Peroxynitrite reduces the endothelium-derived hyperpolarizing factor component of coronary flow-mediated dilation in PECAM-1-knockout mice

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Liu, Yanping, Aaron H. Bubolz, Yang Shi, Peter J. Newman, Debra K. Newman, and David D. Gutterman. Peroxynitrite reduces the endothelium-derived hyperpolarizing factor component of coronary flow-mediated dilation in PECAM-1 knockout mice. Am J Physiol Regul Integr Comp Physiol 290: R57–R65, 2006. First published September 15, 2005; doi:10.1152/ajpregu.00424.2005.—Platelet endothelial cell adhesion molecule 1 (PECAM-1) is capable of transducing signals in endothelial cells exposed to shear; however, the biological consequences of this signal transduction are unknown. Because shear stress elicits flow-mediated dilation (FMD), we examined whether steady-state FMD in mouse coronary arteries (MCAs) is affected in the PECAM-1 knockout (KO) mouse. MCAs were isolated from wild-type (WT) or KO mice and prepared for videomicroscopy, histoﬂuorescence, Western blotting, and immunohistochemistry. FMD was examined in the absence and presence of N-nitro-L-arginine methyl ester (l-NAME) and l-NAME+indomethacin (INDO). FMD was reduced in KO relative to WT MCAs, but the l-NAME-inhibitable portion of FMD was similar between the two. The INDO-sensitive component of FMD was diminished in KO MCAs. In contrast, the residual component of dilation, presumably because of endothelium-derived hyperpolarizing factor (EDHF), was abolished in KO MCAs. Histofluorescence showed relatively more superoxide (O2•−; oxy-ethidium ﬂuorescence) and peroxide production (dihydrorhodamine-123 ﬂuorescence) in KO MCAs at rest. Flow augmented O2•− and peroxide production in WT MCAs but had little effect on KO MCAs. Enhanced nitric oxide generation was observed in arteries from KO mice, accompanied with increased eNOS S1177 phosphorylation. In vessels from KO mice, treatment with ebselen decreased peroxynitrite (ONOO−) formation and improved the reduced FMD, largely due to restoration of the presumed EDHF component. These results suggest that PECAM-1 is necessary for normal FMD in the mouse coronary circulation. In the absence of this adhesion and signaling molecule, ONOO− production is increased concomitant with a reduction in both the EDHF and INDO-sensitive components of FMD.

flow-induced dilation; oxidative stress

Shear stress acting on the endothelial cell apical surface serves as an important signaling mechanism for a variety of cell processes, including stimulated release of vasodilator factors (18, 31, 45), activation of ion channels in the cell membrane, changes in cellular pH, cell proliferation and vascular remodeling, and cytoskeletal rearrangement with cellular realignment (19, 41). Shear or flow-mediated vasodilation is arguably the most important physiological mechanism of vasodilation. It involves stimulated endothelial release of a vasodilating substance such as nitric oxide (NO), prostacyclin, or endothelium-derived hyperpolarizing factor (EDHF). Despite the prominence of shear-mediated vasodilation in numerous arterial beds, the initiating signal transduction mechanisms have not been clearly delineated. Shear responsiveness appears greatest in the most rigid nondeformable cellular regions, where the stress is highest. In endothelial cells, this occurs at the lateral cell-cell junctions (8). In many vascular tissues shear triggers endothelial release of nitric oxide through a complex pathway involving surface glycoprotein distortion, which is transmitted through the endothelial cytoskeleton to the basal membrane at focal adhesions at which tyrosine kinases are activated to increase intracellular calcium and activate nitric oxide synthase (5, 34). It is unclear which cell surface receptors are involved in propagating the shear signal to the underlying cytoskeleton.

Recent evidence suggests that in addition to its role in leukocyte migration, platelet endothelial cell adhesion molecule 1 (PECAM-1), CD31, is an important signaling molecule in endothelial cells. It is concentrated at cell junctions (1), in regions highly susceptible to the effects of shear and has been reported to be colocalized with endothelial nitric oxide synthase (eNOS) in the cell membrane (8, 14). PECAM-1 has also been reported to connect to remote cellular sites through the actin cytoskeleton (reviewed in Ref. 35) and is tyrosine-phosphorylated in response to shear stress (16). Thus the transmembrane protein PECAM-1 is a logical candidate component of the shear stress signaling pathway involved in flow-mediated vasodilation. In fact, recent evidence indicates that PECAM-1 contributes to NO-mediated dilation resulting from rapid changes in flow in skeletal muscle arterioles (2).

In this study, we examined the effect of PECAM-1 deficiency on the dilation of coronary arterioles in response to application of steady state shear stress. We observed an impairment in shear-induced vasodilation in PECAM-1-deficient relative to wild-type vessels, largely due to a reduction in the EDHF-mediated component of dilation. These findings con-

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firm a modulatory role for PECAM-1 in the signaling pathway for flow-mediated dilation of the coronary circulation.

MATERIALS AND METHODS

Preparation of mouse coronary arteries. Eight-week-old male PECAM-1-knock-out (KO) mice that have been backcrossed for >12 generations onto a C57BL/6 background and age- and sex-matched wild-type (WT) mice were maintained in a pathogen-free facility of the Animal Resource Center of the Medical College of Wisconsin. All experiments were approved by the local institutional animal care and use committee at the Medical College of Wisconsin.

PECAM-1 KO and WT mice were anesthetized with tribromoethanol (0.4 mg/kg body wt) (Sigma) intraperitoneally and hearts were removed. Mouse coronary arteries (MCAs) were dissected from the left ventricle of the heart and prepared for videomicroscopy, histofluorescence, or immunohistochemistry.

Videomicroscopy. MCAs were cannulated on glass micropipettes in an organ chamber filled with physiological saline solution (PSS) as described previously (33). Each pipette was attached to an individual pressure reservoir. The PSS was warmed to 37°C, continuously circulated, and bubbled with 21% O2-5% CO2-74% N2. Vessel diameter was recorded after development of spontaneous myogenic tone. When necessary U46619 (10−8 to 10−7 mol/l) was added to achieve 30−50% constriction. Flow was produced by changing the heights of the reservoirs in equal and opposite directions to generate a pressure gradient. Intraluminal diameter was measured in the presence of flow generated by pressure gradients of 20 and 100 cm H2O in the absence and the presence of antagonists. At the end of each experiment, vessels were treated with papaverine (100 µM) to determine the relaxation capacity of the arteriole. All measurements were made at steady state flow achieved 5 min after initiating the pressure gradient.

To identify the mediators responsible for dilation of MCAs in response to shear stress, pharmacological inhibitors were used. Nω-nitro-arginine methyl ester (L-NAME; 100 µmol/l), which inhibits nitric oxide synthase (NOS); indomethacin (INDO; 10 µmol/l), which inhibits cyclooxygenase (COX); or polyethylene glycolated catalase (PEG-CAT), which decomposes hydrogen peroxide, were administered to the vessel chamber after initial measurement of shear-induced vessel dilation, allowing 20−30 min incubation before measuring the effect of antagonist on the shear-induced dilation response. In some cases, sequential time control experiments were performed, which showed that the magnitudes of two consecutive flow-induced dilation responses were similar with no intervening treatment.

In separate experiments, MCAs from each type of mouse were incubated for 24 h at 37°C with ebselen (100 µmol/l, reapplied every 8 h) dissolved in DMEM with 10% fetal bovine serum, 100 U/ml penicillin G and 100 µg/ml streptomycin. After the 24-h incubation, flow-mediated dilation was assessed as described above.

Fluorescence detection of hydrogen peroxide and superoxide. Dichlorodihydrofluorescein diacetate (DCFH-DA) and hydroethidine (HE) (27) were used to evaluate the production of hydrogen peroxide (H2O2) and superoxide (O2−), respectively. Four MCAs from the same mouse were exposed to either no flow (pressurized, 0 gradient), or flow at a pressure gradient of 100 cm H2O. One vessel (not pressurized) served as a control. DCFH-DA (5 µmol/l) and HE (5 µmol/l) were added together in a light-protected chamber for 30 min at 37°C, either during flow or static conditions. Vessel segments were then washed with fresh PSS solution, and removed for fluorescence microscopy. DCF (the product of the reaction of DCFH with peroxide) and Oxy-ethidium (oxy-Etd, the fluorescent product of the reaction of HE with superoxide) were excited at 488 nm and 585 nm, and the emission was measured at 526 and 620 nm, respectively. Fresh untreated (control) and experimental tissues were examined in parallel and images were recorded using the same computer-specified gain and intensity settings. Results are expressed as relative fluorescence intensity of the user-defined vessel region, using a vessel treated with PEG-CAT (500 U/ml) and MnTBAP (300 µmol/l) as a baseline control [where reactive oxygen species (ROS) generation is maximally quenched].

Fluorescence detection of nitric oxide production. MCAs were isolated from WT or KO mice and intraluminally exposed to 4,5-diaminofluorescein diacetate (DAF-2) (1 µmol/l, Molecular Probes), a NO indicator, for 15 min followed by a wash with PSS solution. Production of NO was examined under fluorescence microscopy at excitation 495 nm and emission 515 nm. All fluorescence images were analyzed for intensity of fluorescence within a user-defined region of the arteriolar segment (maximal traceable area of the central portion of the vessel). Artificial autofluorescent regions were manually eliminated from analysis. Relative average fluorescence intensity was normalized for surface area and compared between control and experimental vessels.

Immunohistochemical detection of nitrotyrosine. Nitrotyrosine formation was detected in MCAs for evaluating the production of peroxynitrite (ONOO−). Frozen sections of MCAs (5 µm thick) were cut and mounted onto glass slides. After 5 min of washing with PBS, slides were treated with protein block serum-free solution (DAKO) for 30 min at room temperature. Slides were then incubated for 2 h with monoclonal mouse anti-nitrotyrosine antibodies (Upstate Biotechnology), or native eNOS (Santa Cruz Biotechnology) diluted 1:100,000 in PBS, which was incubated at 4°C overnight. After a 10-min wash with TBST 3 times, membranes were incubated with goat anti-rabbit IgG-HRP (1:10,000 Bio-Rad Laboratories) at room temperature for 1 h. The membranes were developed with ECL-Plus (Amersham) and analyzed with densitometry. Phospho-eNOS levels were expressed as the ratio of phospho-eNOS to total native eNOS band intensities.

Statistics. All data are expressed as means ± SE. Percent dilation was calculated as the percent change from preconstricted diameter to the diameter after flow (maximal diameter was measured after papaverine, 10−4 mol/l). In some cases the L-NAME-sensitive component of dilation was determined by the following calculation: % maximal dilation observed in the absence of inhibitors − % maximal dilation observed in the presence of L-NAME. The indomethacin-sensitive component of dilation was calculated as % maximal dilation observed in the presence of L-NAME + INDO − % maximal dilation observed in the presence of L-NAME alone. The residual component, presumably EDHF-mediated, was calculated according to the formula: % maximal dilation observed in the absence of inhibitors − % maximal dilation observed in the presence of L-NAME + INDO. Data from vessels exposed to flow before and after antagonists were compared using a one-way ANOVA with repeated measures for pressure gradient and condition (antagonist exposure). The relative fluorescence intensities observed in arterioles from WT or KO mice exposed to static conditions, flow, or ACh were compared using a two-way
ANOVA. All differences were judged to be significant at the level of \( P < 0.05 \).

**RESULTS**

Mediators of flow-mediated dilation (FMD) in WT and PECAM-1 KO MCAs: roles of NO, PGs, and EDHF. We first examined the role of traditional endothelium-mediated dilator substances, NO, prostacyclin, and EDHF, in the coronary arteriolar dilation to shear stress. The baseline diameter of MCAs from WT and KO mice was 105 ± 8 μm and 109 ± 8 μm, respectively. The contractile response to the preconstrictor U46619 was similar in MCAs from WT and KO mice and the dose of U46619 used to constrict WT (60 ± 15 nmol/l) and KO (65 ± 13 nmol/l) animals was similar as well. The magnitude of FMD was reduced in KO relative to WT MCAs (Fig. 1, A and B) (% maximum dilation, WT vs. KO, 26 ± 3% vs. 11 ± 4% and 68 ± 7% vs. 38 ± 3% at 20 and 100 cm H2O pressure gradient, respectively; \( n = 5 \), \( P < 0.05 \)). The contribution of NO, vasodilator PGs, and EDHF to FMD was determined by the effect of L-NAME (an NO synthase inhibitor), INDO (a cyclooxygenase inhibitor), and a combination of L-NAME and INDO, respectively. The L-NAME-inhibitable portion of dilation to shear stress was similar in WT and KO animals. A slightly greater portion of the dilation, however, was sensitive to indomethacin in WT vs. KO animals. Surprisingly, the remaining, presumed EDHF, component was abolished in KO MCAs, suggesting that the reduced FMD in KO mice is largely due to the loss of EDHF-mediated dilation (Fig. 1C).

\( \text{H}_2\text{O}_2 \) acts as an EDHF regulating flow-mediated dilation in MCAs of WT but not PECAM-1 KO mice. We have previously demonstrated that \( \text{H}_2\text{O}_2 \) acts as an EDHF in regulating FMD in diseased human coronary arterioles(31); however, the chemical nature of EDHF is dependent upon the vascular bed, species, and experimental conditions (10) and has not been clearly defined in MCAs. Therefore, we examined the extent to which \( \text{H}_2\text{O}_2 \) contributes to FMD in MCAs. Figure 2A shows that FMD of WT MCAs was significantly reduced by intraluminal application of PEG-CAT (500 U/ml), a cell-permeable \( \text{H}_2\text{O}_2 \) scavenger, indicating a critical role for \( \text{H}_2\text{O}_2 \) in FMD in MCAs. PEG-CAT had no effect on FMD in KO MCAs (Fig. 2B). These data demonstrate that the \( \text{H}_2\text{O}_2 \) component of the dilation to shear stress in MCA, which is detectable in wild-type mice, is absent in mice lacking PECAM-1.

![Fig. 1](http://ajpregu.physiology.org/)

![Fig. 2](http://ajpregu.physiology.org/)
Flow increases both $O_2^-$ and $H_2O_2$ generation in WT but not KO MCAs. The finding that PEG-CAT inhibited FMD of WT but not KO MCAs suggested that KO vessels are unable to either produce $H_2O_2$ or respond to $H_2O_2$ that is produced normally during shear stress. We therefore evaluated production of $H_2O_2$ and its precursor, superoxide anion ($O_2^-\cdot$), in resting vessels and those exposed to increased flow. As shown in Fig. 3, in the absence of flow (resting condition) oxy-Etd and DCFH fluorescence intensities, which reflect production rates of superoxide and $H_2O_2$, respectively, were increased in MCAs from KO mice (fold-increase of 4.5 ± 1.6 and 3.5 ± 1.1, respectively) compared with WT ($n = 4$, $P < 0.05$). Flow augmented both $O_2^-$ and $H_2O_2$ formation in WT MCAs with average increases in oxy-Etd and DCF fluorescence intensities of 3.0 ± 1.3- and 3.7 ± 1.3-fold, respectively, in flow relative to no flow conditions. In contrast to WT, flow appeared to reduce both $O_2^-$ and $H_2O_2$ formation in KO MCAs (2.3 ± 1.2 and 3 ± 1.3, respectively); however, this reduction was not statistically significant.

NO production and eNOS serine phosphorylation are enhanced in PECAM-1 KO mice. The production of NO in MCAs from WT or KO mice was determined using DAF-2 fluorescence. Enhanced DAF-2 fluorescence intensity was observed in KO MCAs compared with WT (Fig. 4A) indicating that NO generation was augmented in KO MCAs. The observed increase in NO formation could be due to increased expression or increased activity of the eNOS enzyme. Total heart eNOS expression levels did not differ between WT and KO mice (Fig. 4B, bottom). Therefore, enhanced expression of the enzyme does not account for the increase in NO formation. eNOS can be phosphorylated on a serine residue at position 1177 (pS1177) and on a threonine residue at position 495 (pT495), with the former correlating with increased and the latter with reduced enzyme activity (4). Heart homogenates showed increased levels of pS1177 in KO compared with WT mice (Fig. 4B, $n = 6$, $P < 0.05$). In contrast, pT495 levels were similar in KO and WT mice (Fig. 4C). These data suggest that an increase in eNOS activity, but not an increase in eNOS protein levels, contributes to the observed increase in NO formation in PECAM-1 KO relative to WT mice.

Tyrosine nitration is enhanced in PECAM-1 KO coronary arteries. Because both NO and $O_2^-$ generation were increased in MCA from KO mice, ONOO$^-$, which is formed by reaction of NO with $O_2^-$, was assessed by immunohistochemistry, using antibodies specific for 3-nitrotyrosine (3-NT) to detect the product of ONOO$^-$-mediated nitration of tyrosine residues on cellular proteins. No staining of untreated KO vessels was observed in the absence of primary antibody (negative control), indicating specificity. 3-NT-specific antibodies reacted much more strongly with MCAs derived from KO, relative to WT, mice (Fig. 5). This reactivity was 3-NT-specific, as no staining of KO MCAs was observed in the absence of the primary 3-NT-specific antibody (negative control). Ebselen, a scavenger of ONOO$^-$, reduced immunohistochemical evidence for 3-NT in MCAs derived from KO mice but had no effect on 3-NT antibody reactivity with MCAs obtained from WT animals. These data indicate that ONOO$^-$ formation is augmented in coronary vessels from PECAM-1 KO mice compared with WT animals.

Impaired flow-mediated dilation in KO MCAs can be restored by treatment with a scavenger of ONOO$^-$. To determine whether ONOO$^-$ has an inhibitory effect on FMD, FMD in MCAs was examined after 24 h of incubation with ebselen. Treatment with ebselen enhanced FMD of MCAs derived from
KO mice (Fig. 6A compared with Fig. 1B) to a level similar to that observed with WT vessels (Fig. 1A). The proportion of the FMD of ebselen-treated KO vessels that was sensitive to \textit{L}-NAME and INDO (Fig. 6A) remained the same as was observed in untreated vessels from KO animals (Fig. 1B). Therefore, the improved FMD that was observed upon ebselen treatment of vessels derived from PECAM-1 KO mice was largely due to restoration of the \textit{L}-NAME- and INDO-insensitive component of the dilation, which is presumably mediated by EDHF (Fig. 6C). In addition to scavenging peroxynitrite, ebselen can also act as a peroxidase to destroy hydrogen peroxide. We do not believe ebselen is acting in this fashion in the present study as the catalase-sensitive component of dilation was in fact enhanced after treatment of KO vessels with ebselen (Fig. 6). Overall, these data suggest that enhanced ONOO⁻ generation in PECAM-1 KO mice impairs FMD in MCAs by abolishing the bioactivity of H₂O₂, the presumed EDHF in mouse coronary arteries.

**DISCUSSION**

The novel findings of this study are three-fold. First, steady-state FMD was reduced in MCAs of PECAM-1 KO mice. Second, among the vasodilators responsible for FMD in MCA, which include products of NOS and COX, as well as EDHF, NOS-mediated dilator mechanisms were not impaired, whereas COX-dependent dilation was slightly reduced and dilation attributable to EDHF was absent in PECAM-1-deficient vessels. Third, levels of NO and O₂⁻ were elevated in PECAM-1-deficient MCAs, accounting for formation of ONOO⁻, which contributed to the impaired EDHF and COX components of FMD in PECAM-1-deficient vessels. To our knowledge, this study provides the first evidence that PECAM-1 plays a critical role in shear-mediated dilation in the coronary circulation.

The techniques used to obtain the data limit, to some extent, the strength of the conclusions reached in this study. We used fluorescent dyes to assess levels of NO, O₂⁻ and H₂O₂ and immunohistochemistry to detect nitrotyrosine, a product of ONOO⁻ activity, in WT and PECAM-1-deficient MCAs. This is because quantitative assays for O₂⁻ and H₂O₂ production, such as cytochrome c reduction or ESR, respectively, and Western blots for detection of nitrotyrosine residues are not possible because of the very small size of isolated mouse coronary arterioles. However, fluorescent methods for ROS detection are highly sensitive (3, 39), and when simultaneous measurements are made in experimental and control vessels, they can accurately detect relative differences in amounts of ROS. We assessed total ethidium fluorescence changes in vessels. Recent experimental data indicate that both ethidium bromide and oxy-ethidium (the product of the reaction of superoxide with hydroethidine) contribute to the fluorescence intensity (11, 46). Therefore, some of the increased fluorescence observed may reflect oxidative products other than superoxide. Similarly, immunohistochemical data, although qualitative, clearly provide evidence for increased ONOO⁻ production in PECAM-1 KO, relative to WT, mouse coronary arteries. Coupled with specific pharmacological approaches to reduce particular ROS, such as our use of ebselen to scavenge ONOO⁻, these approaches collectively support the conclusion that ONOO⁻, produced as a consequence of generation of NO and O₂⁻, plays a role in inhibiting the vascular response of PECAM-1-deficient MCAs to shear.

We observed in the present studies that EDHF- and COX-dependent components of FMD could be restored upon treatment of PECAM-1-deficient vessels with the ONOO⁻ scavenger ebselen. With respect to EDHF, we found in these studies that EDHF-dependent (i.e., NOS- and COX-independent) dilation of MCAs in response to shear was due to the production of H₂O₂. Although we did not provide direct evidence for H₂O₂-mediated vascular smooth muscle cell (VSMC) hyperpolarization in MCAs, we demonstrated that both flow-mediated dilation and production of H₂O₂ in WT MCAs was blocked with PEG-CAT. Therefore, H₂O₂, which mediates FMD in human coronary arterioles and elicits hyperpolarizati-
tion in that vascular bed (32) and also mediates endothelium-dependent dilation in mouse and human mesenteric arterioles (29, 30), most likely serves as an EDHF in murine coronary arteries. We propose that ONOO\(^-\)/H\(^{11002}\) generated in PECAM-1-deficient MCAs interfered with the ability of VSMC to respond to H\(_2\)O\(_2\). In addition, ONOO\(^-\)/H\(^{11002}\) generation could also have reduced the ability of vascular endothelial cells to produce H\(_2\)O\(_2\). In this study, we found that flow-induced superoxide generation and vasodilation are reduced in PECAM-1-deficient mice, and these impairments are most likely due to the formation of ONOO\(^-\), which consumes superoxide. These data suggest that in the absence of PECAM-1, superoxide reacts with NO to form ONOO\(^-\), which inhibits the ability of the vessel to respond to H\(_2\)O\(_2\), which functions as an EDHF. These findings indicate an important role of PECAM-1 in protecting the EDHF component of flow-mediated vasodilation. Restoration of the EDHF component by ebselen further supports the idea that peroxynitrite associated with PECAM-1 deficiency is responsible for the impaired EDHF dilation induced by flow.

The mechanism underlying nonresponsiveness of PECAM-1-deficient MCAs to H\(_2\)O\(_2\) may involve inhibition of the opening of KCa channels in VSMC, which have previously shown to be inactivated by ONOO\(^-\) (26). Future studies using electrophysiological techniques to examine membrane potential changes to H\(_2\)O\(_2\) in WT and PECAM-1-deficient mouse coronary arteries should be able to confirm this hypothesis. In

Fig. 5. Immunohistochemical evidence for peroxynitrite formation in PECAM-1-deficient murine coronary arteries. Anti-3-nitrotyrosine reactivity (index of peroxynitrite formation) was more prominent in coronary arteries from KO relative to WT mice (top). Staining of KO vessels with a nonspecific antibody served as a negative control (middle). Ebselen, a scavenger of peroxynitrite, which had no effect on WT vessel immunostaining, markedly reduced anti-3-nitrotyrosine reactivity in KO arteries (bottom).

Fig. 6. Effect of ebselen treatment on flow-mediated dilation of PECAM-1-deficient murine coronary arteries. A: ebselen treatment of KO arteries restored flow-mediated dilation to WT levels (compare with Fig. 1A) and restored the ability of KO arteries to dilate in the presence of L-NAME+INDO (compare with Fig. 1B). B: graphic representation of relative contributions of L-NAME-sensitive (solid bar), INDO-sensitive (open bar), and residual components (gray bar; presumably EDHF) to flow-induced dilation in KO murine coronary arteries before (left) and after (right) ebselen (Eb) treatment. The EDHF-dependent component of flow-mediated dilation was significantly enhanced, whereas the L-NAME- and INDO-sensitive components were unaffected, after ebselen treatment in KO murine coronary arteries.

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the case of COX-dependent vasodilation, on the other hand, it is possible that ONOO generated in PECAM-1-deficient MCAs interferes with the ability of vascular endothelial cells to produce the COX-dependent vasodilator, prostacyclin, rather than with the ability of VSMC to respond to it. This is because previous studies have demonstrated that prostacyclin synthase is exquisitely sensitive to and irreversibly inhibited by ONOO⁻ (47). Future studies, in which prostacyclin production in WT and PECAM-1-deficient MCAs is measured, are required to validate this hypothesis.

The finding that ONOO⁻ generated in PECAM-1-deficient MCAs did not interfere with the ability of these vessels to produce and respond to NO was interesting. This observation is consistent with those of Wigg et al. (42), who showed that in mesenteric and femoral arteries from diabetic rats, endothelium-dependent dilation to ACh was reduced, largely because of a reduction in the EDHF-mediated component, whereas the NO component of the dilation was preserved. It is also consistent with a recent study by Bagi et al. (2), who demonstrated a maintained NO-mediated dilation in skeletal muscle arterioles to shear in PECAM-1 KO mice. However, they found that the early NO-mediated dilation to ramped flow is modulated by PECAM-1. Our observation of an overall reduction in steady-state flow-induced dilation in KO animals may reflect the different vascular bed, in which other mediators, including cyclooxygenase products and EDHF contribute to the dilation. Indeed, the NO component of dilation in the coronary arteries was not different between WT and KO mice, consistent with the findings by Bagi et al. (2).

Multiple substances have been postulated to function as an EDHF in the vasculature, including cytochrome P450 metabolites (6), gap junction (13), and K⁺ (9), depending upon the vascular beds studied and experimental conditions used. In the present study, we cannot exclude the possibility that ebselen incubation may have stimulated production of one of these or another substance that acts as an EDHF. Whether other mechanisms in addition to H₂O₂ are involved in flow-mediated dilation after ebselen treatment will be defined in future studies.

Our finding that ONOO⁻, NO, O₂⁻, and H₂O₂ were all present at higher levels in PECAM-1-deficient relative to WT MCAs suggests that PECAM-1 normally functions to regulate levels of NO and O₂⁻, the precursors for production of ONOO⁻ and H₂O₂, in the coronary vasculature. The mechanisms by which PECAM-1 regulates NO and O₂⁻ levels in MCAs are, however, currently unknown. To regulate NO synthesis, PECAM-1 must regulate the activity of any or all of the three isoforms of NOX, including nNOX, iNOX, and eNOX. The latter two of these are expressed in endothelial cells under normal (43) or pathological conditions (24, 25) and are therefore candidates for the regulatory effects of PECAM-1 described in this study. Our findings that NO was present at higher levels and that eNOX was hyperphosphorylated on a regulatory serine residue in PECAM-1 KO relative to WT MCAs and heart lysates, are consistent with the recent report that PECAM-1 associates with and inhibits the activity of eNOX in human umbilical vein endothelial cells (8). However, the extent to which PECAM-1 associates with and regulates eNOX activity in MCAs is not yet known. We postulate that the increased phosphorylation at pS₁₁₇₇ is responsible for the increased basal NO production in PECAM KO mice.

An alternative explanation for the paradoxical findings of increased NO levels and concomitant reduced FMD in PECAM-1 KO MCAs is that attenuated smooth muscle cGMP production lessens dilation in response to NO. To evaluate this possibility, we measured the basal level of cGMP in aortas from WT and KO, since the small size of MCAs precluded their use for this purpose. Preliminary results from studies of four paired KO and WT vessels revealed that aortas behaved similarly to MCAs in that enhanced NO production and up-regulation of eNOS pS₁₁₇₇ was observed in KO relative to WT aortas; however, the baseline level of cGMP production was not significantly different in aortas from KO vs. WT mice (data not shown). Future studies are required to expand upon these initial observations and will include measurement of guanylyl cyclase expression, stimulated phosphorylation of eNOS and consequent functional effects.

With respect to PECAM-1 as a regulator of O₂⁻ production, O₂⁻ can be derived from several sources, including the mitochondria, NAD(P)H oxidase and uncoupled NOS (44). ONOO⁻, by oxidizing tetrahydrobiopterin, can uncouple NOS and increase formation of O₂⁻ (23). However, our studies provide evidence against uncoupled NOS as the source of O₂⁻ in PECAM-1-deficient vessels, as the NOS component of FMD was maintained and NO-dependent DAF-2 fluorescence was actually enhanced relative to WT in MCAs derived from PECAM-1-deficient mice. Further studies are needed to determine the sources of the increased levels NO and O₂⁻ that we observed in PECAM-1-deficient MCAs and to explore indirect, as well as direct mechanisms for PECAM-1-mediated regulation of the enzymes or pathways responsible for production of these ROS in endothelial cells.

Our findings suggest that PECAM-1 regulates endothelial cell production of vasodilators in response to exposure to shear stress. Shear stress acting on the endothelial cell surface activates a signaling cascade that results in the formation of NO and other endothelium-derived substances that migrate to the underlying vascular smooth muscle to stimulate vasodilation (7, 17, 22). As shear is greatest in areas of endothelial cells with least deformability, molecules located at sites of cell-cell junctions are positioned to most efficiently transduce signals in response to shear stress (8). PECAM-1, which is highly concentrated at junctions between endothelial cells (1, 12, 35), is well positioned to function as a biosensor of shear stresses in the endothelial lining of blood vessels. PECAM-1 is a type I transmembrane protein that contains two tyrosine phosphorylation sites within its cytoplasmic domain that, when phosphorylated, support recruitment of the Src homology 2 domain-containing protein tyrosine phosphatase (SHP-2) (reviewed in Ref. 35). Tyrosine phosphorylation of endothelial cell PECAM-1 in response to application of fluid shear stress or mechanical stretch has been previously reported (16, 21, 28, 37, 40). PECAM-1 tyrosine phosphorylation in response to shear has also been shown to correlate with rapid translocation of SHP-2 from its cytoplasmic distribution to the inner face of the endothelial cell plasma membrane (38). In addition, increases in intracellular calcium, potassium and stretch-activated cation channel opening, and prostacyclin release occur downstream of PECAM-1 engagement on PECAM-1-expressing endothelial cell-like cells (15, 36) in a manner that depends on tyrosine phosphorylation sites within the PECAM-1 cytoplasmic domain (20). It will be important to
determine whether PECAM-1 must become tyrosine phosphorylated and bind SHP-2 to control endothelial cell production of ROS and thereby influence the ability of vessels to dilate in response to shear stress.

FMD in MCA requires production of NO, dilator COX products, and the EHDF, H2O2. Absence of PECAM-1 had no effect on the ROS component of FMD, but reduced the COX and abolished the H2O2 component with an associated increase in ONOO− production. Overall, the peak FMD response was reduced by 50% in PECAM-1-deficient relative to WT MCAs. We conclude that PECAM-1 is necessary for normal FMD in the mouse coronary circulation. In the absence of this adhesion molecule, ONOO− production is increased concomitant with a reduction in both the EDHF and COX-mediated components of FMD.

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