Reply to 'Mechanism for microtsunami-induced intercellular mechanosignalling'

Luo *et al.* reply — We thank He *et al.*[1] for their Correspondence regarding our recent paper[2]. They offer an alternate hypothesis for our observed microtsunami (μtsunami)-initiated Ca^{2+} signalling (μT-ICS) that differs from our claim of mechanical activation of stretch-sensitive G protein-coupled receptors (SS-GPCRs) by shear flow. Instead, they assert that μtsunami exposure initiates the release of Ca^{2+} signalling molecules, including ATP, from the cell immediately below the site of laser radiation. These molecules then diffuse to neighbouring cells and activate purinergic receptors, including those coupled to GPCRs, resulting in a propagating intercellular Ca^{2+} wave. They cite previous studies[3–5] that use femtosecond-laser microbeam irradiation to initiate Ca^{2+} signalling in primary rat hippocampal cells and astrocytes as well as immortalized human kidney (HEK293T) and cervical cancer (HeLa) cells. These Ca^{2+} waves have higher velocities (10–40 μm s^{-1}) and smaller spatial extent (~200 μm radius) as compared with μT-ICS waves (4.5 μm s^{-1} and ~400 μm radius, respectively) reported in our paper. Importantly, our studies employed human vascular endothelial cells (HUVECs) whose physiological function requires mechanosensitivity to hemodynamic shear stress.

The papers cited by He *et al.*[3–5] examine the roles of purinergic P2X (ligand-gated ion channels) and P2Y (GPCR) receptors in femtosecond laser microbeam induced Ca^{2+} signalling. Cells were treated with pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) and Reactive Blue 2 (RB2) to provide broad inhibition of P2X and P2Y receptors, respectively. Because Ca^{2+} signalling was unaffected by PPADS but reduced by more than 70% with RB2, the authors concluded that P2Y receptors play a central role in femtosecond laser induced signalling of astrocytes[5]. However, purinergic receptor subtypes vary significantly between cell lines, as do their susceptibility to antagonists such as PPADS and RB2 (ref. 6). In fact, HUVECs express purinergic receptors $P2X_1$, $P2X_6$, $P2X_7$, and $P2Y_1$ (refs 7,8) that are not susceptible to the action of these compounds[6,8].

He *et al.*[1] suggest that 2-APB inhibition of μT-ICS does not elucidate the underlying signalling mechanisms because 2-APB acts at the IP_3 receptor well downstream of the activation of SS-GPCRs. Our demonstration of 2-APB suppression of Ca^{2+} signalling in response to both exogenous ATP administration[9] and μtsunami exposure[2], left open the possibility that purinergic receptors play a principal role in μT-ICS. However, in Supplementary Fig. 1 of our paper[2], we presented data showing minimal Ca^{2+} signalling in HUVEC cultures when exposed to cell lysate, which presumably contains the signalling molecules released by HUVECs resulting from μtsunami exposure.

To further test the roles of SS-GPCRs in μT-ICS, we designed experiments to seek a differential response between exogenous ATP administration and μtsunami stimulation. We first examined exogenous ATP-initiated Ca^{2+} signalling (ATP-ICS) in HUVECs treated with PPADS and RB2. When administered alone, or in combination (at doses as high as 100 μM) they proved ineffective in suppressing ATP-ICS. This result casts serious doubt as to the effectiveness of PPADS and RB2 to investigate the role of purinergic receptors in HUVECs.

Instead, we administered HUVECs with U-73122, a drug that blocks the activation of phospholipase C (PLC) just downstream of GPCR activation. Treatment with 20 μM U-73122 reduced μT-ICS by nearly 70% but did not significantly reduce ATP-ICS (Supplementary Fig. 1). These results, along with the inhibition of both μT-ICS and ATP-ICS by 2-APB, demonstrates that μT-ICS occurs along the GPCR–PLC–IP_3 pathway. Moreover, we treated HUVECs with 200 ng ml^{-1} pertussis toxin and 100 μM suramin, both of which disrupt G protein coupling with differing efficacy and specificity. In these cases, μT-ICS was reduced by over 87% and 95%, respectively, (Supplementary Figs 2,3) whereas exogenous ATP-ICS was reduced only by 9% and 5%, respectively. These results demonstrate that μT-ICS can be inhibited without significantly affecting ATP-ICS, further supporting our hypothesis that μT-ICS is initiated through the activation of SS-GPCRs by the μtsunami-associated shear stresses.

There are also fundamental differences in the operative physical processes when using an 80 MHz train of highly focused 100 fs pulses of 800 nm radiation[10,11] versus single 500 ps pulses of 532 nm radiation[11–13] used in our study. These differences are comprehensively elucidated in the Supplementary Information, rebutting the assertion made by He *et al.* that a train of femtosecond laser pulses can mimic the mechanical stimulation provided by the μtsunami initiated by a single 500 ps laser pulse. In further support of our contention that μT-ICS is mechanical in nature, we examined the potential 'dose response' relationship between μT-ICS probability and shear stress impulse (SSI) in a manner similar to our previous work, which established such a relationship between SSI and cell fate[13,14]. To test for a similar relationship between μT-ICS probability and SSI we examined the μT-ICS using pulse energies of 2–9 μJ, corresponding to maximum cavitation bubble diameters of 140–270 μm. Figure 1a shows the μT-ICS probability and demonstrates that the spatial extent of μT-ICS (from ~200 μm to ~500 μm) is dependent on the laser pulse energy, which determines the spatial distribution of SSI. Importantly, when consolidating the data of μT-ICS probability

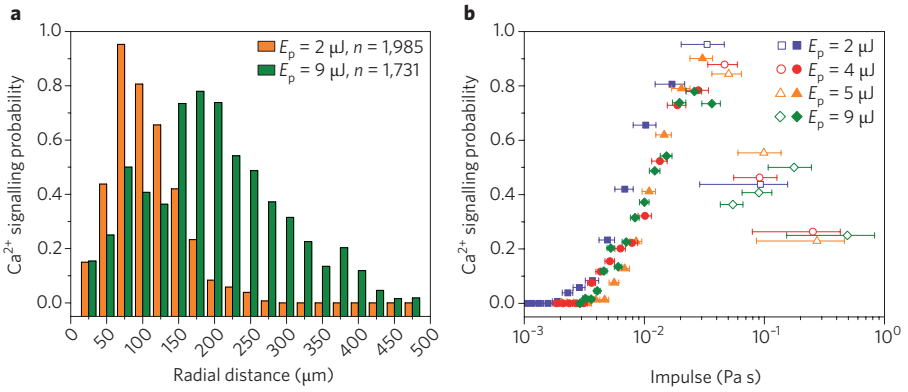

Figure 1 | Mechanical dose-response of μT-ICS. **a**, Probability of μT-ICS versus radial location for laser pulse energies of 2 μJ and 9 μJ showing tunability of the spatial extent of Ca^{2+} signalling. **b**, Signalling probability versus impulse for laser pulse energies ranging from 2–9 μJ. Closed symbols refer to cell locations outside the μCB radius and open symbols refer to regions within the maximum radius of the μCB.

versus shear stress impulse obtained from four different pulse energies, we discover a single 'universal curve' indicating a one-to-one relationship between these two parameters (Fig. 1b). This provides additional evidence that μT-ICS is produced through cellular exposure to SSI. Lastly, we disagree with He *et al.* that slow dynamics of the μT-ICS wave (~5 μm s^{-1}) relative to the impulsive dynamics of the μtsunami makes mechanical stimulation of signalling improbable. The difference in timescale is irrelevant as the kinetics of μT-ICS is dominated by the kinetics of IP_3 generation, molecular transport and signalling within the cell, well downstream from the impulsive shear stress.

Collectively, our new data and analysis supports the hypothesis that μtsunami SSI stimulates the activation of SS-GPCRs that acts along the GPCR–PLC–IP_3 pathway to govern the probability of Ca^{2+} signalling for any given cell in a HUVEC culture. ❐

References

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Justin C. Luo[1,2], Elliot L. Botvinick[1,2,3]* and Vasan Venugopalan[1,2,4]*

[1]Department of Biomedical Engineering, University of California, Irvine, Irvine, California 92697, USA. [2]Laser Microbeam and Medical Program, Beckman Laser Institute, University of California, Irvine, Irvine, California 92697, USA. [3]Edwards Lifesciences Center for Advanced Cardiovascular Technology, University of California, Irvine, Irvine, California 92697, USA. [4]Department of Chemical Engineering and Materials Science, University of California, Irvine, Irvine, California 92697, USA.

*e-mail: ebotvini@uci.edu; vvenugop@uci.edu