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Soon-Gook Hong, … , Marcus Gallagher-Jones, Julia J. Mack

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**Graphical abstract**

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Mechanosensitive membrane domains regulate calcium entry in arterial endothelial cells to protect against inflammation

Soon-Gook Hong,1,2 Julianne W. Ashby,1 John P. Kennelly,2,3 Meigan Wu,1,2 Michelle Steel,1 Eesha Chattopadhyay,1 Rob Foreman,4 Peter Tontonoz,2,3 Elizabeth J. Tarling,1,2 Patric Turowski,5 Marcus Gallagher-Jones,6 and Julia J. Mack1,2

1Department of Medicine, Division of Cardiology, 2Molecular Biology Institute, 3Department of Pathology and Laboratory Medicine, and 4Institute for Quantitative and Computational Biosciences, UCLA, Los Angeles, California, USA. 5UCL Institute of Ophthalmology, University College London, London, United Kingdom. 6Correlated Imaging, Rosalind Franklin Institute, Harwell Science & Innovation Campus, Didcot, United Kingdom.

Endothelial cells (ECs) in the descending aorta are exposed to high laminar shear stress, and this supports an antiinflammatory phenotype. High laminar shear stress also induces flow-aligned cell elongation and front-rear polarity, but whether these are required for the antiinflammatory phenotype is unclear. Here, we showed that caveolin-1–rich microdomains polarize to the downstream end of ECs that are exposed to continuous high laminar flow. These microdomains were characterized by high membrane rigidity, filamentous actin (F-actin), and raft-associated lipids. Transient receptor potential vanilloid (TRPV4) ion channels were ubiquitously expressed on the plasma membrane but mediated localized Ca2+ entry only at these microdomains where they physically interacted with clustered caveolin-1. These focal Ca2+ bursts activated endothelial nitric oxide synthase within the confines of these domains. Importantly, we found that signaling at these domains required both cell body elongation and sustained flow. Finally, TRPV4 signaling at these domains was necessary and sufficient to suppress inflammatory gene expression and exogenous activation of TRPV4 channels ameliorated the inflammatory response to stimuli both in vitro and in vivo. Our work revealed a polarized mechanosensitive signaling hub in arterial ECs that dampened inflammatory gene expression and promoted cell resilience.

Introduction

Blood flow patterns in the aorta are defined by vessel geometry: the curvature of the aortic arch results in low/oscillatory flow, whereas the straight descending aorta experiences high laminar flow (1, 2). The luminal layer of endothelial cells (ECs) in these two regions shows a striking difference in collective cell morphology, where ECs lining the descending aorta are highly elongated and those lining the aortic arch have a cobblestone appearance (3). Endothelial cell alignment with flow induces a well-documented front-rear polarity with respect to the flow direction (4–6), including preferential polarization of the plasma membrane proteins NOTCH1 and vascular endothelial protein tyrosine phosphatase (VE-PTP) to the downstream end of ECs (7–9). ECs in the descending aorta also display a well-known antiinflammatory and atheroprotective phenotype (10), but the underlying mechanisms and connection to front-rear polarity are unknown. Further, the role of plasma membrane polarization for signaling compartmentalization and its link to antiinflammatory signaling in arterial ECs have not been described.

Plasma membrane compartmentalization has long been described as a means to achieve signaling specificity and efficiency. One mechanism for compartmentalization is through the formation of specialized plasma membrane domains, such as lipid rafts and/or caveolae, that can sequester signaling molecules (11, 12). Abundant in ECs, caveolae are known to play an important role in a variety of cellular functions, including signal transduction, endothelial nitric oxide synthase (eNOS) regulation, and calcium (Ca2+) signaling activity (13). In arterial ECs, localized Ca2+ “sparklets” via the ion channel transient receptor vanilloid 4 (TRPV4) have been shown to promote vasodilation by increasing intracellular Ca2+ (14, 15). TRPV4 is a cation-permeable ion channel whose activity is regulated by both direct and indirect mechanical activation (16). TRPV4 activity is known to regulate blood vessel homeostasis by enhancing vasodilation (17, 18). However, the role of endothelial TRPV4-mediated Ca2+ signaling in vascular inflammation is less clear (19–21).

Mechanotransduction is a critical contributor to EC resilience. Notably, laminar blood flow forces promote anti-oxidative, antithrombotic, and antiinflammatory effects that are atheroprotective and prevent EC dysfunction (22). In the face of stressors, cells alter their gene expression, protein synthesis, and signaling activities to restore homeostasis (23). Nevertheless, how flow inhibits inflammatory signaling to promote endothelial resilience and prevent EC dysfunction is incompletely understood (24). Endothelial dysfunction is characterized by a state of activation and...
Front-rear polarization of arterial ECs. ECs lining the descending aorta are highly elongated, and we noted that high laminar flow resulted in a greater than 2-fold increase in EC aspect ratio in the descending aorta compared with the lower arch (Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/JCI175057DS1). To determine whether this elongated morphology was associated with altered caveolar organization, we stained ECs lining the mouse descending aorta for 2 components of caveolar, the plasma membrane-associated proteins caveolin-1 and cavin-1. We oriented the aorta based on the direction of blood flow, and quantified protein distribution by extracting fluorescence intensity in 3 cell segments of equal length, termed “upstream,” “mid-body,” and “downstream” in relation to the direction of flow (Supplemental Figure 1C). We found that the downstream end of descending aortic ECs displayed the highest levels of caveolin-1 and cavin-1 (Figure 1A and Supplemental Figure 1D).

To specifically investigate the connection between flow and protein distribution, we cultured human aortic EC (HAEC) monolayers on Y-shaped chambers and exposed them to unidirectional laminar flow for 48 hours. After 48 hours, monolayers of aligned cells displayed reduced migratory and proliferative behavior. HAECs in the high-flow region (~20 dynes/cm²) collectively aligned with the flow direction and were morphologically elongated, with a 3-fold increased cell aspect ratio compared with HAECs in the low-flow region (~5 dynes/cm²) (Supplemental Figure 1B). Importantly, the range of 5–20 dynes/cm² represents the typical range of shear stress for large conduit arteries in humans (29). In summary, exposure to sustained high laminar flow resulted in the formation of domains at the downstream end of HAECs, characterized by higher membrane rigidity, and the accumulation of raft-type lipids, F-actin aggregation, and caveolae-associated proteins caveolin-1, cavin-2, and cavins-1.

Activation of eNOS and $\text{Ca}^{2+}$ entry occur at the downstream end. Given the known functional connections between caveolin and eNOS (30–32), we hypothesized that polarization of caveolin-1-rich membrane domains led to localized eNOS activation. Indeed, eNOS phosphorylated on serine 1177 (p-eNOS), an active form of eNOS, was found predominantly concentrated at the downstream end of flow-aligned HAECs, with its staining mostly overlapping with that of the polarized caveolin-1 clusters (Figure 2A). Thus, caveolin-1 polarization correlates with localized activation of eNOS at the downstream end of ECs exposed to sustained flow.

Phosphorylation of eNOS on serine 1177 is frequently $\text{Ca}^{2+}$-dependent (33, 34). To examine changes in intracellular free $\text{Ca}^{2+}$, we transfected HAECs with plasmids encoding the $\text{Ca}^{2+}$ reporter GCaMP and exposed them to high laminar flow (Figure 2B and Supplemental Video 1). Segmentation analysis of individual cells revealed that oscillatory $\text{Ca}^{2+}$ influx events were restricted to the downstream end (black) and whole cell (green), which we identified as enriched for caveolin-1 and p-eNOS (Figure 2B and Supplemental Videos 2 and 3). To quantify the prevalence of $\text{Ca}^{2+}$ activity across the monolayer, full-length cells were segmented, and “active” cells were identified as having an index of dispersion greater than 2. Approximately 50% of the cells showed $\text{Ca}^{2+}$ transients over a 30-minute imaging period (Figure 2C), indicating that oscillatory $\text{Ca}^{2+}$ influx events were a sustained response of ECs under high laminar flow. Segmentation analysis of these active cells revealed that transients were restricted to the downstream end in over 70% of the active cells (Figure 2C and Supplemental Figure 3A).

Polarized signaling activity requires high laminar flow. We next compared $\text{Ca}^{2+}$ activity for HAECs in low-flow versus high-flow regions on Y-slides and observed that elevated shear stress cor-
related with increased Ca\(^{2+}\) oscillations. In the low-flow region, cells were less elongated and exhibited fewer Ca\(^{2+}\) oscillations, which were not preferentially localized (Figure 3A and Supplemental Videos 4 and 5). With increasing shear stress, cell morphology changed from cobblestone to elongated, and we observed concomitant enhanced Ca\(^{2+}\) activity with increasingly preferential localization to the downstream end of the elongated cells (Figure 3B, Supplemental Figure 3B, and Supplemental Video 6).
we imaged GCaMP-HAECs over the time course of flow, static, and re-flow conditions, which revealed that the oscillatory Ca\textsuperscript{2+} activity was observed only in the presence of laminar flow (Figure 3C, Supplemental Figure 4D, and Supplemental Video 10).

We next asked whether cell body elongation is sufficient for these the flow-induced phenotypes. To test this, we elongated HAECs in the absence of flow, by culturing them on line-patterned slides to achieve cell aspect ratio similar to those seen with flow (Figure 4A). Indeed, caveolin-1 showed asymmetric distribution at one end of static, elongated cells (Figure 4B). However, in contrast with flow-aligned HAECs, static elongated ECs accumulated caveolin-1 on either end of the cell, resulting in a random pattern. Importantly, polarized p-eNOS was not observed in static elongated ECs (Figure 4C). Accordingly, they did not display Ca\textsuperscript{2+} oscillations as measured by live cell imaging of GCaMP-HAECs (Figure 4D and Supplemental Video 11). Unlike HAECs exposed to high flow, HAECs exposed to low flow or static elongated HAECs did not show asymmetric activation of p-eNOS (Figure 4E). Thus, while cell elongation was sufficient to trigger caveolin-1 clustering at either end of ECs, the sustained presence of high laminar flow was required for coordinated polarization of caveolin-1 within the population and the formation of functional signaling domains.

Localized Ca\textsuperscript{2+} entry occurs via TRPV4/caveolin-1 association at the downstream end. We postulated that Ca\textsuperscript{2+} entry from the extracellular space via plasma membrane channels was required

there was a positive correlation between cell aspect ratio and Ca\textsuperscript{2+} oscillations at the downstream end (Supplemental Figure 3C), suggesting that flow-induced elongation is required for the localized Ca\textsuperscript{2+} signaling.

To capture the process of EC alignment with flow and Ca\textsuperscript{2+} activity, we imaged GCaMP-HAECs over the 48 hours, recording for 10 minutes every hour. We observed that Ca\textsuperscript{2+} activity at the downstream end of the cell was sustained only at the conclusion of flow alignment when cells had established an increased aspect ratio (Supplemental Figure 3D and Supplemental Video 7). Tracking an individual cell over the 48 hours, we noted that the prevalence and location of Ca\textsuperscript{2+} activity were sporadic as the cell changed shape, with localized activity when the cell aspect ratio was greater than 4 (Supplemental Figure 3E and Supplemental Video 8), further indicating that EC alignment with flow sustains the localized Ca\textsuperscript{2+} oscillations at the downstream end. To test whether non-arterial ECs exhibit this signaling behavior in response to flow alignment, we imaged GCaMP-transfected human umbilical vein ECs (HUVECs) under arterial flow (20 dynes/cm\textsuperscript{2}) and observed sustained Ca\textsuperscript{2+} oscillations at the downstream end of elongated cells (Supplemental Figure 4A and Supplemental Video 9). The flow-aligned HUVEC monolayer also displayed a polarized accumulation of caveolin-1 at the downstream end (Supplemental Figure 4, B and C), suggesting a response similar to that seen in arterial ECs. To test the requirement of shear stress for the Ca\textsuperscript{2+} signaling,
for Ca\textsuperscript{2+} activity. Consistent with this, the addition of the Ca\textsuperscript{2+} chelator EGTA blunted these Ca\textsuperscript{2+} oscillations (Figure 5A and Supplemental Figure 5A). Furthermore, Ca\textsuperscript{2+} oscillations at the downstream end continued in the presence of cyclopiazonic acid (CPA) (Supplemental Figure 5B), a sarco/endoplasmic reticulum Ca\textsuperscript{2+}-ATPase inhibitor, suggesting that the observed activity was independent of Ca\textsuperscript{2+} release from intracellular stores. Since the ion channel TRPV4 is implicated in endothelial Ca\textsuperscript{2+} “sparklet” activity in mouse arteries (14), we tested the role of TRPV4 channels by adding the TRPV4-specific antagonist GSK205 during imaging of GCaMP-HAECs under flow and found that it suppressed Ca\textsuperscript{2+} activity (Figure 5B and Supplemental Figure 5C). Considering the role of PIEZO channels as sensors of blood flow (35, 36), we also tested the role of PIEZO1 by adding the stretch-activated cation channel blocker GsMTx-4 (37), which did not affect Ca\textsuperscript{2+} oscillations at the downstream end (Supplemental Figure 5D).

The cumulative data indicate that TRPV4 is required for oscillatory Ca\textsuperscript{2+} entry at polarized caveolin-1-rich domains, where active eNOS was observed in flow-exposed elongated ECs.

Next, we investigated how TRPV4-dependent Ca\textsuperscript{2+} entry was polarized in response to flow conditioning of HAECs. Notably, TRPV4 mRNA and protein levels were indistinguishable in HAECs irrespective of the presence of laminar flow, whereas known flow-responsive genes KLF2 and KLF4 and eNOS protein showed an upregulation at the downstream end of cells where oscillatory TRPV4-mediated Ca\textsuperscript{2+} activity occurs.

Disruption of polarized caveolin-1 abolishes localized Ca\textsuperscript{2+} activity. Cholesterol affects caveolin-1 clustering and functions of cave-
olae, and sequestration of cholesterol using methyl-β-cyclodextrin (MβCD) disrupts caveolar function (39). We treated flow-aligned HAECs with MβCD for 30 minutes (Figure 6A). This effectively depleted the accessible pool of cholesterol (Supplemental Figure 6A) and also disrupted flow-induced plasma membrane polarization of caveolin-1 (Figure 6B). Functionally, this treatment reduced the levels of intracellular nitric oxide (NO) (Figure 6C), further supporting that caveolin-1 polarization directly affects endothelial signaling. Indeed, this also resulted in a loss of localized Ca²⁺ activity under laminar flow (Figure 6, D–F). Furthermore, treatment with the TRPV4-specific agonist GSK1016790A (GSK101; 10 nM) led to Ca²⁺ activity at the ends of elongated ECs exposed to MβCD (Figure 6, D–F), indicating that TRPV4 channels continued to be activatable in the absence of cholesterol. In line with our observation that TRPV4 channel expression was observed across the entire cell surface (Figure 5C), we found that GSK101 stimulated Ca²⁺ entry across the entire plasma membrane (Supplemental Figure 6B). However, higher doses (1 μM) were required to induce Ca²⁺ entry throughout the entire cell body, suggesting that the TRPV4 channel open probability was greater at the ends of flow-elongated ECs.

Inhibition of TRPV4 activity in the presence of laminar flow results in inflammation. We next investigated whether flow-induced TRPV4 activity promotes an anti-inflammatory signaling behavior.
For this, we modulated TRPV4 activity in flow-aligned HAECs by treating them with the TRPV4 antagonist GSK205 or vehicle, and performed transcriptional profiling and confocal imaging (Figure 7A). Inhibition of TRPV4 in flow-aligned HAECs resulted in an altered transcriptional profile after 2 hours (Figure 7, B and C, and Supplemental Figure 7A). Specifically, we noted the emergence of an inflammatory phenotype, as evidenced by Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis and gene set enrichment analysis of the RNA sequencing data (Supplemental Figure 7, B and C). Individual gene expression changes by quantitative PCR (qPCR) confirmed an upregulation of inflammatory genes associated with NF-κB activation, including SELE, VCAM1, and ICAM1 (Figure 7D). Ingenuity Upstream Regulator Analysis in IPA (QIAGEN) of the RNA sequencing data revealed the NF-κB
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sion of the proinflammatory adhesion molecules ICAM-1 and E-selectin (Figure 8A). Inhibition of TRPV4 activity also resulted in reduced NO production (Figure 8B) and increased reactive oxygen species (ROS) generation (Figure 8C), whereas GSK101-mediated activation of TRPV4 enhanced NO production (Supplemental Figure 8B). We confirmed that caveolin-1-rich domains remained polarized during TRPV4 inhibition (Supplemental Figure 8C), suggesting that the domains are present but not signaling. Finally, siRNA-mediated reduction of TRPV4 protein in HAECs

gene RELA, which is the NF-κB p65 subunit, to be the top activated upstream regulator in response to GSK205 treatment (Figure 7E).

In line with the changes in gene expression, 2-hour GSK205 treatment was associated with enhanced nuclear localization of the NF-κB p65 subunit (Figure 7F). By contrast, treatment with GsMTx-4 for 2 hours did not result in nuclear NF-κB p65 (Supplemental Figure 8A), further indicating that the inflammatory response was not associated with PIEZO channels. Moreover, inhibition of TRPV4 activity for 4 hours increased surface expres-
Aortic endothelium showed increased expression of VCAM-1 and nuclear NF-κB p65 in mice treated with GSK205 (Figure 8E). Taken together, these data indicated that TRPV4 activity sustains a pro-vasodilatory and antiinflammatory phenotype in the presence of laminar flow in vitro.

Activation of TRPV4 ameliorates the response to an acute inflammatory stimulus. We considered that activation of TRPV4 could exposed to laminar flow also reduced eNOS levels (Supplemental Figure 8D) and increased nuclear NF-κB p65 (Supplemental Figure 8E). We infer that TRPV4 activity in ECs exposed to laminar flow prevents proinflammatory responses in vitro.

To test the role of TRPV4 activity in vivo, we injected wild-type mice with either GSK205 (10 mg/kg) or vehicle and analyzed the aorta 4 hours later (Figure 8D). En face staining of the aortic endothelium showed increased expression of VCAM-1 and nuclear NF-κB p65 in mice treated with GSK205 (Figure 8E). Taken together, these data indicated that TRPV4 activity sustains a pro-vasodilatory and antiinflammatory phenotype in the presence of laminar flow in vitro and in the aorta in vivo.

**Figure 7. Inhibition of TRPV4 activity under flow induces inflammatory gene expression and NF-κB signaling.** (A) HAEC monolayers were flow-aligned for 48 hours followed by the addition of DMSO (control) or TRPV4 antagonist GSK205 (20 μM) in the presence of laminar flow (20 dynes/cm²). (B) Principal component analysis (PCA) plot of samples based on RNA sequencing analysis. RNA from n = 3 replicates per condition. (C) Heatmap displays top 76 significantly expressed genes between GSK205- and DMSO-treated HAECs. (D) Bar plot of gene expression shows an increase in inflammatory gene expression for GSK205-treated HAECs. Statistics calculated using 2-tailed, unpaired t test. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. (E) Bar plot of the top putative upstream regulators in response to GSK205 treatment as identified by Ingenuity Pathway Analysis (IPA) of the RNA sequencing data. (F) Control and GSK205-treated monolayers were fixed after 2 hours and stained for ZO-1 and the p65 subunit of NF-κB. Nuclear expression of NF-κB p65 was quantified by mean fluorescence intensity. Shown is mean intensity from n = 117 (DMSO) and n = 115 (GSK205) cells analyzed from n = 5 biological replicates and statistics calculated using 2-tailed, unpaired t test. **P < 0.01. Scale bars: 20 μm.
enhance cell resilience and an antiinflammatory response in ECs. Caveolin-1-rich domains remained polarized for at least 30 minutes after removal of flow (Supplemental Figure 9A), so we used flow-aligned HAECs in static conditions to study the effect of activating TRPV4 in the presence of tumor necrosis factor-α (TNF-α) (Figure 9A). Treatment with GSK101 (10 nM) for 30 minutes reduced the expression of proinflammatory genes, including NF-κB inhibitor α (NFKBIA), vascular cell adhesion molecule 1 (VCAM1), C-C motif chemokine ligand 2 (CCL2), selectin E (SELE), and TNF (Figure 9, B and C). Nuclear localization of NF-κB p65 was also attenuated upon activation of TRPV4 (Figure 9D). Changes to ICAM-1 protein expression levels were not observed within this short inflammatory TNF-α stimulation (Supplemental Figure 9B). However, we observed TNF-α–stimulated ROS production, which was attenuated by GSK101 costimulation (Supplemental Figure 9C). In contrast, the inflammatory response to TNF-α was not reduced by activation of PIEZO1 channels with the agonist Yoda1 (Supplemental Figure 10, A–C), further supporting the role of TRPV4 channels in mitigating inflammation. Overall, these data indicate that exogenous TRPV4 activation suppresses the inflammatory TNF-α response in HAECs.
Figure 9. Activation of TRPV4 dampens the endothelial response to an inflammatory stimulus in vitro. (A) Experimental design for static TNF-α treatment (10 ng/mL; 30 minutes) in the presence of GSK101 (10 nM) or DMSO. (B) Gene expression was measured by qPCR for TNF-α-treated monolayers in the presence of DMSO or GSK101. Gene expression of PECAM1, ITGB1, TRPV4, CCN1, NFKBIA, VCAM1, CCL2, SELE, and TNF plotted as mean ± SD. P < 0.05, **P < 0.01 by 2-tailed, unpaired t test; n = 3 biological replicates. (C) Gene expression as in B with mRNA expression plotted as a heatmap of mean expression. (D) After TNF-α treatment with or without GSK101, HAECs were fixed and stained for VE-cadherin and NF-κB (καB p65). Graph represents mean fluorescence intensity in the nucleus from n = 110 (DMSO), n = 79 (TNF-α + DMSO), and n = 87 (TNF-α + GSK101) cells per group from n = 4 biological replicates per condition and statistics calculated using 1-way ANOVA with post hoc Tukey’s multiple-comparison test. ***P < 0.001. Scale bars: 10 μm.

To test whether TRPV4 activation could attenuate the endothelial inflammatory response in vivo, we used a mouse model of acute LPS exposure (1.5 mg/kg). We treated mice with or without GSK101 (10 μg/kg) and isolated the blood and aorta after 4 hours (Figure 10A). LPS treatment led to an increased concentration of inflammatory cytokines in the plasma (Supplemental Figure 11, A and B), a strong upregulation of inflammatory gene expression in EC-enriched RNA from the descending aorta (Supplemental Figure 11, C and D), and substantial VCAM-1 expression on aortic endothelium accompanied by nuclear NF-κB p65 expression (Figure 10, B and C). Notably, Nos3 was also increased in animals cotreated with GSK101 (Figure 10B). En face imaging of the descending aortae of animals cotreated with GSK101 also showed a reduction of endothelial VCAM-1 and nuclear NF-κB p65 expression (Figure 10D). In summary, these data show that TRPV4 activation dampens the endothelial inflammatory response in vitro and in vivo.

Discussion

Our work shows that laminar flow preferentially polarized caveolin-1 to unique signaling domains at the downstream end of arterial ECs. This polarization activated TRPV4 and eNOS in a spatially restricted manner to suppress inflammatory pathways (Figure 10E). Caveolin-1 clustering at the downstream end occurred in response to flow alignment in aortic ECs in vivo and in vitro. We reveal that these clusters define a previously undescribed mechanosensitive domain that enables focal Ca2+ entry for eNOS activation and inhibition of inflammatory signaling. These domains were associated with a distinct pattern of proteins, lipids, and consequently biophysical properties rendering the downstream luminal surface of flow-aligned HAECs notably different from other parts of the cell. It remains unclear how elongation and laminar shear stress induce this local modulation of the plasma membrane, with both cytoskeletal and membrane lipids possible mediators (40, 41). Considering that the lipid phosphatidylinositol-4,5-bisphosphate (PIP2) has been shown to directly regulate TRPV4 activity in brain capillary endothelium (42, 43), the role of PIP2 and other plasma membrane lipids at these domains in response to changes in shear stress remains of interest.

Caveolin-1 and caveolae have been long recognized as important regulators of arterial organization in response to shear stress, in particular with respect to the regulation of vascular tone by both recruitment and modulation of eNOS activity (44–46). Our data indicate that caveolin-1 clustering also plays a role in promoting endothelial cell resilience by dampening inflammatory gene expression. Shear stress has previously been shown to induce caveola formation at the luminal surface of arterial ECs (47, 48); however, polarization of caveolae in this setting has not been shown. Future investigation of how caveolin-1 clustering affects the organization and dynamic redistribution of caveolae and membrane lipid composition will thus be of particular interest, as will be the contribution of the actin cytoskeleton.

Cell polarity is an important regulator of vascular phenotype and function (49). While the apical-basolateral polarity of ECs is clearly defined within the vessel wall, the mechanisms underlying
Compartmentalization has been described for the cell plasma membrane where membrane components cluster due to specific interactions to achieve spatiotemporal control (11, 12, 53). We show that flow elongation enables the polarized accumulation of caveolin-1 in the endothelial plasma membrane to create lipid-rich domains that compartmen-
talize Ca2+ signaling in the presence of flow. While TRPV4-mediated Ca2+ fluxes have been correlated with caveolae microdomains in ECs (54–56), visualization of Ca2+ entry after cell alignment with flow, the subcellular polarization of activity with respect to flow direction, and the mechanosensitive nature of the activity have not been clearly shown. By cycling the applied flow on and off, we revealed that Ca2+ oscillations were only sustained when laminar shear stress was present, thus indicating the mechanosensitivity of the polarized domain. Importantly, since all imaging experi-
ments to subcellular regions and membranes. Compartmentalization has been described for the cell plasma membrane where membrane components cluster due to specific interactions to achieve spatiotemporal control (11, 12, 53). We show that flow elongation enables the polarized accumulation of caveolin-1 in the endothelial plasma membrane to create lipid-rich domains that compartmentalize Ca2+ signaling in the presence of flow. While TRPV4-mediated Ca2+ fluxes have been correlated with caveolae microdomains in ECs (54–56), visualization of Ca2+ entry after cell alignment with flow, the subcellular polarization of activity with respect to flow direction, and the mechanosensitive nature of the activity have not been clearly shown. By cycling the applied flow on and off, we revealed that Ca2+ oscillations were only sustained when laminar shear stress was present, thus indicating the mechanosensitivity of the polarized domain. Importantly, since all imaging experi-
ments were performed after 48 hours of flow exposure, these Ca2+ oscillations were associated with the continuous presence of flow, in contrast to previous observations of Ca2+ spikes at the onset of shear stress (57, 58). Here, we show that TRPV4 channels at the downstream end of arterial ECs produce localized Ca2+ oscillations in the presence of sustained laminar flow. We assert that this compartmentalization of signaling activity is an important feature by which aortic ECs promote pro-vasodilatory and antiinflammatory pathways in the presence of atheroprotective flow.

TRPV4 is a mechanosensitive ion channel (59, 60) shown to associate with endothelial caveolae for Ca2+-dependent eNOS activation in pulmonary arteries (61). However, the modulation of its activity in the plasma membrane under flow was less clear. The physical association of TRPV4 with caveolin-1 at the downstream end of the cell indicated that this activation mechanism plays an important role in restricting Ca2+ entry to this region of the cell. Since inhibiting the TRPV4-mediated Ca2+ influx resulted in reduced NO production and an increase in both ROS and inflammation, our data suggest that endothelial cell resilience is clearly dependent on the presence and activity of these polarized plasma membrane domains. While endothelial TRPV4 activity has been shown to promote vasodilation in arteries (18, 62, 63), its role in vascular inflammation has not been as clearly defined (64). In fact, there are conflicting data regarding the role of TRPV4 activity in the inflammatory cascade (20, 21, 65). Here, we provide evidence that TRPV4 activity under laminar flow is antiinflammatory in aortic ECs. It led to eNOS activation and the production of NO, both indicators of antiinflammatory signaling (66), and ultimately contributed to the dampening of inflammatory gene expression. Therefore, this localized TRPV4 activity is a previously unrealized antiinflammatory mechanism of laminar flow and eNOS activation.

Additional studies are required to determine whether there exists a critical shear stress level or “set point” (67) to initiate and sustain the localized signaling activity. Live cell imaging experiments showed a correlation between EC aspect ratio and the presence of polarized Ca2+ activity, suggesting that there is a shear stress “set point” that promotes both cell body elongation and localized signaling activity. It is interesting to note that flow-aligned ECs displayed variation in cell aspect ratio and caveolin-1 clustering, but the underlying contributors to this heterogeneity are unknown.

Recent work has highlighted variable VCAM-1 and CD36 expression in the mouse aorta (68) and polarized VE-PTP clustering in ECs of the mouse aorta and vena cava (69). Additionally, single-cell RNA sequencing data have revealed 5 distinct populations of ECs exposed to laminar flow (70). It is interesting to consider that these populations may be correlated with different levels of cell resilience capacity, as previous work showed that ECs exhibited variable expression levels of VCAM-1 after exposure to an inflammatory agonist (71). Spatial transcriptomics can be applied to correlate the transcriptional signature of ECs with the presence and activity of polarized Ca2+ signaling domains. Whether there exists a pattern to the distribution of the ECs with these TRPV4 signaling domains is not yet known. It is intriguing to consider that these signaling cells may act as hubs that are spatially distributed across the endothelium to promote vessel resilience.

Considering that reduced eNOS activity and increased superoxide production are known contributors to EC dysfunction (72, 73), investigation into the role of these polarized caveolin-1/TRPV4 signaling domains in human vascular diseases is warranted. If the presence and activity of these microdomains function as an endothelial resilience mechanism, then diminished domain activity may contribute to EC dysfunction in response to cardiovascular risk factors such as diabetes, dyslipidemia, and aging. Furthermore, since the regulation of eNOS activation is finely tuned (74), identifying the downstream pathways directly affected by these signaling domains will be critical for establishing their role in vascular health and disease. Future research will also establish whether additional protective functions of laminar flow are mediated by polarized caveolin-1/TRPV4 microdomains.

In conclusion, we reveal that the anterior-posterior endothelial cell polarity established in response to laminar flow results in mechanosensitive signaling domains that both promote cell resilience and suppress inflammation.

Methods
Further information can be found in Supplemental Methods.

Sex as a biological variable
Our study used both male and female wild-type mice and primary ECs from both male and female donors.

Cell culture and shear stress
Primary HAECs (Cell Applications S304-05a), immortalized Telo-HAECs (ATCC CRL-4052), or primary HUVECs (VEC Technologies lot NCI-1199 pooled from 24 donors) were used. Primary HAECs were used from P4 to P12, and TeloHAECs were used up to P20. Primary HUVECs were used from P5 to P9. For cell culture experiments, MCDB-131 complete medium (VEC Technologies MCDB-131 Complete) or EGM-2 medium (Lonza CC-3162) was supplemented with 10% FBS (Omega USDA certified FBS FB-11). For plating of cells on cell culture dishes, 0.1% gelatin (Stemcell Technologies 07903) coating was used. For primary HAECs (Cell Applications CRL-1590), immortalized Telo-HAECs (ATCC CRL-4052), or primary HUVECs (VEC Technologies lot NCI-1199), we used serum-free Culture Media (CM) (Lonza CC-3162) for primary ECs. HAECs (ATCC CRL-1590), immortalized Telo-HAECs (ATCC CRL-4052), or primary HUVECs (VEC Technologies lot NCI-1199) were used from P5 to P9. For cell culture experiments, MCDB-131 complete medium (VEC Technologies MCDB-131 Complete) or EGM-2 medium (Lonza CC-3162) was supplemented with 10% FBS (Omega USDA certified FBS FB-11). For plating of cells on cell culture dishes, 0.1% gelatin (Stemcell Technologies 07903) coating was used.

For application of shear stress, ECs were seeded in μ-Slide 4.0 Maxi (Ibidi, 80567) or μ-Slides (Ibidi 80126) or Tissue Culture Dishes (Ibidi 80126). Unidirectional laminar flow was applied to confluent monolayers using the ibidi pump system (ibidi 10902). For experiments requiring access to the cells, including atomic force microscopy, cell monolayers were exposed to shear stress in glass-bottom 6-well plates (Cellvizio P06-1.5H-N) or 35 mm glass-bottom FluoroDishes (WPI FD35-100) on an orbital shaker (BenchMark Scientific BT302). A rotation speed of 130 rpm was applied to achieve endothelial cell alignment on the periphery of the well where the flow is unidirectional, while cells were unaligned in the center of the well where flow is multidirectional (75, 76). For cell alignment in the absence of flow, cells were plated on custom-made μ-Slides with micropattern surface (ibidi 83851).

Live cell fluorescence imaging
Membrane fluidity. For visualization of cell plasma membrane fluidity, Laurdan dye (6-dodecanoyl-2,6-dimethylaminophenylacetylene) (Invitrogen D250) was applied at 10 μM to cell monolayers and incubated 30 minutes at 37°C, then washed and imaged as 1× HBSS. For imaging, Zeiss LSM 880 with Chameleon 2-photon laser with Plan-Apochromat x20/0.8 M27 objective and GaAsP photomultiplier tube (PMT) array detector were used. Images were collected using 2-photon excitation.
set to 770 nm, and 2-channel emission detection was set for 400–460 nm and 470–530 nm. The acquisition of generalized polarization images was performed using ImageJ software (NIH) and ImageJ macro GPCalc ratiometric quantification and visualization (77).

**Lipid raft imaging.** Live cell imaging was performed using BODIPY FL C_{12}-Ganglioside G_{1a} (Invitrogen B13950). Cell monolayers were treated with 50 μM BODIPY FL C_{12}-Ganglioside G_{1b} for 20 minutes at 37°C with Hoechst 33342 (AAT Bioquest 19753).

**Calcium imaging.** HAECS and HUVECS were transected with GCaMP plasmid (pPB_CAG_GCamp5g) with PiggyBac construct (PB_Vector) (gift from R. Wollman, UCLA, Los Angeles, California, USA) using Lipofectamine 3000 transfection reagent (Invitrogen L30000001) or TransIT-X2 Dynamic Delivery System (Mirus MIRT6010) in Opti-MEM medium (Gibco 31985062) overnight. Cells were then selected for GCaMP expression with blasticidin (Gibco A113903). GCaMP-expressing HAECS were plated on Y-shaped chamber μ-slides (ibidi 80126), and after reaching confluence, slides were connected to the ibidi pump system for flow conditioning. After 48 hours of unidirectional flow, the Y-slide was connected to Yellow/Green perfusion set (ibidi 10964) modified with non-permeable tubing containing 13 mL of conditioned MCDB-131 (VEC Technologies MCDB-131 WOFBS) with 10% FBS (Omega USDA certified FBS FB-11) for live cell imaging. All steps were completed with minimal light exposure. Fluorescence images were acquired using Zeiss Observer Z1 with Colibri 7 light source, CMOS camera (Photometrics Prime 95), and ZEN Blue software. Images were acquired once every 3 seconds for total imaging time. For the long-term live cell imaging of the flow alignment, the ibidi stage-top incubator system (ibidi 1270) was used in combination with μ-slide 0.2 Luer ibiTest (ibidi 80166) and Yellow/Green perfusion set (ibidi 10964). For observation of changes in calcium activity, chemicals were added to one of the ibidi syringe reservoirs when air pressure was not active. Chemicals used included ethylene glycol tetraacetic acid (EGTA; 1.6 mM; Fisher Scientific NC1280093), Adenosine A1 Receptor Agonist Cyclopiazonic Acid (CPA; 50 μM; Sigma-Aldrich 119135), M-Therapoxotoxin-Gr1a (GmXtX4, 5 μM; Alomone Labs STG-100), Yoda1 (0.5 μM; Tocris 5586), GSK1016790A (10 nM or 1 μM; Sigma-Aldrich 530533), and GSK205 (20 μM; MedChemExpress HY-120691A).

**Nitric oxide imaging.** For quantification of NO production, cells were treated with 5 μM DAF-FM (Invitrogen D23844) for 20 minutes at 37°C with Hoechst 33342 (AAT Bioquest 17535).

**Reactive oxygen species imaging.** For oxidative stress detection, cell monolayers were treated with 5 μM CellROX Deep Red Reagent (Invitrogen C10422) or 5 μM CM-HDCFDA (Molecular Probes, C6827) for 20 minutes at 37°C with or without Hoechst 33342 (AAT Bioquest 17535).

**Membrane cholesterol imaging.** pALD4 was a gift of A. Radhakrishnan (UT Southwestern Medical Center; Addgene plasmid 111026) (78), and ALOD4 was purified and labeled with Alexa Fluor 488 as previously described (79, 80). Visualization of the accessible pool of cholesterol was performed after 30 minutes of treatment with 10 mM methyl-beta-cyclodextrin (MβCD) in 1% lipoprotein-deficient serum (LPDS; Sigma-Aldrich S5519) or 1% LPDS control, followed by flow for 2 hours in complete medium. HAECs were then stained with 20 μg/mL ALD4-F488 for 10 minutes at room temperature followed by 4% paraformaldehyde (PFA; Sigma-Aldrich 322415) fixation and application of 4,6-diamidino-2-phenylindole, dihydrochloride (DAPI) nuclear stain (AAT Bioquest 17507).

After respective dye incubation, cell monolayers were washed with 1× HBSS, then imaged in culture medium or 1× HBSS using Zeiss Colibri LED plus CMOS camera (Photometrics 95B) detection or LSM 900 with Airyscan2 GaAsP-PMT detector for gentle confocal imaging.

**Animals**
Both male and female wild-type C57BL/6J mice were purchased from The Jackson Laboratory (JAX:000664). All mice were fed a chow diet and water ad libitum under a 12-hour light/12-hour dark cycle.

**Aorta en face immunostaining**
Mice were perfused with ice-cold 1× PBS with an incision of the right ari trium to release the blood followed by perfusion with 4% PFA. The thoracic aorta was isolated and postfixed with 0.4% PFA overnight at 4°C. The vessel was then washed 3 times with 1× PBS and permeabilized by incubation with 0.3% Triton X in 2% normal donkey serum (Jackson ImmunoResearch Laboratories 017-000-121) in 1× PBS for 30 minutes at room temperature followed by incubation with primary antibodies overnight at 4°C with gentle agitation. After 1× PBS washes, the vessel was incubated with corresponding secondary antibodies for 2 hours at room temperature followed by washing with 1× PBS 3 times for 10 minutes each. The vessel was then cut longitudinally and mounted in Fluoromount-G (Southern-Biotech 0100-01). Primary antibodies used included ERG (Abcam ab92513), VE-cadherin (R&D AF1002), caveolin-1 (Invitrogen PAI-064), cav-1 (Abcam ab48824), NF-kB p65 (Cell Signaling 8242S), and VCAM-1 (BD Pharmlingen 550547).

**Immunofluorescence and confocal imaging**
For immunostaining, cell monolayers were fixed with 4% PFA for 10 minutes followed by multiple washes with 1× PBS. Samples were then blocked for 2 hours with 10% normal donkey serum (Jackson ImmunoResearch Laboratories 017-000-121) in 1× PBS. Depending on the protein targets of interest, samples were permeabilized with either 0.1% Triton X-100 (Fisher Scientific A16046-0F) or 0.01% digitonin (EMD Millipore 3004100). Primary antibodies were incubated overnight at 4°C in blocking buffer and secondary antibodies applied for 2 hours at room temperature. Primary antibodies used included caveolin-1 (Invitrogen PAI-064, R&D AF5736, or Santa Cruz Biotechnology sc-894), cavin-1 (Abcam ab48824), caveolin-2 (Invitrogen PA5-21927), ICAM-1 (Santa Cruz Biotechnology sc-107), E-selectin (Invitrogen MA-06506), ZO-1 (Invitrogen MA3-39100-A488), NF-kB p65 (Cell Signaling Technology 8242S), p-ceNOSSer1177 (Invitrogen PA5-35879 or Santa Cruz Biotechnology sc-81510), TRPV4 (Alomone Labs ACC-034 or LSBio LS-C401108), and VE-cadherin (R&D AF938). For F-actin staining, Alexa Fluor 555 Phalloidin (Invitrogen A34058) was applied with secondary antibody incubation.

Imaging was performed on a Zeiss LSM 900 confocal microscope equipped with 405 nm, 488 nm, 561 nm, and 640 nm laser lines using Plan-Apochromat objectives (×10, ×20, ×40, or ×63) and Airyscan2 GaAsP-PMT detector. Identical laser intensity settings were applied to all samples being compared with equivalent Z thickness. After acquisition, a maximum-intensity projection of the Z-stack was applied using ZEN Blue 3.5 software (Zeiss). Image processing and quantification of parameters were performed with ZEN Blue software, IMARIS software (Bitplane), ImageJ (NIH), or custom script (see Image analysis section in Supplemental Methods for details).
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Address correspondence to: Julia J. Mack, Department of Medicine, Division of Cardiology, University of California, Los Angeles, 650 Charles E. Young Drive South, Los Angeles, California 90095, USA. Phone: 310.825.5749; Email: jmack@mednet.ucla.edu.

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