of proinflammatory cytokines that may play an important pathophysiologic role in atherogenesis.

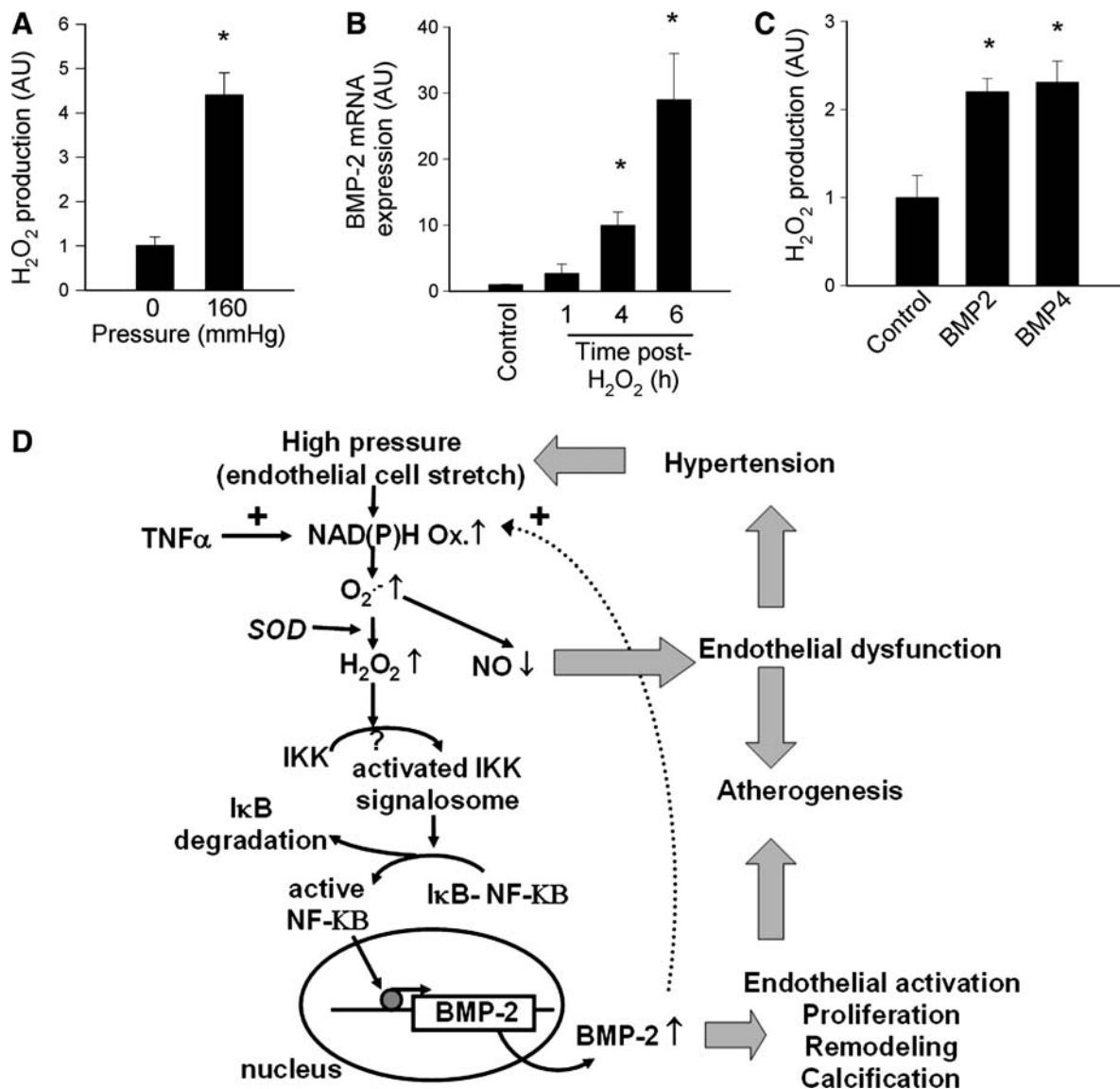


FIG. 1. Mapping the relation between pressure, ROS production, and BMPs in the context of hypertensive vascular remodeling. (A) Exposure of isolated rat femoral arteries to high pressure (160 mm Hg) *in vitro* significantly increases vascular H₂O₂ generation (measured with the homovanillic acid/horseradish peroxidase method). Data are expressed as mean \pm SEM. $n = 4-5$ for each group; * $p < 0.05$ vs. control, # $p < 0.05$ vs. high-pressure treated). (B) Time course of H₂O₂ (10⁻⁴ M)-induced BMP-2 mRNA expression in CAECs (real-time QRT-PCR). Data are expressed as mean \pm SEM ($n = 4$ for each time point). Figures were redrawn based on data presented in reference (21). (C) Treatment of CAECs (24 h) with BMP-2 (10 ng/ml) and BMP-4 (10 ng/ml) significantly increases H₂O₂ production [measured with the DCF fluorescence method (129)]. (D) Proposed scheme for a common redox-sensitive pathway leading to activation and nuclear translocation of NF- κ B in endothelial cells induced by an increased H₂O₂ production due to the activation of the NAD(P)H oxidase by inflammatory cytokines and high pressure. Binding of NF- κ B to its target sequences promotes the expression of BMP-2, which induces proatherogenic phenotypic changes in endothelial cells.

Role of Bone Morphogenic Proteins in Vascular Physiology and Pathophysiology

Bone morphogenetic proteins (BMPs) are members of the TGF- β superfamily. The first hint of the existence of BMPs came from the early clinical observations that bone formed in transplanted tissues. Subsequently it was discovered that a number of tissues possess various "osteogenic proteins," or BMPs, that could induce new bone formation (132). After the purification and sequencing of the first BMPs in the late 1980s

(82, 143), identification of the BMP family grew rapidly, and today it is known to comprise >30 cytokines, based on sequence homology. Although the name BMP is descriptive of one particular function, inducing ectopic bone or cartilage formation, it is quite misleading; BMPs play important roles in diverse cell types. Vascular endothelial and smooth muscle cells both express BMP receptors and secrete BMPs (28, 110, 113, 114, 140). Among them, BMP-2 and BMP-4 (which are closely related by their amino acid sequence and act on the same receptor) have been shown to regulate a host of cellular

functions (110, 114, 140, 153), including cardiovascular development (153), angiogenesis (1, 31, 47), neovascularization in tumors (71), vascular calcification (56, 108–110, 125, 137, 151, 152), and smooth muscle cell chemotaxis in response to vascular injury (140).

Role of BMPs in vascular inflammation and atherogenesis: role of Nox oxidases

Recent studies reported a striking upregulation of BMP-2/4 in atheroprone regions and atherosclerotic lesions (28, 113, 114, 118, 140). With *in vitro* and *ex vivo* approaches, BMP-2/4 also were shown to exert proinflammatory effects by inducing expression of adhesion molecules and enhancing monocyte adhesion (18, 113, 114). Prolonged BMP-4 infusion in C57Bl6 and apolipoprotein-null mice impairs endothelium-dependent vasodilation and induces arterial hypertension in an NAD(P)H oxidase-dependent manner (87). Activation of BMP signaling either by overexpression of the BMP-2/4 in vascular cells or by administration of recombinant BMPs results in endothelial dysfunction, oxidative stress (by activating NAD(P)H oxidases), and an enhanced monocyte adhesiveness to the endothelium (18, 21, 113, 114). Vascular BMP signaling also has been linked to vascular calcification during atherogenesis (28, 52, 108, 110) and calciphylaxis (38), as well as to calcific stenosis of the aortic valves (90, 117, 145).

Aging is known to increase the risk for atherosclerosis by promoting vascular inflammation and upregulating Nox-oxidase-derived ROS production (22). It has been proposed that alterations in the vascular expression/activity of BMP antagonists may lead to unopposed BMP-2 activity in aging (118), which may contribute to age-related oxidative stress and promote arterial calcification. BMP-2/4 is thought to signal primarily by activating the mothers against decapentaplegic (Smad) and mitogen-activated protein kinase (MAPK) pathways, although evidence suggests that BMP-2/4 may also activate NF- κ B (18, 114). In that regard, it is important that aging is known to increase basal NF- κ B activity in the vasculature, which may sensitize endothelial cells to the proinflammatory effects of BMPs (22). Future studies should elucidate the role of BMP-receptor subtypes and the interaction between downstream signaling mechanisms induced by BMPs, which mediate their proinflammatory effects.

Many lines of evidence thus suggest that BMPs may function as proinflammatory, prohypertensive, and proatherogenic mediators in the vessel wall. So far, our understanding of the factors that regulate vascular BMP-2/4 expression supports this premise. For example, we have shown that in coronary arterial endothelial cells, expression of BMP-2 is upregulated by proinflammatory stimuli, such as TNF- α and H₂O₂, by activation of NF- κ B (18, 21). Accordingly, in hyperhomocysteinemia, coronary artery inflammation and upregulation of TNF- α is associated with an increased BMP-2 expression (126). Type 2 diabetes also is associated with induction of BMP-2 expression and activation of BMP-2-dependent Msx2-Wnt programs in the aorta that contribute to aortic calcium accumulation (2). Importantly, aortic BMP-2 expression could be attenuated by treatment with the TNF- α -neutralizing antibody infliximab (2). Because of the central role of hemodynamic forces in atherogenesis, our review focuses on the role of shear stress and pressure/wall tension in regulation of vascular BMP-2/4 expression.

Role of BMPs in vascular development, angiogenesis, and stem cell commitment

Convincing data show that BMPs are involved in embryonic and adult blood vessel formation in health and disease (25, 47, 71, 93, 94, 105, 154). In particular, the involvement of the BMP-regulated Id family of helix-loop-helix transcription factors in angiogenesis has been investigated extensively. Nevertheless, some controversy exists regarding differences between BMP-2 and BMP-4 on vascular cell proliferation and the different roles that these BMPs may play in endothelial and vascular smooth muscle cell physiology. Endothelial progenitor cells express BMP-2/4 and BMP receptors (111). However, published data suggest that BMP-2 enhances commitment of mesodermal cells to a cardiomyogenic fate (65, 80), whereas BMP-4 favors endothelial commitment of embryonic stem cells (138). Such BMP-dependent signaling pathways involved in embryonic development can be reactivated in adults and may thus contribute to the reparative effects of progenitor cell therapy after myocardial infarction and critical leg ischemia.

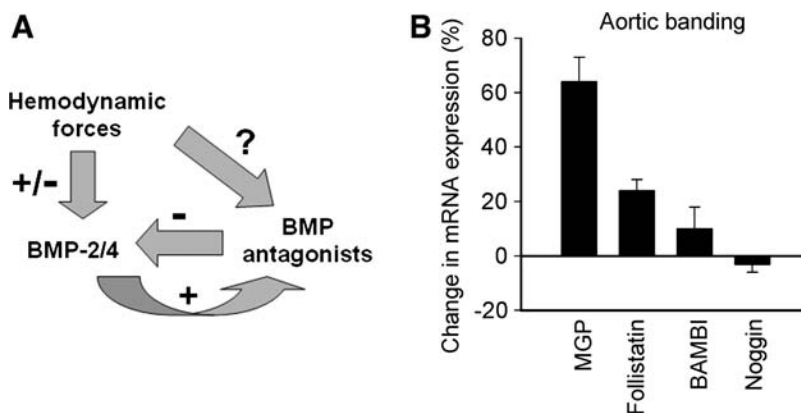
Conversely, the role of BMPs in NAD(P)H oxidase activation during angiogenesis and inflammatory gene expression in endothelial progenitor cells is poorly understood. Endothelial progenitor cells exhibit notable antioxidant defenses (27, 46), yet under conditions of increased oxidative stress, their function seems to be compromised (12, 59). In particular, increased NAD(P)H oxidase activation has been linked to loss of endothelial progenitor cell function and impaired postischemic neovascularization in diabetes (29) and in hypertension (150). It is thought that reduction of stem cell number or activity can contribute to cardiovascular aging and impaired angiogenesis in aged mammals, and considerable evidence indicates that increased production of ROS, in part due to the upregulation of NAD(P)H oxidases, underlies cellular dysfunction in the aged cardiovascular system [reviewed recently elsewhere (22)]. Further studies are evidently needed the better to elucidate the link between BMPs and NAD(P)H oxidase activation in endothelial progenitor cells under these pathophysiologic conditions.

Differential prooxidant and prohypertensive effects of BMPs in the pulmonary and systemic circulation

Genetic analysis of patients with primary pulmonary hypertension indicates that BMP signaling plays an important protective role in the pulmonary circulation. Studies in the last decade have shown that multiple loss-of-function mutations affecting BMP receptors (in both *bmpr2* and *alk1* genes) (26, 57, 70, 83, 123, 124) lead to monoclonal endothelial cell growth in plexiform lesions in patients with idiopathic pulmonary arterial hypertension (73, 134). Although it is now clear that mutations in the BMP receptors can predispose to pulmonary hypertension, why such mutations in the germline (*i.e.*, passed along to all somatic cells from the germinative cells) predispose only the pulmonary arteries but not systemic arteries to hypertensive disease has yet to be resolved.

It is believed that in pulmonary arteries, BMP signaling exerts important vasoprotective effects controlling the balance between proliferation and activation of apoptosis in endothelial and smooth muscle cells (31, 32, 42, 101, 102, 122). Importantly, recent experimental studies demonstrated that

FIG. 2. Interactions between BMPs and their antagonists in hypertension. (A) Proposed scheme showing that hemodynamic forces may regulate the expression of both BMP-2/4 and BMP antagonists in vascular cells, which together will determine BMP activity in the vasculature. (B) High pressure may alter the expression of BMP-binding proteins in coronary arteries of aortic banded rats ($n=3$). Data are expressed as relative changes in mRNA expression, as compared with vessels of sham-operated rats ($n=5$).



the expression and function of the BMP/Smad signaling axis is perturbed in secondary pulmonary hypertension as well (92). Thus, altered BMP signaling may contribute to the pathogenesis of both primary and secondary forms of pulmonary hypertension. In contrast, BMP-2 and BMP-4 function as prooxidant and prohypertensive mediators in systemic arteries (18, 64, 87, 113, 131), suggesting that BMP signaling plays different roles in the pulmonary and systemic circulations. Indeed, recent findings demonstrate that whereas BMP-4 elicits significant endothelial dysfunction and NAD(P)H oxidase activation in the systemic arteries, activating NF- κ B and increasing monocyte adhesiveness, the pulmonary arteries are completely protected from such adverse functional effects (19). We attribute the lack of BMP-4-induced endothelial activation in pulmonary arterial endothelial cells to the superior resistance of these cells to the prooxidant effects of BMP-4 rather than to a lower expression of NAD(P)H oxidase in the pulmonary vasculature (19). Importantly, pulmonary arteries are known to be resistant to atherosclerosis. Moreover, it is likely that differential effects of BMP-4 on NAD(P)H ox-

idase activity are responsible for the selective systemic (but not pulmonary) hypertension induced by high circulating levels of BMP-4 (87). Previous findings showed that in endothelial cells from the systemic circulation, BMPs activate NAD(P)H oxidase *via* a pathway that involves PKC (18), and evidence exists of association of the BMP-receptor complex with PKC (45). Hence, the lack of BMP-4-induced NAD(P)H oxidase activation in pulmonary arterial endothelial cells could be due to the differential role of PKC in BMP signaling in pulmonary and systemic arteries. It is also possible that subsets of BMP receptors and modulators of BMP signaling (BMP antagonists, co-receptors, *etc.*) are expressed differentially in systemic and pulmonary endothelial cells, which would activate discrete signaling pathways (19). Further studies are needed to determine which receptor is responsible for NAD(P)H oxidase activation in endothelial cells from systemic arteries and whether the divergence in BMP-induced ROS generation between systemic and pulmonary cells can be explained by the differential expression of BMP receptors and/or their downstream targets.

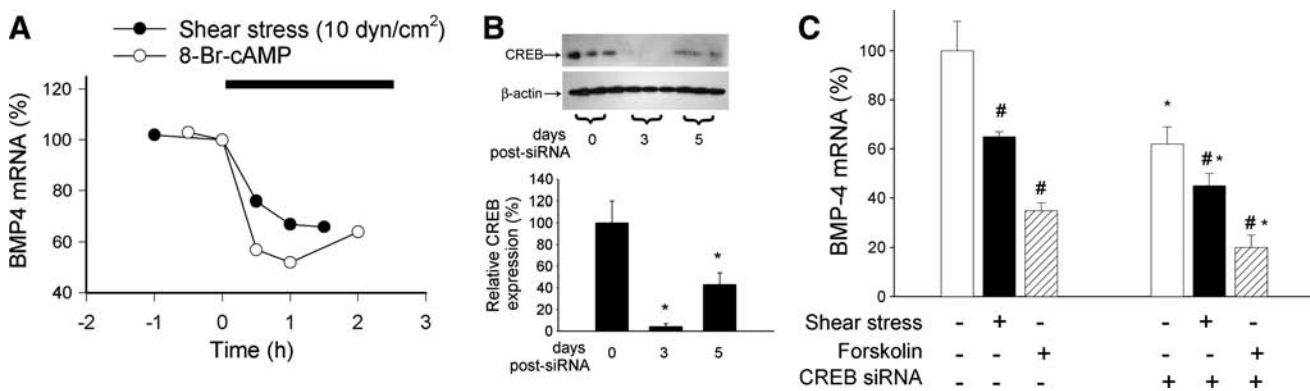
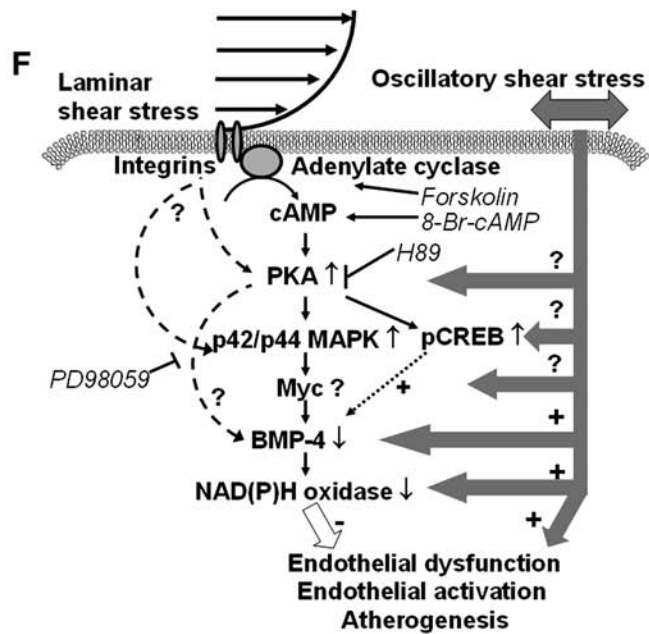
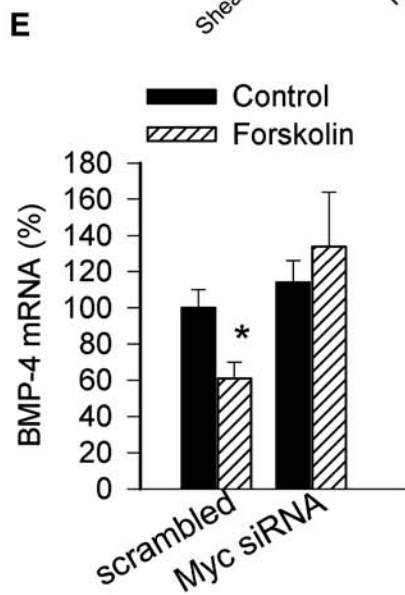
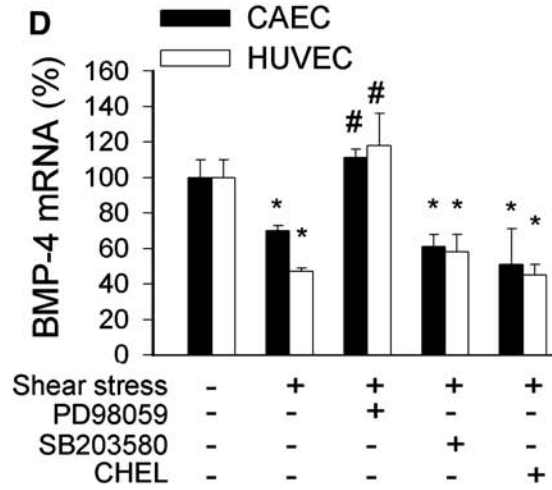
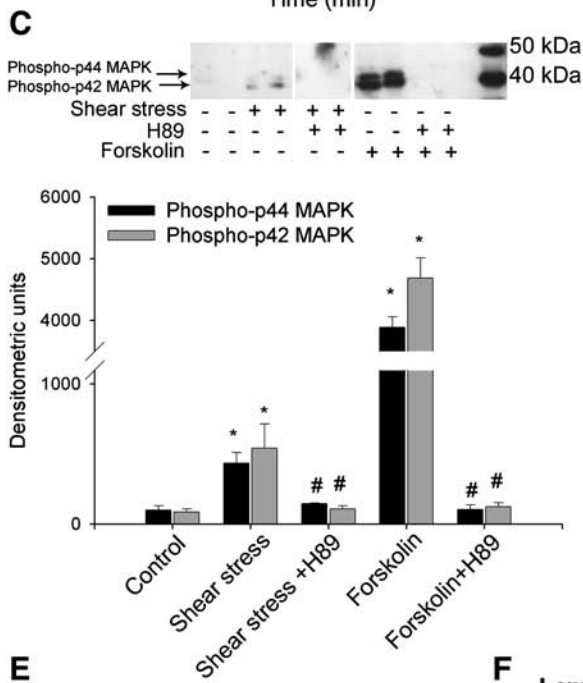
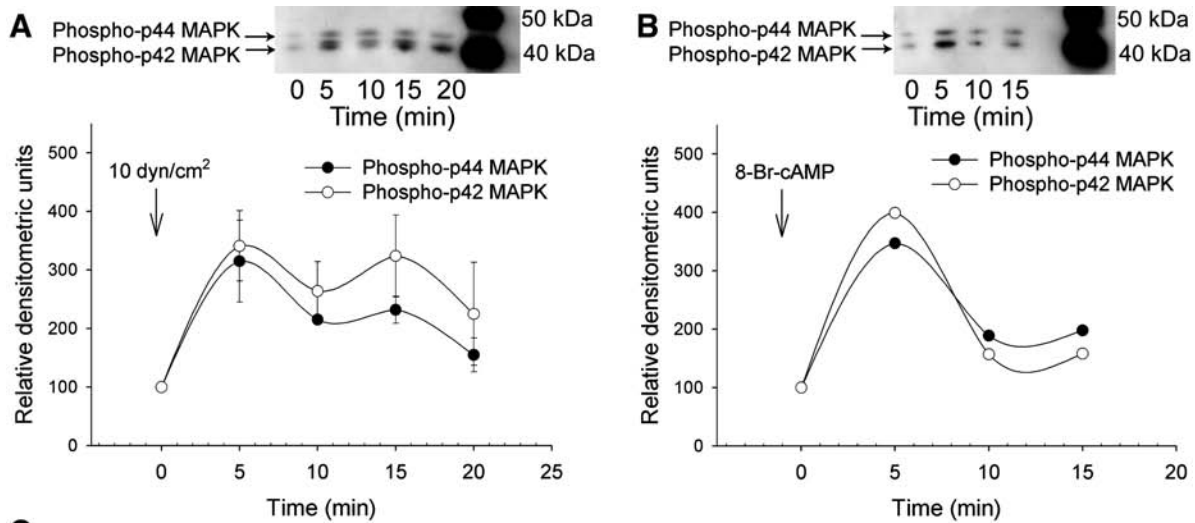


FIG. 3. Downregulation of BMPs by shear stress is independent of CREB. (A) Time course of BMP-4 expression downregulation in HCAECs in response to shear stress (10 dyn/cm²) or a cell-permeable cAMP analogue (0.3 mM 8-Br-cAMP). Analysis of mRNA expression was performed with real-time QRT-PCR. Figure is redrawn based on data from reference (129). (B) Representative Western blot and densitometric data showing the downregulation of CREB in HCAECs [achieved with RNA interference by using proprietary siRNA sequences and the Amaxa Nucleofector Technology; Amaxa, Gaithersburg, MD, as we previously reported (18, 21)] as a function of time after transfection. Data are expressed as mean \pm SEM ($n=3$ for each data point). * $p < 0.05$. (C) Knockdown of CREB did not prevent shear stress-induced (10 dyn/cm², for 2 h) and forskolin-induced (2 h) changes in BMP-4 expression in HCAECs (on day 3 after transfection). Analysis of mRNA expression was performed with real-time QRT-PCR. β -actin was used for normalization. Data are expressed as mean \pm SEM ($n=4$ for each group). * $p < 0.05$ vs. no siRNA, # $p < 0.05$ vs. no shear stress/no forskolin.



Regulation of BMP-2/4 Expression by Pressure/Wall Tension

Regulation of vascular BMP expression by oxidative stress and inflammatory stimuli prompted the speculation that proatherogenic hemodynamic forces that increase endothelial ROS production may also regulate BMP-2/4 expression. We demonstrated that increased wall tension/high intraluminal pressure is sufficient to induce BMP-2 expression *in vitro*, in the absence of vasoactive circulating factors (21). This finding has been confirmed by the observations that BMP-2 is upregulated in forelimb arteries of aortic banded rats, which are exposed to high pressure and exhibit an increased ROS production (128), whereas normotensive arteries located downstream from the coarctation (but exposed to the same circulating factors) exhibit a normal phenotype (21).

Strong evidence indicates that NF- κ B plays a central role in the regulation of endothelial BMP-2 expression. Increased DNA-binding activity of NF- κ B was shown to increase in *in vivo* models of hypertension (3) and in cultured endothelial and smooth muscle cells exposed to cyclic stretch (15, 51). Furthermore, NF- κ B binding site(s) are present in the promoter region of the rat BMP-2 gene and in the 5' flanking region of the BMP-2 gene in mouse chondrocytes (34). The NF- κ B binding site is localized in an evolutionary highly conserved region of the BMP-2 promoter (21), which explains the similar NF- κ B-dependent regulation of BMP-2 expression in human and rat endothelial cells (18, 21). However, the promoter regions of the human and mouse BMP-4 genes differ from their BMP-2 counterparts because they lack an NF- κ B binding site (48). This could account for the preferential regulation BMP-2 by TNF- α and H₂O₂ (18, 21). Hence, TNF- α is known to induce BMP-2 in endothelial cells through NAD(P)H oxidase-derived H₂O₂ production (18). Previous studies by our laboratories and others also demonstrated that high pressure similarly activates NAD(P)H oxidase, increasing vascular O₂⁻ and H₂O₂ production that leads to NF- κ B activation (21, 78, 79) and BMP-2 overexpression (21). It seems that high pressure/cell stretch elicits rapid degradation of the redox-sensitive NF- κ B inhibitor I κ B α (21, 78), which unmasks the nuclear localization sequence on NF- κ B, allowing its nuclear translocation and transcriptional activity. A consequence of pressure-dependent upregulation of BMPs in the vascular wall, downstream of NAD(P)H oxidase and NF- κ B activation, was shown to be endothelial activation (113, 114).

Correspondingly, a recent study demonstrated that prolonged exposure of cultured arteries to high pressure results in enhanced monocyte adhesion to the endothelium (104). Thus, it is likely that pressure-induced upregulation of BMP-2 and other related cytokines (79) will contribute to/enhance endothelial activation in hypertension and atherosclerosis.

Interestingly, pressure- or stretch-induced increased expression of the BMP-related cytokine TGF- β has been documented in blood vessels (119), and mechanosensitive expression of BMPs has been shown previously in bone (86, 95) and chondrocytes (144). Nevertheless, we recently found that TNF- α did not increase BMP-2 expression in vascular smooth muscle cells (18). Because TNF- α elicits comparable NF- κ B activation in endothelial and smooth muscle cells (18), we speculate that differences in factors that lie downstream from NF- κ B (*e.g.*, differential expression of cell-specific coactivators) may be responsible for this phenomenon. It is of note that VEGF was likewise shown to upregulate BMP-2 in cultured human dermal microvascular endothelial cells, but not in lymphatic endothelial cells (133). Although this finding raised the possibility that regulation of BMP-2 expression may differ between endothelial cells from different organs, we found that basal BMP-2 expression was comparable in vessels from various systemic vascular beds (18), and that TNF- α elicited similar BMP-2 induction in human umbilical vein endothelial cells and coronary arterial endothelial cells (18, 21). Hence, BMP-2 induction by mechanical or inflammatory factors may be more complex than initially anticipated.

In various tissues, the actions of BMPs can be antagonized by proteins that block BMP signal transduction at multiple levels, such as pseudoreceptors, inhibitory intracellular binding proteins, and factors that induce BMP ubiquitination. A large number of extracellular proteins that bind BMPs and prevent their association with signaling receptors have emerged, including noggin, chordin, BAMBI (BMP and activin membrane-bound inhibitor), matrix Gla protein (MGP), and follistatin. Many of these BMP-binding proteins can be expressed in vascular tissues (6, 14, 16, 54, 60, 106, 107, 116, 146–149), although their roles in the regulation of vascular BMP signaling is not yet completely understood (131). For example, BMPER (bone morphogenetic protein-binding endothelial cell precursor-derived regulator) is a secreted protein that directly interacts with BMP-2 and BMP-4 and modulates BMP-2/4-induced signaling pathways in endothelial cells (47, 93, 154). MGP is thought to prevent vascular

FIG. 4. Downregulation of BMPs by shear stress implicates ERK1/2 and Myc. (A, B) Representative Western blots and densitometric data (*below*) showing the time course of p42/44 MAP kinase phosphorylation in shear stress-exposed (10 dyn/cm²; A) and 8-Br-cAMP-treated (B) human coronary arterial endothelial cells (HCAECs). (C) Representative Western blot and densitometric data (*below*) showing the effect of pretreatment with the PKA inhibitor H89 (10 μ M) on shear stress-induced (10 dyn/cm², 10 min) and forskolin-induced (10 μ M, for 10 min) phosphorylation of p42/p44 MAP kinase in HCAECs. Data are expressed as mean \pm SEM. **p* < 0.05 vs. static control; #*p* < 0.05 vs. shear stress exposed. (D) Effect of pretreatment with inhibitors of the p42/p44 MAP kinase (PD98059, 10 μ M), p38 MAP kinase (SB203580, 10 μ M), or PKC (chelerythrine, 10 μ M) on shear stress-induced (10 dyn/cm², for 2 h) downregulation of BMP-4 expression in HCAECs and human umbilical vein endothelial cells (HUVECs). Data are expressed as mean \pm SEM. **p* < 0.05 vs. static control; #*p* < 0.05 vs. shear stress exposed. (E) Effect of pretreatment with anti-Myc siRNA on forskolin-induced changes in BMP-4 expression in HCAECs (on day 3 after transfection). Analysis of mRNA expression was performed with real-time QRT-PCR. β -actin was used for normalization. Data are expressed as mean \pm SEM (*n* = 4 for each group). **p* < 0.05. (F) Proposed scheme for the mechanism by which shear stress activates the cAMP/PKA pathway and regulates the expression of BMP-4 in the vascular endothelium. Because BMP-4 elicits endothelial activation and vascular calcification, the model predicts that cAMP/PKA-mediated inhibition of BMP-4 expression contributes to the antiatherogenic and vasculoprotective effects of laminar shear stress.

calcification by binding BMP4 (146) and BMP2 (135, 136). Conversely, the expression of BMP antagonists is regulated by BMPs (14, 121, 147, 148), pointing to a local feedback mechanism to modulate the cellular activities of BMPs. It is likely, though, that increased expression of BMP-2/4 coupled with decreased BMP antagonist expression has additive effects. Atherogenic stimuli such as TNF- α and oxLDL appear to regulate the expression of BMP antagonists in endothelial cells (16), and altered expression of BMP antagonists has been demonstrated in atherosclerotic lesions (107). Importantly, some studies suggest that BMP antagonists such as MGP (Fig. 2) and noggin (54) may be also regulated by hemodynamic forces.

The mechanisms underlying the transcriptional regulation of BMP antagonists in vascular cells have not yet been explored. Studies suggesting that the cAMP/PKA pathway may be involved in the regulation of BMP antagonists MGP (33) and follistatin (141) in nonvascular cells raise the interesting possibility that BMPs and BMP antagonists may be regulated by similar cellular signals.

Regulation of BMP-2/4 Expression by Shear Stress

Expression of BMP-4 in the vasculature is primarily localized to the endothelial cells, which are the primary sensors of changes in shear stress due to altered hemodynamics. At present, it is not well understood whether alterations in shear stress (either *via* direct transmission of the mechanical force by cellular connections and the extracellular matrix or indirectly *via* endothelium-derived paracrine mediators) induce transcriptional changes of BMPs, BMP receptors or modulators of BMP signaling pathways in the smooth muscle cells underlying the vascular endothelium. Nevertheless, the first indication that shear stress may control BMP-4 expression in endothelial cells came from microarray studies conducted to identify proatherogenic genes whose expression may be regulated by laminar flow (7, 113, 114). Solid evidence indicates that atheroprotective laminar flow/shear stress transcriptionally downregulates BMP-4 expression in multiple endothelial cell types from different species, whereas it does not affect BMP-2 transcript levels (20, 113).

Differential regulation of BMP-4 and BMP-2 was also substantiated in *ex vivo* intact artery and *in vivo* animal models of high flow (20), showing that shear stress is an important regulator of BMP-4 expression both in conduit arteries and in microvessels. Shear-dependent regulation of BMP-4 was further emphasized by a recent study showing low transcript levels of BMP-4 in protected, high-shear regions of the aortic valve, in contrast with abundant endothelial expression of BMP-4 in low-flow, calcification-susceptible valvular regions (8). It is known that laminar shear stress activates multiple redox-sensitive signaling pathways in endothelial cells [reviewed in references (74, 75, 77)], but the role of these pathways in shear-dependent regulation of BMP-2/4 expression is not well understood. Instead, in this review, we focus on the recently discovered roles of the shear stress-activated cAMP/PKA and ERK signaling pathways in regulating BMP-4 expression in endothelial cells.

Role of the cAMP/PKA pathway

Recently, we found that inhibition of the cAMP/PKA pathway prevents the downregulation of the BMP-4 gene in endothelial cells exposed to shear stress (20). The inhibitory

effect of shear stress on BMP-4 expression also was mimicked by the exogenous administration of an adenylate cyclase activator, a cAMP analogue, or a PKA activator (Fig. 3) (20). Although shear stress-induced production of cAMP is generally considered to derive from the autocrine effect of prostaglandins, and prostaglandins can downregulate BMP-4 (Ungvari, unpublished observation, 2006), this is likely not to be the case in sheared endothelium because downregulation of BMP-4 is maintained even in indomethacin-treated cells (20). Previous studies using magnetic RGD peptide-coated microbeads demonstrated that shear-induced mechanical activation of integrin- β leads to significant increases in intracellular cAMP (85), probably as a result of direct activation of adenylate cyclase. Moreover, cAMP is known to bind to the regulatory subunit of PKA, activating the enzyme, and recent data suggest that PKA is activated by shear stress (5, 20). A central role of PKA signaling is supported by the findings that pharmacologic or molecular inhibition of PKA prevents shear stress- and forskolin-induced downregulation of BMP-4 (20). It is also important to note that the mammalian BMP-4 gene structure is very similar to the *Drosophila* homologous gene decapentaplegic (*dpp*). During limb development, *dpp* expression is inhibited by the PKA pathway (62), whereas cells lacking PKA exhibit upregulation of *dpp* (99). Thus, PKA-dependent regulatory mechanisms controlling expression of BMP-4 appear to be conserved. Our recent data suggest that the cAMP/PKA pathway primarily decreases the transcription rate of the BMP-4 gene (rather than controlling the mRNA stability) (20).

Finally, despite the similar biologic roles of BMP-4 and BMP-2, the transcriptional regulation of these two cytokines by shear stress is again markedly different; activation of the cAMP/PKA pathway did not affect endothelial BMP-2 expression (20).

We recently reported that in human coronary arterial endothelial cells, both shear stress and cAMP analogues resulted in phosphorylation and activation of CREB, which were prevented by inhibition of PKA (20). The binding of phosphorylated CREB to the cAMP-responsive element (CRE) controls gene transcription (activation or suppression of transcription, depending on the target gene). It is significant that CRE sites have been found in the promoter region of many genes that respond to mechanical stimuli and may also exist in the BMP-4 promoter. On the basis of these considerations, we hypothesized that CREB would participate in BMP-4 regulation (20). Contrary to our prediction, however, we recently found that knockdown of CREB decreased basal BMP-4 expression in endothelial cells but it did not prevent shear stress- or forskolin-induced downregulation of BMP-4 (Fig. 3). These data suggest that CREB is a positive regulator of BMP-4 expression and that the downregulation of BMP-4 by shear stress is mediated *via* a cAMP/PKA-dependent but phospho-CREB-independent signal-transduction pathway.

Role of MAP kinase activation

Previous studies provided ample evidence that exposure of endothelial cells to laminar shear stress activates p42/44 MAPK in a time-dependent manner (63, 100), we recently confirmed (Fig. 4A and C). Growing evidence suggests crosstalk between cAMP/PKA and ERK signaling in various cell types; in coronary arterial endothelial cells, cAMP and

forskolin can activate p42/44 MAPK, and inhibition of PKA prevents both shear stress- and forskolin-induced activation of p42/44 MAPK (Fig. 4C). These results suggest that p42/44 MAPK is a critical downstream effector in the shear stress-activated cAMP/PKA pathway in endothelial cells, in line with the finding that inhibition of p42/44 MAPK (but not the p38 MAPK or PKC) prevented shear stress-induced downregulation of BMP-4 (Fig. 4D). Once activated, p42/44 MAPK is known to translocate to the nucleus and phosphorylate a number of different transcription factors (30), many of which have putative binding sites in the promoter region of the BMP-4 gene. At present, the MAPK-dependent transcription factor(s) lying downstream target of the cAMP/PKA pathway that downregulates the expression of BMP-4 in response to shear stress is unknown. Because knockdown of Myc (but not Sp-1 or p53) apparently attenuates the effect of forskolin on endothelial BMP-4 expression (Fig. 4E) and Myc-binding sites are conserved among the promoter region of human, baboon, cow, bat, mouse, and rat BMP-4 orthologues (66), future studies should characterize the role of Myc in detail in the regulation of BMP-4 expression in endothelial cells.

Role of oscillatory flow and NAD(P)H oxidase activation

In contrast with the effects of laminar shear stress, disturbed flow conditions, such as oscillatory shear stress, upregulate BMP-4 expression in endothelial cells (113, 114). Some evidence suggests that enhanced ROS production by NAD(P)H oxidase contributes to this response (113, 114). Further studies are clearly needed to elucidate the role of the cAMP/PKA pathway, CREB activation, ERK-dependent signaling mechanisms, or a combination of these effects on oscillatory shear stress. The significance of the upregulation of BMP-4 expression by disturbed hemodynamic conditions is also supported by the finding that BMP-4 protein is expressed in the endothelial cells overlying early atherosclerotic lesions but not in those that line uninjured human coronary arteries (113, 114). Based on the findings that ICAM-1 expression is increased in human coronary arteries in endothelial regions that express BMP-4 (113, 114) and that BMP-4 elicits monocyte adhesiveness *in vitro* (18, 113, 114), one can consider BMP-4 a master regulator of mechanosensitive endothelial activation. Many data presented in the present review uphold this hypothesis, which is further supported by a recent report that exposure of the aortic surface to pulsatile shear stress increased expression of the inflammatory markers VCAM-1 and ICAM-1 in a BMP-4-dependent manner (117). Dr. Hanjoong Jo's laboratory (14) recently showed that healthy endothelial cells can co-express BMP antagonists along with BMP-4 in response to disturbed flow conditions, suggesting that these antagonists can play a negative-feedback role against the inflammatory response of BMP-4. It has yet to be elucidated how endothelial expression of BMP antagonists is altered in pathophysiologic conditions (*e.g.*, diabetes, hypercholesterolemia, hyperhomocysteinemia), which are known to promote atherogenesis.

Perspectives

Taken together, we propose that hemodynamic forces (pressure/wall tension and shear stress) regulate expression

of vascular BMP-2/4 in endothelial and smooth muscle cells. As shown in Fig. 1D, high pressure/increased wall tension elicits increases in $[Ca^{2+}]_i$ and PKC activation, stimulating Nox-dependent $O_2^{\cdot -}$ and H_2O_2 production. Increased cytosolic ROS levels lead to NF- κ B activation, which upregulates BMP-2 expression. Conversely, atheroprotective laminar shear stress activates the cAMP/PKA and p42/44 MAP kinase pathway, which reduces BMP-4 expression in endothelial cells (Fig. 4F). Laminar shear stress also stimulates eNOS-dependent production of NO, which exerts vasodilatory, antiproliferative, and antiinflammatory effects on the smooth muscle cells. Because BMP-4 activates NAD(P)H oxidases and elicits endothelial dysfunction, monocyte adhesiveness to the endothelium, and vascular calcification, the model predicts that cAMP/PKA-mediated inhibition of BMP-4 expression contributes to the multifaceted antiatherogenic and vasculoprotective effects of laminar shear stress. In contrast, oscillatory/pulsatile shear stress increases Nox-derived ROS generation, decreasing the bioavailability of NO and upregulating BMP-4. Persistently disturbed hemodynamic conditions may also lead to the upregulation of paracrine signaling systems (including the local renin-angiotensin system and TNF- α production) that may also regulate BMP expression. Further studies are needed the better to elucidate (a) the downstream signaling pathways and target genes induced by BMPs in endothelial cells; (b) the role of BMPs in vascular remodeling induced by changes in the hemodynamic environment; (c) the differential redox-sensitive regulation of BMPs in endothelial and smooth muscle cells, characterizing the factors involved in their transcriptional regulation; and (d) the differences in BMP expression and regulation between systemic arteries (high pressure and atheroprone) and pulmonary arteries (low pressure, resistant to atherosclerosis, affected by BP-receptor mutations in primary pulmonary hypertension).

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Abbreviations

BAMBI, BMP and activin membrane-bound inhibitor; MAPK, mitogen-activated protein kinase; MGP, matrix Gla protein; NOS, nitric oxide synthase; NOX, NAD(P)H oxidase; PKC, protein kinase C; ROS, reactive oxygen species; SOD, superoxide dismutase; TNF- α , tumor necrosis factor- α .

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