Bone Morphogenic Protein 4 Produced in Endothelial Cells by Oscillatory Shear Stress Induces Monocyte Adhesion by Stimulating Reactive Oxygen Species Production From a Nox1-Based NADPH Oxidase

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Abstract—Atherosclerosis is an inflammatory disease occurring preferentially in arterial regions exposed to disturbed flow conditions including oscillatory shear stress (OS). OS exposure induces endothelial expression of bone morphogenic protein 4 (BMP4), which in turn may activate intercellular adhesion molecule-1 (ICAM-1) expression and monocyte adhesion. OS is also known to induce monocyte adhesion by producing reactive oxygen species (ROS) from reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, raising the possibility that BMP4 may stimulate the inflammatory response by ROS-dependent mechanisms. Here we show that ROS scavengers blocked ICAM-1 expression and monocyte adhesion induced by BMP4 or OS in endothelial cells (ECs). Similar to OS, BMP4 stimulated H_2O_2 and O_2^- production in ECs. Next, we used ECs obtained from p47phox$^{-/-}$ mice (MAE-p47$^{-/-}$), which do not produce ROS in response to OS, to determine the role of NADPH oxidases. Similar to OS, BMP4 failed to induce monocyte adhesion in MAE-p47$^{-/-}$, but it was restored when the cells were transfected with p47^{phox} plasmid. Moreover, OS-induced O_2^- production was blocked by noggin (a BMP antagonist), suggesting a role for BMP. Furthermore, OS increased gp91phox (nox2) and nox1 mRNA levels while decreasing nox4. In contrast, BMP4 induced nox1 mRNA expression, whereas nox2 and nox4 were decreased or not affected, respectively. Also, OS-induced monocyte adhesion was blocked by knocking down nox1 with the small interfering RNA (siRNA). Finally, BMP4 siRNA inhibited OS-induced ROS production and monocyte adhesion. Together, these results suggest that BMP4 produced in ECs by OS stimulates ROS release from the nox1-dependent NADPH oxidase leading to inflammation, a critical early atherogenic step. **(*Circ Res*. 2004;95:773-779.)**

Key words: BMP4 ■ oscillatory shear ■ reactive oxygen species ■ monocyte adhesion ■ endothelial cells ■ NADPH oxidase

Vascular endothelial cells (ECs) are constantly exposed to fluid shear stress, the frictional force generated by blood flow over the vascular endothelium. The importance of shear stress in vascular biology and pathophysiology has been highlighted by the focal development patterns of atherosclerosis in hemodynamically defined regions. For example, the regions of branched and curved arteries exposed to disturbed flow conditions including oscillatory shear stress (OS) correspond to "lesion-prone areas" that preferentially develop atherosclerosis.[1,2] In contrast, straight arteries exposed to steady, high levels of laminar shear stress (LS) are relatively well protected from atherosclerotic plaque development.[1,2]

Atherosclerosis is an inflammatory disease preferentially occurring in lesion-prone areas.[2,3] The earliest measurable markers of atherogenesis include expression of inflammatory adhesion molecules such as E-selectin, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), and subsequent monocyte adhesion and recruitment into the lesion-prone areas.[2,4,5] Additional critical atherogenic events include the loss of bioavailable NO production and an increase in reactive oxygen species (ROS) levels, including superoxide (O_2^-), hydrogen peroxide (H_2O_2), and peroxynitrite ($ONOO^-$).[6,7]

The mechanisms by which different flow conditions (LS and OS) prevent or induce inflammation and atherosclerosis have been the topic of intense studies in recent years. The opposite effects of OS and LS are likely to be mediated by both acute regulation of vasoactive factor production and

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chronic changes involving gene and protein expression profiles.[8–15] Several groups, including ours,[8–13] have performed DNA microarray studies in an attempt to systematically identify mechanosensitive genes, which change in response to LS, OS, or turbulent flow, in ECs. Through our DNA microarray studies and the subsequent functional studies, we have discovered a novel mechanism by which OS induces inflammatory responses in ECs: the production of bone morphogenic protein 4 (BMP4).[13] We have shown that exposure of ECs to OS induces BMP4 production, which in turn stimulates ICAM-1 expression and subsequent monocyte adhesion in a nuclear factor κB (NF-κB)–dependent manner.[13] In a more recent study, we found that OS stimulates monocyte adhesion by increasing ROS (O_2^- and its derivatives) levels derived from reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidases in ECs.[16] However, it is not known whether BMP4 that is induced by OS is directly involved in OS-dependent ROS production and subsequent monocyte adhesion.

Here, we examined the hypothesis that BMP4 produced in response to OS induces inflammatory responses (ICAM-1 production and monocyte adhesion) by stimulating ROS production from NADPH oxidases. This hypothesis was tested by using ECs obtained from wild-type (wt; C57BL6) or NADPH oxidase knockout mice in combination with pharmacological and molecular approaches.

Materials and Methods

ECs and Shear Studies

ECs obtained from the thoracic aortas of C57BL6 control (MAE-wt) and $p47^{phox}$-null mice (MAE-$p47^{-/-}$) were used.[16] Human aortic ECs (HAECs; Clonetics) were cultured in EBM-2 bullet kit (Clonetics).[13] Confluent ECs grown in 100-mm tissue culture dishes (Falcon) were exposed to OS (±5 dyne/cm^2) or static control conditions for 1 day using a cone-and-plate device as described by us.[13]

Monocyte Adhesion Assay

After OS or BMP4 treatment, binding of Tamm–Horsfall protein-1 (THP-1) monocytes to MAE cells was determined as described previously.[13,16]

ICAM-1 Expression

Surface expression of ICAM-1 in HAECs was determined by fluorescence-activated cell sorting (FACS) as described by us[13] and in the online supplement.

H_2O_2 and O_2^- Assays

H_2O_2 and O_2^- productions were determined by fluorometry using 2′, 7′-dichlorofluorescein diacetate (DCF-DA; 7.5 μg/mL; Molecular Probes)[17] and electron spin resonance (ESR) spectroscopy using methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine (CMH)[16] as described further in the online supplement.

Small Interfering RNA Experiments

The annealed small interfering RNA (siRNA) duplexes (nox1 [sense: 5′-GGAAUUAUUUUGAGCUCUUtt′, antisense: 5′-AAGAGCUCAAAAUAAUUCCtg]; BMP4 [sense: 5′GGCGACACUUCUACAGAUGtt, antisense: 5′-CAUCUGUAGAAGUGUCGCCtc] and nonsilencing [sense: 5′-UUCUCCGAACGUGUCACGUtt, antisense: 5′-ACGUGACACGUUCGGAGAAtt]) were used with Oligofectamine (Invitrogen), as described further in the expanded Materials and Methods section in the online data supplement available at http://circres.ahajournals.org.

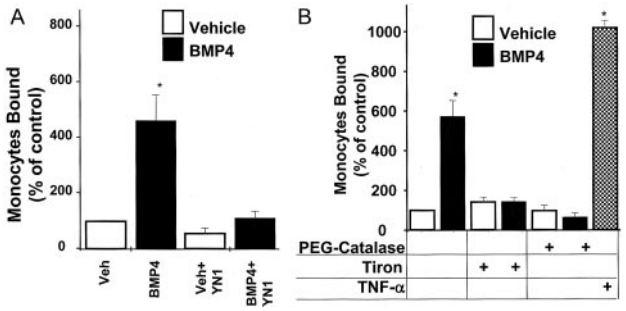

Figure 1. BMP4 stimulates monocyte adhesion in an ICAM-1 and ROS-dependent manner in ECs. A, Confluent MAE-wt cells were treated with BMP4 (20 ng/mL) or vehicle (Veh) for 18 hours. During the last 30 minutes of the incubation, 5 μg/mL of the blocking antibody YN1 was included. B, Confluent MAE-wt cells were preincubated with cell-permeable PEG-catalase (Catalase; 100 U/mL for 18 hours) or Tiron (10 μmol/L for 1 hour). Cells were then incubated with 20 ng/mL BMP4 or vehicle for an additional 18 hours. The numbers of bound THP-1 monocytes (fluorescently labeled) to the treated cells were determined with a fluorescence microscope and expressed as percentage of control (mean±SEM; n=6 in A; n=4 in B; *$P<0.05$. TNF-α (100 U/mL for 2 hours) was used as a control.

Real-Time Quantitative Polymerase Chain Reaction

Real-time polymerase chain reaction (PCR) for nox1, nox2, and nox4 mRNA quantification was performed as described previously[13] and in the online supplement.

Results

Treatment of ECs With BMP4 Stimulates Monocyte Adhesion by ICAM-1 and ROS-Dependent Mechanisms

Recently, we have shown that BMP4 produced in ECs in response to OS acts as an autocrine, inflammatory cytokine, leading to ICAM-1 induction and subsequent monocyte adhesion.[13] In a separate, more recent study, we also have shown that exposure of ECs to OS stimulates ROS production from NADPH oxidases, which in turn results in monocyte adhesion.[16] These 2 findings have prompted the question of whether BMP4 produced in ECs by OS is directly responsible for production of ROS from NADPH oxidases, which then leads to ICAM-1 induction and monocyte binding.

Although we demonstrated previously that OS-induced monocyte adhesion can be prevented by the ICAM-1 blocking antibody (YN1),[13] it was not tested whether YN1 can also block BMP4-induced monocyte adhesion. Treatment of MAE-wt cells with BMP4 for 18 hours stimulated monocyte adhesion by 5- to 6-fold above that of vehicle control, which was completely prevented by YN1 antibody (Figure 1A). This further confirms our previous assumption that ICAM-1 expressed on EC surface plays a critical role in BMP4-induced monocyte adhesion.

Next, we determined whether BMP4-induced monocyte adhesion is regulated in an ROS-dependent manner by treating ECs with ROS scavengers. As shown in Figure 1B, treatment of MAE-wt cells with the cell-permeable polyethylene glycol (PEG)-catalase (converting H_2O_2 to H_2O) and Tiron (scavenging O_2^-) completely blocked BMP4-induced monocyte binding, demonstrating a role for ROS. Tumor

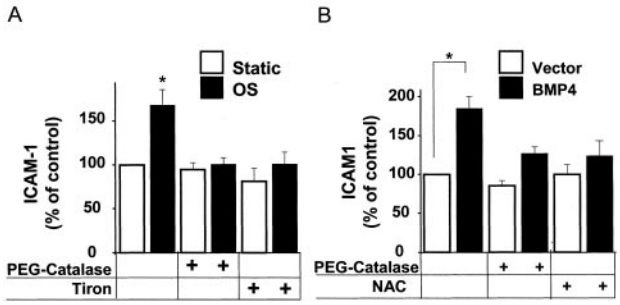

Figure 2. OS and BMP4 induce ICAM-1 expression in an ROS-dependent manner. A, HAECs that were pretreated with PEG-Catalase (100 U/mL for 18 hours), Tiron (10 μmol/L for 1 hour), or vehicle were exposed to OS ($\pm$5 dyne/cm^2) or static conditions for 1 day. B, HAECs were transfected with a BMP4 expression vector or an empty vector control. After transfection, cells were incubated with PEG-catalase (100 U/mL) or *N*-acetyl-cysteine (NAC; 20 mmol/L) for 10 hours. The expression of ICAM-1 on the treated (A and B) HAEC surface was determined by FACS analysis and expressed as percentage of control (mean$\pm$SEM; n=4 each; *$P<0.05$).

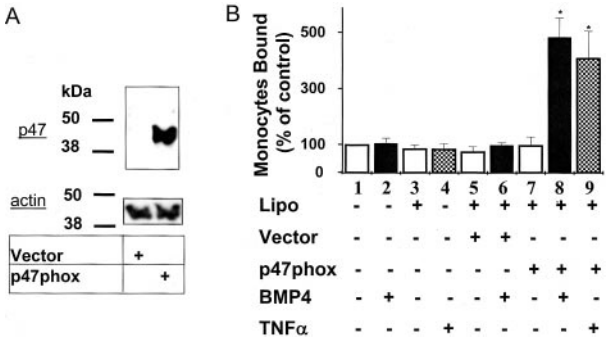

Figure 3. BMP4 stimulates monocyte adhesion in an NADPH oxidase–dependent manner. MAE-p47$^{-/-}$ cells were transfected with p47phox or an empty vector (Vector) control by using Lipofectamine 2000 (Lipo). A, One day after transfection, cell lysates were obtained and analyzed by Western blot using a p47-specific antibody. The same blot was stripped and reprobed with an actin antibody as a protein-loading control. The shown blot is representative of 4 independent experiments. B, Transfected cells were treated with BMP4 (20 ng/mL), TNF-α (100 U/mL), or vehicle control. After 18 hours, cells were washed, and monocyte binding was determined as in Figure 1. The bar graph shows the mean$\pm$SEM (n=4; *$P<0.05$).

necrosis factor-α (TNF-α), a well-known inducer of monocyte adhesion, was used in these studies as a positive control (Figure 1B).

OS and BMP4 Induce ICAM-1 Expression in an ROS-Dependent Manner

Although OS was shown to stimulate monocyte adhesion by inducing ICAM-1 expression on the EC surface in an ROS-dependent manner,[13,18] it was not known whether BMP4 would also induce ICAM-1 by the same mechanism. To address this question, we first determined a role of ROS in OS-induced ICAM-1 expression by using ROS scavengers. Exposure of HAECs to OS for 1 day increased surface expression of ICAM-1 by 68% above that of static conditions (Figure 2A). Inclusion of PEG-catalase or Tiron during shear exposure completely prevented the OS-induced ICAM-1 expression, demonstrating a critical role of ROS in this response (Figure 2A). Next, we determined whether ROS also played an important role in BMP4-dependent ICAM-1 expression on EC surface. As shown previously by us,[13] transfection of BMP4 in HAECs produces prepropeptide (54 kDa) and an active, secreted form (23 kDa) of BMP4, as determined by Western blot analysis using a monoclonal BMP4 antibody (data not shown). BMP4 overexpression stimulated ICAM-1 expression by 84% above that of empty vector control (Figure 2B). BMP4-induced ICAM-1 expression was significantly inhibited by either PEG-catalase or *N*-acetyl-cysteine, suggesting an important role for ROS.

NADPH Oxidases Play a Critical Role in BMP4-Induced Inflammatory Response of ECs

Using MAE-p47$^{-/-}$ cells and the ESR spin trap CMH, we demonstrated that OS stimulates monocyte adhesion by the mechanism dependent on ROS derived from p47phox-based NADPH oxidases.[16] Moreover, we were able to "rescue" the inflammatory responses of MAE-p47$^{-/-}$ cells in response to OS by transfecting them with p47phox cDNA.[16]

Here, we used the same approach to determine whether ROS derived from NADPH oxidases play a critical role in monocyte adhesion to ECs. As shown in Figure 3A, MAE-p47$^{-/-}$ cells expressed no detectable p47phox. However, transfection of the cells with p47phox cDNA[16] led to overexpression of the protein. As shown previously, treatment of MAE-p47$^{-/-}$ cells with BMP4 failed to stimulate monocyte adhesion (Figure 3B, compare lanes 1 and 2). In contrast, overexpression of p47phox protein in the MAE-p47$^{-/-}$ cells rescued the monocyte adhesion response, showing 4- to 5-fold increase above the control (Figure 3B, compare lanes 7 and 8). To ensure the rescued response was specifically attributable to p47phox transfection, several negative controls were performed by treating cells with Lipofectamine alone (Figure 3B, lane 3), TNF-α alone (Figure 3B, lane 4), and Lipofectamine+empty vector with or without BMP4 (Figure 3B, lanes 5 and 6). In addition, as a positive control, treatment of the rescued cells with TNF-α showed $\approx$4-fold increase in monocyte adhesion (Figure 3B, lane 9). These results demonstrate that BMP4 induces monocyte adhesion in an NADPH oxidase–dependent manner.

BMP4 Stimulates O_2^- and H_2O_2 Production in ECs

Because we have shown that OS stimulates production of O_2^- and H_2O_2,[13,19] we next examined whether BMP4 alone could also stimulate the ROS production in MAE cells. As shown in Figure 4A, treatment of MAE-wt cells with BMP4 for 20 hours stimulated O_2^- production by 1.8-fold above control ($P<0.004$), as determined by ESR spectroscopy of 3-methoxycarbonyl-proxyl (CM$^\bullet$). There was also a small (27%), but statistically significant ($P=0.004$) increase in O_2^- production above the control levels after 1 hour of BMP4 treatment. This assay was performed in the presence or absence of superoxide dismutase (SOD) to quantify the SOD-inhibitable amount of CM$^\bullet$, providing further specificity for the assay. In addition, BMP4 increased production of H_2O_2 in mouse aortic ECs (MAECs) by 70% above control ($P<0.05$) and in HAECs by 50% ($P<0.05$), as determined by

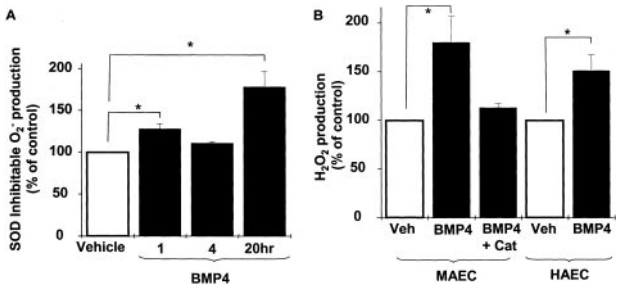

Figure 4. BMP4 stimulates ROS production. A, Immediately after treatment of MAE-wt cells with BMP4 (200 ng/mL) for 1, 4, or 20 hours, cells were trypsinized and reconstituted with the spin trap CMH with or without PEG-SOD (50 U/mL) to determine the specific O_2^- production (mean±SEM; n=3; *$P<0.05$). B, MAE-wt cells that were preincubated with or without PEG-catalase (100 U/mL for 1 day) were treated with BMP4 or vehicle (Veh) for 18 hours. In some studies, HAECs were treated with BMP4 (200 ng/mL) or vehicle for 18 hours. Cells were then washed, incubated with DCF-DA, and the fluorescent intensities determined by fluorometry. Shown is a mean±SEM (n=3; *$P<0.05$).

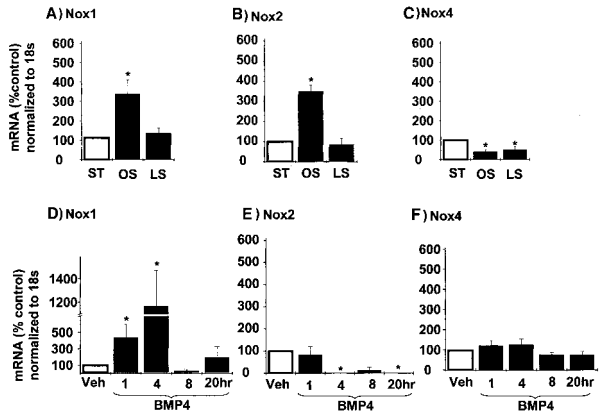

Figure 5. Regulation of nox by OS and BMP4. Total RNAs were extracted from MAECs that were exposed to either OS or LS for 20 hours or BMP4 for 1, 4, 8, or 20 hours (50 ng/mL). Then, mRNA copy numbers of nox1, nox2, and nox4 were quantified by real-time PCR and normalized against 18S copy numbers. Data were expressed as percentage of static (ST) or vehicle (Veh) control and mean±SEM (n=3; *$P<0.05$) are shown.

oxidation of DCF-DA (Figure 4B). BMP4-dependent H_2O_2 production was completely blocked if MAE-wt were incubated with cell-permeable PEG-catalase before and during BMP4 treatment, demonstrating the specificity of the assay (Figure 4B). These results demonstrate that BMP4 alone, much like OS, can stimulate production of O_2^- and H_2O_2 and in ECs.

Differential Regulation of Nox Homologue Expression by OS and BMP4

Next, we examined whether OS and BMP4 increase ROS production by upregulating expression levels of NADPH oxidase catalytic subunits. Because vascular ECs express 3 membrane-bound forms of NADPH oxidases (gp91phox, also known as nox2, and its homologues nox1 and nox4),[20] we examined their expression levels by the quantitative real-time PCR assay. In static cultured MAECs, nox4 mRNA was far more abundant than nox1 and nox2. In static conditions, nox1, nox2, and nox4 copy numbers were 11, 63, and 1242 per 10^8 18S copies, respectively, which is similar to previous findings.[20,21] Next, we determined which nox is regulated by shear stress and BMP4. As shown in Figure 5, OS increased nox1 and nox2 expression. Surprisingly, nox4 levels were significantly reduced by OS (Figure 5C). LS exposure decreased nox4 level by 51% without affecting nox1 and nox2.

BMP4 treatment of MAECs significantly upregulated only nox1 mRNA, whereas nox2 was downregulated, and nox4 was not significantly affected (Figure 5D through 5F). The BMP4 effect on nox1 induction was time dependent, increasing by 1 hour, reaching a maximum by 4 hours, and then returning toward the basal level by 24 hours. In contrast, BMP4 downregulated nox2 to a barely detectable level within 4 hours of treatment (Figure 5E).

The divergent effects of OS (upregulating nox1 and nox2) and BMP4 (upregulating nox1, while downregulating nox2) raise an interesting question regarding which nox would be important in ROS production and subsequent monocyte adhesion in response to OS. Because BMP4 alone can recapitulate OS-induced events from ROS production to monocyte adhesion, we hypothesized that nox1 is responsible for the BMP4-induced ROS production and inflammation. Nox2 may be involved in the proximal pathway leading to BMP4 production. In the next study, the nox1 hypothesis was examined by using an siRNA approach.

Nox1 siRNA Blocks OS-Induced Monocyte Binding

For this study, we transfected MAECs with nox1-specific siRNA and a nonsilencing siRNA (N-si) as a control. As shown in Figure 6A, in static control MAECs, nox1 siRNA (Nox1-si) reduced the nox1 mRNA level by 67% (n=6), whereas N-si did not. In addition, Nox1-si did not affect either nox2 or nox4 mRNA levels (Figure 6C and 6D), demonstrating its specificity for nox1. Next, we examined whether knocking down nox1 mRNA expression in MAECs could block OS-dependent monocyte adhesion. As shown in Figure 6B, Nox1-si completely prevented OS-induced monocyte adhesion compared with the N-si–treated cells (Figure 6B). This result, for the first time, identifies nox1 as a mechanosensitive NADPH oxidase, playing an essential role in the monocyte adhesion response in ECs.

BMP4 siRNA Blocks OS-Induced Monocyte Binding

Some of the evidence supporting the hypothesis that OS induces inflammatory responses in ECs by producing BMP4 is based on the use of the BMP antagonist noggin[13] (supplemental Figure S1). In addition, our current and previous findings[13] have shown that BMP4 alone can recapitulate these OS-dependent inflammatory responses. However, noggin could inhibit not only BMP4 but also other BMP family members, making it difficult to conclude the specific role of BMP4 in the OS-induced inflammatory responses. To address this, we examined the effect of a BMP4 siRNA on the OS-induced monocyte adhesion and ROS production. Treatment of MAECs with 50 nmol/L BMP4 siRNA significantly reduced the mature BMP4 level in the conditioned media by

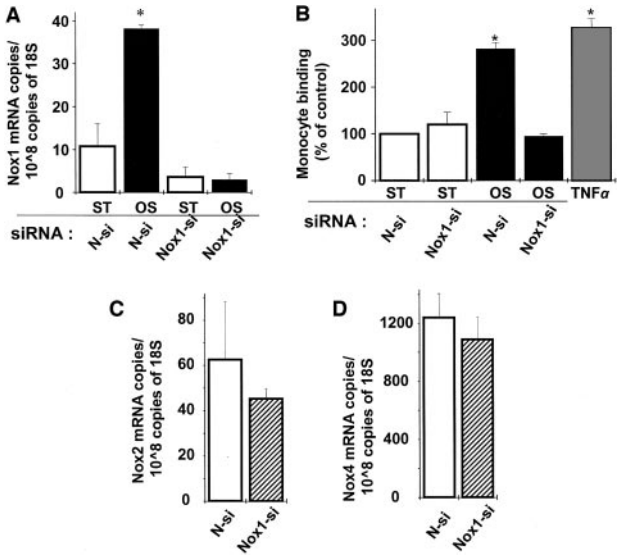

Figure 6. Nox1 silencing prevents monocyte adhesion induced by OS. A and B, MAECs were transfected with 100 nmol/L Nox1-si or N-si. Two days after transfection, cells were exposed to OS or static (ST) for 24 hours, followed by RNA extraction for real-time PCR assays (A) or monocyte binding assays using TNF-α as a positive control (B). Data shown in B are expressed as mean±SEM (n=3; *$P<0.05$). C and D, MAECs were transfected with Nox1-si or N-si, and total RNA was harvested for real-time PCR assays for nox2 (C) and nox4 mRNA quantification (D) as described in A. Data shown in A, C, and D are expressed as mRNA copy numbers of nox1, nox2, and nox4 per 10^8 copies of 18S (*$P<0.05$).

≈80% compared with that of a N-si (Figure 7A). Under the same conditions, BMP4 siRNA but not N-si completely blocked OS-induced monocyte adhesion (Figure 7B). In addition, the BMP4 siRNA significantly reduced the ROS production in both static and sheared cells by 37% and 46% of the respective nonsilencing controls (Figure 7C). These results demonstrate that endothelial BMP4 produced in response to OS is the critical BMP member responsible for ROS monocyte adhesion and ROS production.

Discussion

The novel findings of this study are that (1) BMP4 stimulates ROS production by the mechanisms dependent on p47phox-based NADPH oxidases; (2) ROS produced in response to BMP4 plays a critical role in 2 inflammatory responses of ECs: surface expression of ICAM-1 and monocyte adhesion; (3) OS increases nox1 and nox2 mRNA expression while decreasing nox4; (4) BMP4 increases only nox1 mRNA level; (5) knocking down nox1 blocks OS-induced monocyte adhesion; and (6) knocking down BMP4 inhibits OS-induced ROS production and prevents monocyte adhesion. Collectively, these novel findings strongly suggest that BMP4, produced in ECs in response to OS, acts as a proinflammatory cytokine by stimulating ROS production in a nox1-dependent manner, eventually leading to the monocyte adhesion response.

Atherosclerosis is now well known to be an inflammatory disease preferentially occurring in lesion-prone areas associated with unstable shear stress in branched or curved arteries. In addition, increased levels of ROS have been strongly implicated in atherosclerotic plaque development.[21] Recently, we have shown that chronic exposure (1 day) of ECs to OS stimulates production of ROS (O_2^- and H_2O_2) in an NADPH oxidase–dependent manner,[16,19] which in turn led to an inflammatory response as determined by monocyte adhesion.[16] In our previous study,[16] we found that the monocyte adhesion response of ECs in response to OS was only observed after a chronic exposure (≥18 hours), but not by a shorter exposure (4 hours), suggesting a requirement for de novo synthesis of proteins such as BMP4. Consistent with this idea, we recently provided evidence that BMP4 induction, requiring several hours of OS exposure, is responsible for triggering the monocyte adhesion response in ECs.[13] Furthermore, our current data showing a significant increase in ROS production after 20 hours of BMP4 treatment (Figure 4) may be attributable to an increase in nox1 mRNA level (Figure 5).

More recently, we have shown that MAE-p47$^{-/-}$ cells do not produce O_2^- and fail to induce monocyte adhesion in response to OS.[16] In that study, we were able to rescue or restore OS-dependent O_2^- production and monocyte adhesion by transfecting MAE-p47$^{-/-}$ cells with p47phox vector.[16] These results suggested that OS stimulates p47phox-based NADPH oxidases to produce ROS, which then leads to the subsequent monocyte adhesion response. However, at the time, it was not known whether BMP4 was involved in this ROS-dependent response and which nox type was responsible for this pathway.

In the current study, using the same molecular rescue approach, we were able to restore the BMP4-dependent monocyte adhesion to the MAE-p47$^{-/-}$ cells by re-expressing

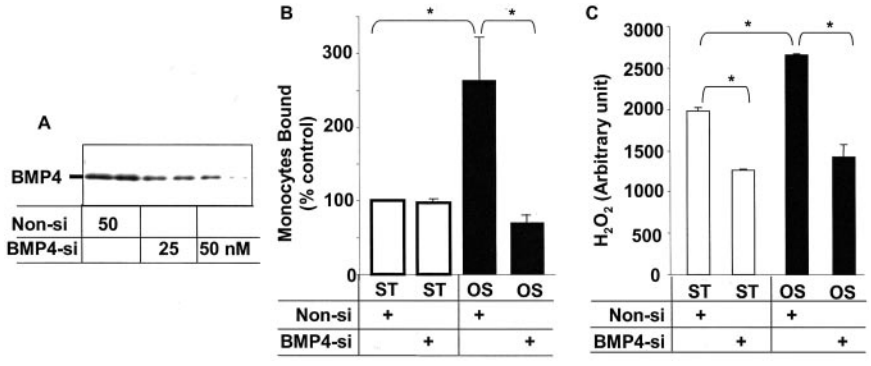

Figure 7. BMP4 siRNA inhibits monocyte adhesion and ROS production induced by OS. A, Three days after transfection of MAECs with BMP4 siRNA (BMP4-si) or N-si (50 nmol/L), the conditioned media were collected and analyzed by Western blot with a BMP4 antibody. B and C, After 3 days of transfection with BMP4-si or N-si (50 nmol/L), MAECs were exposed to OS or static (ST) conditions for 20 hours, and monocyte adhesion and ROS production were determined as described in Figure 4. Data in B are expressed as percentage change over N-si group (mean±SEM; n=3; *$P<0.05$). Data in C represent DCF fluorescence intensity (mean±SEM; n=3 to 4; *$P<0.05$).

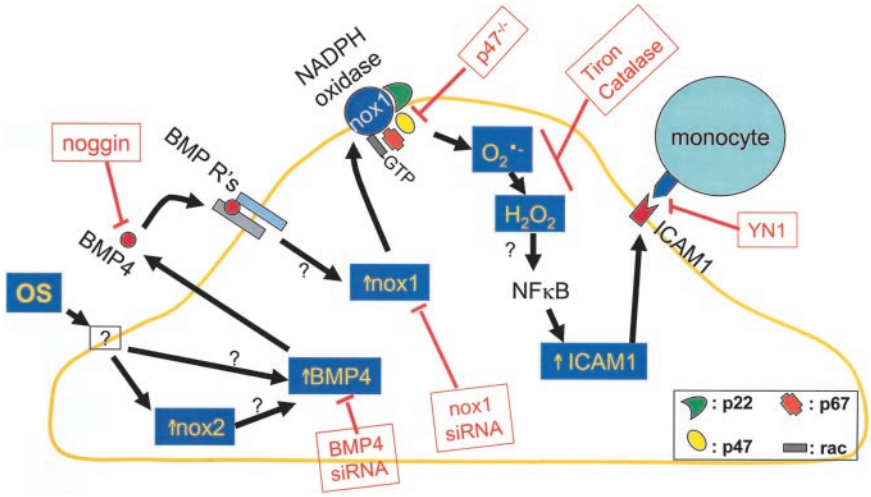

Figure 8. Working Hypothesis, ECs in lesion-prone areas are exposed to disturbed flow conditions including OS. OS induces BMP4 expression, which stimulates production of ROS in a nox1- and p47^{phox}-dependent manner. ROS then stimulates ICAM-1 expression and monocyte binding, leading to foam cell formation and atherosclerosis.

p47phox (Figure 3B). We also directly demonstrated that BMP4 alone can stimulate O_2^- and H_2O_2 production in ECs (Figure 4A and 4B). Moreover, we were also able to block OS-induced ROS production and monocyte adhesion by using either noggin[13] or the BMP4 siRNA (Figure 7; supplemental Figure S1). Together, these results demonstrate a critical role of BMP4 in mediating the OS-dependent ROS production and inflammatory responses.

What is the mechanism underlying ROS production in response to OS and BMP4? It is likely that ROS production stimulated by BMP4 and OS (by upregulating BMP4) would involve activation of enzyme activities and increased expression of the components of NADPH oxidases. ECs express several NADPH oxidases that are composed of multiple subunits. Strong evidence indicates that they express the classical neutrophil oxidase, comprised of gp91phox (also known as nox2) and p22phox in the membrane, and p47phox, p67phox, and Rac in the cytosol.[6,22] Recent work[21,23] and the current study (Figures 5 and 6) have shown that MAECs also express nox1 and nox4. Importantly, p47phox can regulate nox1 and nox2,[24,25] suggesting that these 2 NADPH oxidase homologues are candidates for the OS- and BMP4-dependent oxidase, based on the ability of p47phox to reconstitute the response in p47phox-deficeint MAECs (Figure 3).[16] Although nox1 and nox2 are upregulated by OS, only nox1 was upregulated by BMP4 (Figure 5). Interestingly, Nox1-si blocked OS-induced monocyte adhesion. Together, these results suggest that nox1 plays a role as a distal step after BMP4 induction in response to OS. The role of nox2 is not clear at the moment. It is possible that nox2 may be an upstream regulator of BMP4 induction or, perhaps because of restricted subcellular localization, it may regulate OS-stimulated responses other than monocyte adhesion.

Recently, Hwang et al[23] have shown that OS (created by a parallel plate system) increases nox2 and nox4 mRNA levels in bovine aortic ECs. Although their OS effect on nox2 is consistent with the current study, its effect on nox4 is not. This discrepancy between the 2 studies may be at least partially attributable to the different species (bovine versus mouse), different shear device (parallel plate versus cone-and-plate), and the different shear exposure times (20 hours versus 6 hours). Furthermore, these authors[23] did not examine nox1 levels. Although our study clearly establishes nox1 as the mediator of BMP4-dependent monocyte adhesion in response to OS, the functions of nox2 and nox4 remain to be determined.

In atherosclerotic conditions, nox1, nox2, and nox4 have all been implicated. Human atherosclerotic lesions contain increased levels of ROS mainly because of NADPH oxidases.[21,26,27] Nox4 is abundantly expressed in the intimal, medial, and adventitial layers of nonatherosclerotic and atherosclerotic coronary arteries.[21] Nox2 expression associated with plaque macrophages was also reported in the same study. In contrast, nox1 levels are very low[21] or undetectable in some studies.[28] In a rabbit carotid injury model, an increase in nox2 level was found in the neointima.[28] The most dramatic time-dependent changes in nox types have been demonstrated in a rat carotid balloon-injury model during restenosis development. Immediately after the carotid injury, nox1 level was increased (within 3 days), whereas nox4 level remained unchanged for the first 7 days but went up in the late phase (15 days after the injury). In the same study, a nox2 increase was noted 7 days after the injury. It is also interesting to note that nox1 has been implicated in inflammatory conditions,[29,30] given the role of nox1 in OS and BMP4-induced monocyte adhesion. Although these in vivo studies, especially human atherosclerosis studies, demonstrate the expression of all 3 nox types, it has been difficult to establish their quantitative changes in a cell-specific manner. Our current study begins to demonstrate the changes of each nox type in ECs in response to pathophysiologically important atherogenic stimuli: OS and BMP4.

Based on the current data and literature, we propose that BMP4 is a critical mechanosensitive autocrine cytokine, triggering proinflammatory and proatherogenic responses of ECs by increasing ROS production from NADPH oxidases as described in Figure 8. The ECs in lesion-prone areas experience unstable flow conditions such as low shear stress and OS, which induce BMP4 expression through undefined mechanisms. OS-induced nox2 expression may play a role in this step. BMP4 produced in ECs, acting as an autocrine factor and binding to the BMP receptors, stimulates ROS

(O_2^- and H_2O_2) production by the mechanisms critically dependent on nox1 induction and the activity of nox1- and p47phox-based NADPH oxidase. In addition, ROS derived from NADPH oxidases seems to act as a "kindling" radical, promoting more ROS production from another enzyme: xanthine oxidase.[19] The ROS then initiate the inflammatory cascades, stimulating ICAM-1 expression on the EC surface in an NF-κB–dependent manner, subsequently leading to monocyte adhesion. The monocytes recruited in the lesion-prone areas then become foam cells, eventually leading to atherosclerotic plaques. In contrast, LS in straight arterial regions acts as a potent anti-inflammatory and antiatherogenic force by inhibiting expression of BMP4 and VCAM-1 in ECs.[13]

In summary, the current study provides a novel mechanism involving ROS, derived from the nox1-based NADPH oxidase, through which BMP4 acts as a critical and essential cytokine mediating the proinflammatory and proatherogenic effects of OS. The discovery of BMP4 as a mechanosensitive, proinflammatory cytokine stimulating ROS production provides not only a better understanding of the role of shear stress in vascular biology and pathophysiology but also an opportunity for development of diagnostic and therapeutic approaches.

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Bone Morphogenic Protein 4 Produced in Endothelial Cells by Oscillatory Shear Stress Induces Monocyte Adhesion by Stimulating Reactive Oxygen Species Production From a Nox1-Based NADPH Oxidase

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Expanded Materials and Methods

Endothelial Cells and Shear Studies: Endothelial cells obtained from the thoracic aortas of C57/BL6 control (MAE-wt) and $p47^{phox}$-null mice (MAE-$p47^{-/-}$) were isolated and cultured as described previously[1], and used between passages 4-10. Human aortic endothelial cells (HAEC) purchased from Clonetics were cultured in EBM-2 bullet kit (Clonetics) and used between passages 5-8 as described by us[2]. Confluent endothelial cells grown in 100 mm tissue culture dishes (Falcon) were exposed to OS (± 5 dyn/cm^2 at 1 Hz frequency) or static control conditions for 1 day using a cone-and-plate device as described by us[2].

Monocyte Adhesion Assay: Following OS or BMP4 treatment, binding of THP-1 monocytes to MAE cells was determined as described previously[1,2].

Transfection of p47phox: In some studies, MAE-$p47^{-/-}$ cells were transfected with p47phox expression vector or an empty vector control as described[1].

ICAM-1 Expression: Surface expression of ICAM-1 in HAEC was determined by fluorescence activated cell sorting (FACS) as described by us[2]. In some studies, HAEC were transfected with BMP4 cloned in a bi-cistronic pAdTrack CMV vector or an empty vector, both expressing green fluorescent protein (GFP), using Lipofectamine 2000[2]. As shown previously by us[2], HAEC produced and secreted active form of BMP4 into the culture medium under this condition (20-30% transfection efficiency as determined by fluorescence microscopy). In these experiments, ICAM-1 expression was measured in the red phycoerytherin channel, while the green channel was used to monitor the GFP expression. GFP expression was determined by FACS analysis. Because expression of

GFP was similar among different treatment groups within the same experiment, we did not need to normalize ICAM-1 expression data to GFP level.

H_2O_2 assay: Cells grown in 6-well plates were treated with OS, BMP4 or vehicle control for 18 hrs in the presence or absence of cell-permeable polyethylene glycol (PEG)-catalase (100 U/ml). Cells were then washed once with Hepes buffered saline solution (HBSS) followed by incubation with 7.5 μg/ml 2', 7'-dichlorofluorescein diacetate (DCF-DA, Molecular Probes) in HBSS for 1 hr at 37C^o in the dark[3]. Cells were washed with HBSS, incubated further for an additional 40 min in HBSS, and the DCF fluorescence intensities were measured in a Cytofluor Multi-Well plate reader (excitation at 475 nm and emission at 525 nm).

O_2^- Assay: O_2^- production was determined by electron spin resonance spectroscopy using methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine (CMH) as a spin probe in the presence or absence of PEG-superoxide dismutase (PEG-SOD; 50 U/ml) to quantify the SOD-inhibitable portions of CM• as described by us[1].

Small interference RNA (siRNA) Nox1 silencing experiments. To knockdown mouse nox-1 and BMP4 mRNAs, the annealed siRNA duplexes for nox1 (sense: 5'-GGAAUUAUUUUGAGCUCUUtt -3', antisense: 5'- AAGAGCUCAAAAUAAUUCCtg -3') and for BMP4 (sense: 5'GGCGACACUUCUACAGAUGtt, antisense: 5'-CAUCUGUAGAAGUGUCGCCtc), respectively, were purchased from Ambion. Non-silencing siRNA duplexes (sense: 5'-UUCUCCGAACGUGUCACGUtt-3', antisense: ACGUGACACGUUCGGAGAAtt-3') were purchased from Qiagen. MAEC were transfected at a final siRNA duplex concentration of 25 to 100 nM using Oligofectamine (Invitrogen, Carlsbad, CA, USA) in serum free medium. After 6 hrs, the medium was supplemented with fetal bovine serum (final 10% concentration). Following transfection

(48 hrs for nox1 or 72 hrs for BMP4 siRNA's), the cells were exposed to either oscillatory shear flow (±5 dynes/ cm^2) or static condition for 24 hrs. Nox1 mRNA knock down was confirmed with real-time quantitative PCR. BMP4 knockdown was confirmed by Western blot analysis of conditioned media using serum-free DMEM and cell lysates as we previously described[2].

Real time quantitative polymerase chain reaction (PCR). Real time PCR for nox1, nox2 and nox4 mRNA quantification was carried out as previously described[2]. Briefly, 4 μg of total RNA was reverse transcribed by using random primers and a Superscript-II kit (Life Technology) to synthesize first-strand cDNA. The cDNA was assayed by real time PCR using a LightCycler (Roche). Copy numbers were determined based on standard curves generated with murine standards for nox1, nox2 and nox4 and 18S templates. The following primers were used to amplify mouse nox1 (5' to 3')- forward: CGCTCCCAGCAGAAGGTCGTGATTACCAAGG and reverse: GGAGTGACCCCAATCCCTGCCCCAACCA, for mouse nox2- forward: CCACATACAGGCCCCCTTCAG and reverse: GTTGGGGCTGAATGTCTTCCTCTTT mouse nox4- forward: CTGGTCTGACGGGTGTCTGCATGGTG and reverse: CTCCGCACAATAAAGGCACAAAGGTCCAG. Real-time PCR for nox1, nox2 and nox4 was carried out using the annealing temperature 67ºC, 58ºC and 65ºC, respectively, using recombinant Taq polymerase (Life Technology), and Taq start antibody (Clontech) and SYBR green detection of products. DNA gels were ran to confirm specificity of PCR products.

Materials: Mouse recombinant BMP4 and human recombinant noggin were purchased from R&D Systems, polyethylene glycol (PEG)-catalase, PEG-superoxide dismutase

(SOD) and Tiron were obtained from Sigma. YN1 antibody and Lipofectamine 2000 were purchased from Southern Biotechnology and from InVitrogen, respectively.

Statistical Analysis: Data are reported as average ± SEM obtained from at least 3 independent studies. Statistical significance was assessed by Student's t-test using a Microcal Origin statistical package.

Supplemental Figure S1. The BMP antagonist, noggin, blocks OS-induced O_2^- production.

Confluent MAE-wt cells were treated with OS (± 5 dyn/cm^2) or static controls for 1 day in the presence or absence of noggin (50 ng/ml). Following shear exposure, SOD-inhibitable O_2^- production was determined by ESR using CMH. Shown is a mean ± SEM (n=4, $^*p < 0.05$).

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Figure S1