Downregulation of Bone Morphogenetic Protein 4 Expression in Coronary Arterial Endothelial Cells

Role of Shear Stress and the cAMP/Protein Kinase A Pathway

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Objective—Bone morphogenetic protein 4 (BMP-4) is a transforming growth factor β family member cytokine that exerts proinflammatory effects on the endothelium and is likely to play a role in atherogenesis. Recent studies suggested that atheroprotective levels of shear stress control endothelial BMP-4 expression; however, the underlying mechanisms remained unknown.

Methods and Results—We found that shear stress downregulated BMP-4 expression in human and rat coronary arterial endothelial cells (CAECs) as well as in cultured mesenteric arterioles, although it had no effect on the expression of BMP-2, a related cytokine. In human coronary arterial endothelial cells, 8-bromo-cAMP, the adenylate cyclase activator forskolin, or a cAMP-dependent protein kinase (PKA) activator effectively decreased BMP-4 expression, mimicking the effects of shear stress. Indeed, shear stress induced the nuclear translocation of PKA-c, and inhibition of PKA attenuated the effects of shear stress and forskolin on BMP-4 expression. RNA decay assay and BMP-4 promoter-driven luciferase reporter gene assay showed that cAMP regulates BMP-4 expression at the transcriptional level.

Conclusions—Laminar shear stress and the cAMP/PKA pathway are important negative regulators of BMP-4 expression in the vascular endothelium. Because BMP-4 elicits endothelial activation and dysfunction, hypertension, and vascular calcification, inhibition of BMP-4 expression by shear stress and the cAMP/PKA pathway is likely to exert antiatherogenic and vasculoprotective effects. (***Arterioscler Thromb Vasc Biol.* 2007;27:776-782.)**

Key Words: atherosclerosis ■ inflammation ■ atrioventricular fistula ■ hemodynamic forces ■ endothelium

The transforming growth factor β family member cytokines bone morphogenetic protein 4 (BMP-4) and BMP-2 (a structurally related cytokine that acts on the same receptors) have been shown to play an important role in cardiovascular physiology and pathophysiology, including cardiac and vascular development[1] and angiogenesis, neointima formation, and development of pulmonary hypertension (reviewed elsewhere[2–5]). Recent studies suggest that BMPs are upregulated at athero-prone regions in blood vessels and may contribute to vascular calcification and the development of atherosclerotic plaques.[2,3,6–8] We have also recently shown that chronic BMP-4 infusion in C57Bl6 and apolipoprotein E knockout mice impairs endothelium-dependent vasodilation and induces arterial hypertension by an NADPH oxidase-dependent manner.[9] In vitro and ex vivo studies from our laboratories indicate that activation of BMP signaling by either overexpression of the BMP-4/BMP-2 in vascular cells or administration of recombinant BMPs results in endothelial dysfunction, oxidative stress, and an enhanced monocyte adhesiveness to the endothelium.[2–5] Also, we have demonstrated that BMP expression in coronary arterial endothelial cells is upregulated by oxidative stress and by the proinflammatory cytokine tumor necrosis factor α.[4,5] These lines of evidence suggest that BMPs may function as proinflammatory, prohypertensive, and proatherogenic mediators in the vessel wall.

In the past decades, it has been established that vascular inflammation and atherosclerosis develop in hemodynamically well-defined regions. It is thought that during normal vascular homeostasis laminar shear stress maintains an anti-inflammatory, antiatherogenic phenotype of endothelial cells. In contrast, adverse changes in the hemodynamic environment, in particular a combination of low shear stress and increased wall tension (eg, caused by high pressure), elicit proinflammatory phenotypic changes favoring atherogenesis. Importantly, recent data suggest that expression of BMP-4 and -2 is regulated by hemodynamic forces. We have demonstrated that BMP-2 expression can be upregulated by

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cellular stretch in high pressure-exposed arteries.[4] Mechanosensitive upregulation of BMPs has been also observed in bone, chondrocytes, connective tissues, and urinary bladder smooth muscle cells (reviewed in elsewhere[4]). The first data that shear stress may downregulate BMP-4 expression in endothelial cells came from microarray studies conducted to identify proatherogenic genes whose expression may be regulated by atheroprotective laminar flow.[2,3,10] However, the signaling mechanisms underlying shear stress–induced transcriptional control of BMP-4 and the effect of shear stress on endothelial BMP-2 expression remained completely unknown.

Thus, in the present study, we aimed to contrast the effects of shear stress on expression of BMP-2 and -4 in vitro in human coronary arterial endothelial cells (HCAECs), human umbilical vein endothelial cells (HUVECs), and rat coronary arterial endothelial cells (RCAECs), as well as in vivo models. Previous studies by this and other laboratories[11–13] have identified multiple pathways that are activated by shear stress and have the potential to regulate gene expression. These shear stress–activated mechanisms include activation of NO synthase, cyclooxygenase, endothelial hyperpolarization, and activation of cAMP-dependent protein kinase (PKA) pathway.[13] Thus, in the present study we aimed to elucidate the role of these cellular pathways in shear stress–dependent regulation of endothelial BMP-4 expression.

Methods

Cell Cultures

Primary HCAECs (Cell Applications Inc), HUVECs (Cell Applications Inc), and RCAECs (Celprogen, San Pedro, Calif) were maintained in culture as described.[5] Cells were subject to experiments after passage 4.

Application of Fluid Shear Stress

Confluent endothelial cells, last fed with the complete growth medium, were exposed to fluid shear stress (10 dyne/cm^2)[12,14] using a custom-built shearing device. To test the role of paracrine factors known to be released from the coronary arterial endothelium by shear stress (including NO, prostaglandins [PGs], O_2^-/H_2O_2 and endothelium-derived hyperpolarizing factors), cells were pretreated with the NO synthase inhibitor N^ω-nitro-L-arginine-methyl-ester (L-NAME) (3×10^{-4} mol/L), the cyclooxygenase inhibitor indomethacin (10^{-5} mol/L), polyethylene glycol–superoxide dismutase (200 U/mL) plus polyethylene glycol–catalase (200 U/mL) or depolarizing concentration of KCl (50 mmol/L).

To test the role of the cAMP/PKA pathway, HCAECs were exposed to shear stress or treated with 8-bromo-cAMP (8-Br-cAMP) (a cell permeable cAMP analog; 0.3 mmol/L), forskolin (which activates adenylate cyclase; 30 μmol/L), or N^6-benzoyladenosine-3′,5′-cyclic monophosphate (6Bnz) (a site-selective and membrane-permeant activator of PKA; BIOLOG Life Science Institute, Bremen, Germany), with or without pretreatment with the specific PKA inhibitor H89 (10 μmol/L, for 1 hour).

Vessel Culture and Animal Models of Increased Shear Stress in Arteries and Microvessels

To investigate the effect of shear stress and PKA activation on endothelial BMP-4 expression in intact vessels, first-order mesenteric arterioles were isolated from male Wistar rats (n=10, Taconic Biotechnology) using microsurgery instruments. All animal use protocols were approved by the Institutional Animal Care and Use Committee of the New York Medical Center. The vessels were cannulated on both sides, pressurized to 80 mm Hg, and maintained in organoid culture under minimal intraluminal flow conditions (wall shear stress<0.5 dyne/cm^2) for 24 hours, as described.[15] After this culture period, intraluminal flow was initiated to expose the vessels to a shear stress of 10 dyne/cm^2 for 2 hours. Wall shear stress (WSS) was calculated from the diameter ($2r$, measured by videomicroscopy) and flow data according to the equation $WSS=4\eta Q/\pi r^3$, where η is the viscosity of perfusate (0.007 poise at 37°C), Q is the perfusate flow, and r is the vessel radius.[11] Then the vessels were snap-frozen in liquid nitrogen, and changes in BMP-4 expression were determined. In separate experiments, isolated carotid arteries were cultured in the absence or presence of 8-Br-cAMP or forskolin for 24 hours.

To investigate the effects of increased shear stress in vivo, we used 2 animal models. To study increased shear stress on the endothelial BMP-4 and BMP-2 expression in large vessels, we harvested the thoracic aortas of rats with an abdominal aortocaval fistula (characteristics of the animals have been recently published[16]). This animal model is characterized by a 4-fold increase in aortic blood flow (J.G., unpublished data, 2005).

To study increased shear stress on the endothelial BMP-4 and BMP-2 expression in the microvascular endothelium, we harvested high blood flow-exposed and normal blood flow-exposed, first-order mesenteric arterioles of Bl/6 mice with mesenteric artery ligation according to the method of Loufrani et al.[17] This animal model is characterized by a ≈50% to 66% increase in blood flow in microvessels parallel to the ligation[17] (E.N.T.P.B., unpublished data, 2005).

Quantitative Real-Time Polymerase Chain Reaction

Total RNA from the arteries was isolated with Mini RNA Isolation Kit (Zymo Research, Orange, Calif) and was reverse transcribed using Superscript III RT (Invitrogen) as described previously.[4,5] Real-time RT-PCR technique was used to analyze mRNA expression using the Strategen MX3000, as reported.[4,5] Oligonucleotides used for real-time quantitative RT-PCR (QRT-PCR) are listed in Table I in the online data supplement, available at http://atvb.ahajournals.org.

Immunofluorescent Detection of PKA-c

HCAECs were grown on a coverslip and exposed to shear stress or forskolin (for 10 minutes) as described. Then, cells were fixed in ice-cold methanol and permeabilized, and immunolabeling was performed using a primary antibody against the catalytic subunit of PKA (PKA-c) (BD Pharmingen).

Adenoviral Gene Transfection

Isolated mouse carotid arteries and HCAECs were infected with recombinant adenovirus in serum-free DMEM for 1 hour and then cultured for 24 hours before forskolin or shear stress treatment. Construction of the recombinant adenovirus encoding PKA inhibitor (Ad-PKI) has been described previously.[13] Recombinant adenovirus encoding green fluorescence protein (Ad-GFP) was used as a control.

Detection of CREB Phosphorylation

Phosphorylation of cAMP response element-binding protein (CREB) was detected by Western blotting as described.[5] In brief, HCAECs were exposed to shear stress or treated with forskolin or 8-Br-cAMP for 5 to 20 minutes. The cells were rinsed with PBS, and then hot lysis buffer was added (10 mmol/L Tris, pH 7.4, 1 mmol/L sodium ortho-vanadate, 1% SDS). The lysate was sonicated for 5 seconds. In some experiments, HCAECs pretreated with H89. Equal amounts of protein (50 μg) were electrophoresed on 10% SDS-PAGE gel, transferred to a polyvinylidene fluoride (PVDF) membrane with a semidry blotting system, and labeled with a phosphorylation-specific primary antibody for CREB (Cell Signaling). The membranes were washed with PBS and incubated for 1 hour with sheep anti-rabbit IgG–horseradish peroxidase or donkey anti-mouse IgG–horseradish peroxidase (Amersham) at the final titer of 1:4000. The membranes were developed with ECL-Plus (Amersham).

Transient Transfection and Luciferase Assays

Effect of shear stress and forskolin on CREB activity in HCAECs was tested by a reporter gene assay. We used a CREB reporter comprised of a CRE site upstream of firefly luciferase (CREB-Luc, Stratagene) and a renilla luciferase plasmid under the control of the cytomegalovirus promoter (as an internal control).

To characterize the role of the conserved proximal promoter region of the BMP-4 gene in cAMP-induced downregulation of BMP-4, endothelial cells were transfected with luciferase plasmid constructs containing the evolutionarily conserved proximal region of the BMP-4 promoter (from −715 to +97) or a deletion fragment (−147 to +97; constructs were kindly provided by Dr Chul Geun Kim, Hanyang University, Seoul, Korea[18]) and then exposed to forskolin.

All transfections were performed with Novafector (Venn Nova LLC, Pompano Beach, Fl) following the protocols of the manufacturer. Firefly and renilla luciferase activities were assessed after 42 hours using the Dual Luciferase Reporter Assay Kit (Promega) and a luminometer.

RNA Decay Assay

To determine whether BMP-4 expression is regulated by changes in the mRNA stability, HCAECs were treated with forskolin in the presence or absence of 2.5 μg/mL actinomycin D (to halt transcription). Cells were harvested at 15, 30, 60, and 120 minutes. QRT-PCR was used to profile the decay rate of BMP-4 transcript.

Data Analysis

Data were normalized to the respective control mean values. Data are expressed as mean±SEM. Statistical analyses of data were performed by Student's *t* test or by 2-way ANOVA followed by the Tukey post hoc test, as appropriate. $P<0.05$ was considered statistically significant.

Results

Shear Stress Downregulates the Expression of BMP-4 mRNA in Endothelial Cells

As shown in Figure 1, the steady-state levels of BMP-4 transcript were downregulated by shear stress in each endothelial cell type studied (Figure 1A). In both HCAECs and in RCAECs, shear stress resulted in a rapid downregulation of the BMP-4 transcript and maximal inhibition occurred within 2 hours after initiation of shear stress (Figure 1B and 1C). Recent studies also have shown that shear stress downregulates the endothelial expression of BMP-4 protein as well.[2,3] Shear stress did not affect BMP-2 expression in either cell types (Figure 1D). Expression of BMP-4 increased post–shear stress exposure (98±30% at 8 hours).

We found that BMP-4 expression was significantly decreased in intact mesenteric arterioles exposed to increased flow/shear stress in organoid culture (supplemental Figure IA). Increased flow also resulted in significant downregulation of BMP-4 expression both in mesenteric arterioles of mice with parallel mesenteric vessel ligation (supplemental Figure IB) and in aortas of rats with aortocaval fistula (supplemental Figure IC). In mesenteric arteries, BMP-4 expression tended to increase 1 day after decreasing intraluminal flow by ligation (data not shown). Previous studies also have shown that immunostaining for BMP-4 protein varies between different arterial locations.[2,3] Increased flow/shear stress did not decrease vascular BMP-2 expression in mesenteric arterioles of mice with parallel mesenteric vessel ligation (supplemental Figure IA). BMP-2 expression tended to increase in aortas of rats with aortocaval fistula; however, the difference did not reach statistical significance (supplemental Figure IC).

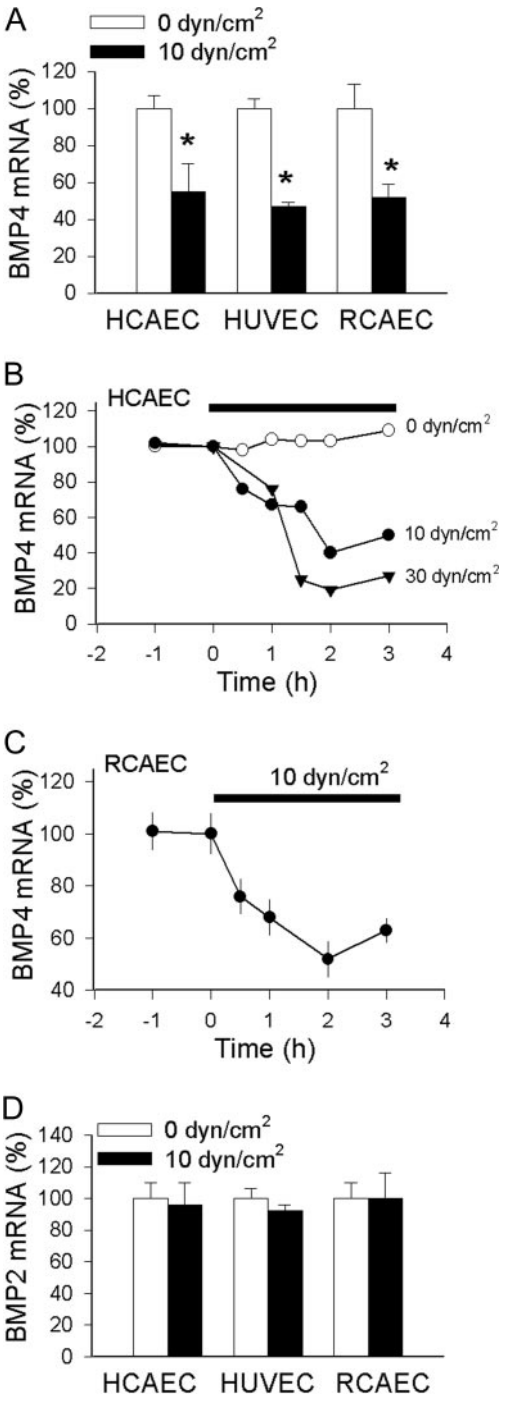

Figure 1. A, Effect of shear stress (10 dyne/cm^2 for 2 hours) on BMP-4 expression in primary HCAECs, HUVECs, and RCAECs. Analysis of mRNA expression was performed by real-time QRT-PCR. β-Actin was used for normalization. Data are mean±SEM (n=4 to 6 for each group). *$P<0.05$. B and C, Time course of shear stress–induced downregulation of BMP-4 expression in HCAECs and RCAECs. D, Effect of shear stress (10 dyne/cm^2 for 2 hours) on BMP-2 expression in HCAECs, HUVECs, and RCAECs.

Inhibition of NO synthesis, PG production and endothelium-dependent hyperpolarization or scavenging of reactive oxygen species did not prevent shear stress–induced down-

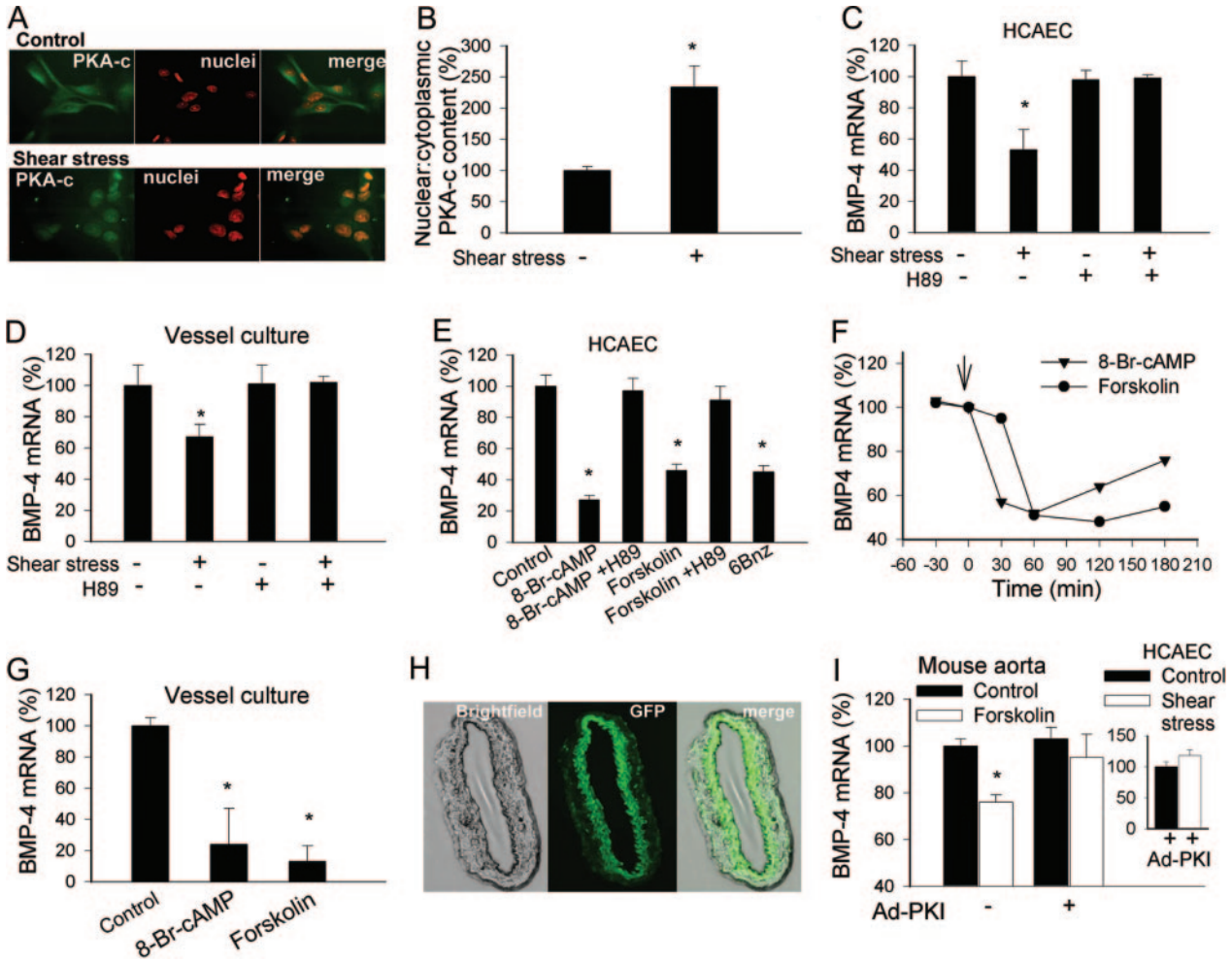

Figure 2. A, Original fluoromicrographs showing cytoplasmic PKA-c staining in static HCAECs (top panels) and increased nuclear PKA-c staining in shear stress-exposed HCAECs (bottom panels). Red indicates nuclear counterstaining. B, Bar graphs show summary data for nuclear-to-cytoplasmic PKA-c content. Data are normalized to the control mean and expressed as mean±SD. C, Effect of pretreatment with the PKA inhibitor H89 (10 μmol/L) on downregulation of BMP-4 expression in primary HCAECs by shear stress (10 dyne/cm^2 for 2 hours). D, Effect of pretreatment with H89 on shear stress–induced (10 dyne/cm^2 for 2 hours) downregulation of BMP-4 expression in cultured, cannulated rat mesenteric arterioles. E, HCAECs were also stimulated with 8-Br-cAMP (0.3 mmol/L), forskolin (10 μmol/L), or 1 mmol/L 6Bnz in the absence or presence of H89 for 2 hours. Data are mean±SEM (n=4 for each group). *$P<0.05$. F, Time course of 8-Br-cAMP– and forskolin-induced downregulation of BMP-4 expression in HCAECs. G, Downregulation of BMP-4 expression in rat carotid arteries in organoid culture (for 24 hours) by 8-Br-cAMP and forskolin. Analysis of mRNA expression was performed by real-time QRT-PCR. β-Actin was used for normalization. Data are mean±SEM (n=4 for each group). *$P<0.05$. H, Cross-sections of a mouse carotid artery treated with a green fluorescence protein (GFP)-expressing adenoviral vector (after 2-day organoid culture). The endothelium was functionally intact as determined by relaxation to acetylcholine. I, Adenovirus-mediated expression of PKA inhibitor (Ad-PKI) prevents forskolin-induced downregulation of BMP-4 in endothelial cells microdissected from mouse carotid arteries. Ad-PKI transfection also prevented shear stress–induced downregulation of BMP-4 in HCAECs (inset). Analysis of mRNA expression was performed by real-time QRT-PCR. β-Actin was used for normalization. Data are mean±SEM (n=4 for each group). *$P<0.05$.

regulation of BMP-4 in HCAECs (supplemental Figure II; see the online data supplement), suggesting that shear stress–induced release of these autocrine/paracrine mediators is unlikely to play a role in this phenomenon.

Role of the cAMP/PKA Pathway in Regulation of BMP-4 Expression

We next examined the possibility that a cAMP/PKA pathway mediated the inhibition of BMP-4 expression by shear stress. Immunofluorescent labeling showed that in static HCAECs, PKA-c was localized predominantly in the cytoplasm, whereas shear stress increased nuclear PKA-c content (Figure 2A), increasing nuclear/cytoplasmic PKA-c fluorescence intensity ratios (Figure 2B). Forskolin also significantly increased nuclear PKA-c content (not shown). The selective PKA inhibitor H-89 (Figure 2C and 2D) or inhibition of the adenylate cyclase (shear stress: 75±6%; 9-cyclopentyladenine plus shear stress: 258±37%) prevented the inhibitory action of shear stress on BMP-4 gene expression in HCAECs and in cultured mesenteric vessels.

As shown in Figure 2E, stimulation of PKA activity by 0.3 mmol/L 8-Br-cAMP (a cell membrane-permeable analog of cAMP), by forskolin (an adenylate cyclase activator), or by 6Bnz (a direct activator of PKA) resulted in marked inhibition of BMP-4 gene expression, which could be prevented by pretreatment with H89. Treatment with H-89 alone did not

affect the steady-state levels of BMP-4 transcript (Figure 2C and 2D). We confirmed that the time course of the effects of 8-Br-cAMP and forskolin (Figure 2F) were similar to that of shear stress (Figure 1F). Endothelial expression of BMP-4 increased after forskolin treatment (93±23% at 8 hour).

We also demonstrated that 8-Br-cAMP and forskolin effectively downregulate BMP-4 expression in cultured arteries (Figure 2G). Forskolin and 8-Br-CAMP did not affect BMP-2 expression either in HCAECs or in cultured vessels (data not shown). To further confirm the role of PKA by using an independent approach, we examined the effect of adenovirus-mediated expression of protein kinase A inhibitor (Ad-PKI). As shown in Figure 2H, in control experiments, adenoviral expression of green fluorescence protein was present in both the endothelium and medial layers of cultured mouse carotid arteries. Using the same method, vessels were transfected with Ad-PKI, which prevented forskolin-induced downregulation of BMP-4 (Figure 2I). Transfection of HCAECs with Ad-PKI also prevented downregulation of BMP-4 by shear stress (Figure 2I, inset) and forskolin (not shown).

One of the downstream mechanisms by which the cAMP/PKA pathway may regulate gene expression involves phosphorylation and activation of cAMP response element binding protein (CREB). We used rVISTA (http://www.gsd.lbl.gov/vista) analysis to confirm the presence of putative cAMP-responsive elements (CRE) in the evolutionarily highly conserved proximal part of the 5′-flanking region of the human BMP-4 gene. As shown in Figure 3A through 3C, shear stress, 8-Br-cAMP, and forskolin induced CREB phosphorylation and CREB activation in HCAECs. H89 inhibited shear stress and cAMP-induced CREB phosphorylation (Figure 3B and 3C), indicating that CREB activation depends on PKA.

BMP-4 Expression Is Regulated at the Level of Transcription

The mRNA decay assay showed that the BMP-4 transcript, similar to many other cytokines and growth factors, is relatively short-lived ($t_{1/2}$=68 minutes in forskolin-stimulated HCAECs), and its expression is primarily regulated at the level of transcription (Figure 4A). Also, forskolin significantly decreased luciferase activity in endothelial cells transfected with a BMP-4 promoter-driven luciferase construct (Figure 4B), supporting the view that the cAMP/PKA pathway primarily decreases transcription rate of the BMP-4 gene, rather than altering mRNA stability. Deletion from position −715 to −147 prevented the significant reduction of promoter activity by forskolin in both HCAECs and HUVECs (Figure 4B), indicating the presence of a binding site regulated by the cAMP/PKA pathway within the evolutionarily conserved proximal region of the BMP-4 gene (Figure 4C).

Discussion

There are 3 important findings in this study. First, we have shown that shear stress transcriptionally downregulates BMP-4 expression in multiple endothelial cell types from different species, whereas it does not affect BMP-2 transcript levels (Figure 1). Differential regulation of BMP-4 and BMP-2 was also evident in high-flow/increased shear stress–exposed intact arteries in vitro and in vessels from animal

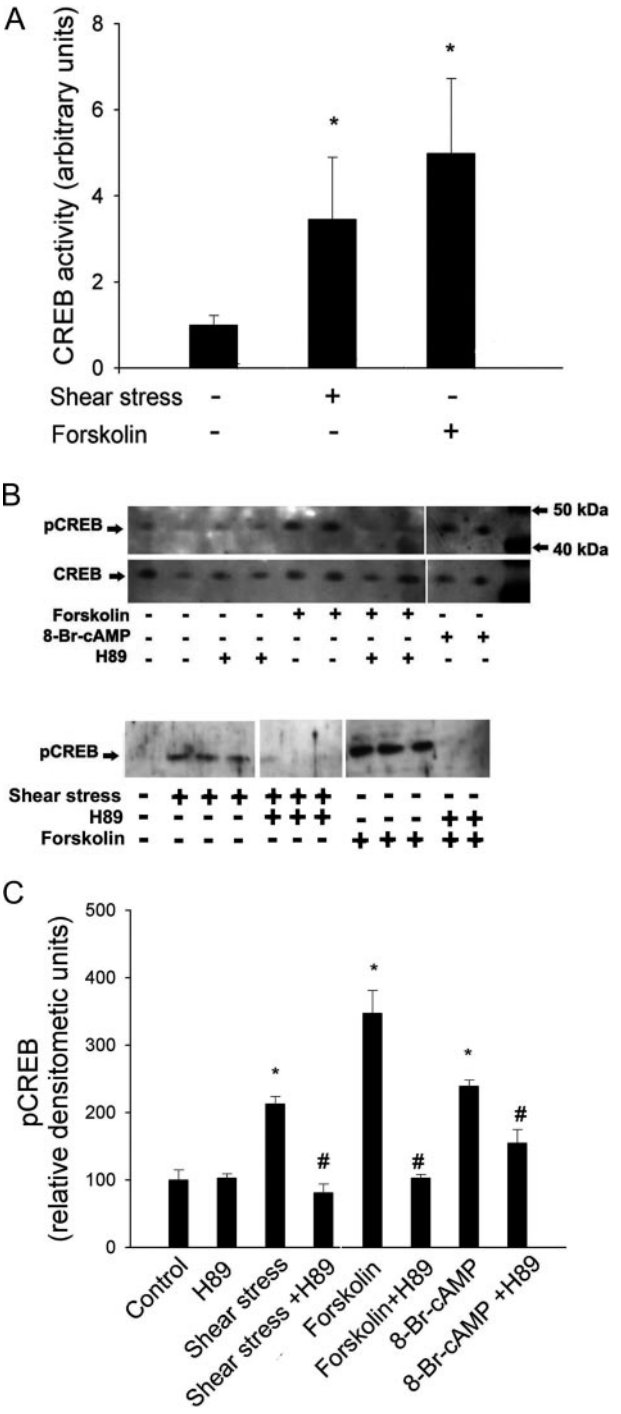

Figure 3. A, Reporter gene assay showing the effects of shear stress (10 dyne/cm^2 for 2 hours) and forskolin (10 mol/L for 2 hours) on CREB reporter activity in HCAECs. Endothelial cells were transiently cotransfected with CRE-driven firefly luciferase and cytomegalovirus (CMV)-driven renilla luciferase constructs followed by shear stress or forskolin stimulation. Cells were then lysed and subjected to luciferase activity assay. After normalization, relative luciferase activity was obtained from 4 to 7 independent transfections. (Data are mean±SD; *P<0.05 vs control.) B and C, Representative Western blots (B) and densitometric data (C) showing the effect of pretreatment with the PKA inhibitor H89 (10 μmol/L) on shear stress–induced (10 dyne/cm^2), forskolin-induced (10 μmol/L), and 8-Br-cAMP–induced phosphorylation of CREB (pCREB) in HCAECs. Representative results of 3 independent experiments are shown. Data are mean±SEM. *P<0.05 vs static control, #P<0.05 vs untreated.

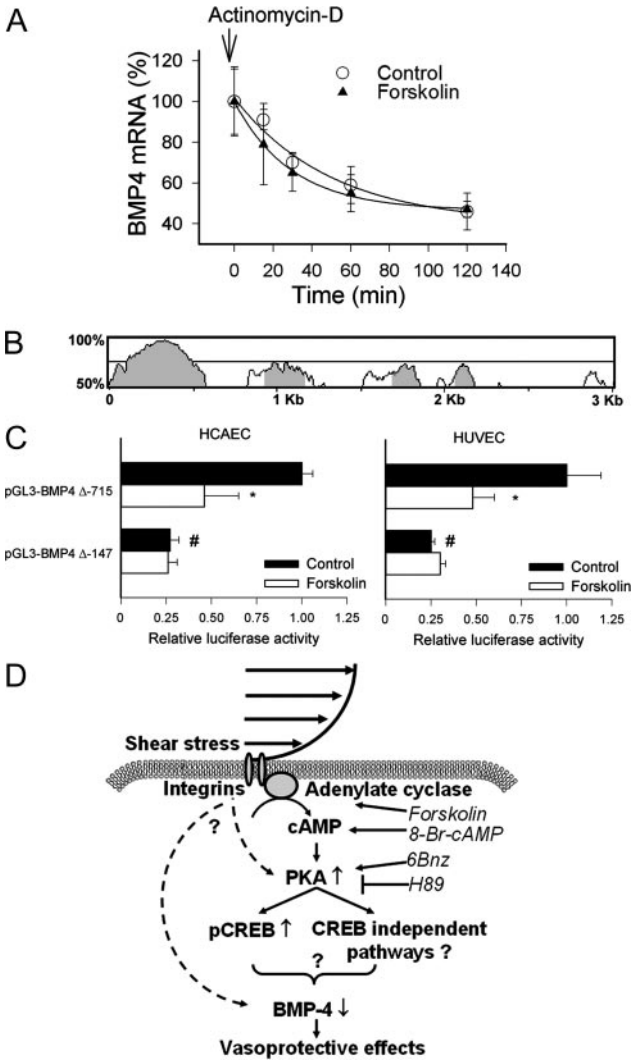

Figure 4. A, Changes in the level of BMP-4 transcript in untreated and forskolin-treated HCAECs after the addition of actinomycin D. B, VISTA plot showing the percent of conservation between the 5′-flanking regions of the rat and human BMP-4 genes. C, HCAECs and human umbilical vein endothelial cells (HUVECs) were transfected with a reporter gene construct containing the 812-kb conserved domain of the 5′-flanking region of the BMP-4 gene or a deleted fragment and then treated with forskolin. The luciferase activity of each construct was expressed as a function of the activity recorded for the parent promoter construct. After normalization, relative luciferase activity was obtained from 4 to 7 independent transfections. (Data are mean±SD; *$P<0.05$ vs control.) D, 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 shear stress.

models of in vivo increased blood flow (supplemental Figure I; see the online data supplement), showing that intraluminal flow/shear stress is an important regulator of BMP-4 expression both in the conduit arteries and microvessels. We would like to acknowledge that in the aortocaval fistula model, other hemodynamic changes may occur (including marked increases in pulse pressure), which may also affect BMP-4 and/or BMP-2[4] expression. Also, we would like to point out that extrapolation of the results obtained in the model systems used to in vivo pathological conditions in humans should be done with great caution, because the flow/shear stress patterns in athero-prone regions of the circulation are more complex (eg, negative flow, oscillating flow, perturbed flow, etc) than modeled in our experiments. The in vivo significance of shear stress regulation of BMP-4 is supported by recent studies showing decreased transcript levels of BMP-4 in protected, high-shear regions of the aortic valve, whereas endothelial cells in calcification-susceptible valvular regions abundantly express BMP-4.[19] Thus, despite the similar biological roles of BMP-4 and BMP-2, the transcriptional regulation of these 2 cytokines is markedly different. Indeed, the promoter regions of the 2 genes do not show any homology.[4] Our present and previous findings show that shear stress primarily controls the expression of BMP-4,[2,3] whereas increased wall tension/high pressure, inflammatory stimuli, and oxidative stress all regulate the expression of BMP-2.[4,5]

The mechanotransduction of shear stress involves activation of a number of parallel pathways that have different biological roles, including the activation of endothelial NO synthase, PG synthesis, H_2O_2 release, endothelium-derived hyperpolarized factor pathways, etc. To examine the role of the major autocrine/paracrine pathways that are activated in response to shearing, we assayed the effect of shear stress on BMP-4 expression in the presence of inhibitors of NO and PG synthesis, free radical scavengers, and KCl (to exclude the role of K^+ induced endothelial hyperpolarization). We found that in HCAEC regulation of BMP-4 expression is independent of the shear-induced release of these paracrine factors (supplemental Figure II; see the online data supplement).

The second important finding of this study is that inhibition of the cAMP/PKA pathway attenuates shear stress-induced downregulation of the BMP-4 gene (Figure 2). Furthermore, exogenous administration of adenylate cyclase activator, cAMP analog, or PKA activator mimicked the effects of shear stress on the inhibition of BMP-4 gene expression in endothelial cells (Figure 2). Production of cAMP has been considered to be attributable to the autocrine effect of shear stress–induced PGs. However, this is likely not to be the case because shear-dependent regulation of BMP-4 was preserved in indomethacin-treated cells (supplemental Figure II; see the online data supplement). Previous studies using magnetic RGD peptide-coated microbeads demonstrated that shear-induced mechanical activation of integrin β leads to significant increases in intracellular cAMP,[20] likely as result of direct activation of adenylate cyclase. 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.[14] Indeed, increasing shear forces (to 10 dyne/cm^2) acting on HCAECs elicited translocation of the catalytic subunit of PKA (PKA-c) into the nucleus[20] (Figure 2A and 2B). A central role of PKA signaling is supported by the findings that pharmacological or molecular inhibition of PKA attenuates cAMP-dependent downregulation of BMP-4 (Figure 2C through 2E and 2I). We next examined the effects of PKA activation on downstream signaling in HCAECs by quantitating whether it phosphorylates and activates the transcrip-

tional regulator CREB. Figure 3 shows that both shear stress and cAMP analogs resulted in phosphorylation and activation of CREB, which were prevented by inhibition of PKA. The binding of pCREB to the 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 it seems that CRE site(s) are also present in the evolutionarily conserved 5′-flanking sequences of the BMP-4 gene.

In theory, PKA may control BMP-4 levels either at the level of transcription or posttranscriptionally. Results from RNA decay assay (Figure 4A) and the luciferase assay (Figure 4C) support the view that the cAMP/PKA pathway primarily decreases the transcription rate of the BMP-4 gene (rather than controlling the mRNA stability). At present, it is unknown whether oscillatory shear stress, which was shown to upregulate BMP-4 expression,[2,3] fails to activate the cAMP/PKA pathway or it activates transcriptional mechanisms that override the negative regulatory effects of cAMP/PKA signaling.

It is important to note that the mammalian BMP-4 gene structure is very similar to the Drosophila homologous gene decapentaplegic (*dpp*). The *dpp* gene is critical for various processes during embryogenesis, including dorsal–ventral specification. It is significant that during limb development, *dpp* expression is inhibited by the PKA pathway,[21] whereas cells lacking PKA exhibit upregulation of *dpp*.[22] Thus, PKA-dependent regulatory mechanisms controlling expression of BMP-4, an evolutionarily ancient and critical gene, seem to be conserved. In future studies, we aim to elucidate the downstream targets of the cAMP/PKA pathway that regulate BMP-4 expression in endothelial cells, including the role of CREB and CREB-independent pathways and the interaction between cAMP/PKA and p42/44 MAPK signaling.[12,23]

Collectively, we propose that laminar shear stress activates the cAMP/PKA pathway, and this pathway regulates the expression of BMP-4 in the vascular endothelium (Figure 4D). Because BMP-4 can elicit endothelial activation and dysfunction, hypertension, 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.

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Disclosures

None.

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ONLINE DATA SUPPLEMENT

Table I. Oligonucleotides for real-time RT-PCR

mRNA targets	*sense*	*Antisense*
human BMP-2	GGTGGAATGACTGGATTG	GCATCGAGATAGCACTG
human BMP-4	CCTTTCCACTGGCTGAC	CACCCACATCCCTCTAC
human CREB	GCTCACGCCTGTAATCCC	CAATTCTGCTGCCTCAACC
human GAPDH	AACGAATTTGGCTACAGC	AGGGTACTTTATTGATGGTACAT
rat BMP-2	TCAAGCCAAACACAAACAG	CGCTAAGCTCAGTGGG
rat BMP-4	GAATAAGAACTGCCGTCG	CCTTGTCGTACTCGTCC
rat β-actin	GAAGTGTGACGTTGACAT	ACATCTGCTGGAAGGTG
mouse BMP-2	ACACAAACAGCGGAAG	AGAGTCTGCACTATGGC
mouse BMP-4	AATAAGAACTGCCGTCG	CCACACCCCTCTACCA
mouse β-actin	AATAAGTGGTTACAGGAAGTC	ATGAAGTATTAAGGCGGAAG

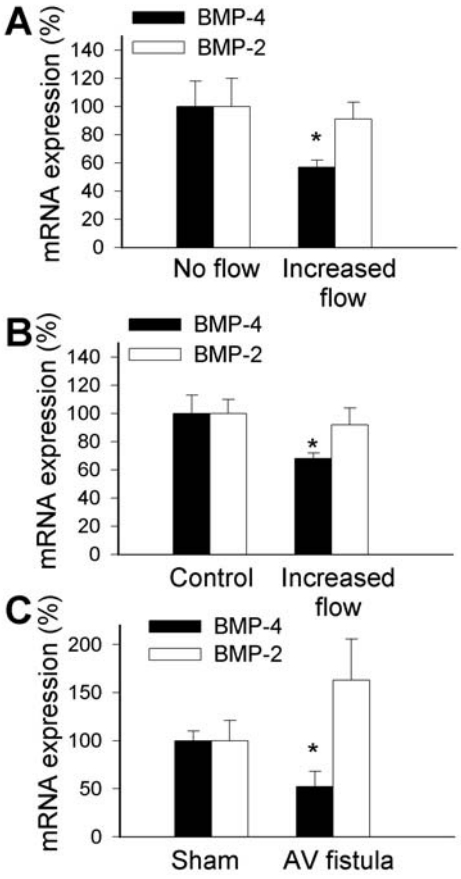

Figure IA: Expression of BMP-4 and BMP-2 mRNA in cultured, cannulated rat mesenteric arterioles with or without shear stress-exposure (10 dyn/cm^2 for 2 h). **B**: Expression of BMP-4 and BMP-2 mRNA in control mesenteric arterioles and increased flow/shear stress-exposed vessels of mice with parallel mesenteric arteriolar ligation. **C:** Expression of BMP-4 and BMP-2 mRNA in increased flow/shear stress-exposed aortas of rats with AV-fistula and vessels of sham operated control rats. Analysis of mRNA expression was performed by real-time QRT-PCR. β-actin was used for normalization. Data are mean ± S.E.M. (n=4-6). *$p<0.05$.

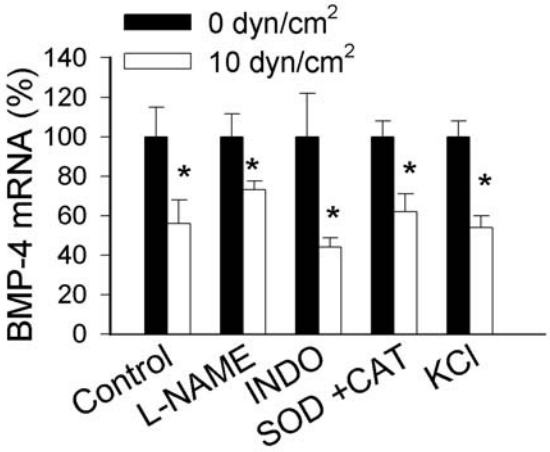

Figure II: Effect of pre-treatment with L-NAME ($3x10^{-4}$ mol/L), indomethacin (10^{-5} mol/L), SOD (200 U/mL) plus catalase (200 U/mL) and depolarizing KCl (50 mmol/L) on down-regulation of BMP-4 expression in primary human coronary arterial endothelial cells (HCAEC) by shear stress (10 dyn/cm^2, for 2 h). Analysis of mRNA expression was performed by real-time QRT-PCR. β-actin was used for normalization. Data are mean ± S.E.M. and are normalized to the mean of no-shear controls (n=4 for each group). *$p<0.05$.

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