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Analysing the shear induced sensitization of mechanosensitive ion channel Piezo-1 in human aortic endothelial cells

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Abstract

Mechanosensitive ion channels mediate endothelial responses to blood flow and orchestrate their physiological function in response to haemodynamic forces. In this study, we utilized microfluidic technologies to study the shear-induced sensitization of endothelial Piezo-1 to its selective agonist, Yoda-1. We demonstrated that shear stress-induced sensitization is brief and can be impaired when exposing aortic endothelial cells to low and proatherogenic levels of shear stress. Our results suggest that shear stress-induced sensitization of Piezo-1 to Yoda-1 is independent of cell-cell adhesion and is mediated by the PI3K-AKT signalling pathway. We also found that

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shear stress increases the membrane density of Piezo-1 channels in endothelial cells. To further confirm our findings, we performed experiments using a carotid artery ligation mouse model and demonstrated that transient changes in blood-flow pattern, resulting from a high-degree ligation of mouse carotid artery, alters the distribution of Piezo-1 channels across the endothelial layer. These results suggest that shear stress influences the function of Piezo-1 channels via changes in membrane density, providing a new model of shear-stress sensitivity for Piezo-1 ion channel.

Graphical Abstract

In this study, we utilized microfluidic technologies to study the shear-induced sensitization of endothelial Piezo-1 to its selective agonist, Yoda-1. Our results suggest that shear stress influences the function of Piezo-1 channels via changes in membrane density, providing a new model of shear-stress sensitivity for Piezo-1 ion channel.

Introduction

Mechanical forces are essential to the development and normal physiology of the cardiovascular system and regulate the movement of pressurised blood through the vasculature to different organs. Every cardiac cycle exerts two main forces on the vessel wall: shear stress and pulsatile pressure. In the lumen of blood vessels, endothelial cells are in direct contact with haemodynamic forces and their ability to sense these forces regulates both short-term vascular tone and long-term vascular remodelling to adjust vessel diameter in accordance with tissue demand (Davies 1995).

In addition to its contribution to normal physiology, the endothelial response to haemodynamic forces plays a key role in the development and progression of atherosclerosis. Early lesions of atherosclerosis develop at arterial branches and inner curvatures of the coronary and carotid arteries, where the blood flow is disturbed, and shear stress is correspondingly low (Ku, Giddens et al. 1985, Nerem 1992). Mechanotransduction in endothelial cells is regulated by a complex mechanosensory machinery allowing the cells to sense and transduce the mechanical forces associated with blood flow (Baeyens, Bandyopadhyay et al. 2016). Endothelial sensitivity to shear stress is regulated by a mechanosensory complex comprising platelet endothelial cell adhesion molecule/ vascular endothelial-cadherin (PECAM/ VE-cadherin) and vascular endothelial growth factor receptor 2 (VEGFR2) (Tzima, Irani-Tehrani et al. 2005).

Mechanosensitive (MS) ion channels are among the fastest class of mechanoreceptors expressed in endothelial cells. As they gate in response to haemodynamic forces, they allow an influx of ions at a rate of 10^6 ions per second (Elam and Lansman 1993). There is accumulating evidence that the activation of MS ion channels plays a central role in the regulation of endothelial responsiveness to flow and different aspects of normal vascular physiology, including vasoregulation in response to an acute change in arterial flow and wall remodelling in response to chronic haemodynamic alteration (Ohno, Gibbons et al. 1993, Ohno, Cooke et al. 1995, Gautam, Gojova et al. 2006, Mendoza, Fang et al. 2010, Hyman, Tumova et al. 2017). Previous studies by ourselves and others have shown that in endothelial cells, shear stress sensitizes the response of the MS ion channels TRPV4 (Baratchi, Almazi et al. 2016) and P2X4 (Yamamoto, Korenaga et al. 2000) to their selective agonists GSK1016790A and

adenosine triphosphate (ATP), respectively. Furthermore, shear stress has been shown to regulate the membrane expression, post-translational modification and function of ion channels, including TRPV4 (Baratchi, Almazi et al. 2016, Soffe, Baratchi et al. 2017), transient receptor potential melastatin 7 (TRPM7) (Oancea, Wolfe et al. 2006) and voltage-dependent potassium channel Kv1.5 (Boycott, Barbier et al. 2013).

Piezo-1 is a newly discovered cation-selective MS channel with ~2500 amino acids (Coste, Mathur et al. 2010). The polypeptides form a homotrimeric channel in the plasma membrane with membrane embedded propeller-like structure that mediates mechanosensitivity (Coste, Mathur et al. 2010, Saotome, Murthy et al. 2018, Wang, Chi et al. 2018). Piezo-1 has been reported as a sensor of shear stress in endothelial cells and essential for lymphatic valve development (Nonomura, Lukacs et al. 2018) and endothelial alignment to the direction of the flow, linking this ion channel to the regulation of the vascular architecture (Li, Hou et al. 2014, Ranade, Qiu et al. 2014). Notably, a global knockout of Piezo-1 in mice is embryonically lethal (Li, Hou et al. 2014, Ranade, Qiu et al. 2014). Recent studies have identified the different protein regions of Piezo-1 that are involved in mechanosensitivity (Zhang, Chi et al. 2017, Wang and Xiao 2018); however, the mechanism by which shear stress regulates the contribution of Piezo-1 to physiological function is poorly understood. Piezo-1 channel activity is activated by the small-molecule Yoda-1, which stabilizes the open conformation of the channel and reduces its mechanosensitivity threshold (Syeda, Xu et al. 2015).

The aim of this study was to take advantage of microfluidic technologies to study the shear stress-induced sensitization of Piezo-1 to its selective agonist (Yoda-1) in human aortic cells. Our hypothesis was that shear stress regulates the sensitivity of Piezo-1 to Yoda-1 via change in membrane expression. Using microfluidic models, we analyse the response of endothelial cells at various concentrations of Yoda-1 and under controlled static and dynamic shear stress profiles. We systematically characterize a newly described mechanism of shear-induced sensitization of Piezo-1 to Yoda-1 and identify the signalling pathways involved (**Figure 1**). Furthermore, using a partial carotid artery ligation model, we confirmed the effect of shear stress and change in hemodynamics on the distribution of Piezo-1 across the endothelium.

Figure 1. Schematic image showing the summary of experimental setting and results reported in this study.

Results

Shear stress activates Piezo-1 and sensitizes the response of Piezo-1 to Yoda-1

To determine how Piezo-1 channels respond to shear stress, we initially examined the mechanosensitivity of human embryonic kidney 293T (HEK293T) cells stably expressing Piezo-1-mCherry (Piezo1-mCherry-HEK293T) in response to varying levels of shear stress (0.6–20 dyne/cm²) and measured changes in intracellular calcium level ([Ca²⁺]_i). Shear stress greater than 2 dyne/cm² caused a significant increase in the [Ca²⁺]_i of Piezo-1-mCherry-HEK293T cells, but not in the parental cell line ($p < 0.001$) (**Figure 2a-h**). An increase in shear stress from 2 to 6 dyne/cm² significantly decreased the maximum response time from 158 ± 6.7 sec to 105 ± 3.2 sec ($p < 0.0001$) (**Figure 2i**). Exposure of cells to 3 cycles of shear stress with short intervals between (duration of each pulse was 200 sec with intervals of 60 sec between each pulse) caused a [Ca²⁺]_i profile dominated by an early peak and the [Ca²⁺]_i peak was significantly ($p < 0.001$) reduced during subsequent pulses (**Figure 2j-k**).

Next, we measured the effect of shear stress on the Piezo-1 response to its selective agonist, Yoda-1: Piezo-1-mCherry-HEK293T cells were exposed to constant shear stress of 2 or 20 dyne/cm² in the presence of 100 nM–10 μM of Yoda-1. For all experimental conditions, cellular response to Yoda-1 was higher and response time was decreased in presence of 20 dyne/cm² ($p < 0.001$) (**Figure 3a-d**). Followed by this Piezo-1-mCherry-HEK293T cells and human aortic endothelial cells (HAECs) were exposed to a step-wise increase in shear stress from 2 to 20 dyne/cm² at various concentrations of Yoda-1. In both cell types, $[Ca^{2+}]_i$ was minimal in response to shear stress when stimulated with 100 nM Yoda-1; however, in the presence of 1–10 μM of Yoda-1 an increase in shear stress from 2 to 20 dyne/cm² sensitized the response of Piezo-1 to Yoda-1, increased the fraction of the responding cells and reduced the response time of Piezo-1 to Yoda-1 (**Figure 3a-h**, Supplementary 1a–d). Overall, the quantitative analysis showed a correlation between $[Ca^{2+}]_i$ and an increase in shear stress in the presence of 1–10 μM of Yoda-1.

To further examine the roles of calcium release and calcium influx in the shear-induced sensitization of Piezo-1 to Yoda-1, HAECs were treated with either BAPTA-AM (10 μM) (Wang, Wu et al. 2019), a chelator of the intracellular Ca^{2+} stores, or thapsigargin (1 μM), a selective Ca^{2+} ATPase inhibitor. BAPTA-AM and thapsigargin abolished the shear-induced sensitization completely and partially ($p < 0.001$), respectively (**Figure 3f**). Further, the removal of free calcium from the buffer (using 2 mM EGTA) or the inhibition of MS channels (using 2.5 μM GSMTx or 30 μM ruthenium red) completely and partially ($p < 0.001$), respectively, abolished the shear-induced sensitization of Piezo-1 to Yoda-1 (**Figure 3f**).

Figure 2. Piezo-1-mCherry-HEK293T cells are sensitive to shear stress. (a-f) Representative single-cell profiles of changes in $[Ca^{2+}]_i$ in Piezo-1-mCherry-HEK293T cells after exposure to the indicated levels of shear stress (a-e) and in (f) parental non-transfected HEK293 cells (NT-HEK293) in the presence of 10 dyne/cm² shear stress; fluorescent traces from at least 25 individuals cells (grey lines) were recorded and used to derive mean values (red line) under each experimental condition. (g) Summary graph showing the peak increase in $[Ca^{2+}]_i$ level Piezo-1-mCherry-HEK293T (Piezo-1) and NT-HEK293 cells (NT) after exposure to 10 dyne/cm² shear stress. (h) Summary graphs showing the maximum increase in $[Ca^{2+}]_i$ level and (i) the peak response times of the Piezo-1-mCherry-HEK293T cells after exposure to shear stress of 0.6–20 dyne/cm². (j) Response of Piezo-1-mCherry-HEK293T to 3 cycles of shear stress with short intervals (duration of each pulse was 200 sec with intervals of 60 sec between each pulse). (k) Summary graph showing max. response of Piezo-1-mCherry-HEK293T after exposure to 3 cycles of shear stress. (h-k) The data summarize the results of 4 independent experiments (at least 25 cells were analyzed in each experiment).

Figure 3. Shear stress sensitizes the response of Piezo-1 to Yoda-1 dependent on Ca^{2+} influx and release from intracellular stores. (a-c) Representative single-cell profiles of changes in $[\text{Ca}^{2+}]_i$ in HAECs in response to the indicated step-wise change in shear stress and in the presence of the indicated levels of Yoda-1. Fluorescent traces from at least 30 individual cells (grey lines) were recorded and used to derive mean values (red line) under each experimental condition. (d) Summary of data from 4 independent experiments. (e-f) Comparative analysis showing the dynamics of Piezo-1-mCherry-HEK293T responses to 1 μM and 10 μM of Yoda-1 in the presence of 2 or 20 dyne/cm^2 . (g) Summary graph showing the response time of Piezo-1-mCherry-HEK293T to different concentrations of Yoda-1 in the presence of 2 and 20 dyne/cm^2 . (h) Summary graph showing that blocking of the Ca^{2+} influx via the depletion of Ca^{2+} from the buffer or the treatment of cells with ruthenium red ([RR], 30 μM), GsMTx4 (2.5 μM), the inhibition of Ca^{2+} released from internal stores via treatment of cells with BAPTA-AM (10 μM) or thapsigargin (1 μM) blocked the shear-induced sensitization of Piezo-1 to Yoda-1. The data in (d-h) represent 4

independent experiments and were analyzed using ordinary two-way ANOVA and Tukey's multiple comparison test (***, $p < 0.001$ and ****, $p < 0.0001$).

Shear-induced sensitization is brief, while long-term exposure to shear stress controls the sensitivity of endothelial cells

To determine the duration of shear-induced sensitization, HAECs were pre-exposed to 20 dyne/cm² of shear stress for 1 min. Next, the cells were exposed either immediately or after 2 to 10 min of static rest to a stepwise increase in shear stress from 2 to 20 dyne/cm² in the presence of 5 μ M Yoda-1 (**Figure 4a-f**). Consistent with the data presented in Figure 2, pre-exposure of cells to 20 dyne/cm² shear stress sensitized the response of Piezo-1 to 5 μ M of Yoda-1 at 2 dyne/cm² of shear stress (**Figure 4b**). This sensitization declined rapidly, as exposing the cells to 2 min of static rest before exposure to a step-wise increase in shear stimulation significantly decreased their sensitivity to Yoda-1 (**Figure 4c**). Sensitization was completely abolished after 10 min of static rest (**Figure 4d-e**). Overall, the shear-induced sensitization of Piezo-1 to Yoda-1 was reduced by 1.32 ± 3.2 -fold after 2 min ($p = 0.013$), 1.39 ± 0.8 -fold after 5 min ($p < 0.001$) and 1.58 ± 1.34 -fold after 10 min ($p < 0.0001$) of static rest (**Figure 4f**).

To examine the effects of atheroprotective versus atheroprone shear-stress levels on shear-induced sensitization of Piezo-1 to Yoda-1, the endothelial cells were cultured for 24 h under shear stress of 20 dyne/cm² (representing atheroprotective shear-stress levels) or 4 dyne/cm² (representing low and atheroprone shear-stress conditions) overnight followed by a sensitization assay. Endothelial cells cultured under flow representing 20 dyne/cm² maintained their sensitivity to the increase in shear stress. Conversely, endothelial cells cultured under low shear stress of 4 dyne/cm² lost their sensitivity to shear stress. Overall, the endothelial cells cultured at 20 dyne/cm² showed 1.7 ± 1.42 fold ($p < 0.001$) more sensitivity to shear stress than those cultured at 4 dyne/cm² (**Figure 4g-i**).

Figure 4. Shear-induced sensitization is brief and the overnight flow condition controls endothelial mechanosensitivity. (a) Representative single-cell profiles of changes in $[Ca^{2+}]_i$ in HAECs in response to the indicated step-wise change in shear stress and in the presence of the indicated levels of Yoda-1. (b) Pre-exposing HAECs to 20 dyne/cm² sensitized the response of Piezo-1 to 5 μ M of Yoda-1 at 2 dyne/cm². (c) In the presence of a 2–10 min static rest condition, HAECs lost their sensitivity to shear stress. (f) Summary graph of the data shown in (a-e). (g-h) Sensitization assays and single-cell profiles of HAECs; the cells maintained their sensitivity to shear stress when cultured under an atheroprotective flow of 20 dyne/cm² but lost their sensitivity to shear stress when cultured under low and atherogenic shear-stress levels. (i) Summary graph of the data shown in (g-h). The data in (f) and (i) summarize the results of 4 independent experiments (at least 20 cells were analyzed in each experiment); in (f) ordinary one-way ANOVA and in (i) Student's t-test were used to analyze the data (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

Sensitization of Yoda-1 by shear stress is independent of cell–cell adhesion and is mediated via the phosphatidylinositol 3-kinase (PI3K)/AKT signalling pathway

Next, we examined the interplay between cell–cell adhesion and the PI3K/AKT/FAK signalling pathway in the regulation of shear-induced sensitization of Piezo-1 to Yoda-1 (**Figure 5a**). HAECs were seeded both at low density such that the majority of cells did not interact with neighbouring cells and at semi-confluent density to ensure that each cell was in contact with at least one neighbouring cell. Under both experimental conditions, shear stress sensitized the response of Piezo-1 to Yoda-1 (**Figure 5b-c**). These experiments indicate that the sensitization is independent of cell–cell adhesion. Furthermore, to determine the role of the PI3K/AKT signalling pathway, HAECs were pre-treated with either a PI3K inhibitor (wortmannin, 100 nM) and an AKT inhibitor (GSK690693) for 30 min before the shear stress–induced sensitization assay. All three inhibitors significantly reduced the shear stress–induced sensitization of Piezo-1 to Yoda-1 ($p < 0.001$) but did not blocked the sensitisation (**Figure 5d**). Overall, the above results show that shear stress–induced sensitization is independent of cell–cell adhesion but requires activation of the PI3K/AKT signalling pathway.

Figure 5. Shear stress–induced sensitization of Piezo-1 to Yoda-1 is independent of cell–cell adhesion and PI3K/AKT activity. (a) Common mechanosensory pathways in endothelial cells that are dependent on cell–cell adhesion and the

PI3K/AKT signalling pathway. (b-c) Representative confocal microscopy images and summary of experiments determining the effect of cell-cell contact on endothelial responses to shear stress. (d) Sensitization assays of HAECs in the presence or absence of 100 nM wortmannin or 1 μ M GSK690693. The data in (c-d) represent 4 independent experiments and were analyzed using ordinary two-way ANOVA and Tukey's multiple comparison test (****, $p < 0.0001$).

Shear stress-induced sensitization coincides with increase in the membrane expression of Piezo-1 and is dependent on components of the cytoskeleton and dynamin

Previously we found that shear stress sensitizes the response of TRPV4 to its selective agonist and this coincides with changes in the membrane expression of TRPV4 (Baratchi, Almazi et al. 2016, Soffe, Baratchi et al. 2017). Similarly, shear stress was reported to increase the membrane expression of TRPM7 in HEK293 cells (Oancea, Wolfe et al. 2006) and Kv1.5 in arterial myocytes (Boycott, Barbier et al. 2013). In the present study, we examined the effect of shear stress on the near membrane expression of Piezo-1 using total internal reflection microscopy (TIRFM) to illuminate the fluorophore within the 250 nm interface of the glass coverslip and the plasma membrane with minimal background (Fish 2009). We measured the density of the Piezo-1 channels near the plasma membrane, as described previously (Baratchi, Almazi et al. 2016). Shear stress increased the near membrane expression of the Piezo-1 channels (**Figure 6a-b**). Furthermore, the inhibition of clathrin-mediated exocytosis by the small-molecule dynamin inhibitor (Dyngo-4a) reduced the effect of shear stress on the membrane expression of Piezo-1 and completely abolished the shear stress-induced sensitization of Piezo-1 to Yoda-1 (**Figure 6a-c**).

The cytoskeleton machinery plays an important role in pre- and post-exocytosis events (Porat-Shliom, Milberg et al. 2013) and previous research suggested a dynamic association between the cytoskeleton and Piezo-1 activity (Nourse and Pathak 2017). Therefore, we next examined the effect of shear stress on the interaction of Piezo-1-mCherry with actin using confocal microscopy. We found that shear stress increased the association of Piezo-1-mCherry with β -actin ($p < 0.001$, $n = 3$, 60 cells in each experiment) and that within 10 min after shear-stress stimulation, the interaction of

Piezo-1 with the actin cytoskeleton significantly decreased (**Figure 6d-e**). The inhibition of microtubule polymerization with colchicine and of actin polymerization with cytochalasin D completely and partially, respectively, eliminated the shear stress-induced sensitization of Piezo-1 to Yoda-1 ($p<0.0001$ and $p=0.01$, respectively) (**Figure 6f**).

Figure 6. Shear stress increases the evanescence field intensity of Piezo-1-mCherry and improves the interaction of Piezo-1-mCherry in a dynamin-dependent manner. (a–b) Representative TIRFM images and summary graph of Piezo-1-mCherry-HEK293T cells that were static or had been exposed to 4 and 20 dyne/cm² or were pre-treated with Dyngo-4a (60 μM) and exposed to 20 dyne/cm². (c) Sensitization assays of HAECs that were not subject to pre-treatment (the control group) and those pre-treated with 60 μM Dyngo-4a for 30 min. (d–e) Confocal microscopy images and summary data showing the colocalization of Piezo-1-mCherry and β-actin that were static, had been exposed to 20 dyne/cm² for 30 sec or had been

exposed to 20 dyne/cm² for 30 sec after which they were given a 10 min rest; a Pearson correlation coefficient was used to measure the degree of colocalization. The data in (d) summarize the results of 3 independent experiments (at least 60 cells were analyzed in each experiment). (f) Sensitization assays of HAECs that were not subject to pre-treatment (the control group) and those pre-treated with 200 nM cytochalasin D or 10 μ M colchicine for 24 h. The data summarize the results of 4 independent experiments and in (b) and (e) were analyzed using ordinary one-way ANOVA and in (c) and (f) with ordinary two-way ANOVA and Tukey's multiple comparison test (at least 30 cells were analyzed in each region of shear stress).

To confirm our *in vitro* finding that changes in shear stress controlled the rapid redistribution of Piezo-1, we induced a shear-stress equivalent to a carotid artery stenosis of ~80% for 10 min by suturing the carotid artery of mice and analyzing the section prepared from the sutured region of the artery by immunohistochemistry. Upstream of the ligation, the majority of Piezo-1 channels were located at the basal membrane of the endothelial cells (**Figure 7a and a'**). Conversely, downstream of the ligation, the Piezo-1 channels were predominantly located in the cytosolic regions of the endothelial cells (**Figure 7b and b'**). Furthermore, at the ligation site, the Piezo-1 channels were located at the basal membrane (**Figure 7c and c'**). Thus, both laminar flow (e.g. at sites upstream of the ligation) and high shear stress (at the site of ligation) were associated with Piezo-1 located at the basal membrane. However, low shear stress and a disturbed flow (downstream of the ligation site) were associated with cytosolic location of Piezo-1. These differences in location as a function of shear stress *in vivo* were statistically significant (**Figure 7d-e**). Additionally, we found that shear stress reduced changes in the thickness of the endothelium (**Figure 7f**).

Figure 7. Shear stress regulates the distribution of Piezo-1 across the endothelial layer. (a-c) Representative immunostaining images of mice carotid artery sutured to create 80% ligation, and density of Piezo-1 across the endothelial layer, labelled with PECAM-1; red arrow in (a) indicates the suture placed to create stenosis. (a'-c') Representative graphs showing the distribution of Piezo-1 across the endothelium, PECAM-1 positive, shown by dashed lines in (a-c) respectively. (d-f) Summary graphs showing the basal vs luminal ratio of Piezo-1, thickness of endothelium and membrane vs cytosolic ratio of Piezo-1 upstream of the ligation, at the ligation site and downstream of the ligation; the data in (d-f) summarize 3 independent experiments (20 regions were analyzed in each experiment) and were analyzed using ordinary one-way ANOVA and Tukey's multiple comparison test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

Discussion

In this study, we describe a new mechanism by which shear stress increases the sensitivity of Piezo-1 to its selective agonist, Yoda-1. We show that this shear stress-induced sensitisation is brief and is independent of cell-cell adhesion and the activation of PI3K/AKT signaling pathway but is clathrin-mediated. We also show that endothelial sensitivity to shear stress changes in cells that have been cultured under athero-promoting shear-stress levels. Furthermore, we show that this event coincided with an increase in the near membrane density of Piezo-1 channels and was dependent on dynamin (a component of vesicular fusion) and an intact cytoskeleton. Thus, shear stress modulates the function of Piezo-1 channels and tunes the contribution of this ion channel into the different physiological functions.

Physiologically, endothelial cells are exposed to various levels of shear stress depending on the vascular beds. For example, in the arteries shear stress is in the range of 10 to 70 dyne/cm², while in the venous system shear stress is in the range of 1 to 6 dyne/cm² (Nigro, Abe et al. 2011). In comparison, at lesion prone regions of arteries, formed at bifurcations and branch points, the shear stress experienced by the endothelial cells is in the range of 0 to 6 dyne/cm² (Zarins, Giddens et al. 1983).

In the present study, we have found that shear stress-induced sensitization is brief and decays in less than 3 min, and that endothelial cells, which have been cultured under an atheroprotective shear stress of 20 dyne/cm², are sensitive to an increase in shear stress. Conversely, endothelial cells cultured under an atherogenic shear stress of 4 dyne/cm² are not sensitive to shear stress elevation.

Our results indicates that the mechanical sensitivity of Piezo-1 in endothelial cells is dependent on the shear stress conditions. This finding is in line with previous studies showing that shear stress influences the expression and function of several endothelial mechanoreceptors (Chien 2007), particularly the activity and expression of the Piezo-1 channel (Li, Hou et al. 2014, Ranade, Qiu et al. 2014).

Endothelial cell-cell adhesion and the activation of mechanosensory complexes of PECAM-1/VE-cadherin/VEGFR2 are known to control the transduction of mechanical stress (Tzima, Irani-Tehrani et al. 2005). In the present study, we have found that the activation of the PI3K/AKT signalling pathway is important in controlling the shear stress-induced sensitivity of Piezo-1 in endothelial cells however

it is independent of cell-cell adhesion. Thus, the mechanotransduction of endothelial cells is a complex hierarchy that involves different processes. Furthermore, our TIRFM data suggest that shear stress increases the near membrane density of Piezo-1 channels, as well as the interaction of Piezo-1 with the actin cytoskeleton in endothelial cells. This finding is aligned with the previous reports showing that in situ the Piezo-1 channel response is heavily controlled by the cortical cytoskeleton (Gottlieb, Bae et al. 2012, Bavi, Richardson et al. 2019).

The sensitivity of the Piezo-1 redistribution to specific inhibitors suggests that this is dependent on the clathrin-mediated exocytosis pathway. To extend our observations of Piezo-1 expression in endothelial cells *in vivo*, we assessed the distribution of Piezo-1 across the endothelial layer in the carotid artery after using a suture to create short-term stenosis. We found that differences in shear stress at various points in the artery relative to the stenosis site affect Piezo-1 distribution across the endothelial layer. The effects of shear stress on the membrane expression of ion channels TRPV4 (Baratchi, Almazi et al. 2016, Soffe, Baratchi et al. 2017), TRPM7 (Oancea, Wolfe et al. 2006) and Kv1.5 (Boycott, Barbier et al. 2013) were examined previously.

Endothelial Piezo-1 contributes to flow-induced ATP release and subsequent activation of AKT, endothelial NOS, vascular tone and blood pressure; and Yoda-1 (Piezo-1 agonist) induces vasorelaxation by mimicking the effect of shear stress on endothelial cells (Wang, Chennupati et al. 2016). Furthermore, endothelial the Piezo-1 response to elevated pressure is linked to endothelial barrier disruption (Friedrich, Hong et al. 2019).

The shear stress-induced sensitization of Piezo-1 to its agonist, Yoda-1, and the cytoskeleton-dependent migration of Piezo-1 from internal stores to the near membrane, presented here, provide new insights on how shear stress regulates and directs Piezo-1 contribution into different functional responses of the vasculature. The identified processes are relevant to pathologies when shear stress and pressure are altered, and can be targeted for therapeutic interventions.

Methods

Reagents and buffers

For calcium imaging experiments, cells were incubated in a buffer containing Hanks' Balanced Salt Solution (HBSS) (Life Technologies, VIC, Australia) containing 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1 mM magnesium chloride (MgCl_2) and 2 mM calcium chloride (CaCl_2), adjusted to pH 7.4. A calcium-free buffer was prepared by supplementing 2 mM ethylene glycol tetraacetic acid (EGTA) in HBSS.

Yoda-1 and Dyngo-4a were purchased from Tocris Bioscience, Bristol, UK. Ruthenium red, colchicine, thapsigargin, cytochalasin D, wortmannin and GSK690693 were purchased from Sigma, St. Louis, MI. BAPTA-AM was purchased from Molecular Probes, Oregon, USA.

All drugs were dissolved according to the supplier's instructions (0.1% v/v dH_2O or 0.1% v/v DMSO) and diluted in HBSS prior to experimentation, and corresponding vehicles (0.1% v/v dH_2O or 0.1% v/v DMSO) were used as controls (Baratchi, Almazi et al. 2016).

Cell culture

Human aortic endothelial cells (HAECs) were grown in endothelial basal medium 2 (EBM-2) media supplemented with the SingleQuots™ kit (Lonza, Maryland, USA) following the supplier's instructions. Early passage cells (passages 2–8) were used for the experiments. Piezo-1 mCherry HEK293T stable cell lines were generated as reported previously (Cox, Bae et al. 2016, Maneshi, Ziegler et al. 2018) and cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and 6 $\mu\text{g}/\text{ml}$ puromycin.

In vitro generation of shear stress and live-cell imaging of intracellular calcium ($[\text{Ca}^{2+}]_i$)

Shear-stress experiments were conducted using previously described protocols (Baratchi, Tovar-Lopez et al. 2014, Baratchi, Almazi et al. 2016). HAECs grown on coverslips were loaded with a Fluo-4 AM ester (Life Technologies, VIC, Australia) prepared in HBSS buffer, followed by mounting onto the microfluidic channel sealed

with a mechanical clamp. Care was taken to eliminate air bubbles in the channel. Details of the numerical design and microfabrication method have been reported (Nahavandi, Baratchi et al. 2014).

The microfluidic device loaded with HAECs was transferred to the stage of a Nikon A1 confocal laser scanning inverted microscope (Nikon Instruments, Inc., New York, USA) for calcium imaging experiments and was connected to a Genie Touch syringe pump (Kent Scientific Corporation, Connecticut, USA). The flow was applied through a polyvinyl chloride calibrated tube (1.02 mm ID; Gilson Inc., Wisconsin, USA) connected to the outlet of the microchip.

For each experiment, loaded cells were excited with a 488 nm laser using the Nikon A1 laser scanning confocal microscope (Nikon Instruments, Inc., New York, USA) with an inverted Nikon Eclipse Ti-E as the base microscope, equipped with a stage-top incubator allowing temperature regulation. Fluorescence emission was detected using a photomultiplier tube following a 525/50 nm band-pass filter and a PlanFluor 10× objective with a numerical aperture of 0.30. Calcium influx was measured as an increase in the fluorescence intensity of Fluo-4 AM and normalized to the fluorescence intensity of resting cells. All imaging experiments were performed at 37 °C unless otherwise stated.

Immunocytochemistry for colocalization and total internal reflection fluorescence microscopy (TIRFM)

Colocalization studies used the Piezo-1 mCherry HEK293T cell line fixed with 4% paraformaldehyde after exposure to the appropriate conditions. Cells were washed with PBS and stained with mouse anti β -actin for 16 h at 4 °C and goat anti mouse-Alexa 647 for 2 hrs at 37°C for detection of polymerized actin cytoskeleton.

For TIRFM, HAECs were fixed with 4% paraformaldehyde followed by washing with PBS. Nonspecific binding was blocked with 5% BSA in PBS and cells were probed with rabbit anti-Piezo-1 antibody (NBP1-78446) (Novus Biologicals, Colorado, USA) directly conjugated to Alexa-647. Immunofluorescence was detected at 647 nm laser using TIRFM and images were analyzed as described previously (Baratchi, Almazi et al. 2016, Soffe, Baratchi et al. 2017).

Carotid artery stenosis surgery in mice, tissue processing and histological analysis of mice tissue

3 male mice on a C57BL/6J background were anaesthetized by intraperitoneal injection of a ketamine (100 mg/kg) and xylazine (10 mg/kg) mixture. An incision was made in the neck of each mouse and the right common carotid artery was dissected from circumferential connective tissues. The 80–90% stenosis was obtained by placing a 9-0 braided polyester fibre suture around the carotid artery together with a 150 μ m needle that was tied to it and later removed. The experiments were approved by the Alfred Medical Research Education Precinct (AMREP) Animal Ethics Committee (project E/1581/2015/B). The stenosis was placed for 1 h until culling.

After the animals were sacrificed by ketamine/xylazine overdose, blood was withdrawn by heart punch followed by left ventricle perfusion with 10 mL PBS at pH 7.4 under physiological pressure. After perfusion, the right carotid artery was embedded in an optimal cutting temperature compound (Sakura Finetek), frozen over dry ice and stored at -80°C until sectioning. Longitudinal cryosections of 6 μ m thickness were prepared using a cryostat (Zeiss MICROM HM 550). Sections were immunostained with anti-Piezo-1 antibody (Novus Biologicals, Colorado, USA) and PECAM-1 fluorophore labelled antibodies, and mounted in mounting medium.

Data analysis

For calcium imaging, $[\text{Ca}^{2+}]_i$ levels were measured from the entire endothelial cell. The cell area was measured by automatically analyzing the area enclosed by a region of interest (ROI) drawn around single endothelial cells using NIS Element software (Nikon Instruments Inc., New York, USA). To quantify changes in $[\text{Ca}^{2+}]_i$ the average intensity of several ROIs was measured and results are reported as the ratio F_1/F_0 . At least 30 cells within each shear-stress region were analyzed. To measure the distribution of Piezo-1 across the endothelium, we have assessed the intensity profile of the Piezo-1 staining across the PECAM-1 positive region of the arteries by drawing 10 consecutive lines with 2-pixel distance apart, on each side of the artery experiencing the similar shear stress levels, using the NIS Element software. The average intensity of Piezo-1 staining for luminal, basal and cytosolic regions was used to obtain the basal/luminal and membrane/cytosolic ratios. The length of the PECAM-

1 positive regions was used to obtain the thickness of endothelium for each shear stress conditions. Data are shown as mean $\pm$ standard error of the mean (SEM) of at least 4 independent experiments. For statistical analysis, Student's t-test and one-way and two-way ANOVA were performed using GraphPad Prism 7 (GraphPad Software Inc., California, USA) and $p < 0.05$ was considered to be significant.

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Author contributions

AL conducted experiments and analysed the results. YCC and CDC conducted experiments and wrote the manuscript. AJ and KP wrote the manuscript. SB designed the study, performed experiments, analysed the results, wrote the manuscript and supervised the work.

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Conflict of interest:

The authors declare that they have no conflicts of interest with the contents of this article.

Data Availability Statement:

The data that support the findings of this study are available from the corresponding author upon reasonable request.

References

- Baeyens, N., C. Bandyopadhyay, B. G. Coon, S. Yun and M. A. Schwartz (2016). "Endothelial fluid shear stress sensing in vascular health and disease." *J Clin Invest* **126**(3): 821-828.
- Baratchi, S., J. G. Almazi, W. Darby, F. J. Tovar-Lopez, A. Mitchell and P. McIntyre (2016). "Shear stress mediates exocytosis of functional TRPV4 channels in endothelial cells." *Cell Mol Life Sci* **73**(3): 649-666.
- Baratchi, S., F. J. Tovar-Lopez, K. Khoshmanesh, M. S. Grace, W. Darby, J. Almazi, A. Mitchell and P. McIntyre (2014). "Examination of the role of transient receptor potential vanilloid type 4 in endothelial responses to shear forces." *Biomicrofluidics* **8**(4): 044117.
- Bavi, N., J. Richardson, C. Heu, B. Martinac and K. Poole (2019). "PIEZO1-Mediated Currents Are Modulated by Substrate Mechanics." *ACS Nano* **13**(11): 13545-13559.
- Boycott, H. E., C. S. Barbier, C. A. Eichel, K. D. Costa, R. P. Martins, F. Louault, G. Dilanian, A. Coulombe, S. N. Hatem and E. Balse (2013). "Shear stress triggers insertion of voltage-gated potassium channels from intracellular compartments in atrial myocytes." *Proc Natl Acad Sci U S A* **110**(41): E3955-3964.
- Chien, S. (2007). "Mechanotransduction and endothelial cell homeostasis: the wisdom of the cell." *Am J Physiol Heart Circ Physiol* **292**(3): H1209-1224.
- Coste, B., J. Mathur, M. Schmidt, T. J. Earley, S. Ranade, M. J. Petrus, A. E. Dubin and A. Patapoutian (2010). "Piezo1 and Piezo2 are essential components of distinct mechanically activated cation channels." *Science* **330**(6000): 55-60.
- Cox, C. D., C. Bae, L. Ziegler, S. Hartley, V. Nikolova-Krstevski, P. R. Rohde, C.-A. Ng, F. Sachs, P. A. Gottlieb and B. Martinac (2016). "Removal of the mechanoprotective influence of the cytoskeleton reveals PIEZO1 is gated by bilayer tension." *Nature Communications* **7**(1): 10366.
- Davies, P. F. (1995). "Flow-mediated endothelial mechanotransduction." *Physiol Rev* **75**(3): 519-560.
- Elam, T. R. and J. B. Lansman (1993). *Mechanosensitive Ion Channels in Vascular Endothelial Cells. Ion Flux in Pulmonary Vascular Control*. E. K. Weir, J. R. Hume and J. T. Reeves. Boston, MA, Springer US: 277-285.
- Fish, K. N. (2009). "Total internal reflection fluorescence (TIRF) microscopy." *Current protocols in cytometry* **Chapter 12**: Unit12.18-Unit12.18.
- Friedrich, E. E., Z. Hong, S. Xiong, M. Zhong, A. Di, J. Rehman, Y. A. Komarova and A. B. Malik (2019). "Endothelial cell Piezo1 mediates pressure-induced lung vascular hyperpermeability via disruption of adherens junctions." *Proceedings of the National Academy of Sciences* **116**(26): 12980-12985.
- Gautam, M., A. Gojova and A. I. Barakat (2006). "Flow-activated ion channels in vascular endothelium." *Cell Biochem Biophys* **46**(3): 277-284.

Gottlieb, P. A., C. Bae and F. Sachs (2012). "Gating the mechanical channel Piezo1: a comparison between whole-cell and patch recording." *Channels (Austin)* **6**(4): 282-289.

Hyman, A. J., S. Tumova and D. J. Beech (2017). "Piezo1 Channels in Vascular Development and the Sensing of Shear Stress." *Curr Top Membr* **79**: 37-57.

Ku, D. N., D. P. Giddens, C. K. Zarins and S. Glagov (1985). "Pulsatile flow and atherosclerosis in the human carotid bifurcation. Positive correlation between plaque location and low oscillating shear stress." *Arteriosclerosis* **5**(3): 293-302.

Li, J., B. Hou, S. Tumova, K. Muraki, A. Bruns, M. J. Ludlow, A. Sedo, A. J. Hyman, L. McKeown, R. S. Young, N. Y. Yuldasheva, Y. Majeed, L. A. Wilson, B. Rode, M. A. Bailey, H. R. Kim, Z. Fu, D. A. Carter, J. Bilton, H. Imrie, P. Ajuh, T. N. Dear, R. M. Cubbon, M. T. Kearney, R. K. Prasad, P. C. Evans, J. F. Ainscough and D. J. Beech (2014). "Piezo1 integration of vascular architecture with physiological force." *Nature* **515**(7526): 279-282.

Maneshi, M. M., L. Ziegler, F. Sachs, S. Z. Hua and P. A. Gottlieb (2018). "Enantiomeric A β peptides inhibit the fluid shear stress response of PIEZO1." *Scientific Reports* **8**(1): 14267.

Mendoza, S. A., J. Fang, D. D. Gutterman, D. A. Wilcox, A. H. Bubolz, R. Li, M. Suzuki and D. X. Zhang (2010). "TRPV4-mediated endothelial Ca²⁺ influx and vasodilation in response to shear stress." *American journal of physiology. Heart and circulatory physiology* **298**(2): H466-H476.

Nahavandi, S., S. Baratchi, R. Soffe, S. Y. Tang, S. Nahavandi, A. Mitchell and K. Khoshmanesh (2014). "Microfluidic platforms for biomarker analysis." *Lab Chip* **14**(9): 1496-1514.

Nerem, R. M. (1992). "Vascular fluid mechanics, the arterial wall, and atherosclerosis." *J Biomech Eng* **114**(3): 274-282.

Nigro, P., J. Abe and B. C. Berk (2011). "Flow shear stress and atherosclerosis: a matter of site specificity." *Antioxid Redox Signal* **15**(5): 1405-1414.

Nonomura, K., V. Lukacs, D. T. Sweet, L. M. Goddard, A. Kanie, T. Whitwam, S. S. Ranade, T. Fujimori, M. L. Kahn and A. Patapoutian (2018). "Mechanically activated ion channel PIEZO1 is required for lymphatic valve formation." *Proceedings of the National Academy of Sciences* **115**(50): 12817-12822.

Nourse, J. L. and M. M. Pathak (2017). "How cells channel their stress: Interplay between Piezo1 and the cytoskeleton." *Seminars in cell & developmental biology* **71**: 3-12.

Oancea, E., J. T. Wolfe and D. E. Clapham (2006). "Functional TRPM7 channels accumulate at the plasma membrane in response to fluid flow." *Circ Res* **98**(2): 245-253.

Ohno, M., J. P. Cooke, V. J. Dzau and G. H. Gibbons (1995). "Fluid shear stress induces endothelial transforming growth factor beta-1 transcription and production. Modulation by potassium channel blockade." *J Clin Invest* **95**(3): 1363-1369.

Ohno, M., G. H. Gibbons, V. J. Dzau and J. P. Cooke (1993). "Shear stress elevates endothelial cGMP. Role of a potassium channel and G protein coupling." *Circulation* **88**(1): 193-197.

Porat-Shliom, N., O. Milberg, A. Masedunskas and R. Weigert (2013). "Multiple roles for the actin cytoskeleton during regulated exocytosis." *Cellular and molecular life sciences: CMLS* **70**(12): 2099-2121.

Ranade, S. S., Z. Qiu, S.-H. Woo, S. S. Hur, S. E. Murthy, S. M. Cahalan, J. Xu, J. Mathur, M. Bandell, B. Coste, Y.-S. J. Li, S. Chien and A. Patapoutian (2014). "Piezo1, a mechanically activated ion channel, is required for vascular development in mice." *Proceedings of the National Academy of Sciences* **111**(28): 10347-10352.

Ranade, S. S., Z. Qiu, S. H. Woo, S. S. Hur, S. E. Murthy, S. M. Cahalan, J. Xu, J. Mathur, M. Bandell, B. Coste, Y. S. Li, S. Chien and A. Patapoutian (2014). "Piezo1, a mechanically activated ion channel, is required for vascular development in mice." *Proc Natl Acad Sci U S A* **111**(28): 10347-10352.

Saotome, K., S. E. Murthy, J. M. Kefauver, T. Whitwam, A. Patapoutian and A. B. Ward (2018). "Structure of the mechanically activated ion channel Piezo1." *Nature* **554**(7693): 481-486.

Soffe, R., S. Baratchi, M. Nasabi, S.-Y. Tang, A. Boes, P. McIntyre, A. Mitchell and K. Khoshmanesh (2017). "Lateral trapezoid microfluidic platform for investigating mechanotransduction of cells to spatial shear stress gradients." *Sensors and Actuators B: Chemical* **251**: 963-975.

Syeda, R., J. Xu, A. E. Dubin, B. Coste, J. Mathur, T. Huynh, J. Matzen, J. Lao, D. C. Tully, I. H. Engels, H. M. Petrassi, A. M. Schumacher, M. Montal, M. Bandell and A. Patapoutian (2015). "Chemical activation of the mechanotransduction channel Piezo1." *Elife* **4**.

Tzima, E., M. Irani-Tehrani, W. B. Kiosses, E. Dejana, D. A. Schultz, B. Engelhardt, G. Cao, H. DeLisser and M. A. Schwartz (2005). "A mechanosensory complex that mediates the endothelial cell response to fluid shear stress." *Nature* **437**(7057): 426-431.

Wang, M., Y. Wu, Y. Yu, Y. Fu, H. Yan, X. Wang, T. Li, W. Peng and D. Luo (2019). "Rutaecarpine prevented ox-LDL-induced VSMCs dysfunction through inhibiting overexpression of connexin 43." *European Journal of Pharmacology* **853**: 84-92.

Wang, S., R. Chennupati, H. Kaur, A. Iring, N. Wettschureck and S. Offermanns (2016). "Endothelial cation channel PIEZO1 controls blood pressure by mediating flow-induced ATP release." *The Journal of clinical investigation* **126**(12): 4527-4536.

Wang, Y., S. Chi, H. Guo, G. Li, L. Wang, Q. Zhao, Y. Rao, L. Zu, W. He and B. Xiao (2018). "A lever-like transduction pathway for long-distance chemical- and mechano-gating of the mechanosensitive Piezo1 channel." *Nature Communications* **9**(1): 1300.

Wang, Y. and B. Xiao (2018). "The mechanosensitive Piezo1 channel: structural features and molecular bases underlying its ion permeation and mechanotransduction." *J Physiol* **596**(6): 969-978.

Yamamoto, K., R. Korenaga, A. Kamiya and J. Ando (2000). "Fluid shear stress activates Ca(2+) influx into human endothelial cells via P2X4 purinoceptors." *Circ Res* **87**(5): 385-391.

Zarins, C. K., D. P. Giddens, B. K. Bharadvaj, V. S. Sottiurai, R. F. Mabon and S. Glagov (1983). "Carotid bifurcation atherosclerosis. Quantitative correlation of plaque localization with flow velocity profiles and wall shear stress." *Circ Res* **53**(4): 502-514.

Zhang, T., S. Chi, F. Jiang, Q. Zhao and B. Xiao (2017). "A protein interaction mechanism for suppressing the mechanosensitive Piezo channels." *Nat Commun* **8**(1): 1797.

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