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Forum Review

Role of NADPH Oxidases in Disturbed Flow- and BMP4-Induced Inflammation and Atherosclerosis

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ABSTRACT

Atherosclerosis is an inflammatory disease, occurring preferentially in branched or curved arterial regions exposed to disturbed flow conditions including oscillatory shear stress (OS). In contrast, straight portions exposed to undisturbed laminar shear stress (LS) are relatively lesion free. The opposite effects of atheroprotective LS and proatherogenic OS are likely to be determined by differential expression of genes and proteins, including redox regulating factors. OS induces inflammation via mechanisms involving increased reactive oxygen species (ROS) production from the NADPH oxidases. Through a transcript profiling study and subsequent verification and functional studies, the authors discovered that OS induces inflammation by producing bone morphogenic protein 4 (BMP4) in endothelial cells. BMP4 stimulates expression and activity of NADPH oxidase requiring p47phox and Nox-1 in an autocrine-like manner. The NADPH oxidase activation by BMP4 then leads to ROS production, NF-κB activation, intercellular adhesion molecule 1 (ICAM-1) expression, and subsequent increased monocyte adhesivity of endothelial cells. It is proposed that endothelial NADPH oxidases play a critical role in disturbed flow- and BMP4-dependent inflammation, which is the critical early atherogenic response occurring in atheroprone areas. This emerging field of shear stress, BMP4, NADPH oxidases, inflammation, and atherosclerosis is reviewed. *Antioxid. Redox Signal.* 8, 1609–1619.

ATHEROSCLEROSIS OCCURS PREFERENTIALLY IN HEMODYNAMICALLY DISTURBED ATHEROPRONE AREAS

ATHEROSCLEROSIS IS THE MAJOR CONTRIBUTING FACTOR to cardiovascular disease and involves the interaction of multiple risk factors that include hypercholesterolemia, hypertension, diabetes, and smoking (15, 67, 85, 100). One of the characteristics of atherosclerosis is its focal developmental patterns in branched and curved areas of the arteries, even though most of the risk factors such as systemic levels of serum cholesterol are not locally limited. One of the most pertinent factors that determine the focal atherosclerotic plaque development in these areas is believed to be disturbed flow dynamics.

The blood vessel wall is exposed to several mechanical forces, blood pressure, shear stress, and circumferential stress during the cardiac cycle. Compressive stress is due to blood pressure. Circumferential stress occurs in response to the pulsatility of blood flow. Shear stress is the tangential frictional force imparted on endothelial cells caused by blood flow (14, 30, 79). Shear stress is particularly important to the vascular endothelium. Endothelial cells are in direct contact with the blood and are therefore continually exposed to a dragging force as this fluid moves over them.

Branched, bifurcated, and curved arteries, such as the lesser curvature of the ascending aorta, the outer wall across the apex of the carotid sinus, and the left descending coronary arteries, experience "disturbed" shear stress conditions. It is in these regions (atheroprone regions) where early atherosclerotic lesions are preferentially found (14, 30, 56). Due to complex

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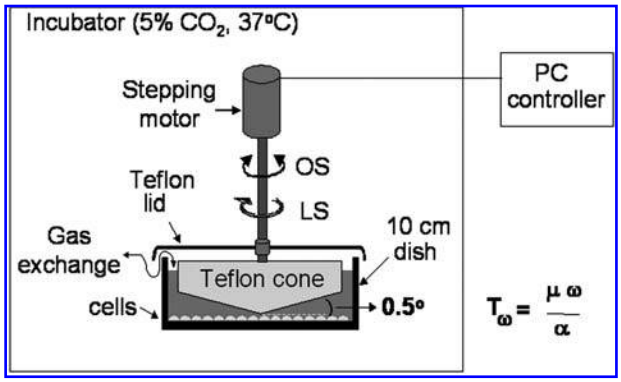

FIG. 1. Cone-and-plate shear device. Shown is a schematic of the modified cone-and-plate shear apparatus (32). The cone has a fixed 0.5° angle and is machined out of Teflon block. The entire shear system except for the PC controller unit is housed in a humidified tissue culture incubator (5% CO_2, 37°C). The cone is rotated back and forth or unidirectionally through an in-house computer program and a stepping motor to generate OS and LS, respectively, or to mimic *in vivo* flow profiles. The shear stress level (Tω) experienced by endothelial cells is controlled by viscosity (μ), angular velocity (ω), and is inversely proportional to cone angle (α). The shear system is designed to be used with a 10 cm tissue culture dish (Falcon) and the Teflon lid is designed to allow free gas-exchange between the cells and the incubator environment, while preventing contaminations.

arterial geometry and pulsatile blood flow during the cardiac cycle, these lesion-prone areas experience disturbed shear conditions including temporal and spatial gradients of wall shear stress over relatively short distances, flow reversal, and flow separation, leading to a low time-averaged shear stress with flow reversal and oscillation (OS) (12, 30, 56). In contrast, straight portions of arteries are exposed to relatively uniform, well-developed stable laminar flows (LS) and are well protected from atherosclerotic plaque development (14, 30, 56).

To investigate *in vivo* correlations that endothelial cells act as mechanosensors of local hemodynamic conditions, several *in vitro* systems have been developed (84). Devices such as the parallel plate flow chamber, vertical step flow chamber, cone-and-plate, and modified cone-and-plate shear apparatus have allowed for controlled experiments on cultured endothelial cells (6, 12, 39, 84, 90). Although we previously used a parallel plate flow chamber (51), we have been preferentially using the modified cone-and-plate shear apparatus for the past 6–7 years, as shown in Fig. 1 (32, 90).

The mechanisms by which undisturbed and disturbed flows act as potent atheroprotective and proatherogenic forces, respectively, have been the subject of intense studies in this field. This review will focus on emerging evidence that shear stress is an essential regulator of inflammatory response in endothelial cells, which is the critical early step in atherogenesis.

ATHEROSCLEROSIS IS AN INFLAMMATORY DISEASE

Atherosclerosis is now viewed as an inflammatory disease, preferentially occurring in the lesion-prone areas exposed to disturbed and low shear stress (67, 77, 85, 94). The earliest measurable markers of atherogenesis include expression of inflammatory adhesion molecules such as E-selectin, vascular cell adhesion molecule-1 (VCAM-1), and intracellular adhesion molecule-1 (ICAM-1), and subsequent monocyte adhesion and recruitment into the lesion-prone areas (11, 26, 85). Repeated insults of endothelial cells to various atherogenic risk factors lead to endothelial dysfunction and increased expression of adhesion molecules, including ICAM-1, VCAM-1, and E-selectin in the lesion-prone areas (11, 67).

Circulating blood monocytes then bind to these adhesion molecules, migrate beneath endothelium, engulf lipids, and transform into macrophage-derived foam cells, eventually becoming the site of advanced atherosclerotic plaques (67, 85). Evidence from mouse atherosclerosis models lacking expression of ICAM-1 or VCAM-1 has shown the importance of both adhesion molecules (8, 11). Each adhesion molecule appears to show different sensitivity toward the humoral and mechanical stimuli. For example, ICAM-1 expression seems to be regulated mainly by unstable shear stress (78). On the other hand, VCAM-1 expression is highly sensitive to LS and hypercholesterolemic conditions. VCAM-1 expression is upregulated by high-fat diet in the low-density lipoprotein receptor knockout mouse model of atherosclerosis (11, 78). Endothelial cells cultured under OS or no flow (static) conditions express easily detectable amounts of VCAM-1. LS exposure, however, significantly inhibits VCAM-1 expression by the NO-dependent mechanism (90, 96). Increased plasma levels of C-reactive protein and soluble ICAM-1 and VCAM-1 are also well-known inflammatory markers (85). Additional critical atherogenic events such as the loss of bioavailable NO production and an increase in reactive oxygen species (ROS) levels, including superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and peroxynitrite ($ONOO^-$) (11, 26), may also be viewed as a part of inflammation either directly or indirectly.

OSCILLATORY SHEAR STRESS IS PROATHEROGENIC WHILE LAMINAR SHEAR IS ATHEROPROTECTIVE

Shear stress controls cellular structure and function, including regulation of vascular tone and diameter, vessel wall remodeling, hemostasis, and inflammatory responses, as summarized in Fig. 2 (14, 23, 59, 95). Exposure of endothelial cells to LS has been shown to induce atheroprotective responses by inhibiting thrombosis, adhesion of platelets and monocytes to endothelium, and apoptosis of endothelial cells (95). In contrast, exposure to OS induces pro-inflammatory and proatherogenic responses including thrombosis, leukocyte adhesion, and apoptosis of endothelial cells (95). Mediators of these responses include intracellular ions (Ca^{2+} and K^+), vasoactive molecules (NO and $O_2^{\bullet -}$), growth factors, and adhesion molecules (14).

Vasoactive factors released from endothelial cells in response to LS and OS have profound effects on the function of smooth muscle cells as well. For example, NO and TGFβ released by LS inhibits smooth muscle cell proliferation, whereas angiotensin II (AngII), PDGF, and endothelin-1 stimulate it (95). The list of vasoactive factors produced in endothelial cells in re-

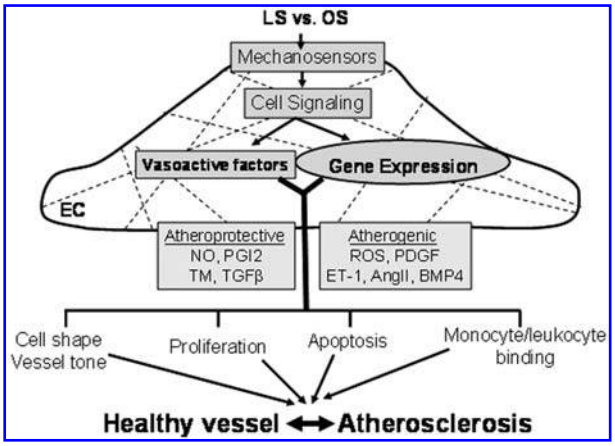

FIG. 2. Differential effects of LS and OS on endothelial cell function and atherosclerosis. Shown is a schematic diagram of an endothelial cell with cytoskeletons. LS and OS are recognized by endothelial mechanosensing systems including cytoskeleton, integrins, cell–cell junction, caveolae, cell surface glycocalyx, G-proteins, and ion channels. Mechanosignals then initiate signaling cascades regulating production of vasoactive factors. LS and OS stimulate production of atheroprotective and atherogenic factors, respectively, and the balance determines whether the arterial wall maintains healthy vessels or develop atherosclerotic plaques. PGI2, prostacyclin; TM, thrombomodulin; ET-1, endothelin-1.

sponse to different shear conditions is likely to grow. Indeed, the opposite effects of LS and OS may be determined in part by differential expression of vasoactive genes and proteins (2, 29).

Induction of the mechanosensitive genes is likely to be mediated by transcription factors (*e.g.,* NF-κB, SMAD, AP-1, early growth response-1, *c*-Jun, *c*-fos, and *c*-myc) (47, 55, 57, 86, 93) that are regulated by upstream signaling molecules including the family of mitogen activated protein kinases (MAPKs), extracellular signal regulated kinase (ERK), *c*-Jun N-terminal kinase (JNK), p38 MAP kinase (p38 MAPK), and Big MAP kinase-1 (47, 55, 57, 88, 93, 99). NF-κB (comprised of two subunits p65 and p50) is retained in the cytosol by being bound to the inhibitor of κB (IκB) in basal state. Upon activation in response to NF-κB-activating signals, IκB is phosphorylated and subsequently degraded. This releases NF-κB to translocate to nucleus and stimulate transcription.

OSCILLATORY SHEAR STRESS INCREASES REACTIVE OXYGEN SPECIES AND INFLAMMATION IN AN NADPH-OXIDASE DEPENDENT MANNER IN ENDOTHELIAL CELLS

Oxidative stress has been implicated in the development of numerous diseases, including premature aging, cancer, neurodegenerative diseases, and atherosclerosis (28). The reactive oxygen species (ROS) superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and peroxynitrite ($ONOO^{\bullet-}$) contribute significantly to such stress and have been intimately linked to atherogenesis via inflammatory responses that result from disturbed flow conditions (17, 31, 34). We have previously shown that OS increases ROS levels in endothelial cells, whereas LS reduces ROS compared to static controls. OS stimulation of ROS leads to ICAM-1 expression and monocyte adhesion, an early and critical atherogenic event (49, 89).

ROS arise from several sources in the endothelium, including NADPH oxidase, xanthine oxidase, mitochondrial oxidase, cytochrome P450, and uncoupled nitric oxide synthase. NADPH oxidase and xanthine oxidase contribute most significantly to the oxidative state. Accumulating $O_2^{\bullet-}$ and H_2O_2 produced by these oxidases can further shift the redox balance by reducing the bioavailability of $NO^{\bullet-}$, which is transformed to $ONOO^{\bullet-}$. Together these ROS can mediate inflammatory pathways via increased expression of adhesion molecules, NF-κB activation, and monocyte adhesion (92, 98). In addition, ROS generated by the endothelium promote atherogenic signaling mechanisms in the vascular wall. It has been shown that NADPH oxidase-dependent oxidative stress induces smooth muscle cell proliferation (7, 36). Also, reduction of $NO^{\bullet-}$ bioavailability results in hypertension by attenuating endothelium-dependent vessel dilation (76). $O_2^{\bullet-}$ and the $NO^{\bullet-}$ derivative $ONOO^{\bullet-}$ act to directly oxidize lipid proteins, specifically LDL to oxLDL (38, 62). oxLDL can promote apoptosis, smooth muscle cell proliferation, and also act as a powerful monocyte chemoattractant, upregulating proatherogenic molecules such as MCP-1, IL-1β, LOX-1, and PAI-1 (10, 61, 80, 82).

NADPH oxidase was originally found in macrophages, that are the major source of O_2^- in the circulatory system. However, vascular endothelial cells, smooth muscle cells, and fibroblasts also express functional leukocyte-type NADPH oxidases, termed nonphagocytic NADPH oxidases (Nox) (35, 52). The phagocytic NADPH oxidase is comprised of a membrane compartment, flavocytochrome b_{558}, consisting of $gp91^{phox}$ (now termed as Nox2) and $p22^{phox}$, and several regulatory cytosolic subunits including $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and the small GTPase rac1 or rac2 (65). Similar to their leukocyte counterpart, nonphagocytic NADPH oxidases are composed of membrane compartments and cytosolic components. Five Nox proteins have been found (Nox1, 2, 3, 4, and 5) (5, 91). Unlike phagocytic NADPH oxidase, which is mainly located in cellular membranes, recent studies have suggested some of the nonphagocytic NADPH oxidases may exist in a preassembled form in the perinuclear region of the endothelial cell, as well as in the cytoskeleton and caveolae of the vascular smooth muscle cell (40, 64).

Endothelial and vascular NADPH oxidases can be activated by numerous mechanical or biochemical stimuli, including shear stress, pulsatile stretch, angiotensin II, low density lipoprotein, thrombin, TNF-α, PDGF, endothelin, and arachidonic acid to induce atherosclerotic development (18, 25, 33, 41, 42, 44, 45, 48, 70, 89, 101). Other studies, which show that NADPH oxidase-derived $O_2^{\bullet-}$ can mediate hypercholesterolemia and diabetes, further underline the regulatory role of $O_2^{\bullet-}$ in the development of atherosclerosis-related disease pathologies (37). Furthermore, angiotensin II-mediated NADPH-derived $O_2^{\bullet-}$ production can lead to vascular hypertension (33, 81). Angiotensin II and PDGF stimulated NADPH-derived $O_2^{\bullet-}$ production results in increased production of MCP-1, an important chemoattractant molecule (4, 70). Therefore, it is evident that the ROS derived from NADPH oxidases of the vascular wall are important in atherosclerosis.

DIFFERENTIAL EFFECT OF LAMINAR AND OSCILLATORY SHEAR STRESSES ON ENDOTHELIAL GENE EXPRESSION PROFILES

The mechanisms by which different flow conditions—high levels of undisturbed LS and disturbed flow conditions including low and OS—prevent or induce inflammation and atherosclerosis have been the topic of intense studies in recent years. Exposure of endothelial cells to different flow conditions is likely to turn on or off genes and proteins, which in turn are responsible for the atheroprotective or atherogenic responses. For example, transcriptional regulation of platelet-derived growth factor, endothelial NOS, Cu/Zn superoxide dismutase (SOD), cyclooxygenase-2, and other shear-sensitive genes has been well described (18, 23, 30, 46, 47, 50, 68, 83, 99). Furthermore, several groups, including us (2, 3, 15, 29, 73, 90), have carried out DNA microarray studies to systematically identify mechanosensitive genes, which change in response to LS, OS, or turbulent flow, in endothelial cells. Through our DNA microarray studies and the subsequent functional studies, we have discovered one mechanosensitive gene, BMP4, that acts as a potent inflammatory cytokine (90). These findings are summarized in this review.

BMP4 PRODUCED IN ENDOTHELIAL CELLS IS A NOVEL MECHANOSENSITIVE PRO-INFLAMMATORY CYTOKINE

The gene chip result was confirmed by several independent methods at the levels of mRNA (quantitative real-time PCR) and protein (Western blot analysis and immunohistochemistry) in cultured endothelial cells, as well as in human coronary arteries. BMP4 protein is easily detected in endothelial cells cultured under static conditions. Expression is increased further by OS (1 day) and inhibited by LS exposure (1 day) (90). The significance of the shear-sensitive BMP4 expression was also supported by the finding that BMP4 protein is expressed in the endothelial cells overlying foam cell lesions, but not in the normal "minimally diseased" areas, of the human coronary arteries (90). Furthermore, ICAM1 staining, but not VCAM-1, is selectively increased in the similar endothelial patches expressing BMP4. While the immunohistochemical detection of BMP4 protein needs additional studies such as an *in situ* mRNA hybridization to examine whether BMP4 is indeed produced by endothelial cells, these results are consistent with previous findings reported in another study using the human carotid arteries with atherosclerosis (26).

BMP4, BMP RECEPTORS, AND SIGNALING

BMP4 and BMP antagonists

BMP was originally discovered as a bone-inducing protein (66). Its critical and diverse role roles now include embryonic development, patterning, cartilage formation, and cell differentiation (43, 72). The BMPs are members of the transforming growth factor-β (TGF-β) super family. This super family consists of TGF-βs, inhibins, bone morphogenetic proteins, growth differentiation factors, antimullerian hormone, activins, and myostatin (72). More than 30 BMP proteins have been identified, and the BMP2/4 and BMP5/6/7 classes are the best characterized members.

BMP4 has been identified as a protein that induces ectopic bone and cartilage formation when implanted in rats (72). It is synthesized as a 408 amino acid precursor (pre-pro-precursor) that is proteolytically cleaved in the Golgi apparatus by pre-proconvertases that recognize the motif RRXR (24), such as furin (9), leaving a C-terminal mature protein (116 amino acids) that has seven conserved cysteine residues. Unlike TGF-β, BMPs are secreted as active proteins and their activities are counterbalanced by secreted antagonists, such as noggin and chordin (71). BMP4 directly binds with high affinity to chordin (K_d = 300 p*M*) or noggin (K_d = 20 p*M*) (102). Binding of BMP4 to noggin or chordin prevents it from binding to the cognate receptor. Although BMP7 can also interact with noggin, it does so with a very low affinity (13). Noggin and chordin do not bind TGF-β1 or activin (27). Due to its relatively specific effect, noggin has been used as a valuable tool to dissect BMP4 function in cells and tissues (89).

BMP4, and the closely related member BMP2 proteins, have been found previously in calcified atherosclerotic plaques (22), and they play a critical role in vascular calcifications involving medial smooth muscle cells (1, 19, 20). However, their regulation and functional importance in endothelial cells and in atherosclerosis are just beginning to be appreciated, as will be detailed in the following section (54, 89, 90). Endothelial cells express BMP6, while vascular smooth muscle cells express BMP2 and BMP6 (97). BMP6 stimulates angiogenesis by activating *id* (inhibitor of differentiation) (97). BMP4 is expressed both in arterial endothelial cells and smooth muscle cells in human coronary arteries, as well as in cultured human aortic endothelial cells (HAEC) and mouse aortic endothelial cells (MAEC).

BMP receptors and intracellular signaling of BMPs

There are two types of signaling receptors specific for BMPs: BMPR-I and BMPR-II, and both required for signaling (13). Three BMP type I receptors, BMPR-IA (also known as ALK3, Activin-Like Kinase-3), BMPR-IB (ALK6), and ALK2, as well as three type II receptors [BMPR-II, Activin Receptor II (ActRII), and ActRIIB] have been identified (54). Although somewhat variable depending upon species and vascular bed origins, endothelial cells from mouse arteries as well as cultured murine and bovine aortic endothelial cells express both type I (ALK2, 3, and 6) and type II BMPRs (97). Unlike their well-known effects in bone formation and embryonic development, the functional importance of BMPRs in vascular wall is not clear. One notable exception is in vascular smooth muscle cells, where the loss-of-function mutations of BMPR-II have been linked to familial primary pulmonary hypertension and sporadic primary pulmonary hypertension in humans (16). In endothelial cells, transfection with constitutively active mutants of ALK2, ALK3, and ALK6 has been shown to stimulate expression of *id* gene and angiogenic responses (97).

BMPs bind to BMPR-II, inducing homodimerization and autophosphorylation (63). The dimerized type II receptor phosphorylates the BMPR-I, activating its kinase activity. Then, type I receptors phosphorylate SMAD proteins. In response to BMP4, SMAD-1, 5, or 8 proteins (also known as receptor-smad or R-smad) are phosphorylated, while TGFβ phosphorylates SMAD-2 and –3. Upon phosphorylation, SMAD-1 associates with SMAD-4 (co-smad) and the complex translocates to the nucleus where it can regulate transcription factors or bind directly to DNA (21) and regulate gene expression. SMAD-6 and –7 are inhibitory proteins, competing with SMAD-1, -5, and -8 and blocking BMP signaling. Interestingly, it has been shown previously that LS induces expression of two inhibitory signaling molecules, SMAD 6 and 7 (94), providing an additional mechanism by which LS prevents BMP4-dependent responses. Other signaling pathways, such as c-Jun, c-Ha-Ras, and c-Raf, have been also implicated in BMP4 signaling in *Xenopus* (64). As discussed below, we identified a novel pathway involving NF-κB mediating OS- and BMP4-dependent inflammatory responses in endothelial cells (90).

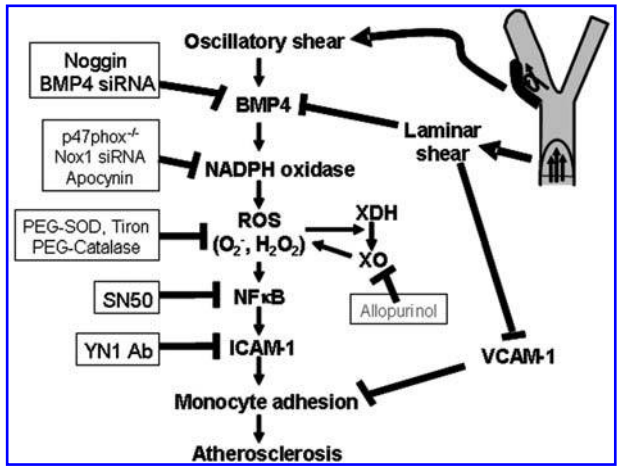

FIG. 3. Role of BMP4 as the mechanosensitive and inflammatory cytokine—overall working hypothesis. The endothelial cells exposed to disturbed shear stress such as OS (*dark line*) in the lesion-prone areas produce BMP4. BMP4 then induces ICAM-1 expression and monocyte adhesion in the ROS and NF-κB-dependent manner. Monocytes recruited to the lesion-prone areas result in foam cell lesion formation, eventually leading to atherosclerotic plaque development. In contrast, LS inhibits inflammatory and atherogenic responses by inhibiting BMP4 expression and by directly downregulating VCAM-1 expression. Shown on the *left side* in *boxes* as well as allopurinol are major inhibitory strategies used for each signaling step.

ROLE OF BMP4 AS A MECHANOSENSITIVE PRO-INFLAMMATORY CYTOKINE IN ENDOTHELIAL CELLS

The selective expression of BMP4 protein in endothelial cells overlying foam cell lesions (an early form of atherosclerotic lesions) prompted a speculation that BMP4 may be involved in the inflammatory responses observed in lesion-prone areas (90). We tested this hypothesis by examining adhesion molecule expressions on endothelial surface and adhesion of THP-1 monocytes to endothelial cells with or without shear stress, BMP4, and various inhibitors. Figs. 3 and 4 are overviews of proposed pathways leading to shear-dependent inflammation and atherosclerosis. We found that OS stimulates monocyte adhesion by specifically inducing ICAM-1 without affecting VCAM-1 and E-selectin levels. Interestingly, treatment of endothelial cells with BMP4 alone increased ICAM-1 induction and monocyte adhesion response. Furthermore, OS-induced ICAM-1 and monocyte adhesion response were completely prevented by inhibiting BMP4 production in endothelial cells by noggin (the BMP antagonist) or the BMP4 siRNA (90). The essential role of ICAM-1 in OS- and BMP4-induced monocyte adhesion was demonstrated further by showing the complete inhibitory effect of a ICAM1 blocking antibody (YN1 Ab) (90). Together, these findings demonstrate the essential role of BMP4 mediating the OS-induced inflammatory responses in endothelial cells.

The relative importance of ICAM-1 and VCAM-1 in atherosclerosis has been controversial. Current evidence, however, from human and mouse studies, as well as cell culture studies,

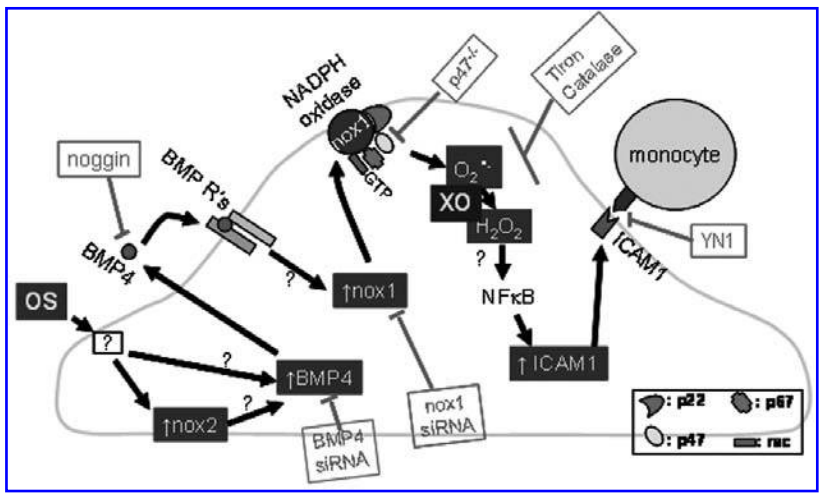

FIG. 4. Role of NADPH oxidase in shear stress- and BMP4-induced inflammatory response in endothelial cells. Shown is the schematic diagram of an endothelial cell with a monocyte binding to the ICAM-1 in resposne to OS. The endothelial cells exposed to OS produce VMP4, which stimulates production of ROS in a Nox1- and $p47^{phox}$-dependent. ROS then stimulates ICAM-1 expression and monocyte binding, leading to foam cell formation and atherosclerosis.

support the importance of both adhesion molecules. Endres *et al*. reported that the early atherosclerotic lesions found in the outer wall of human carotid artery bifurcations showed increased expression of ICAM-1, but not VCAM-1 and E-selectin (26). However, expressions of VCAM-1 and E-selectin did increase in advanced atherosclerotic plaques (26). These seemingly conflicting results on ICAM-1 and VCAM-1 have been reported in mouse atherosclerosis models as well. Cybulsky *et al.* (11) showed that VCAM-1, but not ICAM-1, expression was upregulated by high-fat diet in low density lipoprotein (LDL) receptor$^{-/-}$ mice. In contrast, Nakashima *et al*. (78) reported that only ICAM-1, but not VCAM-1, expression was upregulated in a disturbed flow-dependent manner in lesion-prone areas such as the aortic sinus, while VCAM-1 expression was robustly increased by high-fat diet feeding in ApoE$^{-/-}$ mouse. Evidence from other mouse atherosclerosis models lacking expression of ICAM-1 or VCAM-1 has shown the importance of both adhesion molecules as well (8, 11). Therefore, while both VCAM-1 and ICAM-1 are important in the pathogenesis of atherosclerosis, ICAM-1 expression in lesion-prone areas seems to be regulated mainly by oscillatory shear stress, while VCAM-1 seems to be more responsive to LS as well as high cholesterolemic conditions.

We speculate that disturbed flow conditions prime endothelial cells to be in inflamed state, and that they become the sites of atherosclerosis development if there exist other pro-atherogenic conditions such as hypercholesterolemia, hypertension, smoking, diabetes, etc. Our unpublished studies in ApoE-null mice fed with high-fat diet indeed confirmed that ICAM-1 expression is much more prevalent in the atheroprone area that are exposed to disturbed flow conditions (*i.e.*, the greater curvature of the aortic arch) than that of the atheroprotected lesser curvature.

Molecular mechanisms responsible for OS and BMP4-induced inflammatory responses—role of NF-κB and ROS pathways

Evidence supporting the role of NF-κB in OS and BMP4-dependent inflammatory responses was provided by studies using NF-κB inhibitors and a direct NF-κB assay in endothelial cells (90). The NF-κB translocation inhibitor (SN50) and the proteosome inhibitor MG132 completely prevented ICAM-1 expression induced by BMP4. Also, BMP4 treatment stimulated NF-κB activity as measured by NF-κB-SEAP construct expressing a secreted form of placental alkaline phosphatase driven by four κB sequences in tandem.

OS and BMP4 stimulate inflammation by ROS-derived from NADPH oxidases

Recently, we have shown that exposure of endothelial cells to OS stimulates ROS production from NADPH oxidases, which in turn results in monocyte adhesion (49). Hwang *et al*. has shown that oscillatory shear stress increases Nox2 and Nox4 mRNA level after 4 and 8 h of exposure. (48) Similarly, our group has shown that Nox1 and Nox2 were increased after 24 h of shear (89). These findings prompted the question of whether BMP4 produced in endothelial cells by OS is directly responsible for production of ROS from NADPH oxidases, which then leads to ICAM-1 induction and monocyte binding. Several lines of results have provided definitive evidence proving the hypothesis. The initial evidence was based on pharmacological approaches using ROS scavengers. Treatment of MAE-wt cells with *N*-acetylcysteine (the general ROS chelator), the cell-permeable PEG-catalase (converting H_2O_2 to H_2O), PEG-superoxide dismutase (PEG-SOD), and Tiron (scavenging $O_2^{\bullet -}$) completely blocked OS- and BMP4-induced monocyte binding and ICAM-1 expression, demonstrating a role for ROS (49, 89). While it is a highly useful approach, the chelator approach suffers from the specificity problems of the currently available agents. Therefore, we have developed a combined genetic and molecular approach using MAEC obtained from NADPH oxidase knockout mice lacking p47phox (MAE-p47$^{-/-}$ cells) (49). The p47phox is a critical component of NADPH oxidases (87). We have shown that chronic exposure of MAEC (obtained from a normal C57Bl6 mouse line) to OS stimulates ROS ($O_2^{\bullet -}$ and H_2O_2) productions, whereas chronic LS exposure shuts down ROS production to a significantly lower level (49). In contrast, MAE-p47$^{-/-}$ cells do not produce ROS in response to OS or BMP4, which can be "rescued" to produce ROS by transfecting them with p47phox cDNA (49, 89). Using the similar "rescue" approach in MAE-p47$^{-/-}$ cells, we were able to demonstrate that OS and BMP4 stimulate monocyte adhesion by the mechanism dependent on ROS derived from p47phox-based NADPH oxidases (49).

Once produced by OS from NADPH oxidases in endothelial cells, ROS could then act as a "kindling" radical, promoting more ROS production from other enzymes such as xanthine oxidase (XO) (74, 75). Recently McNally *et al*. (74) showed that OS exposure stimulates ROS production in endothelial cells that can be inhibited by XO inhibitors oxypuriol and tungsten. In addition, they found that protein levels and activity of XO were markedly reduced in MAE-p47$^{-/-}$ cells, compared with MAEC. Interestingly, transfection of MAE-p47$^{-/-}$ cells with a vector encoding p47^{phox} increased XO protein levels (74) and restored OS-induced ROS production (49). Further studies showed that exposure of endothelial cells with either OS or H_2O_2 induced conversion of xanthine dehydrogenase (XDH) to XO, thereby favoring production of ROS using hypoxanthine or xanthine substrates catalyzed by XO (74, 75). In addition to XO, eNOS and NADPH oxidases themselves can be further activated to produce additional ROS. For example, treatment of endothelial cells with H_2O_2 activates NADPH oxidases (75) and uncouples eNOS by oxidizing its cofactor tetrahydrobiopterin (58), resulting in additional ROS production from the cells.

Nox1 is the NADPH oxidase mediating OS- and BMP4-dependent ROS production

Next, we examined whether OS and BMP4 increase ROS production by upregulating expression levels of NADPH oxidase catalytic subunits (49). Vascular endothelial cells express three membrane-bound forms of NADPH oxidases (Nox1, 2, and 4) (60). In static cultured MAEC, Nox4 mRNA is far more abundant than Nox1 and 2. In static conditions, Nox1, Nox2, and Nox4 mRNA copy numbers are 11, 63, and 1,242 per 10^8 18S copies, respectively, which is consistent to other

findings (60, 88). OS increases Nox1 and Nox2 expression. Surprisingly, Nox4 levels were significantly reduced by OS. LS exposure decreased Nox4 level by 51%, without affecting Nox1 and 2. In contrast, Hwang *et al*. have shown that OS increases Nox2 and Nox4 mRNA levels after 4 and 8 h of exposure (47). This difference may be due to different cell types used and shear systems used. More importantly, however, BMP4 upregulates only Nox1 mRNA, while Nox2 is downregulated, and Nox4 is not significantly affected (49). Furthermore, the findings that the knockout of p47phox in MAEC completely blocks both OS- and BMP4-induced ROS production, ICAM-1 induction, and monocyte adhesion provides support for Nox1 (p47phox-dependent enzyme) but not for Nox4 (p47phox independent) (69).

These results showed that Nox1 is the only Nox in our cells that is upregulated by both OS and BMP4, suggesting that it may be responsible for the OS- and BMP4-induced ROS production and monocyte adhesion. Indeed, we found that Nox1 gene knockdown using a specific siRNA, but not a nonsilencing siRNA, prevents both ROS production and monocyte adhesion induced by OS (89), demonstrating the critical role of Nox1 as the mechanosensitive NADPH oxidase. While 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.

SUMMARY AND WORKING HYPOTHESIS

Based on the current data and literature, we propose that BMP4 is a critical mechanosensitive autocrine cytokine, triggering pro-inflammatory and pro-atherogenic responses of endothelial cells by increasing ROS production from NADPH oxidases, as described in Figs. 3 and 4. The endothelial cells in lesion-prone areas experience unstable flow conditions such as low and OS (Fig. 3), which induce BMP4 expression through undefined mechanisms. OS-induced Nox2 expression may play a role in this step (Fig. 4). BMP4 produced in endothelial cells, acting as an autocrine factor and binding to the BMP receptors, stimulates ROS ($O_2^{\bullet -}$ 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, XO. The ROS then initiate the inflammatory cascades, stimulating ICAM-1 expression on the endothelial cell surface in a 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 anti-atherogenic force by inhibiting expression of both BMP4 and VCAM-1 in endothelial cells (90).

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 pro-inflammatory and pro-atherogenic effects of OS. The discovery of BMP4 as a mechanosensitive, pro-inflammatory 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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ABBREVIATIONS

BMP4, bone morphogenetic protein 4; eNOS, endothelial nitric oxide synthase; H_2O_2, hydrogen peroxide; HAEC, human aortic endothelial cell; ICAM-1, intracellular adhesion molecule-1; LDL, low density lipoprotein; LS, laminar shear; MAEC, mouse aortic endothelial cell; NADPH; nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; Nox, nonphagocytic NADPH oxidase; $O_2^{\bullet -}$, superoxide; $ONOO^{\bullet -}$, peroxynitrite; OS, oscillatory shear; ROS, reactive oxygen species; SMAD, Sma + Mad (Mothers against decapentaplegic); VCAM-1, vascular cell adhesion molecule-1; XDH, xanthine dehydrogenase; XO, xanthine oxidase.

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