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Graphical abstract

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

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Conflict-of-interest statement:

The authors have declared that no conflict of interest exists.
Abstract

Endothelial cells (ECs) in the descending aorta are exposed to high laminar shear stress, and this supports an anti-inflammatory phenotype. High laminar shear stress also induces flow-aligned cell elongation and front-rear polarity, but whether these are required for the anti-inflammatory 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-type 4 (TRPV4) ion channels were ubiquitously expressed on the plasma membrane but mediated localized Ca\(^{2+}\) entry only at these microdomains where they physically interacted with clustered Caveolin-1. These focal Ca\(^{2+}\) bursts activated endothelial nitric oxide synthase (eNOS) 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 dampens inflammatory gene expression and promotes 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 show 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 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 anti-inflammatory and athero-protective phenotype (10), but the underlying mechanisms and connection to front-rear polarity are unknown. Furthermore, the role of plasma membrane polarization for signaling compartmentalization and its link to anti-inflammatory signaling in arterial ECs has 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 endothelial cells, caveolae are known to play an important role in a variety of cellular functions, including signal transduction, eNOS regulation, and calcium (Ca²⁺) signaling activity (13). In arterial ECs, localized Ca²⁺ ‘sparklets’ via the ion channel transient receptor potential cation channel subfamily V member 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 Ca\textsuperscript{2+} 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, anti-thrombotic, and anti-inflammatory effects that are athero-protective 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 pro-inflammatory signaling (25, 26). Although the inflammatory response can serve as a protective reaction to harmful stimuli, dysregulated vascular inflammation is a pathological driver of cardiovascular disease (27, 28).

Here, we investigated the role of sustained laminar flow on arterial EC signaling and found a distinct polarization of signaling activity, asymmetrically concentrated at the downstream end of cells. Polarization included membrane lipids, F-actin, and Caveolin-1 associated with TRPV4 channels. In the presence of high shear stress, localized Ca\textsuperscript{2+} oscillations via TRPV4 channels are sustained at these domains and contribute to eNOS activation and anti-inflammatory gene expression. Our studies revealed a spatially-restricted signaling domain in arterial ECs that is both mechanosensitive and anti-inflammatory. The findings suggest that high laminar flow sustains a lateral polarity of ECs that is defined by flow direction through the asymmetric distribution of membrane
components that regulate signaling activity. Furthermore, the data indicate that this signaling activity is an endothelial resilience mechanism and therefore offers a potential target for a therapeutic approach to reverse EC dysfunction and treat vascular inflammation.
Results

Front-rear polarization of arterial endothelial cells

ECs lining the descending aorta are highly elongated, and we noted that high laminar flow resulted in a greater than two-fold increase in EC aspect ratio in the descending aorta compared to the lower arch (Supplemental Figure 1A). To determine if this elongated morphology was associated with altered caveolae organization, we stained ECs lining the mouse descending aorta for two components of caveolae, 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 three 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; 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 h. After 48 h, 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 three-fold increased cell aspect ratio compared to 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 line with in vivo observations, Caveolin-1 and Cavin-1 showed high
concentration at the downstream end of HAECs exposed to high flow (Figure 1B; Supplemental Figure 1E).

We examined the broader cell surface asymmetries of flow-aligned HAECs by atomic force microscopy (Supplemental Figure 2A). This revealed that the cell’s downstream end was considerably stiffer (average Young’s modulus of 7.0 kPa) compared to the upstream end (average Young’s modulus of 5.3 kPa) (Supplemental Figure 2B). Furthermore, Laurdan dye imaging revealed asymmetry of the membrane fluidity across the ECs, where the downstream end typically exhibited a higher generalized polarization (GP) indicating higher membrane rigidity compared to the upstream end (Figure 1C). Membrane fluidity is a result of the distribution and composition of lipids within the bilayer. Accordingly, the fluorescent probe BODIPY FL C5-Ganglioside GM1 was strongly enriched at the downstream end of flow-aligned HAECs (Figure 1D), suggesting the presence of liquid-ordered or lipid raft domains in regions with enriched Caveolin-1.

F-actin also displayed more densely webbed clusters at the downstream end of flow-aligned HAECs (Figure 1E). Importantly, these downstream F-actin clusters correlated with high density Caveolin-1 staining (Figure 1F). This was further highlighted for Caveolin-1 and F-actin by 3D surface rendering (Figure 1G). Caveolin-2 was also observed to accumulate at the downstream end with Caveolin-1 and F-actin clusters (Supplemental Figure 2, C–E). Markedly, the Caveolin-1 cluster size was larger for HAECs exposed to high flow compared to low flow (Supplemental Figure 2F) and the cluster size increased ca. six-fold within these polarized downstream areas in the presence of high flow (Figure 1H). 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, Caveolin-2, and Cavin-1.

 Activation of eNOS and Ca\(^{2+}\) entry occur at the downstream end

Given the known functional connections between caveolae 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 Ca\(^{2+}\) dependent (33, 34). To examine changes in intracellular free Ca\(^{2+}\), we transfected HAECs with plasmids encoding the Ca\(^{2+}\) reporter GCaMP and exposed them to high laminar flow (Figure 2B; Movie 1). Segmentation analysis of individual cells revealed that oscillatory Ca\(^{2+}\) influx events were restricted to the downstream end, which we identified as enriched for Caveolin-1 and p-eNOS (Figure 2B; Movies 2 & 3). To quantify the prevalence of Ca\(^{2+}\) activity across the monolayer, full-length cells were segmented, and ‘active’ cells were identified as having an index of dispersion (IoD) greater than 2. Approximately 50% of the cells showed Ca\(^{2+}\) transients over a 30 min-imaging period (Figure 2C), indicating that oscillatory 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; Supplemental Figure 3A).
Polarized signaling activity requires high laminar flow

We next compared Ca\(^{2+}\) activity for HAECs in low flow versus high flow regions on y-slides and observed that elevated shear stress correlated with increased Ca\(^{2+}\) oscillations. In the low-flow region, cells were less elongated and exhibited less Ca\(^{2+}\) oscillations, which were not preferentially localized (Figure 3A; Movies 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; Movie 6). In fact, there was a positive correlation between cell aspect ratio and Ca\(^{2+}\) oscillations at the downstream end (Supplemental Figure 3C), suggesting that flow-induced elongation is required for the localized Ca\(^{2+}\) signaling.

To capture the process of EC alignment with flow and Ca\(^{2+}\) activity, we imaged GCaMP-HAECs over the 48 h, recording for 10 min every hour. We observed that Ca\(^{2+}\) 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; Movie 7). Tracking an individual cell over the 48 h, we noted that the prevalence and location of Ca\(^{2+}\) activity was sporadic as the cell changed shape, with localized activity when the cell aspect ratio was greater than 4 (Supplemental Figure 3E; Movie 8), further indicating that EC alignment with flow sustains the localized Ca\(^{2+}\) 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 endothelial cells (HUVECs) under arterial flow (20 dynes/cm\(^2\)) and observed sustained Ca\(^{2+}\) oscillations at the downstream end of elongated cells (Supplemental Figure 4A; Movie 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 similar configuration as seen in arterial ECs. To test the requirement of shear stress for the Ca\(^{2+}\) signaling, we imaged GCaMP-HAECs over the time course of flow, static and re-flow conditions, which revealed that the oscillatory Ca\(^{2+}\) activity was observed only in the presence of laminar flow (Figure 3C; Supplemental Figure 4D; Movie 10).

We hypothesized that cell body elongation is sufficient for these 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\(^{2+}\) oscillations as measured by live cell imaging of GCaMP-HAECs (Figure 4D; Movie 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\(^{2+}\) entry occurs via TRPV4/Caveolin-1 association at the downstream end*

We postulated that Ca\(^{2+}\) entry from the extracellular space via plasma membrane channels was required for Ca\(^{2+}\) activity. Consistent with this, the addition of the Ca\(^{2+}\)
chelator EGTA blunted these Ca^{2+} oscillations (Figure 5A; Supplemental Figure 5A). Furthermore, Ca^{2+} oscillations at the downstream end continued in the presence of cyclopiazonic acid (CPA) (Supplemental Figure 5B), a sarco-endoplasmic reticulum (ER) Ca^{2+}-ATPase inhibitor, suggesting that the observed activity was independent of Ca^{2+} release from intracellular stores. Since the ion channel TRPV4 is implicated in endothelial Ca^{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^{2+} activity (Figure 5B; 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^{2+} oscillations at the downstream end (Supplemental Figure 5D). The cumulative data indicate that TRPV4 is required for oscillatory Ca^{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^{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 in response to flow (Supplemental Figure 5, E and F). Importantly, unlike Caveolin-1, Caveolin-2 or Cavin-1, TRPV4 was uniformly distributed across the plasma membrane and did not display any apparent polarization (Figure 5C).

TRPV4 interacting with Caveolin-1 leads to its activation in lung endothelium (38). To determine if this association also occurred in flow-conditioned HAECs, we used a
proximity ligation assay (PLA). We observed specific in situ PLA spots when combining antibodies to Caveolin-1 and TRPV4 or Cavin-1, but not Histone H3 or with individual antibodies alone (Supplemental Figure 5G). Appreciably, TRPV4/Caveolin-1 PLA spots were strongly enhanced in HAECs exposed to high flow and also clearly accumulated at the downstream end of the elongated cells. Cells in the low-flow region did not show a difference in the distribution of PLA spots (Figure 5D). Combined, these data indicated that TRPV4 associated with Caveolin-1 clusters in the presence of high flow at the downstream end of cells where oscillatory TRPV4-mediated Ca^{2+} activity occurred.

Disruption of polarized Caveolin-1 abolishes localized Ca^{2+} activity

Cholesterol affects Caveolin-1 clustering and functions of caveolae, and sequestration of cholesterol using methyl-β-cyclodextrin (MβCD) disrupts caveolae function (39). We treated flow-aligned HAECs with MβCD for 30 min (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 NO (Figure 6C), further supporting that Caveolin-1 polarization directly affects endothelial signaling. Indeed, this also resulted in a loss of localized Ca^{2+} activity under laminar flow (Figure 6, D–F). Furthermore, treatment with the TRPV4-specific agonist GSK1016790A (GSK101; 10 nM) led to Ca^{2+} activity at the ends of elongated ECs exposed to MβCD (Figure 6, D–F; Movie 12), 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 Ca^{2+} entry across the entire plasma membrane (Supplemental Figure 6B). However, higher doses (1 µM) were
required to induce Ca$^{2+}$ 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 h (Figure 7, B and C; Supplemental Figure 7A). Specifically, we noted the emergence of an inflammatory phenotype, as evidenced by KEGG pathway analysis and gene set enrichment analysis (GSEA) of the RNA sequencing data (Supplemental Figure 7, B and C). Individual gene expression changes by 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 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 h GSK205 treatment was associated with enhanced nuclear localization of the NF-κB p65 subunit (Figure 7F). By contrast, treatment with GsMTx-4 for 2 h 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 h increased surface expression of the pro-inflammatory 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 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 pro-inflammatory responses in vitro.

To test the role of TRPV4 activity in vivo, we injected wildtype mice with either GSK205 (10 mg/kg) or vehicle and analyzed the aorta 4 h 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 anti-inflammatory phenotype in the presence of laminar flow in vitro and in the aorta in vivo.

*Activation of TRPV4 ameliorates the response to an acute inflammatory stimulus*

We considered that activation of TRPV4 could enhance cell resilience and an anti-inflammatory response in ECs. Caveolin-1-rich domains remained polarized for at least 30 min 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 alpha (TNFα) (Figure 9A). Treatment with GSK101 (10 nM) for 30 min reduced the expression of pro-inflammatory genes, including nuclear factor-kappa-B-inhibitor alpha (*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 TNFα stimulation (Supplemental Figure 9B). However, we observed TNFα-stimulated ROS production, which was attenuated by GSK101 co-stimulation (Supplemental Figure 9C). In contrast, the inflammatory response to TNFα was not reduced by activating 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 TNFα response in HAECs.

To test whether TRPV4 activation could attenuate the endothelial inflammatory response in vivo, we employed 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 h (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 (Supplemental Figure 11E). Co-treatment of animals with GSK101 suppressed LPS-induced aortic endothelial inflammatory gene expression, including that of NF-κB pathway genes Rel, Rela and Relb (Figure 10, B and C). Notably, Nos3 was also increased for animals co-treated with GSK101 (Figure 10B). En face imaging of the descending aortas of animals co-treated 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 (Fig. 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 Ca\(^{2+}\) 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 to 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 the lipid phosphatidylinositol-4,5-bisphosphate (PIP\(_2\)) has been shown to directly regulate TRPV4 activity in brain capillary endothelium (42, 43), the role of PIP\(_2\) 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 recruiting and modulating eNOS activity (44-46). Our data indicates 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 caveolae 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 endothelial cells is clearly defined within the vessel wall, the mechanisms underlying the establishment and maintenance of a lateral polarity in the aorta are poorly understood (50, 51). Intracellular organelles, the Golgi apparatus and the microtubule-organizing center (MTOC), are often used as indicators of endothelial planar polarity with respect to flow direction (4-6). However, after development, ECs in the descending aorta frequently display the Golgi apparatus positioned at the side of the nucleus (52), indicating neither an upstream nor downstream polarization. Our current finding of polarized signaling domains, along with other reports of asymmetrically distributed plasma membrane proteins (7-9), suggest that the EC surface may serve as a direct read-out of blood flow direction and, therefore, the lateral polarity of the arterial endothelial cell.

Cells use the compartmentalization of signaling events to sustain important physiological functions and prevent crosstalk across pathways. For this, they concentrate specific biochemical components 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 Ca^{2+} signaling in the presence of flow. While TRPV4-mediated Ca^{2+} fluxes have been correlated to
caveolae microdomains in ECs (54-56), visualization of Ca\textsuperscript{2+} entry after cell alignment with flow, the sub-cellular polarization of activity with respect to flow direction, and the mechanosensitive nature of the activity has not been clearly shown. By cycling the applied flow on and off, we revealed that Ca\textsuperscript{2+} oscillations were only sustained when laminar shear stress was present thus indicating the mechanosensitivity of the polarized domain. Importantly, since all imaging experiments were performed after 48 h of flow exposure, these Ca\textsuperscript{2+} oscillations were associated with the continuous presence of flow, in contrast to previous observations of Ca\textsuperscript{2+} spikes at the onset of shear stress (57, 58). Here, we show that TRPV4 channels at the downstream end of arterial ECs produce localized Ca\textsuperscript{2+} 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 anti-inflammatory pathways in the presence of athero-protective flow.

TRPV4 is a mechanosensitive ion channel (59, 60) shown to associate with endothelial caveolae for Ca\textsuperscript{2+}-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 Ca\textsuperscript{2+} entry to this region of the cell. Since inhibiting the TRPV4-mediated Ca\textsuperscript{2+} influx resulted in reduced NO production and an increase in both ROS and inflammation, our data suggests 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 anti-inflammatory in aortic ECs. It led to eNOS activation and the production of NO, both indicators of anti-inflammatory signaling (66), and ultimately contributed to the dampening of inflammatory gene expression. Therefore, this localized TRPV4 activity is a previously unrealized anti-inflammatory 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 Ca\textsuperscript{2+} 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 has 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 Ca\textsuperscript{2+} 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, which 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

Sex as a biological variable

Our study used both male and female wildtype mice and primary endothelial cells from both male and female donors.

Cell culture and shear stress

Primary HAECs (Cell Applications #S304-05a), immortalized TeloHAECs (ATCC #CRL-4052) or primary HUVECs (VEC Technologies lot #NCI-1199 pooled from 24 donors) were used. Primary HAECs were used from P4-P12 and TeloHAECs were used up to P20. Primary HUVECs were used from P5-P9. For cell culture experiments, MCDB-131 complete media (VEC Technologies #MCDB-131 Complete) or EGM-2 media (Lonza #CC-3162) was supplemented with 10% FBS (Omega USDA certified FBS #FB-11). For plating cells on cell culture dishes, 0.1% gelatin (Stemcell #07903) coating was first applied. Cells were cultured in a 37 °C incubator with 5% CO₂.

For application of shear stress, endothelial cells were seeded in ibidi µ-Slide 0.4 Luer ibiTreat (ibidi #80176) or y-shaped ibiTreat µ-slides (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 (Cellvis #P06-1.5H-N) or 35mm 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 are unaligned in the center of the well where flow is multi-directional (75, 76).
For cell alignment in the absence of flow, cells were plated on customized ibidi µ-slides with micropattern surface (ibidi GmbH #83851).

**Live cell fluorescence imaging**

*Membrane fluidity:* For visualization of cell plasma membrane fluidity, Laurdan dye (6-Dodecanoyl-2-Dimethylaminonaphthalene) (Invitrogen #D250) was applied at 10 μM to cell monolayers and incubated 30 min at 37°C then washed and imaged in 1X HBSS. For imaging, Zeiss LSM 880 with Chameleon 2-photon laser with Plan-Apochromat 20x/0.8 M27 objective and GaAsP PMT array detector were used. Images were collected using two-photon excitation set to 770 nm, and two-channel emission detection was set for 400 – 460 nm & 470 – 530 nm. The acquisition of generalized polarization (GP) images was performed using the ImageJ (National Institutes of Health) software and ImageJ macro GPCalc ratiometric quantification and visualization (77).

*Lipid raft imaging:* Live cell imaging was performed using BODIPY™ FL C₅-Ganglioside G₉₁ (Invitrogen #B13950). Cell monolayers were treated with 50 μM BODIPY™ FL C₅-Ganglioside G₉₁ for 20 minutes at 37°C with Hoescht 33342 (AAT Bioquest #17535).

*Calcium imaging:* HAECs and HUVECs were transfected with GCaMP plasmid (pPB_CAG_GCamp5g) with PiggyBac construct (PB_Vector) (gift from R. Wollman, UCLA, Los Angeles, CA) using Lipofectamine 3000 transfection reagent (Invitrogen #L3000001) or TransIT-X2® Dynamic Delivery System (Mirus #MIR6010) in Opti-MEM media (Gibco #31985062) overnight. Cells were then selected for GCaMP expression with Blasticidin (Gibco #A1113903). GCaMP expressing HAECs were plated on y-shaped chamber µ-slides (ibidi #80126) and after reaching confluence, slides were connected to ibidi pump system for flow conditioning. After 48 h 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 95B) and ZEN Blue software. Images were acquired once every 3 s 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 ibiTreat (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 include ethylene glycol tetra-acetic acid (EGTA, 1.6 mM; Fisher Scientific #NC1280093), Adenosine A1 Receptor Agonist N\textsuperscript{6}-Cyclopentyladenosine (CPA, 50 µM; Sigma-Aldrich #119135), M-Theraphotoxin-Gr1a (GsMTx4, 5 µM; Alomone Labs #STG-100), Yoda 1 (0.5 µM; Tocris #5586), GSK1016790A (10 nM or 1 µM; Sigma-Aldrich #530533), GSK205 (20 µM; MedChemExpress #HY-120691A).

**Nitric oxide imaging:** For quantification of nitric oxide production, cells were treated with 5 µM DAF-FM (Invitrogen #D23844) for 20 min 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-H\textsubscript{2}DCFDA (Molecular Probes, C6827) for 20 min at 37°C with or without Hoechst 33342 (AAT Bioquest #17535).
Membrane cholesterol imaging: pALOD4 was a gift of A. Radhakrishnan (UT Southwestern, Dallas, TX; Addgene plasmid #111026) (78), and ALOD4 was purified and labelled with Alexa Fluor 488 as previously described (79, 80). Visualization of the accessible pool of cholesterol was performed after 30 min treatment with 10 mM methyl-beta-cyclodextrin (MβCB) in 1% lipoprotein-deficient serum (LPDS, Sigma #S5519) or 1% LPDS control, followed by flow for 2 h in complete media. HAECs were then stained with 20 μg/mL ALOD4-488 for 10 min 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 1X HBSS then imaged in culture media or 1X HBSS using Zeiss Colibri LED plus CMOS camera (Photometrics 95B) detection or LSM900 with Airyscan2 GaAsP-PMT detector for gentle confocal imaging.

Animals

Both male and female wildtype 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/dark cycle.

Aorta en face immunostaining

Mice were perfused with ice cold 1X PBS with an incision of the right atrium to release the blood followed by perfusion with 4% PFA. The thoracic aorta was isolated and post-fixed with 0.4% PFA overnight at 4 °C. The vessel was then washed three times with 1X PBS and permeabilized by incubating with 0.3% Triton-X in 2% normal donkey serum
(Jackson Immuno Research Laboratories #017-000-121) in 1X PBS for 30 min at room temperature followed by incubation with primary antibodies overnight at 4 °C with gentle agitation. After 1X PBS washes, the vessel was incubated with corresponding secondary antibodies for 2 h at room temperature followed by washing with 1X PBS three times for 10 min each. The vessel was then cut longitudinally and mounted in Fluoromount-G (SouthernBiotech #0100-01). Primary antibodies used: ERG (Abcam #ab92513), VE-Cadherin (R&D #AF1002), Caveolin-1 (Invitrogen #PA1-064), Cavin-1 (Abcam #ab48824), NF-kB p65 (Cell Signaling #8242S), VCAM-1 (BD Pharmingen™ #550547).

**Immunofluorescence and confocal imaging**

For immunostaining, cell monolayers were fixed with 4% PFA for 10 min followed by multiple washes with 1X PBS. Samples were then blocked for 2 h with 10% normal donkey serum (Jackson Immuno Research Laboratories #017-000-121) in 1X 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 Milipore #3004100). Primary antibodies were incubated overnight at 4 °C in blocking buffer and secondary antibodies applied for 2 h at room temperature. Primary antibodies used: Caveolin-1 (Invitrogen #PA1-064 or R&D #AF5736 or Santa Cruz #sc-894), Cavin-1 (Abcam #ab48824), Caveolin-2 (Invitrogen #PA5-21927), ICAM-1 (Santa Cruz #sc-107), E-Selectin (Invitrogen #MA1-06506), ZO-1 (Invitrogen #MA3-39100-A488), NF-kB p65 (Cell Signaling #8242S), p-eNOSSer1177 (Invitrogen #PA5-35879 or Santa Cruz #sc-81510), TRPV4 (Alomone Labs #ACC-034 or LSBio #LS-C401108), VE-Cadherin (R&D #AF938). For F-actin staining, Alexa Fluor™ 555 Phalloidin (Invitrogen #A34055) was applied with secondary antibody incubation.
Imaging was performed on a Zeiss LSM 900 confocal microscope equipped with 405nm, 488nm, 561nm and 640nm laser lines using Plan-Apochromat objectives (10x, 20x, 40x or 63x) 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 was performed with ZEN Blue software (ZEISS), IMARIS software (Bitplane), ImageJ (NIH) or custom script (see section on Image analysis in Supplemental Material for details).

Statistics
Statistical analysis was performed using GraphPad Prism software. The results are presented as mean ± SD. Depending on how many conditions were compared, either two-tailed independent t-test analysis or one-way analysis of variance (ANOVA) with Tukey's post hoc multiple comparisons test was conducted. The Pearson correlation coefficient (r) was used to measure the strength of a linear association between two variables. P < 0.05 was considered statistically significant for all analyses.

Drawings
Schematics in Figures 1B, 2B, 2C, 3A, 3B, 4A, 4C, 5A, 5B, 5E, 6A, 6E, 6F, 7A, 8D, 10A, 10E and Supplemental Figures 1A, 1B, 1C, 1E, 2A, 3A, 4A, 4B were created with BioRender.com.

Study approval
All mouse experiments were approved by the Institutional Animal Care and Use Committee (IACUC) and Animal Review Committee (ARC) at UCLA.
Data availability

Values for all data points in graphs are reported in the Supporting Data Values file. Sequencing data have been deposited in the NCBI’s Gene Expression Omnibus database (GSE255770). Custom code used for image analysis is available via public GitHub repository link: https://github.com/marcusgj13/endoSeg.
Author contributions

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References


Figure 1: Membrane polarization and Caveolin-1-enriched microdomains in aortic ECs exposed to laminar flow.

(A) Confocal imaging of Caveolin-1 in the endothelium of mouse descending aorta. Individual cells were segmented into 3 equal-length regions (upstream, mid and downstream) and staining intensity determined in each segment for 216 cells from \( n = 4 \) aortas. Graph displays mean ± SD with data analyzed by one-way ANOVA and post hoc Tukey’s multiple comparisons test. For (B–H), confluent monolayers of HAECs were exposed to laminar shear stress (20 dynes/cm\(^2\)) for 48 h then imaged. (B) HAECs were stained for Caveolin-1, ZO-1 and DAPI then segmented into 3 equal-length regions and Caveolin-1 staining intensity quantified for each subcellular region for 79 cells from \( n = 4 \) biological replicates. Graph displays mean ± SD with data analyzed by one-way ANOVA and post hoc Tukey’s multiple comparisons test. (C) Representative generalized polarization (GP) color-coded image to determine membrane fluidity. Higher GP was observed at the downstream end (yellow arrow) compared to upstream end (blue arrow).
(D) Staining live cells with BODIPY FL C₅-Ganglioside G₄M₁ showed polarized accumulation of signal at the downstream end (arrows). (E) Fixed cells showed a higher density of F-actin staining at downstream ends (arrows). (F) Imaging F-actin, Caveolin-1, ZO-1, and nuclei highlights the presence of Caveolin-1 and F-actin at the downstream end, and the formation of F-actin web-like features (arrows). (G) 3D surface rendering of the downstream end of a cell showed high density of Caveolin-1 where F-actin accumulates. (H) Representative image of Caveolin-1, ZO-1 and nuclear staining used for Caveolin-1 cluster analysis. The cluster index was determined in subcellular segments and plotted as means ± SD for n = 4 biological replicates. Data was analyzed by one-way ANOVA and post hoc Tukey’s multiple comparison test. ****P < 0.0001.
Figure 2: eNOS phosphorylation and Ca\(^{2+}\) oscillations occur at the downstream end in the presence of high laminar flow.

For (A–C), confluent monolayers of HAECs were exposed to laminar shear stress (20 dynes/cm\(^2\)) for 48 h. (A) Representative image of flow-aligned HAECs stained for Caveolin-1 and eNOS phosphorylated on serine 1177 (p-eNOS). Individual channel images, displayed in Rainbow Lookup Table (LUT), highlight the accumulation of signal for both Caveolin-1 and p-eNOS at the downstream end (arrowheads). (B) Imaging of Ca\(^{2+}\) activity in live HAECs expressing GCaMP. Fluorescence intensity over time is plotted for 15 min for one full-length cell using 3 defined regions of interest. Note that Ca\(^{2+}\) oscillations are observed exclusively at the downstream end; blue arrow indicating one Ca\(^{2+}\) peak. Corresponding time sequence is displayed for indicated timepoints. (C) All cells within an imaging field of view were outlined and ID’ed. GCaMP signal was extracted over 30 min for n = 6 independent experiments and analyzed for Ca\(^{2+}\) activity. Approximately 50 % of cells had Ca\(^{2+}\) activity (index of dispersion, IoD, greater than 2). From 730 cells (n = 3 independent experiments), active cells were further segmented into 3 equal-length segments for the upstream, mid-body and downstream regions. Of the active cells, over 70% had Ca\(^{2+}\) activity restricted to the downstream end.
Figure 3: Ca\(^{2+}\) activity at downstream end is enhanced with increased shear stress.

(A) To model variation in flow, GCaMP HAECs were seeded on y-shaped slides and exposed to unidirectional laminar flow for 48 h prior to imaging. Ca\(^{2+}\) activity was imaged in low-flow (~5 dynes/cm\(^2\)) and high-flow regions (~20 dynes/cm\(^2\)). Cell segmentation and extraction of the fluorescence intensity over time showed enhanced activity at the downstream end of cells in the high-flow region. IoD plots show data from 177 low-flow and 98 high-flow cells across n = 3 biological replicates. (B) Region of flow convergence on the y-shaped slide experiences increasing shear stress levels. Cells exposed to high-flow were morphologically aligned and exhibited more Ca\(^{2+}\) spikes (in yellow) compared to cells in the low-flow area (marked by red outline). (C) Representative GCaMP intensity trace showing 90 min of Ca\(^{2+}\) activity at the downstream end of flow-aligned HAEC in the presence of flow (30 min; 20 dynes/cm\(^2\)), static (30 min; 0 dynes/cm\(^2\)) and re-flow (30 min; 20 dynes/cm\(^2\)) conditions.
Figure 4: High laminar flow is required for localized signaling activity.

(A) HAECs were seeded on either non-patterned chambers and flow aligned (20 dynes/cm²) or seeded on line-patterned chambers and cultured statically. After 48 h, cells were analyzed and aspect ratio calculated for n = 4 biological replicates. Shown are means ± SD. ns, not significant by two-tailed, unpaired t test. (B–C) HAECs were elongated statically on the line-patterned chamber and stained for Caveolin-1, p-eNOS and DAPI. Representative images of the staining are shown in (B), with segmentation analysis from 26 cells shown in (C). Graphs show intensity displayed as means ± SD and analyzed by one-way ANOVA with post hoc Tukey’s multiple comparisons test. ns, not significant, *P < 0.05, **P < 0.01. (D) Ca²⁺ activity was recorded for GCaMP-expressing
HAECs that were cultured statically on line-patterned chambers. Representative live cell recording of intensity trace for one cell over 10 min showed a lack of localized activity. (E) HAECs were cultured statically on the line-patterned chamber or y-shaped slide for 48 h. Representative images of p-eNOS staining using equivalent imaging conditions to compare the signal intensity across conditions. The p-eNOS signal is displayed using false color Rainbow Lookup Table (LUT) to highlight the clustered regions of staining in cells under high flow.
Figure 5: Localized Ca^{2+} entry requires TRPV4 channel activity and occurs in areas of TRPV4/Caveolin-1 association.

For (A–B), confluent monolayers of GCaMP transfected HAECs were exposed to laminar shear stress (20 dynes/cm²) for 48 h prior to live cell imaging. (A) Representative GCaMP intensity trace showing Ca^{2+} activity at the downstream end after the addition of EGTA (1.6 µM) to chelate calcium ions in culture media. (B) Representative GCaMP intensity trace showing Ca^{2+} activity at the downstream end after the addition of the TRPV4 antagonist GSK205 (20 µM). For (C–D), HAECs were seeded on y-shaped slides and exposed to unidirectional laminar flow for 48 h. Immunofluorescence was compared for cells in low-flow (~5 dynes/cm²) and high-flow regions (~20 dynes/cm²). (C) TRPV4 protein staining showed no difference for low flow versus high flow regions. Representative images from n = 3 biological replicates; statistics calculated by two-tailed, unpaired t test show no significance, ns, between regions. Quantifying the subcellular distribution of expression indicated that TRPV4 was not polarized under flow. Shown are means ± SD from 44 low-flow and 57 high-flow cells across 4 biological replicates. (D)
Representative images of proximity ligation assay (PLA) to detect TRPV4 and Caveolin-1 association (magenta puncta) in low-flow and high-flow regions for n = 4 replicates. Shown are puncta/cell with means ± SD and statistics calculated using unpaired, two-tailed t test; ***P < 0.001. Additional segmentation analysis showed that TRPV4/Caveolin-1 PLA puncta preferentially occurred in the downstream end only for cells exposed to high flow. 38 cells were analyzed for the low-flow region and 93 cells were analyzed for the high-flow region from n = 4 biological replicates. Data was analyzed by one-way ANOVA and post hoc Tukey’s multiple comparisons test; ns, not significant, *P < 0.05 and ***P < 0.001.
Figure 6: Cholesterol depletion abolishes polarized signaling.

(A) Experimental design for cholesterol depletion of HAECs. MβCD was used to deplete plasma membrane cholesterol. (B) Flow-aligned HAECs were treated with MβCD for 30 min then fixed and stained for Caveolin-1 and DAPI. MβCD treatment abolished Caveolin-1 polarization as shown by intensity plots of representative cells from the control and MβCD treated groups. (C) NO production was visualized via DAF-FM loaded flow-aligned monolayers of control and MβCD treated HAECs. Shown are mean DAF-FM fluorescence intensities ± SD for n = 3 biological replicates and statistics calculated using two-tailed, unpaired t test; **P < 0.01. (D) GCaMP imaging of the cholesterol-depleted cells under flow (20 dynes/cm²) for 20 min showed lack of Ca²⁺ activity. Displayed are time-dependent images of a representative cell and the corresponding intensity trace for the downstream end. At t = 20 min (blue arrow), the TRPV4 agonist GSK1016709A (GSK101,
10 nM) was added to the flowing culture media. This led to an immediate Ca\textsuperscript{2+} burst as seen in the image at 20.1 min. (E) Overall, only 13% of the cells depleted for cholesterol were active in the initial 20 min of imaging. The number of active cells increased to 75% following the addition of GSK101. (F) IoD heatmaps show Ca\textsuperscript{2+} activity following cholesterol depletion and subsequent GSK101 addition for n = 244 cells.
Figure 7: Inhibition of TRPV4 activity under flow induces inflammatory gene expression and NF-κB signaling.

(A) HAEC monolayers were flow aligned for 48 h 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 two-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 RNAseq data. (F) Control and GSK205 treated monolayers were fixed after 2 h 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 two-tailed, unpaired t test; **P < 0.01.
Figure 8: Inhibition of TRPV4 activity induces an inflammatory phenotype.

(A) Control and GSK205 treated HAECs were fixed after 4 h and stained for DAPI, ICAM-1 and E-Selectin. Shown are representative images and mean intensities ± SD from n = 5 biological replicates and statistics calculated using two-tailed, unpaired t test; ****P < 0.0001. (B) Quantification of NO production in live cells by DAF-FM imaging after 2 h of treatment. Shown are representative images and mean intensities ± SD from n = 4 biological replicates and statistics calculated using two-tailed, unpaired t test; **P = 0.0023. (C) After 2 h, control and GSK205 treated monolayers were incubated with CellROX probe and imaged to quantify reactive oxygen species (ROS) production. Shown are representative images and mean intensities ± SD from n = 6 biological replicates and statistics calculated using two-tailed, unpaired t test; ****P < 0.0001. (D) Experimental design for in vivo inhibition of TRPV4 activity via IP injection of GSK205 (10 mg/kg) or DMSO (volume equivalent) as control. After 4 h, mice were euthanized, and aortas collected for en face imaging. (E) Confocal imaging of abdominal aortas from mice injected with GSK205 or DMSO. Shown are representative images and associated quantification of VCAM-1 and nuclear NF-κB p65 staining intensity. Graphs represent...
mean intensities ± SD from n = 4 mice and statistics calculated using two-tailed, unpaired t test; *$P < 0.05$. 
Figure 9. Activation of TRPV4 dampens the endothelial response to an inflammatory stimulus in vitro.

(A) Experimental design for static TNFα treatment (10 ng/mL; 30 min) in the presence of GSK1016790A (GSK101; 10 nM) or DMSO. (B) Gene expression was measured by qPCR for TNFα 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 two-tailed, unpaired t test. (n = 3 biological replicates). (C) Gene expression as in (B) with mRNA expression plotted as a heat map of mean expression. (D) Following TNFα treatment +/- GSK101, HAECs were fixed and stained for VE-Cadherin and NF-κB p65. Graph represents mean fluorescence intensity in the nucleus from n = 110 (DMSO), n = 79 (TNFα+DMSO), n = 87 (TNFα+GSK101) cells per group from n = 4 biological replicates per condition and statistics calculated using one-way ANOVA with post hoc Tukey’s multiple comparisons test; ***P < 0.001.
Figure 10. Activation of TRPV4 attenuates endothelial response to an inflammatory stimulus in vivo.

(A) Experimental design for in vivo LPS treatment (1.5 mg/kg) in the presence of GSK101 (10 µg/kg) or DMSO in wild-type mice. (B) Gene expression of aortic endothelium quantified by qPCR of mRNA isolated from the descending aorta of mice injected with LPS + DMSO (n = 3) or LPS + GSK101 (n = 4). Gene expression plotted as mean ± SD; ns, not significant, *P < 0.05 by two-tailed, unpaired t test. (C) Aortic endothelial gene expression plotted as heat map for inflammatory genes measured by qPCR in (B). (D) En face staining of abdominal aortas from mice injected with LPS + DMSO or LPS + GSK101. Shown are representative images and associated quantification of VCAM-1 staining intensity and NF-κB p65 nuclear intensity. Graphs represent mean intensities ± SD from n = 4 mice and statistics calculated using two-tailed, unpaired t test; *P < 0.05. (E) Graphical model describing how laminar flow supports localized TRPV4 activation by polarized Caveolin-1 rich microdomains, which leads to Ca2+ entry, eNOS activation, NO production and inhibition of NF-κB mediated transcription. Inhibition of TRPV4 signaling at these domains results in vascular inflammation.