Role of caveolae in shear stress-mediated endothelium-dependent dilation in coronary arteries

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Aims	Caveolae are membrane microdomains where important signalling pathways are assembled and molecular effects transduced. In this study, we hypothesized that shear stress-mediated vasodilation (SSD) of mouse small coronary arteries (MCA) is caveolae-dependent.
Methods and results	MCA (80–150 μm) isolated from wild-type (WT) and caveolin-1 null (Cav-1$^{-/-}$) mice were subjected to physiological levels of shear stress (1–25 dynes/cm^2) with and without pre-incubation of inhibitors of nitric oxide synthase (L-NAME), cyclooxygenase (indomethacin, INDO), or cytochrome P450 epoxygenase (SKF 525A). SSD was endothelium-dependent in WT and Cav-1$^{-/-}$ coronaries but that in Cav-1$^{-/-}$ was significantly diminished compared with WT. Pre-incubation with L-NAME, INDO, or SKF 525A significantly reduced SSD in WT but not in Cav-1$^{-/-}$ mice. Vessels from the soluble epoxide hydrolase null (Ephx2$^{-/-}$) mice showed enhanced SSD, which was further augmented by the presence of arachidonic acid. In donor–detector-coupled vessel experiments, Cav-1$^{-/-}$ donor vessels produced diminished dilation in WT endothelium-denuded detector vessels compared with WT donor vessels. Shear stress elicited a robust intracellular Ca^{2+} increase in vascular endothelial cells isolated from WT but not those from Cav-1$^{-/-}$ mice.
Conclusion	Integrity of caveolae is critical for endothelium-dependent SSD in MCA. Cav-1$^{-/-}$ endothelium is deficient in shear stress-mediated generation of vasodilators including NO, prostaglandins, and epoxyeicosatrienoic acids. Caveolae plays a critical role in endothelial signal transduction from shear stress to vasodilator production and release.
Keywords	Caveolae • Shear stress • Coronary artery • Soluble epoxide hydrolase • Calcium

1. Introduction

Caveolae are 50- to 100 nm cholesterol- and sphingolipid-rich flask-shaped invaginations of the plasma membrane found in most cell types of the body that form in the presence of the structural scaffolding protein, caveolin.[1] Caveolae form plasma membrane microdomains which act as platforms for assembling signal transduction molecules that include receptors, ion channels, and enzymes such as kinases and phosphatases.[2,3] Three isoforms of caveolin are known: caveolin-1 (Cav-1), caveolin-2, and caveolin-3.[1] In the vasculature, caveolae are abundant in endothelial cells and Cav-1 is the major coat protein that is required for caveolae formation in the endothelium.[1–3] Genetic ablation of Cav-1 (Cav-1$^{-/-}$) attenuates both pressure- and agonist-induced vasoconstriction, and decreases agonist-induced vasodilation, suggesting that caveolae play an important role in the regulation of vascular

reactivity.[4,5] Caveolae are also postulated to be mechanosensors and function as mechanotransducers in the vascular endothelium.[6]

Uniquely situated at the interface between luminal haemodynamic forces and the underlying smooth muscle, the vascular endothelium transduces mechanical and chemical signals into vasoreactivity by the release of vasoactive molecules, which govern vascular tone and tissue perfusion. Shear stress is a potent endothelium-dependent physiological vasodilatory signal that regulates most tissue beds.[7,8] Shear stress-mediated vasodilation (SSD) is known to involve major endothelium-dependent vasodilators including nitric oxide (NO),[9] prostacyclin (PGI$_2$),[10] and endothelium-derived hyperpolarizing factors (EDHFs).[10] Epoxyeicosatrienoic acids (EET), the cytochrome P450 (CYP) epoxygenase metabolites of arachidonic acid (AA), are a major form of EDHF in coronary circulation.[11] In this study, we examined the role of

caveolae in mediating SSD through NO, prostaglandins (PG), and EET signalling in mouse coronary arteries (MCA).

2. Methods

2.1 Animals

Male wild-type (WT) and Cav-1$^{-/-}$ mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA) at 10 weeks of age. The epoxide hydrolase null (Ephx2$^{-/-}$) mice were generated as previously described.[12] Handling and care of animals, as well as all animal procedures, were conducted in conformity with the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH Publication no. 85–23, revised 1996) and approved by the Institutional Animal Care and Use Committee, Mayo Foundation.

2.2 Preparation of mouse coronary arteries and SSD

Mouse coronary arteries were prepared as previously described.[13] Briefly, mice were anaesthetized with sodium pentobarbital (100 mg/kg, intraperitoneal). Their hearts were rapidly excised and placed in ice-cold Krebs' solution (in mmol/L): NaCl 118.3, KCl 4.7, CaCl$_2$ 2.5, MgSO$_4$ 1.2, KH$_2$PO$_4$ 1.2, NaHCO$_3$ 25, and dextrose 11.1, pH 7.4. Isolated small MCA (80–150 μm in diameter) were mounted in a vessel chamber filled with Krebs' solution, secured between two borosilicate glass micropipettes, and placed on the stage of an inverted microscope (CK40, Olympus) that was coupled to a CCD camera (OLY-105, Olympus) and a video micrometer (VIA-100, Boeckeler Instruments, Tucson, AZ, USA). The vessel lumen was filled with Krebs' solution and maintained at a constant intraluminal pressure of 80 mmHg using a syringe microinjection pump and a pressure-servo controller (Living Systems, Burlington, VT, USA) as shown in Supplementary material online, *Figure S1* and is similar to that used by other investigators.[14] Incremental levels of shear stress (1, 5, 10, 15, 20, and 25 dynes/cm^2) were applied to each vessel through the microinjection pump with flow rates calculated according to the following equation:

$$Q = \frac{\pi D^3 \tau}{32 \eta}$$

where Q is the flow rate, D is the vessel diameter, τ is the shear stress, and η is the viscosity of fluid. Examples of vessel diameter measurements using videomicroscopy are shown in the Supplementary material online, *Figure S2*. Vessel diameters were monitored and measured continuously. Flow rates were adjusted to achieve the next level of shear stress based on the diameter reached.

2.3 Pharmacological interventions

Vessels were equilibrated for 60 min in oxygenated (21% O$_2$, 5% CO$_2$, balanced with N$_2$, 37 °C) Krebs' solution. Endothelin-1 (ET-1, up to 10 nmol/L) was applied to pre-contract the vessels to 50–70% of the passive diameter. ET-1 produced similar diameter changes in vessels from WT (76.0 ± 4.0 μm, $n = 22$) and Cav-1$^{-/-}$ (77.9 ± 7.2 μm, $n = 18$). The effects of ET-1 were sustained for at least 30 min which was the usual duration of the shear stress experiments (vessel diameters were 66.0 ± 4.3 μm 5 min after ET-1 exposure and were 67.7 ± 4.7 μm after 30 min, $n = 6$, $P = $ NS). Results on the % vessel pre-contraction by ET-1 before shear stress or other vasodilation interventions are tabulated in Supplementary material online, *Table S1*. Dilation in response to different physiological levels of shear stress was compared between WT and Cav-1$^{-/-}$ vessels in the presence and absence of 100 μmol/L $N(\omega)$-nitro-L-arginine methyl ester (L-NAME, eNOS inhibitor), 10 μmol/L indomethacin (INDO, cyclooxygenase (COX) inhibitor), and 10 μmol/L SKF 525A (SKF, CYP epoxygenase inhibitor). Incubation with these inhibitors did not produce any significant effects on vessel diameters (Supplementary material online,

Table S2). At the end of each experiment, vessels were constricted with 100 mmol/L KCl and then maximally dilated with a Ca^{2+}-free solution. Vessels were not acceptable for experiments if they showed leaks, failed to constrict by 50% to 10 nmol/L ET-1 or to 100 mmol/L KCl, or failed to dilate to zero Ca^{2+} or to nitroprusside. The vasodilatory response was calculated as a percentage of the maximum diameter as defined by the following equation:

$$\text{Vasodilation (\% maximum)} = \left[\frac{D_{SS} - D_{ET}}{D_{MAX} - D_{ET}} \right] \times 100\%$$

where D_{SS} is the vessel diameter at a specific level of shear stress, D_{ET} is the vessel diameter after application of ET-1 in the absence of shear stress, and D_{MAX} is the vessel diameter in zero Ca^{2+} Krebs' solution in the absence of shear stress but in the presence of ET-1. Continuous recordings of vessel diameters were performed using a real-time edge-detection system (V94 Living Systems Instrumentation) that detected and tracked the luminal vessel diameter. Signal output was acquired by a 16-bit data acquisition system (DIGI-DATA 1321A, Axon Instruments, Foster City, CA, USA) with a sampling frequency of 2 kHz and no filter, and was continuously recorded using Axoscope software. In this study, each vessel was used for only one intervention/condition, so different sets of vessels were used for determination of the effects of endothelium denudation or pre-incubation of pharmacological agents.

In some vessels, the endothelium was denuded by slowly perfusing 3–5 mL of air through the lumen of unpressurized vessels. The effectiveness of endothelium denudation was verified by demonstrating that the vessel: (i) failed to dilate to 1 μmol/L acetylcholine; (ii) constricted normally to ET-1; and (iii) dilated normally to 100 μmol/L sodium nitroprusside. In the donor–detector-coupled vessel experiments, the donor vessels had intact endothelium, whereas the detector vessels were denuded of endothelium. Donor and detector vessels were mounted in separate chambers connected by a glass micropipette (Supplementary material online, *Figure S1B*).

2.4 Measurements of intracellular calcium in endothelial cells

Vascular endothelial cells were obtained by explantation from control and Cav-1$^{-/-}$ mouse aortas using Matrigel enriched with endothelial growth factors.[15] The outgrowing cells were dissociated by dispase (10 U/mL), collected, and passaged in endothelial growth medium-2 (EGM-2, Lonza) supplemented with 5% foetal bovine serum, fibroblast growth factor, vascular growth factor, insulin-like growth factor, ascorbic acid, and hydrocortisone. The isolated cells were used within four passages and were characterized by immunostaining with antibodies against von Willebrand factor (1:200 dilution), an endothelial marker, and with antibodies against α-actin (1:200 dilution), a smooth muscle marker. Results showed that >85% of the cells in the preparation were endothelial cells.

For calcium imaging, cells were seeded on a slide and loaded with fura-2 AM (3 μmol/L, Invitrogen) for 30 min at 37°C. The slide was then assembled in a shear stress chamber similar to that previously described[16] and placed on the stage of an inverted Olympus IX71 microscope. The cells were exposed to 11 dynes/cm^2 of shear stress and the intracellular calcium fluorescence signals were measured as a ratio of fluorescence intensities at 510 nm from excitations of 340/380 nm using a Hamamatsu ORCA-R2 CCD camera with a Sutter LB-LS/17 light source. Background fluorescence was subtracted and the calcium signal (F) was normalized to baseline fluorescence (F0) and expressed as a ratio (F/F0) using MetaFluor software.

2.5 Materials

Chemicals were purchased from Sigma-Aldrich Co. (St Louis, MO, USA).

2.6 Statistical analysis

All data are expressed as mean ± SEM. Vasodilation is expressed as the percentage of change in vessel diameter relative to the maximum vessel

diameter measured in the presence of a Ca^{2+}-free solution. Vasodilation data on key findings expressed as the change in vessel diameter, which is considered to be the most accurate representation of SSD, are provided in Supplementary material online, *Figures S3, S5,* and *S6*. Drug concentration at half maximum effect (EC_{50}) was determined by curve fitting using Origin software (OriginLab Corp., Northamptonm, MA, USA). Efficacies of endothelium-dependent vasodilation were compared by area-under-the-curve calculations. One-way ANOVA was used to compare multiple groups and statistical significance was defined as $P < 0.05$.

3. Results

3.1 Shear stress-induced endothelium-dependent vasodilation is impaired in Cav-1$^{-/-}$ coronary arteries

SSD in MCA was significantly diminished in vessels from Cav-1$^{-/-}$ mice. Shear stress of 25 dynes/cm^2 resulted in a 92.2 $\pm$ 0.7% dilation in WT vessels ($n = 22$) but only a 41.8 $\pm$ 1.5% dilation in Cav-1$^{-/-}$ coronaries ($n = 18$, $P < 0.001$) (*Figure 1A* and *C*). Removal of the endothelium reduced SSD significantly to 22.3 $\pm$ 3.3% at 25 dynes/cm^2 in WT ($n = 8$, vs. endothelium intact, $P < 0.001$) and to 14.9 $\pm$ 1.3% in Cav-1$^{-/-}$ ($n = 8$, vs. vessels with intact endothelium, $P < 0.001$) (*Figure 1B* and *C*). Vasodilation expressed as vessel diameter change in micrometres is shown in Supplementary material online, *Figure S3A* and *B*. Similar results were obtained when caveolae formation was disrupted by treatment with methyl-β-cyclodextrin (Supplementary material online, *Figure S3C* and *D*). These results suggest that SSD in MCA is endothelium- and caveolae-dependent.

3.2 Impaired endothelium-dependent vasodilator pathways in Cav-1$^{-/-}$ coronary arteries

Animal studies have shown that SSD is mediated by the release of endothelium-dependent vasodilators including NO, PG, and EETs.[8,17,18] SSD by 25 dynes/cm^2 was significantly reduced after individual incubation with L-NAME (100 μmol/L), INDO (10 μmol/L), and SKF 525A (10 μmol/L), from 92.2 $\pm$ 0.7% ($n = 22$) to 61.0 $\pm$ 2.8% ($n = 6$), 69.1 $\pm$ 1.7% ($n = 8$), and 77.6 $\pm$ 1.7% ($n = 12$), respectively in WT ($P < 0.001$ for all three inhibitors vs. without drug pre-treatment), but had no effects in Cav-1$^{-/-}$ vessels (*Figure 2*). Representative continuous tracings of SSD after pre-incubation with L-NAME, INDO, and SKF 525A in WT and Cav-1$^{-/-}$ MCA are shown in Supplementary material online, *Figure S4*. These results suggest that all three vasodilator pathways are important in mediating SSD in normal MCA with a quantitative contribution in the order of NO > PG > EETs, but their effects are lost in Cav-1$^{-/-}$ vessels. After incubation with L-NAME, INDO, and SKF 525A together, vasodilation produced by shear stress was at a level comparable to that of Cav-1$^{-/-}$ vessels (*Figure 2D*). These results suggest that the endothelium of Cav-1$^{-/-}$ mice is deficient in the generation and release of shear stress-induced vasodilators.

3.3 Shear stress-induced dilation of coronary arteries from Ephx2$^{-/-}$ mice

To further delineate the role of EETs in SSD, we used coronary arteries from soluble epoxide hydrolase (sEH) null (Ephx2$^{-/-}$) mice.[12] sEH converts EETs to dihydroxyeicosatrienoic acids and is responsible for removal of EETs thereby diminishing their beneficial cardiovascular properties.[19] In Ephx2$^{-/-}$ mice, the effects of EETs should be

potentiated. In the presence of eNOS and COX inhibition with L-NAME and INDO, shear stress of 25 dynes/cm^2 produced dilations of 46.2 $\pm$ 3.3% ($n = 8$) dilation in WT vessels but 70.7 $\pm$ 3.4% ($n = 6$) in Ephx2$^{-/-}$ coronary arteries ($P < 0.001$) (*Figure 3A*). Endothelium denudation reduced SSD in both WT and Ephx2$^{-/-}$ to the same level (*Figure 3A*). Results with vasodilation expressed in vessel diameter change are shown in Supplementary material online, *Figure S6A*. These results indicate that the levels of EETs are important in mediating SSD.

After a 15 min incubation with 10 μmol/L AA, in the presence of L-NAME and INDO, SSD by 25 dynes/cm^2 was augmented significantly by AA in both WT and Ephx2$^{-/-}$ MCA to 80.2 $\pm$ 1.3% ($n = 10$, $P < 0.001$ vs. without AA) and 84.7 $\pm$ 3.8% ($n = 4$, $P < 0.05$ vs. without AA), respectively (*Figure 3B* and *C*). These results confirmed that EETs are important mediators of SSD and their contribution can be enhanced by AA supplementation.

3.4 Impaired acetylcholine- and arachidonic acid-induced dilation in Cav-1$^{-/-}$ coronary arteries

We further assessed the ability of AA to produce dilation in mouse vessels in the absence of sheer stress. We found that AA dilated WT MCA in a concentration-dependent manner with an EC_{50} of 3.46 μM. At 10 μmol/L, AA produced 55.3 $\pm$ 6.1% ($n = 4$) dilation in WT MCA (*Figure 4A*). However, the ability of AA to produce dilation in Cav-1$^{-/-}$ MCA is significantly diminished. At 10 μmol/L, AA produced only 21.0 $\pm$ 4.6% ($n = 4$, $P < 0.001$ vs. WT) dilation in Cav-1$^{-/-}$ MCA (*Figure 4A*). Vasodilation expressed in vessel diameter change is shown in Supplementary material online, *Figure S6B*.

To further examine the non-shear stress-dependent endothelial function, we determine the effects of acetylcholine (ACh) on the dilation of WT and Cav-1$^{-/-}$ MCA. In WT vessels, ACh produced a concentration-dependent vasodilation with an EC_{50} of 0.27 μM. At 1 μmol/L, ACh produced 52.7 $\pm$ 3.3% dilation in WT vessels ($n = 7$). The effects of ACh were significantly blunted in Cav-1$^{-/-}$ vessels producing only 21.0 $\pm$ 1.6% dilation ($n = 6$, $P < 0.001$ vs. WT) at 1 μmol/L (*Figure 4B*). Vasodilation expressed in vessel diameter change is shown in Supplementary material online, *Figure S6C*. These results indicate that both shear stress-dependent and -independent endothelial functions are abnormal in Cav-1$^{-/-}$ coronary arteries.

To determine whether the vasodilation abnormalities were endothelial or smooth muscle in origin, we determine the effects of sodium nitroprusside (SNP) on WT and Cav-1$^{-/-}$ vessels. SNP produced similar dilations in WT and Cav-1$^{-/-}$ MCA (*Figure 4C*), suggesting that the ability of the contractile elements to produce vasodilation is intact, supporting that the vascular abnormalities associated with the absence of caveolae are endothelium-related.

3.5 Donor-detector-coupled vessels studies confirmed deficiency in shear stress-induced vasodilation by Cav-1$^{-/-}$ vessels

To confirm the deficiency in shear stress-induced generation and release of vasodilators in Cav-1$^{-/-}$ vessels, we performed experiments with donor−detector coupled vessels (Supplementary material online, *Figure S1B*). Donor vessels had intact endothelium, whereas detector vessels had endothelium removed. With WT donor vessels, 25 dynes/cm^2 induced 45.9 $\pm$ 3.5% vasodilation in WT detector vessels that were denuded of endothelium ($n = 6$). In contrast, Cav-1$^{-/-}$ donor vessels only induced 26.8 $\pm$ 2.3% vasodilation in WT detector vessels
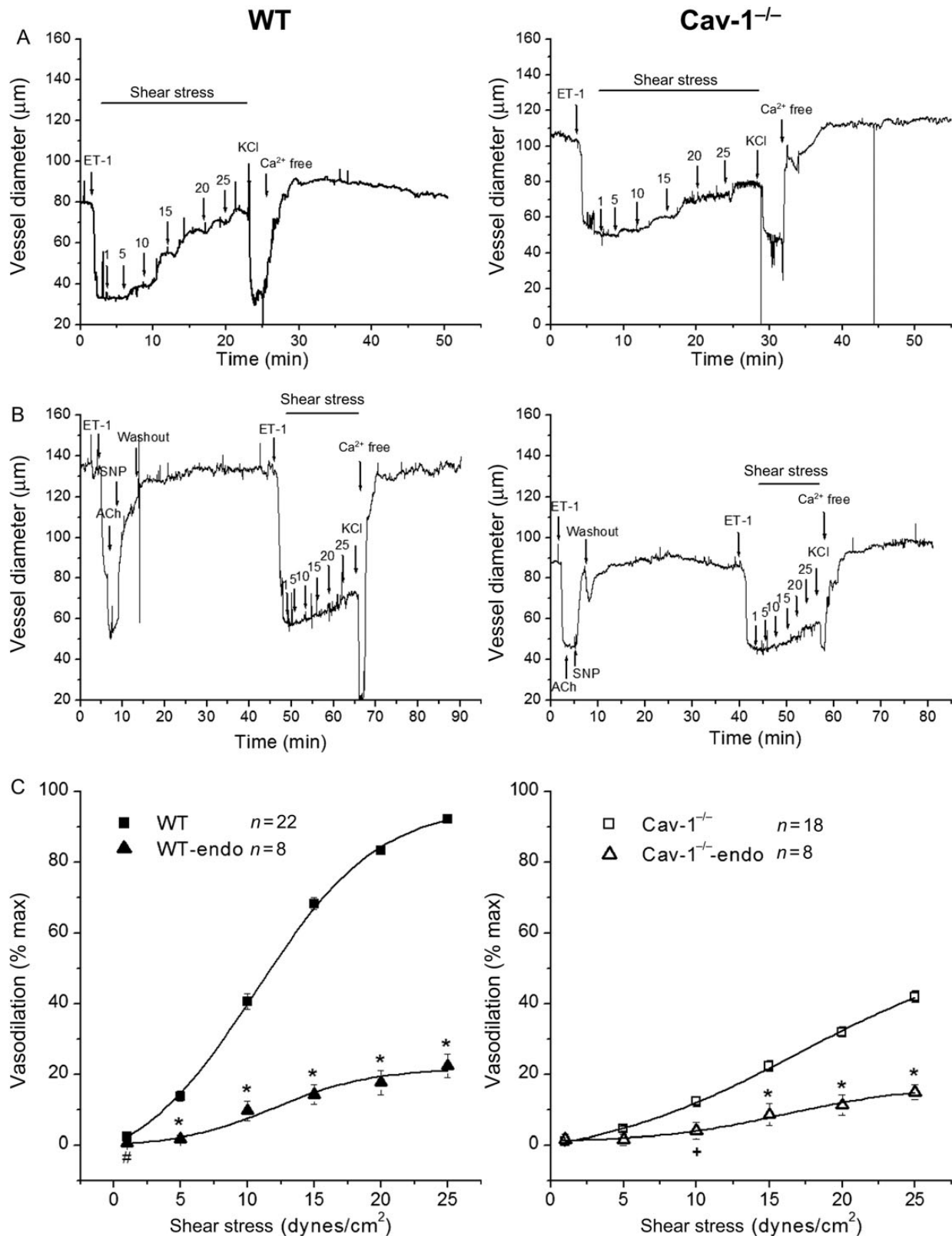
Figure 1 Role of caveolae and effects of endothelium denudation on SSD. (*A*) Representative continuous recordings of vessel diameters of isolated coronary arteries from WT (left panel) and Cav-1$^{-/-}$ (right panel) mouse vessels were pre-contracted with ET-1 followed by shear stress-induced dilation. At the end of the experiments, vessels were exposed to 100 mmol/L KCl and then to zero Ca^{2+} Kreb' solution. (*B*) Endothelium-denuded WT (left panel) and Cav-1$^{-/-}$ (right panel) mouse coronary arteries failed to dilate in response to acetylcholine (ACh 1 μmol/L) while the effects of sodium nitroprusside (SNP 100 μmol/L) remained intact. After endothelium removal, shear stress-induced dilation was significantly attenuated in both WT and Cav-1$^{-/-}$ vessels. (*C*) Group data showing SSD in WT ($n = 22$) and Cav-1$^{-/-}$ mice ($n = 18$) and the effects of endothelium denudation. WT-endo and Cav-1$^{-/-}$-endo are vessels without endothelium, $n = 8$ for both, $^{\#}P < 0.05$, $^{+}P < 0.01$, and $^{*}P < 0.001$ vs. vessels with intact endothelium.
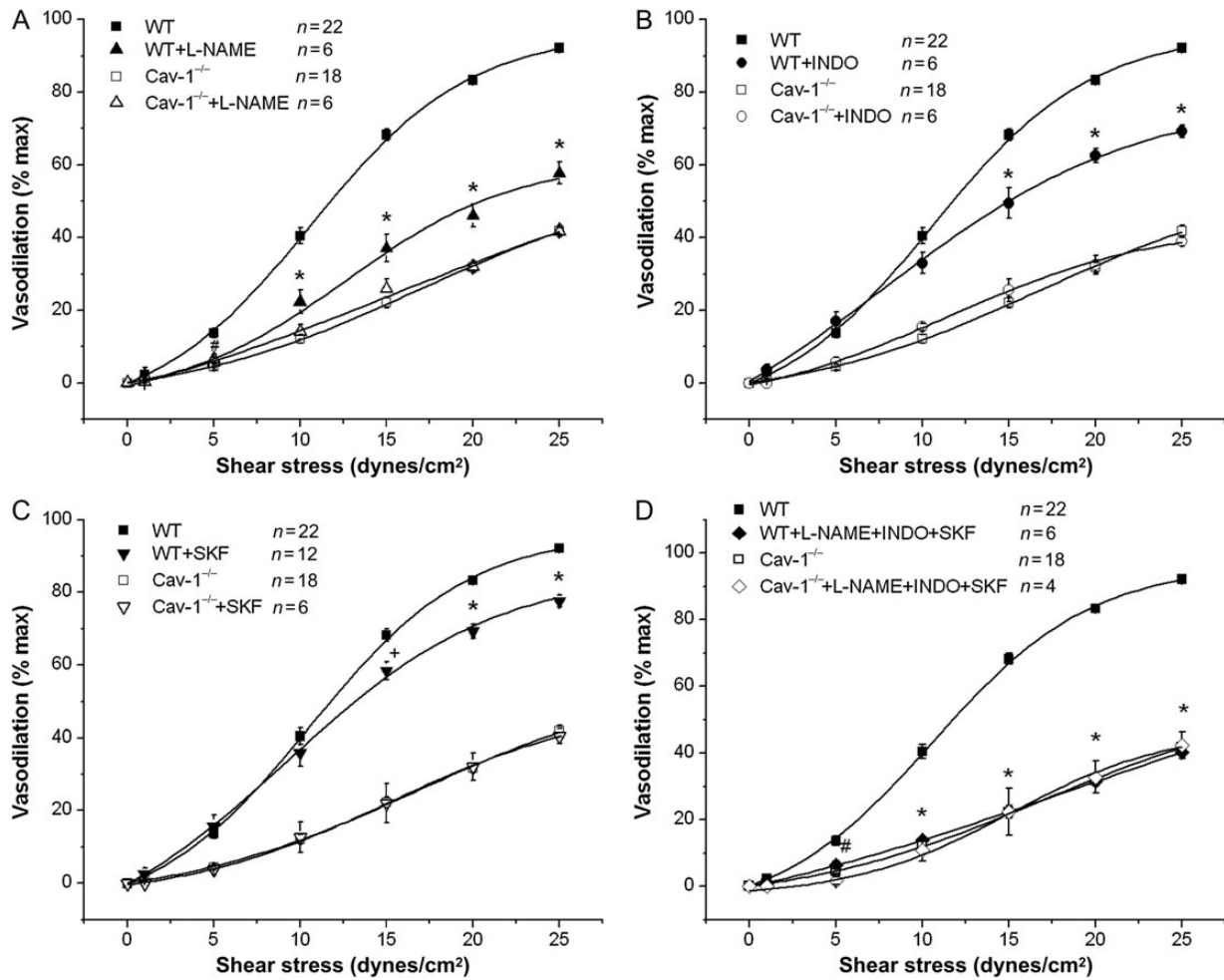
Figure 2 Effects of eNOS, COX, and CYP inhibition on SSD. Vessels were pre-treated with (*A*) L-NAME (100 μmol/L) for 30 min, with (*B*) Indomethacin (10 μmol/L) for 30 min, with (*C*) SKF 525A (10 μmol/L) for 60 min, and with (*D*) L-NAME + INDO + SKF 525A. #$P < 0.05$, +$P < 0.01$, and *$P < 0.001$ vs. no treatment.

($n = 7$, $P < 0.001$ vs. WT donor vessels) (*Figure 5*). These results confirmed that Cav-1$^{-/-}$ MCA are deficient in generating and/or releasing vasodilators in response to shear stress.

3.6 Deficient shear stress-induced calcium response in Cav-1$^{-/-}$ endothelial cells

To determine the mechanisms that underlie the endothelial dysfunction in Cav-1$^{-/-}$ MCA, we measured the intracellular Ca^{2+} response to shear stress in endothelial cells explanted from WT and Cav-1$^{-/-}$ aortas. Endothelial cells explanted from mouse aortas were immunohistochemically positive for von Willebrand factor but negative for α-actin which is positive for vascular smooth muscle cells (*Figure 6A*). Endothelial cells from WT vessels showed a robust increase in intracellular Ca^{2+} upon exposure to 11 dynes/cm^2 of shear stress. In contrast, endothelial cells from Cav-1$^{-/-}$ vessels showed a significantly blunted response, indicating that the absence of caveolae has profound effects on Ca^{2+} homeostasis in endothelial cells in response to shear stress (*Figure 6B*).

4. Discussion

We have made several important observations in the present study. First, endothelium-dependent SSD of MCA is dependent on the integrity of caveolae. Secondly, shear stress-mediated activation of NO, PG, and EETs is caveolae-dependent. Thirdly, the contribution of EETs to SSD can be augmented by retardation of degradation or by supplementation with AA. Fourthly, in the absence of caveolae, endothelial function is abnormal with blunted Ca^{2+} response to shear stress. These novel important findings have significant physiological and clinical relevance.

Shear stress is an important physiological mechanical stimulus that regulates endothelial and vascular function. Endothelial cells are capable of transducing haemodynamic forces into intracellular signalling events.[20] Our study shows that physiological levels of shear stress produce potent vasodilation in MCA, while vessels from Cav-1$^{-/-}$ mice only generate 38% of the response as that in WT (*Figure 1*). This finding reinforces the contention that caveolae may serve as the mechanical sensors that transduce the effects of shear stress.[6,21]

While NO has been found to be the dominant vasodilator in SSD in some previous reports,[22,23] our study showed that in normal mouse
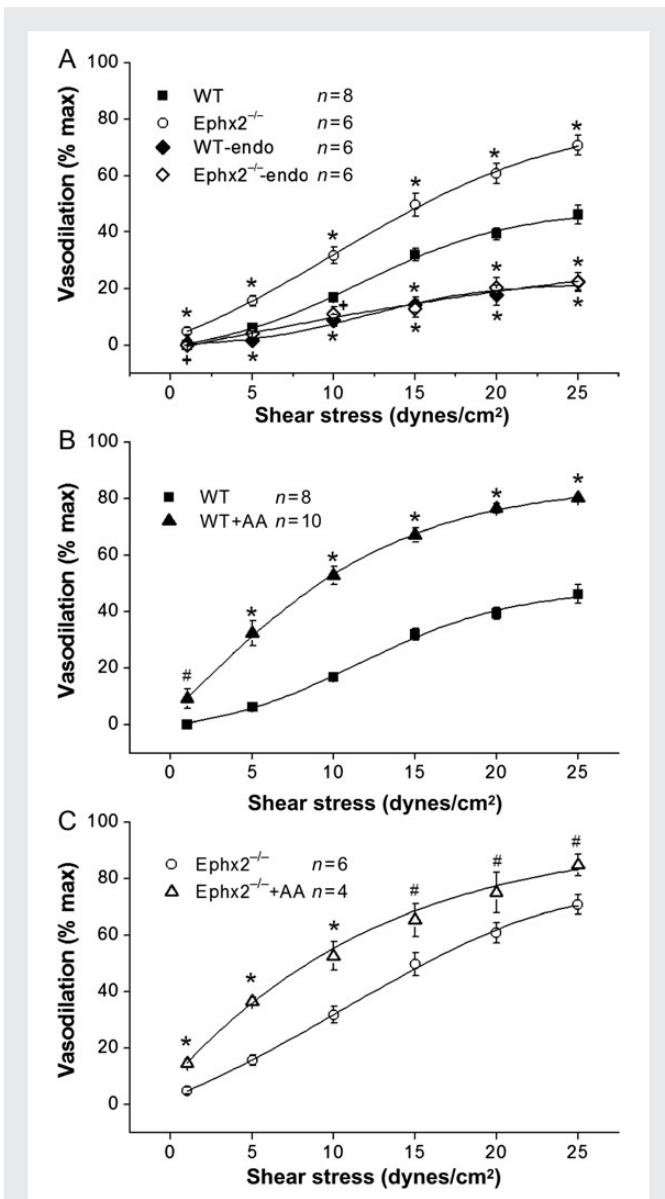
Figure 3 Role of soluble epoxide hydrolase on SSD. (*A*) In the presence of L-NAME (100 μmol/L) and INDO (10 μmol/L), the same level of shear stress produced greater vasodilation in Ephx2$^{-/-}$ compared with WT vessels (WT $n = 8$ and Ephx2$^{-/-}$ $n = 6$, *$P < 0.001$ vs. WT). After endothelium denudation, SSD was significantly reduced in both Ephx2$^{-/-}$ and WT mice ($n = 6$ for both Ephx2$^{-/-}$-Endo and WT−Endo; +$P < 0.01$ and *$P < 0.001$ vs. vessels with intact endothelium). In the presence of arachidonic acid (AA 10 μmol/L) in vessels pre-incubated with with L-NAME and INDO, SSD is increased both in WT ($n = 10$) (*B*) and in Ephx2$^{-/-}$ vessels ($n = 4$) (*C*) #$P < 0.05$ and *$P < 0.001$ vs. no AA).

Figure 4 Impaired endothelial function in Cav-1$^{-/-}$ coronary arteries. (*A*) AA-induced (10^{-10} to 10^{-4} mol/L) vasodilation in WT and Cav-1$^{-/-}$ coronary arteries with and without intact endothelium. #$P < 0.05$, +$P < 0.01$, and *$P < 0.001$ for Cav-1$^{-/-}$ vs. WT vessels. ##$P < 0.05$, ++$P < 0.01$, and **$P < 0.001$ for WT vessels denuded of endothelium (WT-endo) vs. WT vessels. (*B*) ACh-induced (10^{-10} to 10^{-4} mol/L) vasodilation in WT and Cav-1$^{-/-}$ coronary arteries. +$P < 0.01$ and *$P < 0.001$ for Cav-1$^{-/-}$ vs. WT vessels. (*C*) Sodium nitroprusside-induced (10^{-10} to 10^{-4} mol/L) vasodilation in WT and Cav-1$^{-/-}$ coronary arteries.

coronary arteries, SSD involves all three major vasodilator pathways, which generate NO, PG, and EETs. eNOS is known to be a target of mechanotransduction mechanisms involving endothelial surface molecules such as integrin.[24] Integrin activation phosphorylates eNOS at serine-1179, which results in eNOS activation and enhancement of NO production.[25] Shear stress-induced activation of COX-mediated generation of PG is less well-understood. Studies have shown that flow-me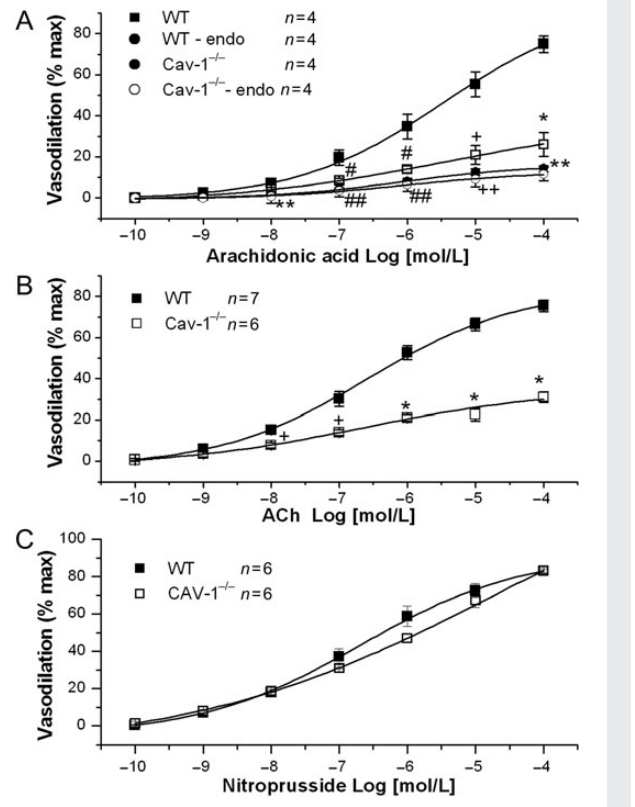diated vasodilation in eNOS-deficient mice is compensated by enhanced release of PG, and SSD is completely abolished by inhibitors of NO and PG.[26] Other studies have reported that COX-2 is essential for both the shear stress response and maintenance of flow-mediated vasodilation in the absence of eNOS.[27] The cross-talk between NO and PG is essential to maintain normal vasotone under physiological conditions. We found that the effects of eNOS and COX inhibition do not completely abolish SSD, which requires the additional inhibition of CYP epoxygenase. EETs are CYP epoxygenase metabolites of AA and have been shown to be EDHFs in a variety of vascular beds, including coronary arteries.[10,28] Recent studies have shown that shear stress stimulates the release of EETs from endothelium, which directly hyperpolarizes smooth muscle in rat mesentery arteries.[29] EETs dilate human coronary arteries through activation of BK channels.[11]

The quantitative contributions of these vasodilators are in the order of NO > PG > EETs in approximate ratios of 5:2:1 based on area-under-the-curve calculations for the endothelium-dependent vasodilations. These findings are different from previous reports that showed NO as the sole shear stress-induced vasodilator. The discrepancy in results may be due to differences in species,[23] the different methods of baseline vasoconstriction,[22] and the absence of caveolae
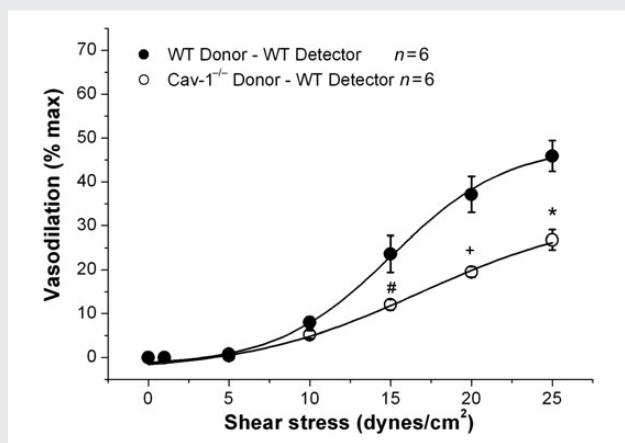
Figure 5 Effect of shear stress-induced coronary vasodilation in donor-detector-coupled vessels with WT and Cav-1$^{-/-}$ as donor vessels and endothelium-denuded WT vessels as detectors. SSD in detector vessels with WT donor vessels (closed circle) and Cav-1$^{-/-}$ donor vessels (open circle). $^{\#}P < 0.05$, $^{+}P < 0.01$, and $^{*}P < 0.001$ vs. WT donor vessels.
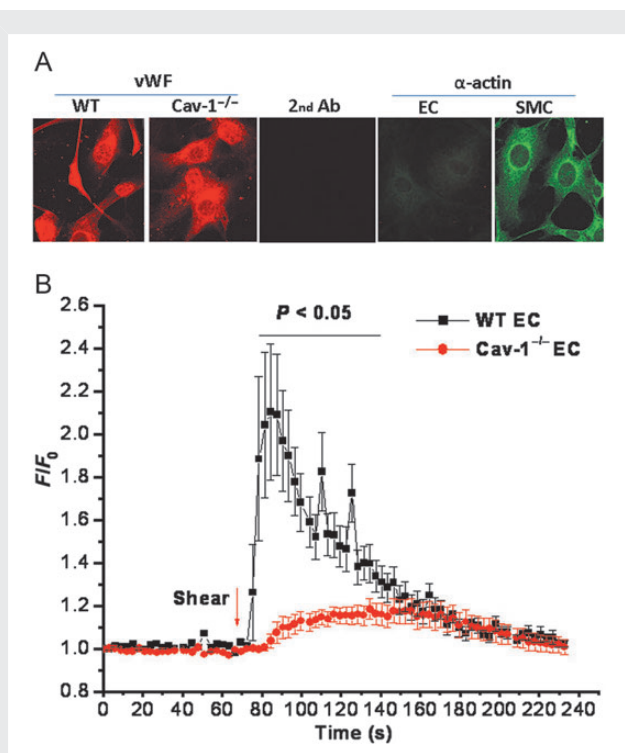

Figure 6 Impaired shear stress-induced Ca^{2+} response in vascular endothelial cells from Cav-1$^{-/-}$ mice. (*A*) Characterization of endothelial cells explanted from WT and Cav-1$^{-/-}$ aortas. Endothelial cells were stained with antibodies against the endothelial cell marker, von Willebrand factor (vWF, red), and the smooth muscle cell marker, α-actin (green). (*B*) Increase in intracellular Ca^{2+} in response to shear stress (11 dynes/cm^2) in fura-2 loaded endothelial cells from WT ($n = 7$) and Cav-1$^{-/-}$ ($n = 8$) vessels. Statistical difference between WT and Cav-1$^{-/-}$ cells was indicated by the bar above the tracings with $P < 0.05$.

in non-endothelial cells in the current study. However, non-NO dilators are involved in SSD is confirmed in Ephx2$^{-/-}$ vessels that the contribution of EETs to shear stress-induced endothelium-dependent vasodilation is augmented two-fold. Similarly, in the presence of L-NAME and INDO, supplementation with AA resulted in a 2.4-fold enhancement of SSD. These results suggest increased levels of AA may augment SSDs possibly through increased production of vasodilators that are non-prostaglandin metabolites of AA, possibly EETs.

It is rather striking that in the absence of caveolae, all three major vasodilator generating pathways lose their effectiveness in responding to shear stress. eNOS is targeted to caveolae and binding with Cav-1 inhibits eNOS and SSD through NO signalling is abnormal in Cav-1$^{-/-}$ vessels.[2] In addition, basal eNOS phosphorylation of serine 1176, a key regulatory site of phosphorylation by many kinases including Akt, AMP kinase, and PKA, was reduced in Cav-1$^{-/-}$ vascular extracts, suggesting that flow activation of upstream kinases may be impaired.[30]

PG including PGI$_2$ are also important endothelium-derived vasodilator released as a result of mechanotransduction by shear stress.[27] In WT coronary arteries, COX-mediated generation of vasodilators accounts for about 25% of SSD. In human vascular endothelial cells, prostacyclin synthase (PGIS) is targeted to caveolae and colocalize with Cav-1 and the production of PGI$_2$ in caveolae is important for the mediation of PGI$_2$ cellular function.[31,32] Our results suggest that the integrity of caveolae is critical in PG signalling. This is consistent with a previous observation that production of COX metabolites in caveolae is important for mediation of PG cellular function.[31,32]

EETs are EDHFs that produce vasodilation via activation of K$^+$ channels, which in turn leads to membrane hyperpolarization and vasorelaxation through closure of Ca^{2+} influx pathways.[33,34] Under basal conditions, shear stress-induced CYP epoxygenase-mediated vasodilation accounts for about 13% of endothelium-dependent vasodilation in WT coronary arteries (*Figure 2*), which is absent in Cav-1$^{-/-}$ vessels. CYP enzymes are not known to be targeted to caveolae microdomains. These findings raise the possibility that caveolae-dependent regulation of the vasodilator pathways may be mediated through a more fundamental mechanism than simple physical localization within caveolae microdomains. We speculate that in Cav-1$^{-/-}$ vessels, SSD is impaired due to loss of the spatial organization that is critical for the efficient generation of shear stress-induced vasodilators and for the coupling between vasodilators and effectors such as BK channels. In addition, our finding that shear stress-induced Ca^{2+} response in endothelial cells from Cav-1$^{-/-}$ vessels is profoundly abnormal elucidates a fundamental mechanism that underlies the shear stress-induced vascular dysfunction in Cav-1$^{-/-}$ coronary arteries.

Experiments involving donor–detector-coupled vessels confirmed that Cav-1$^{-/-}$ MCA are deficient in the generation and release of shear stress-induced endothelium-dependent vasodilators (*Figure 5*). With WT donor vessels, detector vessels showed a dose-dependent response to increasing levels of physiological shear stress with 45.9 ± 3.5% dilation by 25 dynes/cm^2. In comparison, the ability of Cav-1$^{-/-}$ donor vessels to produce shear stress-induced dilation in detector vessels was significantly diminished (*Figure 5*).

Intracellular Ca^{2+} is a major determinant of vascular endothelial function.[35] The dynamic spatiotemporal control of intracellular Ca^{2+} levels in vascular endothelial cells facilitates the modulation of multiple signalling pathways. Shear stress is the most potent stimulus in the activation of endothelial NOS and this process is Ca^{2+} dependent.[36] In addition, Ca^{2+} activates kinases that phosphorylate specific serine residues and augment eNOS activities. Production of AA-derived vasodilators is

also Ca^{2+} dependent. AA from intracellular stores in phospholipids is released by the rate-determining hydrolytic action of phospholipase A2 which is activated by Ca^{2+}.[37] AA serves as a precursor for eicosanoids including PGI_2 through the cyclooxygenase pathway and EETs through the CYP pathway.[38] Hence, disturbance in intracellular Ca^{2+} homeostasis as a result of loss of caveolae would affect the generation of NO, PG, and EET from all three vasodilator pathways. In support of our findings, a previous study showed that flow-induced Ca^{2+} response in endothelial cells starts at the caveolae and propagates as Ca^{2+} waves through the entire cell.[39] The shear stress-induced endothelial Ca^{2+} increase is dependent on extracellular Ca^{2+} entry.[40] The major Ca^{2+} entry pathways including the voltage-gated Ca^{2+} channels, the transient receptor potentialchannels, and the store-operated Ca^{2+} entry Orai channels are known to be localized to lipid rafts/caveolae.[41] Loss of the caveolae microdomains may result in the abnormal activation of the Ca^{2+} entry mechanisms. Our findings in this study provide further support that caveolae are mechanosensors and are important in mediating shear stress-induced endothelial function, but other mechanisms of mechanosensing are also known including the role of endothelial cell glycocalyx.[42]

In this study, we have presented compelling evidence that shear stress-induced coronary vasodilation is caveolae-dependent. Although we are unable to measure directly the generation of vasodilators in the mouse coronary arteries, our results suggest that with the loss of caveolae, NO, PG, and EET-mediated SSD is abolished. In addition, in the absence of caveolae, shear stress fails to elicit a normal endothelial intracellular Ca^{2+} increase which is critical for the generation of vasodilators. Our results support the notion that caveolae are critical determinants that transduce haemodynamic signals into vascular responses.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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