Examination of the role of transient receptor potential vanilloid type 4 in endothelial responses to shear forces

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(Received 13 May 2014; accepted 5 August 2014; published online 15 August 2014)

Shear stress is the major mechanical force applied on vascular endothelial cells by blood flow, and is a crucial factor in normal vascular physiology and in the development of some vascular pathologies. The exact mechanisms of cellular mechano-transduction in mammalian cells and tissues have not yet been elucidated, but it is known that mechanically sensitive receptors and ion channels play a crucial role. This paper describes the use of a novel and efficient microfluidic device to study mechanically-sensitive receptors and ion channels *in vitro*, which has three independent channels from which recordings can be made and has a small surface area such that fewer cells are required than for conventional flow chambers. The contoured channels of the device enabled examination of a range of shear stresses in one field of view, which is not possible with parallel plate flow chambers and other previously used devices, where one level of flow-induced shear stress is produced per fixed flow-rate. We exposed bovine aortic endothelial cells to different levels of shear stress, and measured the resulting change in intracellular calcium levels ($[Ca^{2+}]_i$) using the fluorescent calcium sensitive dye Fluo-4AM. Shear stress caused an elevation of $[Ca^{2+}]_i$ that was proportional to the level of shear experienced. The response was temperature dependant such that at lower temperatures more shear stress was required to elicit a given level of calcium signal and the magnitude of influx was reduced. We demonstrated that shear stress-induced elevations in $[Ca^{2+}]_i$ are largely due to calcium influx through the transient receptor potential vanilloid type 4 ion channel. © 2014 AIP Publishing LLC.
[\[http://dx.doi.org/10.1063/1.4893272\]](http://dx.doi.org/10.1063/1.4893272)

INTRODUCTION

The inner surface of all blood vessels in the cardiovascular system is lined with endothelial cells. These cells play a crucial role in cardiovascular homeostasis and vascular tone by responding to the physical and chemical signals in the flowing blood.¹ These cells are biological mechano-transducers, which sense and convert mechanical forces associated with blood flow such as shear stress into biochemical signals to communicate with adjacent vascular smooth muscle cells and thus they control contraction and relaxation of blood vessels.

There is compelling evidence to show that the molecular mechanism of mechano-transduction involves ion channels, but it may also involve mechano-sensitive (MS) receptors, enzymes, and intracellular signalling. Several candidate eukaryotic ion channels respond to mechanical stimuli. These include the degenerin/epithelial sodium channels (DEG/ENaC), several transient receptor potential (TRP) channels, two-pore domain potassium (K2P) channels, MscS-like channels,² and piezo channels.³ For example, mechano-sensitive ion channels have been proposed to be important in Ca^{2+} dependent nitric oxide release in heart,⁴ and pressure mechano-transduction in bladder urothelial cells.⁵

TRP channels are the major class of calcium permeable channels on endothelial cells, and there is evidence linking the regulation of $[Ca^{2+}]_i$ to vasodilation in response to mechanical stimulation.² Among the family of TRP channels, a growing body of data supports a role for transient receptor potential vanilloid type 4 (TRPV4) in mechano-sensation, as reviewed elsewhere.^{6,7} In particular, it has been proposed that TRPV4 may function as a signal transducer that responds to shear stress.^{8–10} However, due to technical limitations of current techniques to study mechano-sensitive ion channel activity in endothelial cells significant questions remain regarding the identity of molecules involved and the their mechanism of action.

Patch clamp electrophysiology is a valuable approach for studying the biophysics and pharmacology of MS ion channels. However, it has some limitations in study of the effect of mechanical forces on ion channels. For example, endothelial cells are small and flat and difficult to patch.² Electrophysiology in general is a low throughput, high information-content analysis method, as measurements are performed on single cells.²

To overcome these limitations, sophisticated optical techniques have been developed that rely on calcium sensitive dyes.^{11,12} In order to analyse shear stress induced mechano-transduction by optical methods, new macroscale devices have been developed. One example is the parallel plate flow chamber, which has been used by several groups to study the response of cellular populations to shear stress.^{13,14} Parallel plate flow chambers have also been used to study the effect of shear stress on expression of a vascular cell adhesion molecule (VCAM-1) proliferation in endothelial cells,^{15,16} and also to study the role of p120-Catenin in morphological changes of endothelial cells growing under shear stress.¹⁷ Flow chambers with variable geometries have been used to apply a shear gradient over endothelial cells,¹⁸ while a T-shaped chamber has been used to mimic the shear gradient at the arterial bifurcation.¹⁹ The cone-plate viscometer is another approach that produces shear stress by rotating the shallow cone with respect to the stationary base plate.^{20,21} Magnetic tweezers have also been used to selectively study the response of individual cells to shear stress.^{22,23} One limitation of these methods is that it is not possible to independently observe the effect of multiple levels of shear simultaneously. Furthermore, these methods are often difficult to customize, require large numbers of cells and volumes of chemicals and are therefore not suitable for cells that are rare or for measurements that are inherently variable or that require multiple conditions to be tested.

Microscale technologies have emerged as powerful tools for biological discovery, since they offer precise control over the environmental conditions such as flow rate, shear stress, temperature, and exposure to receptor agonists and antagonists.^{24,25} Moreover, they allow parallel experimentation, integration with other systems, short reaction times, and versatility in design.²⁶

So far, two strategies have been used to obtain controlled shear stress in microfluidic systems. The first is the use of devices which possess multiple, independent channels that enable cells to be exposed to different levels of shear and/or experimental compounds. Such devices allow higher throughput and are suitable for use over extended periods. One example is a microfluidic device with eight parallel microchannels providing a range of shear stress from 0.5 to 50 dyn cm⁻², which has been used to study the effect of shear stress on platelets.²⁷ However, a limitation of the design of this device is that it does not allow analysis of multiple shear stresses in one field of view and is therefore not ideal for multi-point, comparative analysis of mechano-sensitive ion channels. Another strategy is the use of channels with varying geometry that allow analysis of multiple levels of shear in a small area, while other experimental conditions remain the same.^{28–30} For example, Wang *et al.*³⁰ integrated an oblique barrier into a parallel channel to generate a shear gradient along the flow. In order to adopt this strategy, one must be able to calculate the amount of shear that each cell is experiencing. Additionally, due to the gradual change in the channel width, its dimensions do not permit simultaneous viewing of the effect of the highest and lowest shear stresses in one field of view, at the highest resolutions possible.

Studies of mechano-transduction at the micro- and nano-scale should benefit greatly from the ability to apply precise mechanical stimulation to cells to mimic *in vivo* conditions while also enabling high resolution fluorescent imaging of intracellular signals, like calcium. Therefore, an important aim of this work is to implement varying geometry strategy^{28–30} to

create a microfluidic device with a wide range of shear stresses with clear boundaries, which can be monitored in one field of view to enable multiple observations in one run.

We have developed a microfluidic device with three independent diverging-converging microchannels that enable the analysis of a range of physiological and pathophysiological shear stresses with clear boundaries in one field of view. Additionally, the diverging-converging design allows comparison of the effect of pressure on cells under the same level of shear stress. In particular, these features are important to study the role of the calcium-permeable TRP channels, as changes in $[Ca^{2+}]_i$ occurs within the range of a few milliseconds to tens of seconds, following stimulation and no previously reported devices enable the study of ion channels with this precision. We have used this device to study the response of bovine aortic endothelial cells (BAECs) to shear stress enabling, for the first time, the identification of a role for TRPV4 in BAEC mechano-sensitivity.

Our observations show that BAECs respond to shear stress with a rapid increase in $[Ca^{2+}]_i$, which depends on the applied shear and temperature. In this paper, we provide direct evidence for the role of TRPV4 in the shear stress response of aortic endothelial cells. Furthermore, this report is the first evidence showing that in addition to shear, pressure affects the dynamics of calcium signalling (but not the amplitude of the response).

MATERIALS AND METHODS

Design, fabrication, and numerical characterization of the microfluidic device

The microfluidic channel used in our investigation was 12 mm long and incorporated a contoured shape so that the channel width constricted and then expanded to increase and then decrease the shear stress induced along the bottom surface of the channel where cells were cultured. The height of the channel was 80 μm , while its width varies from 2000 μm to 500 μm along the contracted region (Figure 1(a)).

The microdevice was fabricated from Polydimethylsiloxane (PDMS) (Sylgard) cast against a patterned mould formed using standard photolithography techniques as follows. A 4-in. silicon wafer was spin coated with SU8-3050 (MicroChem Corp.) photo-resist using a spread cycle of 300 rpm and 100 rpm s^{-1} for 10 s and a development cycle of 1000 rpm and 300 rpm s^{-1} for 30 s, in order to achieve a film of 100 μm thickness with good uniformity. A cycle of edge bead removal was conducted using edge bead removal solvent. The SU8 coated wafer was soft-baked by ramping the temperature at 6 $^{\circ}\text{C min}^{-1}$ starting from 23 $^{\circ}\text{C}$ and holding at 100 $^{\circ}\text{C}$ for 30 min to dry out the solvents. The SU8 film was exposed with a mask pattern for 1 min of UV on an MJB3 contact mask aligner with a wavelength of 360 nm and a power of 8 mW cm^{-2} . The mask was fabricated from a printed film (2400 dpi). After the UV exposure, the patterns were cross-linked by baking on a hotplate for 1 min at 65 $^{\circ}\text{C}$ and 4 min at 95 $^{\circ}\text{C}$. The exposed and cross-linked film was slowly cooled to room temperature. The unexposed SU8 was developed for 8–12 min with periodic manual agitation to remove the unexposed material. After developing the SU8 pattern, the wafer was cleaned with isopropanol and then rinsed with de-ionised (DI) water. A final hard bake was performed by heating the sample to 120 $^{\circ}\text{C}$ for 3 h, in order to improve and strengthen the cross-linked SU8 pattern and extend the service life. The SU8 pattern was then ready for use as a mould for casting PDMS channels.

PDMS was prepared at ratio of 10:1 and degassed for 30 min. The mixture was poured onto the previously made SU8 mould. The PDMS was cured in an oven at 100 $^{\circ}\text{C}$ for 20 min and then peeled from the SU8 mould and a 5 mm inlet reservoir hole was made using a biopsy punch. For the outlet connection to the syringe pump, a 1 mm biopsy punch was used. After both holes were punched, the PDMS channel was placed directly onto a glass slide of 22 $\times$ 22 mm glass coverslips (Thermo Fisher Scientific) coated with cultured cells. Adhesion was achieved simply due to the low surface energy of PDMS. In order to improve the sealing between the PDMS and glass slide, a clamp was designed and fabricated to provide homogeneous pressure and avoid fluid leakage. The clamp was fabricated using a milling machine from a 4 mm Perspex (PMMA) sheet, using computer aided manufacture software (CAD/CAM Galaad) (Figure 1(b)).

FIG. 1. Figures show (a) schematic of the microchannel with contracting-expanding geometry, (b) PDMS channels sealed with mechanical clamps to avoid leaking, (c) contours of velocity and shear stress along the channel at a flow rate of $10 \mu\text{l min}^{-1}$ obtained by numerical simulations, and (d) plot of velocity and shear stress along the middle of the channel at a flow rate of $10 \mu\text{l min}^{-1}$ obtained by numerical simulations.

A series of computational fluid dynamics simulations were conducted to predict the flow field and obtain the shear stress induced on cells. The simulations were performed using the finite-volume based ANSYS Fluent 6.3 software (Canonsburg, PA, USA).

The liquid was assumed to be incompressible and Newtonian. The Reynolds number based on the hydraulic diameter of the channels ($Re = \rho \cdot U \cdot D / \mu$, where ρ is the density of liquid, U is the average velocity, D is the hydraulic diameter of the channel across its contracted region, and μ is the dynamic viscosity of liquid) varies from 0.58 to 5.8 for the applied flow rates ranging from 10 to $100 \mu\text{l min}^{-1}$, indicating the laminar characteristics of the flow. Under these conditions, the following differential equations governing the balance of mass and momentum (also called continuity and Navier-Stokes equations) were solved within the channel:

$$\nabla \cdot \vec{U} = 0, \quad (1)$$

$$\rho(\vec{U} \cdot \nabla)\vec{U} = -\nabla P + \mu \nabla^2 \vec{U}, \quad (2)$$

in which $\vec{U}$ and P are the velocity and pressure of the liquid, respectively. The boundary conditions included the zero pressure at the inlet, a suction flow rate at the outlet, and no-slip boundary conditions along the bottom, top, and side walls of the channel. The second order upwind

scheme was used to discretize the convective and diffusive fluxes in the momentum equation. Pressure-Velocity coupling is obtained by the SIMPLE-Consistent (SIMPLEC) algorithm.³¹

Numerical simulations indicate that the flow velocity follows a parabolic profile across the cross section of the channel. At a flow rate of $10 \mu\text{l min}^{-1}$, a maximum velocity of 1.25 mm s^{-1} is obtained along the middle plane of the channel ($z = 40 \mu\text{m}$), which increases to 5.4 mm s^{-1} at the contracted region (Figures 1(c) and 1(d)). The shear stress at the bottom of the channel ($\tau_{xz} = \frac{\partial U_x}{\partial z}|_{z=0}$) follows the trend of the velocity, and increases linearly from 0.5 to 2.1 dyn cm^{-2} across the contracted region (Figures 1(c) and 1(d)).

Reagents and buffers

Imaging was conducted with the cells immersed in Hanks' balanced salt solution (HBSS) buffer containing: 140 mM NaCl, 5 mM KCl, 10 mM HEPES, 11 mM D-glucose, 1 mM MgCl_2 , 2 mM CaCl_2 , and 2 mM probenecid, adjusted to pH 7.4. Calcium free buffer was HBSS buffer with 2 mM EGTA (ethylene glycol tetraacetic acid) added CaCl_2 omitted. Stock solutions of TRPV4-selective agonist GSK1016790A (Sigma-Aldrich, St. Louis, MO), non-selective TRP antagonist ruthenium red (Sigma-Aldrich, St. Louis, MO), TRPV4-selective antagonist HC067047 (Santa Cruz Biotechnology, Santa Cruz, CA) and sarcoendoplasmic reticulum calcium transport ATPase (SERCA) inhibitor, thapsigargin (Sigma-Aldrich, St. Louis, MO) were dissolved in dimethyl sulfoxide and diluted in HBSS prior to experimentation.

Cell culture

BAECs³² (a kind gift from Professor Peter Little, RMIT University) were subcultured in DMEM (Dulbecco's Modified Eagle's medium) supplemented with 10% fetal bovine serum (FBS) and $100 \mu\text{g ml}^{-1}$ of penicillin and streptomycin. HEK293 T-REx (Life Sciences) cell lines stably expressing human TRPV4 were generated. Cells were grown in tetracycline-free DMEM media containing 10% FBS, hygromycin ($50 \mu\text{g/ml}$), and blasticidin ($5 \mu\text{g/ml}$). TRP channel expression was induced using $1 \mu\text{g/ml}$ of tetracycline 4 h before experiments. The negative control cells were HEK293 T-REx cells that were not transfected. For experimental work, cells were seeded onto $22 \times 22 \text{ mm}$ glass coverslips (Thermo Fisher Scientific) at a density of 50 000 cells/coverslip one day before the experiments.

For inhibitor studies, cells were incubated for 30 min with ruthenium red ($1 \mu\text{M}$), HC067047 ($0.01\text{--}1 \mu\text{M}$), Thapsigargin ($1 \mu\text{M}$), or vehicle (0.1% dimethyl sulfoxide control) before each assay. Appropriate concentrations of drugs were chosen from concentration response curves, or from the available literature.

Western Blots

Proteins were separated by 10% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and transferred to a PVDF (polyvinylidene difluoride) membrane at 400 mA for 1 h using a CriterionTM Blotter (BioRad, Gladesville, NSW, AUS) and blocked with 5% BSA (bovine serum albumin) for 1 h at room temperature. Membranes were probed rabbit anti TRPV4 (LS-A8583) and mouse anti GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) (sc-166574) overnight followed by an 1 h incubation at room temperature with HRP (horseradish peroxidase)-conjugated rabbit (sc-2385) and mouse (sc-2371) secondary antibodies, respectively. The bands were visualised with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Scoresby, VIC, AUS) and imaged using the ChemiDOCTM XRS+ (BioRad, Gladesville, NSW, AUS).

Shear experiment and live cell imaging of intracellular calcium ($[\text{Ca}^{2+}]_i$)

Before each experiment, a coverslip with attached BAECs was loaded for 30 min with $2.5 \mu\text{M}$ Fluo-4AM ester at 37°C in HBSS buffer. After incubation with Fluo-4AM, the BAECs were washed twice with warm HBSS and left to de-esterify for a further 30 min. Care was taken to eliminate air bubbles in the channel during loading of the coverslip. For experiments,

the microfluidic device loaded with BAECs was transferred to the stage of a Nikon A1 confocal laser scanning inverted microscope and was connected to a syringe pump (Genie Touch, Kent Scientific Corporation). The polyvinyl chloride calibrated tubing (1.02 mm ID; Gilson, Inc.) was connected to the outlet of the microchip for perfusion at a flow rate of $50 \mu\text{l min}^{-1}$, unless otherwise stated, on the subconfluent layer of BAECs. For each experiment, loaded cells were excited with a 488 nm laser using a Nikon A1 laser scanning confocal microscope (Nikon Instruments, Inc., Melville, USA) with an inverted Nikon Eclipse Ti-E as the base microscope, which was equipped with 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 $\times$ objective with a numerical aperture of 0.30. Calcium influx was measured as an increase in the fluorescent intensity of Fluo-4AM and normalized to the fluorescent intensity of resting cells. At the end of each experiment, the cells were stained with propidium iodide (PI) ($10 \mu\text{g ml}^{-1}$) and only experiments with more than 80% viable cells were analysed. All imaging experiments were performed at 37°C unless otherwise stated.

Data analysis

$[\text{Ca}^{2+}]_i$ levels were measured from the entire endothelial cell. The cell area was measured by automatically analysing the area enclosed by a region of interest drawn (ROIs) around single endothelial cells using NIS element viewer (Nikon Instruments Inc.). To quantify changes in $[\text{Ca}^{2+}]_i$, the average intensity of several ROIs has been measured and results are reported as ratio of F1/F0.

All cells within the area with the same shear were analysed. Data are shown as mean $\pm$ standard deviation of at least three independent experiments and 30 cells have been analysed in each. For statistical analysis Student's t-test or one way ANOVA was performed using Prism 6 (GraphPad software) and $P < 0.05$, was considered to be significant.

RESULTS AND DISCUSSION

Effect of shear stress on $[\text{Ca}^{2+}]_i$

Shear stress in large arteries typically varies from 5 to 20 dyn cm^{-2} .³³ The presence of a physiological level of shear stress is necessary for endothelial cells to elongate, maintain barrier function, express vasodilator, and anti-thrombic factors, as well as to maintain vascular health.³³ In contrast, less than 4 dyn cm^{-2} shear stress promotes the development of atherosclerosis that is associated with reduction in endothelial cell functions such as nitric oxide synthase activity, vasodilation, and endothelial cell repair.¹⁷

In our experiments, shear stress varied smoothly along the curved region of the microfluidic channel; for example, from 0.6 to 2.1 dyn cm^{-2} at a flow rate of $10 \mu\text{l min}^{-1}$, while it varies from 6 to 20 dyn cm^{-2} at a flow rate of $100 \mu\text{l min}^{-1}$ (Figure 1(c)). Accordingly, the shear change over each $100 \mu\text{m}$ of the contraction/expansion region is obtained as $0.076 \text{ dyn cm}^{-2}$ at a flow rate of $10 \mu\text{l min}^{-1}$ while increases to 0.76 dyn cm^{-2} at a flow rate of $100 \mu\text{l min}^{-1}$. Thus, the microfluidic channel provided a range of shear stresses from physiological to pathophysiological, depending on the flow rate.

For these experiments, cells were grown on a coverslip for 24 h and then the coverslip was transferred to the microchannels. To prevent leakage of imaging buffer, clamps were used to seal the glass coverslips against the PDMS channels, as shown in Figure 1(b). The data presented here corresponds to the initial, converging part of the channel unless otherwise stated.

Under the described experimental conditions, shear mediated an elevation in the measured $[\text{Ca}^{2+}]_i$ in BAECs, which increased linearly as a function of shear stress. Flow rates of either 10 or $100 \mu\text{l min}^{-1}$ were used to create laminar shear stress of $0.65\text{--}20.5 \text{ dyn cm}^{-2}$ (Figure 2(a)), which is further detailed in the supplementary material Fig. S1 (Ref. 47) and supplementary Movie-1 [see Fig. 2(a) (Multimedia view)]. The relationship between the changes in the fluorescence intensity to shear stress is plotted in Figure 2(b) with a half maximal effect of 5 dyn cm^{-2} with 1.25 ± 0.02 fold increase in $[\text{Ca}^{2+}]_i$ ($p < 0.01$), and was saturated at 10.5 dyn cm^{-2} with 1.43 ± 0.02 ($p < 0.01$) fold increase in $[\text{Ca}^{2+}]_i$ compare to the resting cells.

FIG. 2. Quantitative analysis of shear stress-induced calcium signalling in BAECs grown on a coverslip. (a) Figures show the distribution of shear along the selected regions of the microchannel (top), cells within these regions have been used for analysis (middle and bottom). Graphs show (b) elevation of [Ca²⁺]_i in response to shear stresses ranging from 0.6 to 20.5 dyn cm⁻². Statistical significance compared to resting cells is indicated by ** for P < 0.01. (c) Dynamics of calcium signalling at different shear stresses of 0.6, 2.0, 6.5, and 20.5 dyn cm⁻². At the flow rates of 10 and 100 $\mu\text{l min}^{-1}$, shear stress increases by 0.1 and 1 dyn cm⁻², respectively across the 100 μm bands. (Multimedia view) [URL: <http://dx.doi.org/10.1063/1.4893272.1>]

Furthermore, shear stress affects the dynamics of calcium signalling; cells started to respond to shear of 0.65 dyn cm⁻² after 20 s, while increasing the shear stress to 6.5 dyn cm⁻² increased the sensitivity of cells and reduced the response time to less than 5 s (Figure 2(c)).

These results show that at a low level of shear (0.6–2 dyn cm⁻²) it takes a longer time for BAECs to sense the shear, and the peak amplitude of [Ca²⁺]_i is at least 0.1 ± 0.03 fold lower ($p < 0.05$, $n = 30$, for cells at 2 dyn cm⁻²) than the cells at a relatively higher level of shear, close to their physiological conditions (6–20 dyn cm⁻²).

Analysing the profile of individual BAECs exposed to shear stress of 20 dyn cm⁻² indicated two distinct responses within the cells. Some cells responded to shear stress with a transient increase in [Ca²⁺]_i which returned to base line levels after reaching a plateau. Alternatively, some cells responded to shear stress by exhibiting a steady increase in [Ca²⁺]_i which did not return to baseline in the time period measured (see supplementary material Fig. S2 (Ref. 47)).

Diverging and converging geometry along the channel provides us with two regions with similar levels of shear stress and different pressure (see supplementary material Fig. S3 (Ref. 47)). Using this characteristic of the device and comparing the effect of pressure on [Ca²⁺]_i of cells under similar levels of shear stress, we found no significant difference in the peak amplitude of response. However, presence of higher pressure within the converging regions of the channel accelerated the response time of BAECs to shear stresses of 2, 6, and 10 dyn cm⁻².

The effect of pressure on the shear stress response was not evident at 16 dyn cm^{-2} (see supplementary material Fig. S4 (Ref. 47)).

Effect of temperature on shear stress-induced calcium release

Temperature is an important influence on cellular responses. For example, temperature is a significant modulator of ion channel function that affects single channel conductance, gating, permeability, and ligand binding affinity.^{34–36} However, many studies have investigated the effect of mechanical stress at room temperature,^{28,37,38} which could alter the channel responses. This is particularly important when considering that many TRP channels are temperature sensitive. For example, TRPV4 is activated in the warm temperature range above 27°C , and it has been demonstrated that slight elevation of experimental temperatures to approximate body temperature, around 37°C , can modulate a variety of intracellular signals.³² At 37°C , TRPV4 is regulated by shear stress, hypotonic cell swelling, and chemical signals in a PKC (Protein kinase C) dependent and independent manner; while at room temperature TRPV4 activation by these stimuli is through a PKC independent pathway only.^{39,40}

We have demonstrated here that the cellular response to shear stress is indeed temperature dependent. Comparing the effect of shear on BAECs showed that increasing the temperature increased the sensitivity of cells and reduced the required level of shear to activate these cells from 10.5 dyn cm^{-2} at 22°C to 3.5 dyn cm^{-2} at 37°C ; and also increased the peak level of $[\text{Ca}^{2+}]_i$ flux from 1.05 ± 0.01 fold at 22°C to 1.2 ± 0.02 fold at 37°C ($n = 4$) ($P < 0.05$) fold at 37°C (Figure 3(a)).

When comparing the individual cells, we found that not all cells were responsive to shear stress, indicating that there might be some variability in activation threshold of cells within the

FIG. 3. Quantitative analysis of shear stress induced calcium signalling at 22°C and 37°C . Graphs show (a) increase of $[\text{Ca}^{2+}]_i$ at 22°C and 37°C , in response to 2.05 and 10.5 dyn cm^{-2} shear stresses, (b) percentage of activated cells in response to shear stress at 22°C and 37°C at 10.5 dyn cm^{-2} . Data is representative of three different experiments and 30 cells were analysed in each experiment.

cellular population. Further, comparing the effect of temperature on cellular response to shear showed that under a constant shear of 10.5 dyn cm^{-2} , elevating the temperature from 22°C to 37°C increased the percentage of activated cells from $10.23\% \pm 2.36\%$ ($n=4$) to $47.00\% \pm 8.8\%$ ($n=4$) ($P < 0.01$) (Figure 3(b)).

The data presented here suggests that temperature plays a significant role in BAECs response to shear stress and subsequent changes of $[\text{Ca}^{2+}]_i$, although it is likely that the effect is not solely due to effects on TRPV4.

Importance of TRPV4 in BAECs response to shear stress

TRPV4 is a non-selective cation channel that can be activated by mechanical stimulation.⁶ Recent studies have proposed that the TRPV4 may play a role in calcium entry and regulating vasodilatation caused by mechanical stimulation in murine aortic vascular endothelial cells.^{41,42} Additionally, it has been shown that TRPV4 in MDCK (Madin-Darby canine kidney) cells are responsive to shear stress and that this response is modulated by channel phosphorylation at the Tyrosine 110 residue.⁹ We hypothesized that TRPV4 is likely to play a role in regulation of the response to shear stress in BAECs. To verify this possibility, we first determined the expression of TRPV4 in BAECs using western blotting and also functional assay of TRPV4 activation using the specific agonist, GSK1016790A.⁹

FIG. 4. Quantitative analysis of BAECs' calcium response to GSK1016790A. (a) Western blot analysis of TRPV4 expression in BAECs, (b) images show BAECs loaded with Fluo-4AM before and after exposure to 5, 15, and 50 nM GSK. Scale bar is $250 \mu\text{m}$. (c) Increase in $[\text{Ca}^{2+}]_i$ in response to different concentrations of GSK1016790A, ranging from 0, 5, 15 and 50 nM, shown as fold-increase in Fluo-4AM fluorescence intensity. Arrows indicate the time GSK1016790A was administered. (d) Calcium influx in response to 15 nM GSK1016790A is dependent on extracellular calcium concentration dependently inhibited the increase in $[\text{Ca}^{2+}]_i$ after shear stress stimulation but did not completely block it. Statistical significance compared to vehicle control is indicated by: a for $1 \mu\text{M}$ HC067047; b for 100 nM HC067047 ($p < 0.01$).

The western blot analysis revealed a band at approximately 120 kDa, the same size that is expected for TRPV4, confirming the expression of TRPV4 in BAECs (Figure 5(a)). Application of the TRPV4 agonist GSK1016790A (15 nM) to BAECs via the microchannel inlet induced a sustained rise in $[Ca^{2+}]_i$, that reached its peak after 1 min, consistent with activation of TRPV4.

Decreasing the concentration of GSK1016790A delayed the onset of calcium influx and its peak (Figures 5(b) and 5(c)). In the absence of extracellular calcium, GSK1016790A failed to evoke calcium influx, an effect that was reversed by re-introducing normal calcium-containing HBSS (Figure 5(d)). This indicates that the elevation in $[Ca^{2+}]_i$ is from an extra cellular source and is not release from intracellular stores. Shear stress induced a graded calcium response in

FIG. 5. Quantitative analysis of BAECs' calcium response in presence of ruthenium red or in the absence of extracellular Ca^{2+} (No $[Ca^{2+}]_o$). Graphs show that removal of calcium from the buffer or treatment of cells with ruthenium red, reduced (a) the effect of shear stress on $[Ca^{2+}]_i$ level and (b) the percentage of cells activated in response to shear stress. Statistical significance compared to vehicle control is indicated by: a for 1 μ M ruthenium red; b for Ca^{2+} free buffer ($p < 0.01$). (c) Depletion of Ca^{2+} from intracellular stores did not inhibit the effect shear stress on $[Ca^{2+}]_i$ level. Normal perfusion buffer and calcium free buffer were as described in materials and methods. Data are representative of three different experiments and 30 cells were analysed in each experiment and we compared No $[Ca^{2+}]_o$ or ruthenium red to the shear stress control.

BAECs. Furthermore, here we have shown that aortic endothelial cells express TRPV4 and respond to the selective TRPV4 agonist, GSK1016790A. In order to investigate the importance of TRPV4 in regulation of the aortic endothelial cell response to shear stress, we treated BAECs with the selective TRPV4 antagonist, HC067047 (1 nM–1 μ M).⁹

HC067047 (1 μ M) partially reduced the shear stress-induced calcium influx from 1.24 ± 0.02 to 1.08 ± 0.01 fold ($n=4$) ($P < 0.01$) (at 16 dyn cm^{-2}). This confirms the role of the TRPV4 channel activation in the BAECs response to shear stress (Figure 4(e)). This experiment also indicated that not all of the response to shear is due to opening of TRPV4.

In order to study if shear stress could potentiate the response of BAECs to agonist, we applied 15 nM GSK1016790A which induces a submaximal response in BAECs and measured changes in $[\text{Ca}^{2+}]_i$ at a flow rate of $20 \mu\text{l min}^{-1}$ or $100 \mu\text{l min}^{-1}$. Increasing the flow rate from 20 to $100 \mu\text{l min}^{-1}$ increased the maximum response of BAECs to GSK1026790A observed 90 s after application of flow, from 1.28 ± 0.038 (fluorescence units) at $20 \mu\text{l min}^{-1}$ ($P < 0.01$) to 1.69 ± 0.08 at $100 \mu\text{l min}^{-1}$ and it also delayed the time taken for the cells to respond (see supplementary material Fig. S5 (Ref. 47)). These effects could be due to the shear stress sensitising (or priming) the TRPV4 ion channels. Overall, these data show the importance of TRPV4 in BAECs response to shear stress and also indicates that shear stress is affecting the profile of TRPV4-induced calcium influx in response to GSK1016790A.

Source of intracellular calcium

The selective TRPV4 antagonist HC067047, did not completely inhibit the observed shear stress-evoked elevation of $[\text{Ca}^{2+}]_i$. We hypothesised that there might be another source of calcium involved, either from intracellular stores (i.e., endoplasmic reticulum) or from extracellular calcium entering through another calcium permeable ion channel. In order to assess the potential involvement of other calcium permeable ion channels or other TRPV family members, we either removed extracellular Ca^{2+} or treated cells with the non-selective TRPV pore blocker, ruthenium red (1 μ M).⁹ Furthermore, using the SERCA inhibitor thapsigargin (1 μ M), we depleted the Ca^{2+} from intracellular stores and measured the change in $[\text{Ca}^{2+}]_i$ upon stimulation with shear stress.

The removal of extracellular calcium abolished the increase in $[\text{Ca}^{2+}]_i$ upon application of shear stress from 1.57 ± 0.14 to 1.08 ± 0.03 fold change above baseline ($n=3$) ($P < 0.01$), (16 dyn cm^{-2}), and reduced the number of activated cells from $45.2\% \pm 5.7\%$ to $15.3\% \pm 1.6\%$ ($n=3$) ($P < 0.05$) (16 dyn cm^{-2}) (Figures 5(a) and 5(b)). Treatment of cells with ruthenium red (1 μ M) decreased the amplitude of BAECs response to shear stress from 1.57 ± 0.14 to 1.13 ± 0.06 ($n=3$) ($P < 0.01$) (16 dyn cm^{-2}), and reduced the percentage of activated cells from $45.2\% \pm 5.7\%$ to $5\% \pm 3.2\%$ ($n=3$) ($P < 0.01$) (16 dyn cm^{-2}), compared to the vehicle control (Figure 4) but did not completely abolish influx of $[\text{Ca}^{2+}]_i$, suggesting another (non-TRP channel mediated) mechanism of Ca^{2+} influx is also present in these cells. Treatment of cells with thapsigargin did not affect shear stress-evoked $[\text{Ca}^{2+}]_i$ elevation (Figure 5(c)). Together, these data provide evidence that influx of extracellular calcium, probably through a non-TRP ion channel, is also important in the BAECs response to shear stress and that release of calcium from intracellular stores is not an important part of this mechanism.

CONCLUSION

The goal of the current study was to investigate the role of the mechanically sensitive ion channels TRPV4 in BAECs using a novel microfluidic device. We designed and fabricated a device that enabled observation of rapid signalling events within cells exposed to different levels of shear stress in one field using intracellular calcium imaging. The ability to grow cells on disposable coverslips reduced the possibility of contamination and increases the throughput compared to other devices. We measured changes in $[\text{Ca}^{2+}]_i$ in BAECs in response to mechanical stimulation to investigate the underlying mechanisms of the cellular response to shear stress. This methodology could readily be extended to other optical imaging assays, such as imaging

of the distribution of ion channel-green fluorescent protein fusion proteins (or derivatives) or bioluminescence resonance energy transfer (BRET) assays.

BAECs responded to shear stress with an increase in $[Ca^{2+}]_i$, which was dependent on the magnitude of shear, confirming their mechano-sensitivity. These responses were temperature dependent, such that increased temperature reduced the amount of shear needed to produce a given calcium signal. While this result was not unexpected, it is important, since many reports on the response of TRPV4 and other ion channels to shear or mechanical stimulation, have been performed at room temperature.^{43,44}

TRPV4 has previously been shown to be a receptor operated ion channel that can be modulated by shear stress.^{8–10} Activity of TRPV4 in vascular endothelium contributes to the regulation of vascular tone. For example, it has been shown that TRPV4 activation by osmotic swelling leads to the metabolism of vasoactive endothelial factors that causes vascular relaxation.⁴⁵ Here, we have shown that TRPV4 is expressed in a cell line derived from bovine aortic endothelial cells and plays a role in their response to shear stress. The response of BAECs to shear stress was dependent on extracellular calcium and incubation of cells with either a specific antagonist, HC67047, or a non-specific TRP ion channel pore blocker, ruthenium red, significantly diminished the $[Ca^{2+}]_i$ response, indicating that the BAECs response to shear stress is mediated in part by the influx of extracellular calcium through TRPV4. This is important as endothelial cell dysfunction in responding to shear stress is related to cardiovascular disease⁴⁶ and understanding the role of TRPV4 in these cells might help us to better understand and treat some forms of cardiovascular pathophysiology.

The current findings demonstrate that our microfluidic system has important advantages over previously reported approaches. It allows analysis of multiple conditions in one experiment, reducing experimental variability.

In addition, the use of confocal microscopy provides information that would be difficult to obtain using electrophysiology in that it has higher throughput and it is possible to simultaneously record calcium signals from many cells under similar or different levels of shear stress.

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