Human endothelial cells size-select their secretory granules for exocytosis to modulate their functional output

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Essentials:

- The secretory granules of endothelial cells release cargo to support multiple processes.
- Each cell contains granules in a range of sizes, correlating with haemostatic activity.
- Some agonists recruit an actin ring to induce exocytosis of the longest granules.
- Such exocytic selectivity provides a novel layer of control over endothelial output.

Abstract:

Background. The secretory granules of endothelial cells, Weibel-Palade bodies, are released in response to numerous extracellular signals. Their cargo is critical to many vascular functions including haemostasis and inflammation. This presents a fundamental problem; how can these cells initiate tailor-made responses from the release of a single type of organelle, each with similar cargo? Each cell contains Weibel-Palade bodies in a wide range of sizes, and we have shown that experimentally-shortening these organelles disproportionately reduces their ability to initiate haemostasis *in vitro*, leaving leukocyte recruitment unaffected. Could the production of this range of sizes underpin differential responses?

Objectives. To determine whether different agonists drive the exocytosis of different sizes of Weibel-Palade bodies.

Methods. We used a high-throughput automated unbiased imaging workflow to analyse the sizes of Weibel-Palade bodies within HUVECs before and after agonist activation to determine changes in organelle size distributions.

Results. We found that a subset of agonists differentially evoke the release of the longest, most pro-haemostatic organelles. Inhibiting the release of these longest organelles by just 15% gives a fall of 60% in an assay of secreted VWF function.

Conclusions. The size-selection of granules for exocytosis represents a novel layer of control, allowing endothelial cells to provide diverse responses to different signals via the release of a single type of organelle.

Keywords: Endothelial Cells/Exocytosis /Organelle Size /von Willebrand Factor/Weibel-Palade Bodies

Introduction:

The release of secretory granules controls physiologically-critical processes, thus regulation of secretory responses is essential. However, how this is managed in cells where exocytosis provides diverse responses is not fully understood. Processes regulated via release of endothelial secretory granules, Weibel-Palade bodies (WPBs), are numerous and diverse [1–3]: the endothelium responds to about 30 agonists that communicate the need to repair damage by initiating haemostasis, respond to invading pathogens by eliciting an inflammatory response, as well as regulating angiogenesis and vascular tonicity [2,4]. Such a range of functions indicates a likely need to provide tailored responses to different agonists, but how is this achieved? One layer of control is at exocytosis; the magnitude of response can be regulated via the number of exocytic events, the mode of fusion - e.g. kiss and run or full fusion of single or multiple granules - and in some systems by differential packaging (and exocytosis) of secretory cargo [5]. However, these forms of regulation may still be insufficient in more complicated scenarios and may not be universally available. In the case of the endothelium,

modulating exocytic magnitude and mode of fusion cannot easily generate appropriate outputs to support the demonstrably wide range of functions these cells are capable of controlling.

A feature of WPBs is that their length (i.e. size) is non-normally distributed and ranges ten-fold in steps from 0.5 to over 5µm. This is determined by the extent of linear co-packaging of pre-sized, half-micron-long quanta of their major cargo protein, von Willebrand Factor (vWF), which drives the formation of these organelles [6]. WPB size is set at the *trans*-Golgi and endothelial cells have considerable influence over this feature; their size is controlled by the size of Golgi cisternae that determines vWF quantal size, by the level of expression of vWF that controls the number of quanta and thus the probability of quanta co-packaging, and by the degree of linkage of the Golgi ribbon; unlinking the Golgi into separate mini-stacks precludes co-packaging, giving shortened, 'mini' WPBs [6]. Finally, we recently reported that the number of quanta at the *trans*-Golgi are also controlled by environmental cues, acting via AMP-activated protein kinase (AMPK), that control flux through the secretory pathway thus linking physiological signals to WPB size [7].

Initiating haemostasis depends on plasma-membrane-anchored vWF strings acting as mechanosensitive binding platforms for the recruitment of platelets and plasma vWF [8,9]. Importantly, the effect of changes in length of WPBs on the function of vWF is amplified as compared to other cargoes [6,8]. Cells with fewer long WPBs exhibit disproportionately diminished length and number of VWF strings. Interestingly, the substantial fall in pro-haemostatic capacity does not simply reflect a reduction in total release of vWF (this is not changed or can even be increased) and is rather a failure in string assembly.

We, and others, have previously found that an actin ring is recruited at a subset of exocytic events where it is required for the efficient release of vWF [10–12]. Acute inhibition of actin or PKC can block ring-dependent release [10,13]. Ring recruitment specifically aids the release of vWF, whilst having little effect on release of smaller molecules stored with WPBs [13]. Subsequently we found that agonists differentially recruit the ring [13]. Potentially actin ring recruitment might act as a means of fine-tuning the endothelial response, to bias it towards increased pro-haemostatic functioning.

We therefore addressed the hypothesis that endothelial cells exploit the large range of WPB lengths by size-selecting those that undergo exocytosis, to increase control over output. Our experiments show that some agonists can preferentially evoke exocytosis of longer WPBs by recruitment of an actin ring. This introduces a novel layer of control over the functioning of endothelial secretory output.

Methods:

Cell culture and nucleofection:

Human umbilical vein endothelial cells (HUVECs) were derived from pooled donors and purchased from Lonza or Promocell. HUVECs were cultured in M199 (Gibco), with 20% fetal calf serum (Biosera), 10 U/ml heparin and 30 µg/ml endothelial cell growth supplement (both from Sigma). siRNA against Luciferase (5'-CGUACGCGGAAUACUUCGA[dT][dT]-3') and vWF (5'-GGGCUCGAGUGUACCAAAA[dT][dT]-3') were purchased from Eurofins. siRNA (25pmol) was delivered via nucleofection using the program U-001 (Lonza). Cells were typically assayed 48 hours after nucleofection.

Antibodies and reagents:

Rabbit anti-vWF (cat. No. A0082) and rabbit anti-vWF-HRP which stain both processed and unprocessed forms of vWF were from DAKO (termed 'total vWF'), two anti-vWF antibodies against a neo-epitope exposed at the carboxy terminus of the propeptide upon furin cleavage [14] to visualise processed vWF (termed 'pro-vWF'), were used in this study This allowed visualisation of only that vWF packaged into WPBs (thus excluding background staining of ER-localised vWF), the first was a kind gift from Dr T Carter (St. George's University London) the second was produced by Eurogentec. Hoechst 33342 was from Life Technologies. Cytochalasin E (CCE) was purchased from Sigma, GÖ6976 was from TOCRIS. Histamine was from Enzo Life Sciences (cat. No. ALX-550-132), Phorbol 12-Myristate 13-Acetate (PMA), adrenaline and 3-isobutyl-1-metylxanthine (IBMX) were from Sigma, Thrombin was from Calbiochem.

WPB release assay:

HUVECs were grown in 96 well plates (Nunc) for two days. Confluent monolayers were washed in pre-warmed release medium (M199 with 0.2% BSA and 10mM HEPES), and where necessary incubated with or without CCE (0.5µM) or PKC inhibitor GÖ6976 (1µm) for 10 minutes. Cells were stimulated with histamine (100µM), PMA (100ng/ml), thrombin (1U/µl), adrenaline (100µM) or IBMX (10µM) either alone or in combination for 10 - 30 minutes before fixation in 4% paraformaldehyde (PFA). All incubations prior to fixation were carried out at 37°C. Cells were permeabilised in 0.2% Triton X-100, blocked in 5% bovine serum albumin for 10 minutes each. Staining for pro-vWF was done using anti vWF antibody followed by anti-rabbit antibody conjugated to Alexa Fluor 488nm and the nucleus with Hoechst 33342.

Exocytic site labelling assay:

Exocytic sites were labelled as described [13] using a modified method from Knop and Gerke [15]. Briefly, HUVECs were grown in 96 well plates and when confluent, incubated overnight in either DMSO, acetate pH 6.4 medium or nocodazole (1µg/ml). Cells were prepared as for WPB release assay before incubation in the presence of anti-vWF (DAKO) in the presence or absence of agonist for 10 minutes. Cells were fixed as described above. vWF was visualised by incubation with anti-rabbit antibody conjugated to Alexa Fluor 488nm.

High throughput image acquisition and segmentation:

The Opera high-content screening confocal microscope (PerkinElmer) was used to acquire images from cells grown on 96 well plates stored in PBS using a 40x air objective (NA 0.6). Imaging was carried out at room temperature. Nine fields of view were acquired per well and at least eight wells per condition used. Images were converted from flex to 16 bit tiff and analysed using a custom-designed program using version 2.7 of the Python programming language with the scikit-image library. For segmentation of exocytic sites image noise was reduced with a Gaussian blurring with a sigma value not impacting the image resolution. A binary mask was created using a threshold value obtained from Moment-preserving thresholding [16]. Adjacent exit sites were split using the marker-based watershed flooding algorithm. For segmentation of WPBs a local adaptive threshold was applied [6]. Segmented objects beneath the resolution limit of the optical system were removed and morphometric measurements taken. The automated segmentation was validated by comparison to a gold standard set of nine images annotated manually. Data analyses were performed in RStudio version 1.1.423.

Secretion assay and ELISA:

HUVECs manipulated to alter the population of small WPBs by being grown overnight in DMSO, acetate pH 6.4 medium or nocodazole (1μg/ml) [6] or by nucleofection of siRNAs against either Luciferase or vWF were washed in release medium as for WPB release assays. Cells were then stimulated, in the presence or absence of CCE (0.5μM), with either histamine, histamine in combination with adrenaline and IBMX or PMA and media recovered. vWF levels in releasate and lysate was determined by ELISA. 96 well plates (Thermo Fisher Scientific) were coated overnight with anti-vWF (total vWF, DAKO) diluted in PBS. Plates were blocked with 1xTEB (1% Triton x-100, 0.2% fish skin gelatin, 1mM EDTA in PBS) for one hour at room temperature. Samples (loaded in duplicate) were incubated for one hour at room temperature in 1xTEB. Following three washes in 1xTEB plates were incubated with anti-vWF antibody conjugated to HRP. HRP activity was visualised using *o*-phenylenediamine and read using a Molecular devices plate reader (Versa Max) over 30 minutes at 425nm.

vWF plasma recruitment assay:

HUVECs grown to confluence onto μ-slides (Ibidi, Munich, Germany) with a 5mm-wide channel were maintained at 37°C and slides attached to a syringe pump (Harvard Apparatus, Holliston, MA, USA) to draw fluid over cells at a constant wall shear stress of 0.25MPa (2.5 dynes/cm^2). Cells were rinsed with Hanks balanced salt solution (HBSS, Life Technologies containing Ca^{2+}, Mg^{2+} and 0.2% BSA) for 2 minutes before being stimulated with histamine in combination with adrenaline and IBMX in the presence or absence of CCE (0.25μm) for five minutes in HBSS followed by normal human pooled plasma for a further five minutes. Cells were fixed with 4% PFA under decreasing rates of flow and subsequently stained for surface-bound vWF with rabbit anti-vWF (DAKO) followed by Alexa 488-conjugated secondary antibody. The nucleus was stained with Hoechst. Samples were imaged by confocal microscopy (Leica TCS SPE) using a 40x (NA 1.15) oil objective. Maximum intensity projections were generated in Fiji and the total area of vWF staining quantified.

Statistical Analysis:

Statistical analyses were performed in GraphPad Prism version 7. Statistical significance was assessed using Student's T-test for two sample datasets. Where more than two samples were compared statistical significance was assessed using one- or two- way ANOVA followed by Dunnet's or Sidak's multiple comparison tests respectively. All tests were two-tailed.

Results:

Some agonists evoke the release of large WPBs

Do endothelial agonists [2–4] cause the release of differently sized WPBs? To test this we measured the lengths of thousands of organelles remaining within human umbilical vein endothelial cells (HUVECs) after activation, using an unbiased, high-throughput imaging approach [6]. WPBs were identified by staining for their main constituent, the processed form of vWF (pro-vWF), and automatically segmented (Figure 1A) to search for a change in the length distribution of WPBs after exocytosis. All agonists cause a decrease in the number of WPBs per cell; if a random selection of organelles is released, the length distribution of those remaining will not differ from controls. If even some size selection occurred, we will find a differential loss of smaller, or larger, organelles (cartooned in Figure 1B). Shortening the population of WPBs to cause a loss of ~40% of WPBs longer than 2μm is sufficient to cause a catastrophic fall in haemostatic function of the released vWF [8], highlighting the importance of any differential release.

HUVECs were stimulated with a range of agonists for 10 (Figure 1C) or 30 minutes (Figure 1D). We found that by 10 minutes, both Phorbol 12-myristate 13-acetate (PMA), and histamine combined with adrenaline and 3-isobutyl-1-methylxanthine (IBMX) consistently resulted in the differential release of long WPBs, reflected by a fall in the proportion of longer organelles remaining (Figure 1Cii). After 30 minutes stimulation histamine alone as well as thrombin/adrenaline/IBMX also resulted in release of long WPBs. PMA and Histamine/adrenaline/IBMX each stimulated release of a similar number of organelles but markedly differed in their propensity to release long organelles, thus the loss of many organelles is not itself sufficient to drive size selection (Figure 1Dii). We also titrated the concentration of PMA, histamine/adrenaline/IBMX and histamine and measured the number of exocytic sites (Supplementary Fig. 1A) and the lengths of the WPBs remaining (Supp. Fig. 1B) and find that agonist responses are saturated at the concentrations used in Figs 1C-D and that even at higher concentrations histamine still does not produce a significant selective response following 10 minutes stimulation.

How do different agonists produce such a selective response? WPBs are known to utilise several modes of exocytosis, including kiss and run, where only small molecules are released (excluding vWF) and full fusion, cumulative or compound exocytosis, in which all cargos can be released [17–20]. A significant proportion of WPBs undergoing full fusion also recruit an actomyosin ring, likely providing force to aid extrusion of the large (20,000+ kDa) vWF concatamers [10,11] that assemble within WPBs into multi-concatameric assemblies that combine into strings after exocytosis. We recently found that agonists differentially recruit this ring, the most efficient of which are histamine/adrenaline/IBMX and PMA, whilst thrombin does not utilise an actin ring to any significant extent [13]. We therefore hypothesised that ring recruitment may promote release of long WPBs.

An actin ring is required for the exocytosis of long WPBs

Is the actin ring needed for release of longer WPBs? We tested this hypothesis using the PKC inhibitor GÖ6976 as this has been previously found to perturb actin ring recruitment downstream of PMA stimulation [13]. Incubation with the inhibitor does not significantly affect the number of WPB/cell in the absence or presence of stimulation (Figure 2Ai). However, after activation with PMA for 10 minutes, the presence of GÖ6976, prevents the release of longer WPB, since the proportion of long WPBs remaining post-stimulation does not differ from unstimulated cells (Fig 2Aii). Interestingly, GÖ6976 does not block the release of long WPBs when cells are stimulated with histamine alone (Sup. Fig. 2B), or in combination with adrenaline and IBMX (Sup. Fig. 2A), suggesting that while the recruitment of an actin ring confers the ability to select long WPBs for exocytosis, that recruitment may operate via both PKC-dependent and independent mechanisms.

Cells with smaller WPBs exhibit reduced actin ring usage

Our data show that firstly, organelle size selection does occur in endothelial cells, and is thus likely an important mechanism by which cells can modulate the haemostatic response, and secondly that longer WPBs may not be released if ring assembly is perturbed. WPB size is modulated in response to environmental cues via AMPK [7] and changes in vWF expression [8], which is affected by vascular-bed-specific environmental cues [21]. Is the ring still used if WPBs are all small? We investigated the use of the ring in cells with mini-WPBs; generating shortened organelles by unlinking Golgi mini-stacks via treatment with nocodazole (to depolymerise microtubules), or acetate-containing medium at pH 6.4 (to lower the pH in the cytosol) (Figure 2B, 2C, Supp Fig 3) [6,8]. To independently confirm the

relationship of WPB size and ring usage, we used an assay developed to measure the area of WPB exocytic events [13,15], that shows larger exocytic sites correlating with actin ring use [13]. This confirmed that both shortening treatments reduced the number of large exocytic sites in response to all agonists (Figure 2Ci). Interestingly, this is coincident with pH 6.4 treatment increasing overall exocytic activity (Figure 2Cii) [8].

We also tested the effect of short WPBs on vWF secretion. We noted that GÖ6976 and CCE (which is less selective but also inhibits ring formation) both inhibit the release of vWF in PMA-stimulated cells [10,13]. If this inhibition reflects loss of actin ring function, and the ring is indeed required for the release of long WPBs then exocytosis in cells with shorter WPBs should be less affected by these treatments (Figure 2D). Cells grown for 24 hours in medium at pH 6.4 containing acetate to generate 'mini-WPBs' (the proportion of vWF area covered by long WPBs (length >2µm) was reduced by approximately half), were stimulated with different agonists in the presence or absence of GÖ6976 (Figure 2Ei, Supp. Figure 4A) or CCE (Figure 2Eii, Supp. Figure 4B) and the amount of vWF released measured. We calculated the efficacy of the two inhibitors by dividing the amount of vWF released from inhibitor-treated cells by the amount released in the corresponding untreated cells to give a fold inhibition (a value of less than 1 indicates less vWF was released from cells treated with inhibitor than controls. A value of 1 indicates the inhibitor had no effect on release, whilst greater than 1 indicates that more was released from cells incubated with inhibitor than control cells).

Results confirmed the prediction that the degree to which GÖ6976 and CCE inhibit release in control cells strongly correlates with actin ring recruitment and a propensity to release long WPBs. PMA, the agonist most associated with exocytosis of long WPBs and with ring recruitment, is most inhibited by both drugs; vWF release falling by approximately 50% in control cells (Figure 2Ei and 2Eii, grey bars and Supp. Figure 4), whereas, histamine, the least associated with exocytosis of long WPBs, is not inhibited by either drug. Histamine/adrenaline/IBMX, in line with previous data (Supp. Figure 2A), is not inhibited by GÖ6976, but release is reduced to about 30% of control cells upon CCE treatment. This is likely because histamine activation may not require PKC (Lorenzi 2008). We thus find that in cells with mini-WPBs, blocking the actions of the actin ring has a minimal effect on vWF release. Histamine/adrenaline/IBMX-stimulated cells with mini-WPBs are no longer inhibited by CCE treatment, whilst PMA-stimulated cells with mini-WPBs are significantly less affected by either inhibitor treatments than control cells (vWF release is inhibited 50% in control cells and only ~25% in cells with mini-WPBs for both drugs) (Figure 2E). Indeed, in all cases cells with mini-WPBs show no significant change in the amount of vWF released compared to control untreated cells (Sup. Figure 4). These data are entirely consistent with our conclusion that the actin ring is specifically required for the release of long WPBs.

VWF expression varies between vascular locations *in vivo* [22]. To model the effects of changes in vWF levels we titrated vWF siRNA to give a 60% depletion in vWF protein levels (Figure 3A), reducing the fraction of long WPBs by 25% (Figure 3B) [8]. We then measured the amount of vWF released following stimulation with PMA, histamine/adrenaline/IBMX and histamine in the presence or absence of CCE (Figure 3C). No change was seen in the total amount of vWF released following agonist stimulation in control cells suggesting that whilst there is a bias towards long WPBs being released following PMA and histamine/adrenaline/IBMX stimulation, if these are not available shorter WPBs will be released. Again, we find that even a small change in WPB size makes PMA and histamine/adrenaline/IBMX-

stimulated cells less sensitive to CCE treatment, further indicating the use of the actin ring is most necessary for release of long WPBs.

Failure to release long WPBs inhibits haemostatic functioning

What are the functional consequences of reducing the release of the longest WPBs? We have previously shown that cells with small WPBs produce disproportionately fewer and shorter strings and recruit less plasma vWF and platelets (both necessary for haemostasis) [6,8]. We therefore hypothesised that inhibiting the release of long WPBs, which numerically make up only a small proportion of exocytic events following stimulation, would also have a disproportionate effect on function. We previously found that using an acute low dose of CCE to inhibit actin ring formation in activated cells under flow results in the production of significantly shorter vWF strings [13]. We now stimulated cells with histamine/adrenaline/IBMX under flow in the presence or absence of CCE and assessed the ability of agonist-activated cells to recruit plasma vWF (Figure Di). A highly significant reduction in the recruitment of plasma vWF occurs when CCE is used to inhibit the actin ring to inhibit the release of long WPBs (Figure 3D). Thus loss of the differentially increased release of longer WPBs at exocytosis by some agonists, even to a relatively modest extent (histamine/adrenaline/IBMX results in around a ~15% fall in long WPBs compared to unstimulated cells (Figure 1Dii)) has a significant impact on haemostatic function: a 60% fall in the recruitment of plasma VWF to cells.

Discussion:

This investigation into exocytosis of WPBs in primary human endothelial cells reveals that some agonists evoke the selective release of content from larger WPBs (summarised in supplementary table 1), providing a novel layer of secretory control. Automated high-throughput imaging of thousands of organelles (>150,000 per condition; distributed between 16 units of analysis) combined with biochemical measurements of endothelial secretion shows that WPB size influences both likelihood and mode of exocytosis in response to different agonists. We have also recently demonstrated cellular control of WPB size in response to environmental cues, co-ordinated by AMPK [20]: coupling cellular machineries that control organelle size to those supporting a range of exocytic modes allows for highly-differentiated functional responses to be generated by endothelial cells from the release of a single type of organelle (Figure 4).

Endothelial agonists can broadly be divided into two classes, those that act by raising intracellular levels of calcium and those using cyclic AMP (cAMP). However, how approximately 30 different agonists can facilitate different responses following endothelial activation is not clear. Whilst it has been proposed that cAMP-agonists, by only targeting peripheral organelles [23], can provide differential responses, it is unlikely that this would provide such an output since all WPBs are packed with similar sets of cargo (noting that the content of newly forming WPBs in any cell will be simultaneously affected by changes in expression of certain cargos, e.g. IL-1β triggering upregulation of IL-8 (Utgaard 1998, Wolff 1998) or Interleukin-4 and oncostatin M upregulating P-selectin (Yao 1996)). Our new findings show how a range of agonists can indeed provide differentiated responses but through exploiting the functional differentiation generated by a range of granule sizes, rather than as previously suggested, by different contents for individual WPBs [24].

Several cell types produce secretory granules of different sizes – albeit none on anything like the ten-fold scale of difference seen in the endothelium. These include the dense-core granules of neuroendocrine cells, and secretory granules of mast and pancreatic acinar cells amongst others [25–28]. It is therefore possible that other secretory cells also exploit size differences to modulate output. While difficulty in measuring the size of spherical organelles makes investigation of this question technically challenging in most other cell types, a link between granule size and exocytic mode has been demonstrated in PC12 neuroendocrine cells. Here, isoforms of the calcium sensor synaptotagmin localise to differently-sized granules which in turn use different modes of exocytosis [29]. Further, a preference for full fusion over kiss-and-run was also seen in larger vesicles from lactotrophs stimulated with sphingosine [30]. Our data, from a much more explicitly size-driven system, complement these examples and suggest that using granule size to regulate secretory function may be widespread. However, since for most cargos, the functional effect of size selection will only be proportional to the amount released, and the range of sizes is much smaller, the functional consequences of this phenomenon may not be so significant. Further, size selection can only arise where a fraction of granules are used at activation; where all organelles are simultaneously used (such as in extreme compound exocytosis [31]), this strategy would be moot.

How granule size is sensed for selection is unclear. Studies have demonstrated that differential sensitivity to calcium can be used to release distinct sub-populations of granules [32]. This, in principle could operate by differential localisation of fusion machinery to sub-groups of granules. However, while differential localisation of SNAREs [33] potentially provides a mechanism for differential exocytosis, this does not solve the problem of how SNAREs are differentially delivered to organelles distinguished only by their size. Whilst WPB length is highly variable, the width of these organelles is remarkably uniform, thus, features such as membrane curvature are unlikely to act as differentiators between differently-sized WPBs. Furthermore, WPBs fuse with the plasma membrane at their tips which would also preclude the possibility that an increased number of SNARE molecules are present on longer organelles.

In vivo the likelihood that endothelial activation results from a single agonist is highly unlikely. For example adrenaline is always present in plasma [34]. Thus, our findings of differences in vWF release between single and combined agonists are likely important in understanding how endothelial activation occurs in a physiological setting. Whilst PMA itself is not a physiological agent, the similar results we find with histamine/adrenaline/IBMX suggest that PMA can still be a useful tool to identify underlying mechanism. Although it has been shown that agonists may act synergistically to promote the release of vWF [13,35], most work has examined the effects of a few agonists in isolation, such that downstream signalling pathways activated by multiple agonists are largely unexplored. Given that *in vivo*, endothelial activation resulting from a single agonist is unlikely, this is surprising. Here we show that addition of adrenaline and IBMX (which stimulate cAMP production) to either histamine or thrombin (calcium-raising agents) promotes release of longer WPBs above either alone. Variations in the levels of agonists such as raised adrenaline in a 'fight or flight' response could thus amplify an individual's response to an injury causing endothelial activation. Conversely, inhibiting recruitment of the actin ring should specifically inhibit the release of the most thrombotic subset of organelles: patients undergoing vascular surgery, or suffering from other thrombosis-related disorders might benefit from prophylactic use of such therapies. In another physiological context, vWF plays a role in cancer metastasis [36–38] where increased levels of vWF is associated with poor prognoses in acute

myeloid leukaemia and distant metastases in lung cancer patients [39,40]. Altogether, maximum flexibility in regulating vWF release could prove therapeutically beneficial in numerous conditions.

In summary, we have identified a new layer of regulation that may allow endothelial cells to maximise the range of functional outputs that can occur following activation. The resulting highly tuneable system can not only respond to a variety of cues, but also offers several checkpoints that could potentially reduce the likelihood of aberrant activation occurring. We have outlined a system in which granule size, exocytic mode and agonist-specific targeting have been shown to be coupled together to influence measured functional output. WPB size is becoming increasingly apparent as a key player in modulating endothelial output and this work provides another part of the mechanism by which this arises.

Author contributions

Jessica J. McCormack; Conceptualization, Data Analysis, Investigation, Methodology, Writing original draft, reviewing and editing. Kimberly Harrison-Lavoie; Investigation, Data Analysis. Daniel F. Cutler; Conceptualization, Funding acquisition, Project administration, Supervision, Writing original draft, reviewing and editing.

Disclosure of conflict of interest.

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Figure legends

Figure 1: Agonists can select sub-populations of Weibel-Palade bodies based on organelle size.

(A, C-D) HUVECs were grown in 96 well plates and either unstimulated or stimulated with different agonists (PMA, Histamine (Hist), Thrombin, adrenaline (Ad), IBMX either alone or in combination as indicated, for 10 (C) or 30 (D) minutes before being fixed and stained for pro-vWF and the nucleus with Hoechst (A). Up to 144 images from 16 wells were acquired per condition at 40x magnification and WPBs segmented using a custom-designed programme (Segmentation). Scale bar is 25μm. (B) Cartoon illustrating assay used to compare the effects of different agonists on WPB length distributions. Cells contain a population of WPBs of different lengths (long WPBs over 2μm in length are in grey and WPBs shorter than 2μm in yellow) which can be represented as in example histograms. Upon agonist stimulation WPBs will be lost from cells. If a random selection of organelles is released the length distribution will not change (left cell). The selection of longer (middle cell) or shorter (right cell) WPBs will result in the disproportionate loss of the longer or shorter WPBs. This can be seen in histograms (blue bars indicate the distributions following agonist overlaid with the example distribution from unstimulated cells). This can also be represented by looking specifically at the proportion of WPBs which are long, defined as those longer than 2μm (dashed red line on histograms). To compare between multiple treatments the proportion of the area covered by WPBs length greater than 2μm is calculated as a fraction of the total area covered by all WPBs. Disproportionate loss of long WPBs will result in a fall in the area covered by WPBs over 2μm and loss of many shorter WPBs will result in an increase in this value. Following either 10 (C) or 30 (D) minutes of stimulation the total number of WPBs segmented per cell (Ci, Di) and the fraction of the area covered by long WPBs (Cii, Dii) was calculated per image, and the mean of all images per well plotted (N=16 wells). Error bars are standard error of the mean (SEM). Dotted red lines are unstimulated mean. Statistical significance was assessed with one-way ANOVA with Dunnet's multiple comparison test. ** P≤0.01, *** P≤0.001, **** P≤0.0001. A representative experiment is shown from N=4-7.

Figure 2: The targeting of large WPBs is dependent on recruitment of an actin ring at exocytosis

(A) HUVECs were grown in 96 well plates and either untreated or treated with PKC inhibitor (GÖ6976) to inhibit formation of the actin ring. Subsequently cells were either unstimulated or stimulated with PMA for 30 minutes to target the release of long WPBs. Cells were fixed and stained for pro-vWF and 72 images acquired from 8 wells per condition. WPBs were segmented and the total number of WPBs segmented per cell (Ai) and the fraction of the area covered by long WPBs (length greater than 2μm) (Aii) calculated per image. The mean of all images per well is plotted with bars representing SEM. A representative experiment shown from N=3 experiments. (B-C) HUVECs were treated with either DMSO, or either pH 6.4 medium or nocodazole for 24 hours to generate mini-WPBs and either fixed and stained for vWF (B) or stimulated in the presence of anti-vWF before fixation and staining for vWF (C). Images were acquired at 40x magnification, scale bar represents 10μm. The area (Ci) or number (Cii) of exocytic sites was measured from 72 images per condition across 8 wells and the average per well plotted. (Ci) The proportion of sites greater than $2\mu m^2$ was calculated and normalised to DMSO controls for each agonist. A representative experiment is shown from N=3 experiments. Bars are SEM. (D) Schematic showing the proposed use of the actin ring by long WPBs (grey). (E) The amount of vWF released from control cells and cells incubated for 24 hours with pH 6.4 medium to generate mini-WPBs in the presence and absence of GÖ6976 (Ei) or CCE (Eii) was assessed by ELISA following stimulation with histamine, histamine/adrenaline/IBMX and PMA. The amount of vWF released from cells treated with GÖ6976 or CCE is normalised to vWF release from control cells to give the fold inhibition of each treatment (1 (dashed line) indicates the inhibitor has no effect on vWF release, <1 indicates that the inhibitor has reduced vWF release compared to control untreated cells). The mean fold inhibition was calculated from N=3-5 independent experiments. All bars represent SEM. Statistical

significance was assessed with two-way ANOVA with Sidak's multiple comparison test on N=8 wells (A, C) or N=4-5 (Ei) and N=3-4 (Eii) independent experiments. *P<0.05, ** P≤0.01, *** P≤0.001, **** P≤0.0001, ns = not significant.
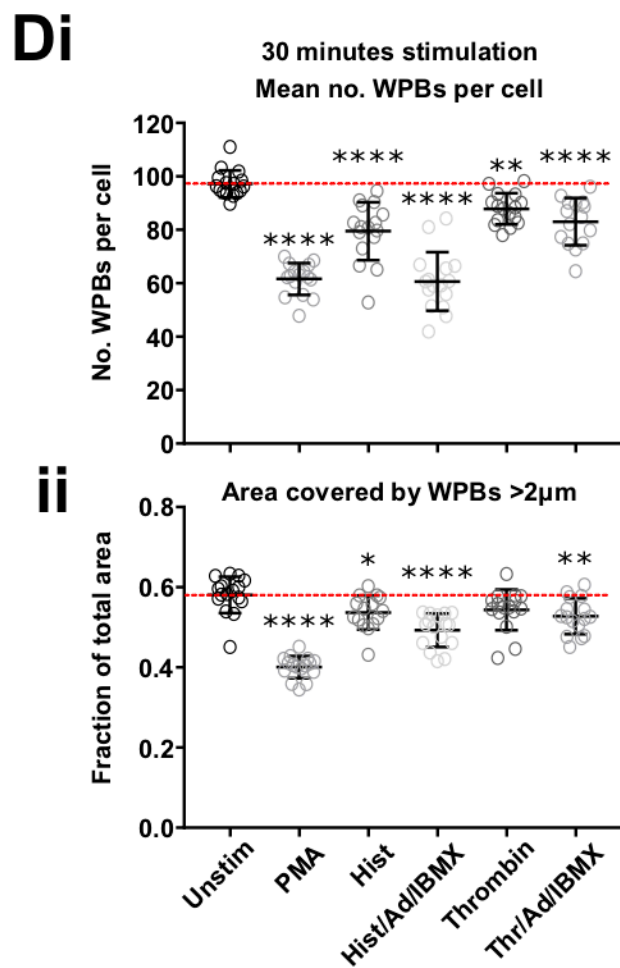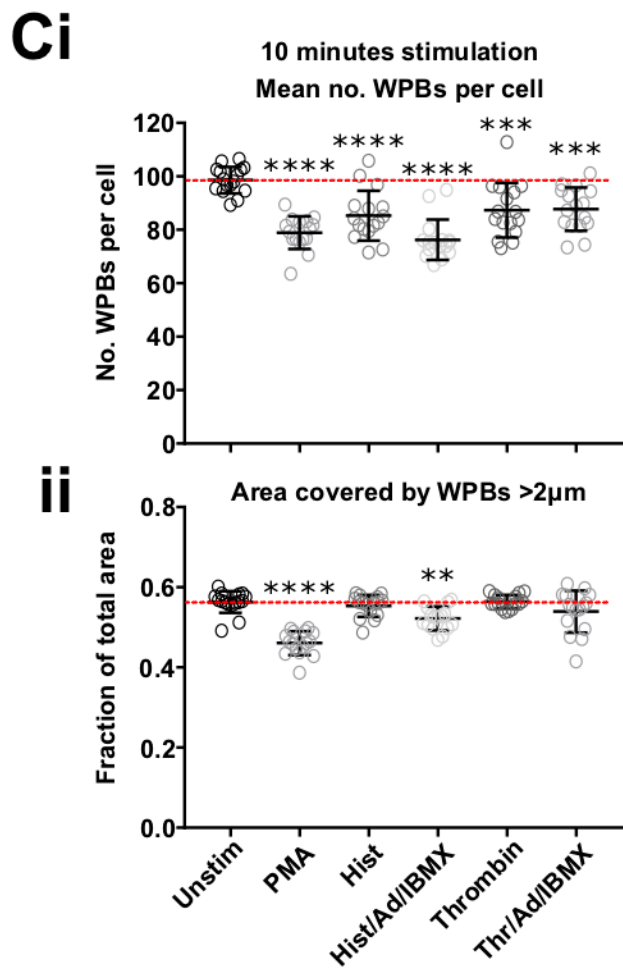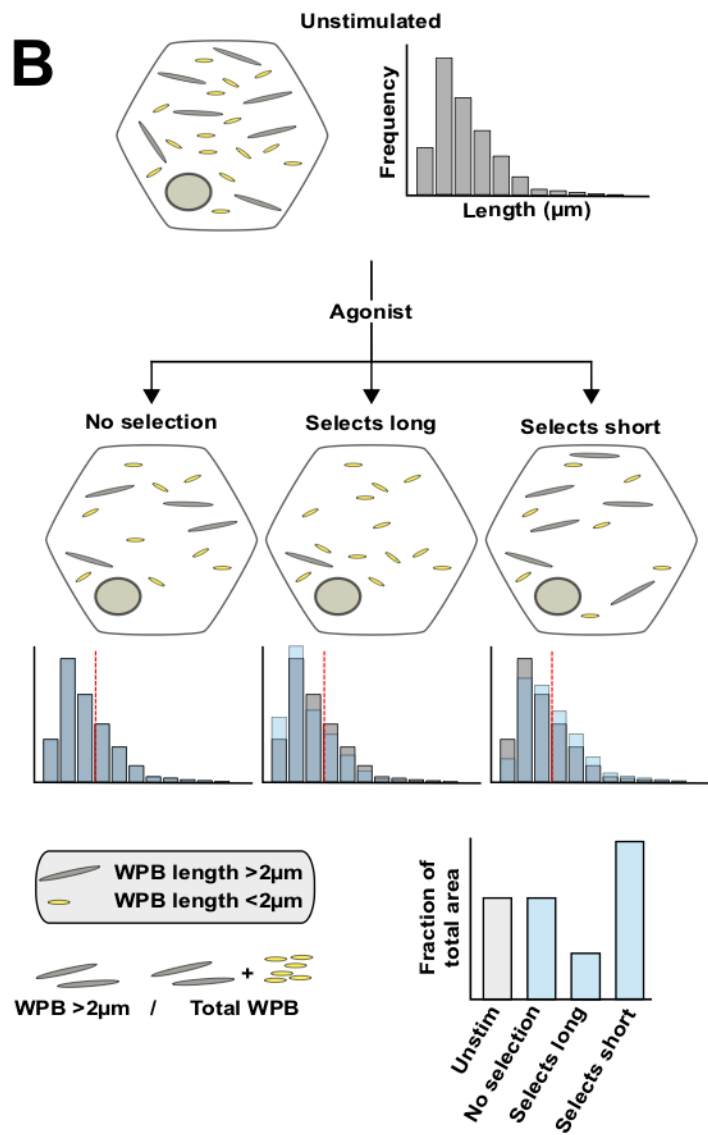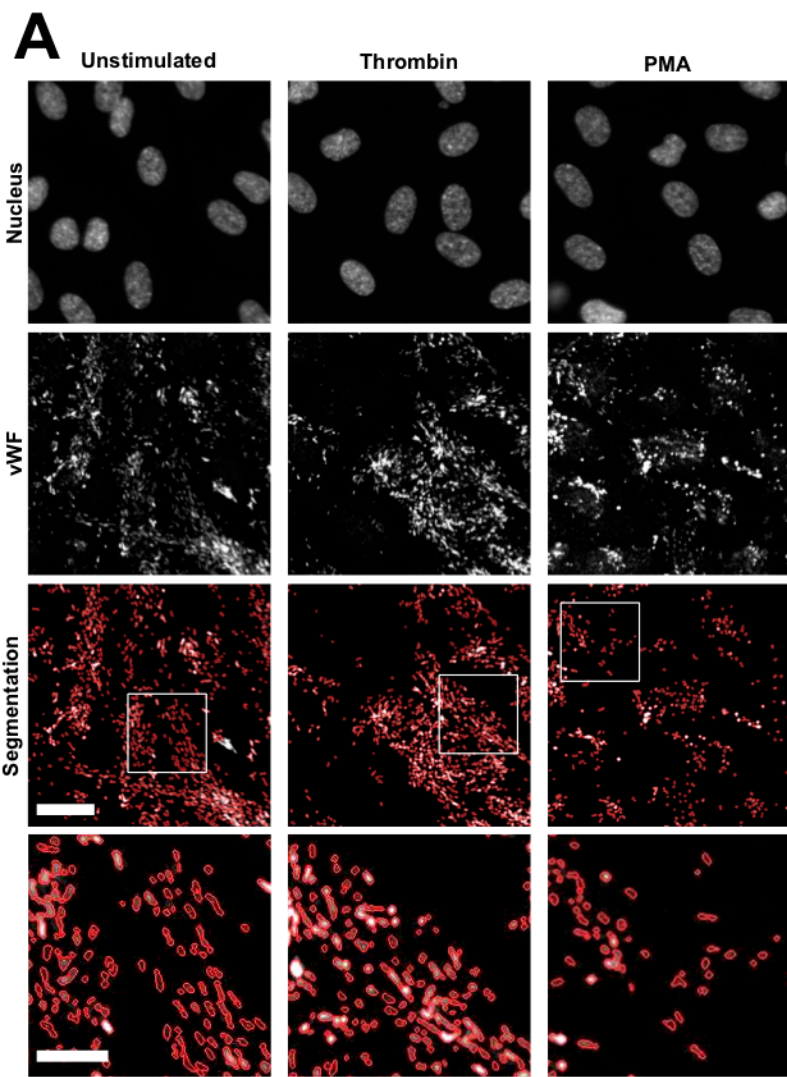
Figure 3: Modulating the WPB population alters exocytic mode

HUVECs were nucleofected with siRNA against Luciferase (Luc) or vWF. (A) The total amount of vWF was measured by ELISA from N=12 independent experiments (bars are SEM). (B) The mean area covered by WPBs with length greater than 2µm was calculated. A representative experiment is shown from N=6 independent experiments (bars are SD). (C) The mean percentage of vWF released from Luciferase and vWF-depleted cells in the presence and absence of CCE was assessed by ELISA following stimulation with PMA (Ci), histamine/adrenaline/IBMX (Cii) and histamine (Ciii) (from N=4 independent experiments). Bars represent SEM. D) HUVEC monolayers under flow were stimulated with histamine in combination with adrenaline and IBMX in the presence of human pooled plasma and in the presence or absence of CCE. (Di) Schematic of flow assay workflow. (Dii) Cells fixed and stained for external vWF. Images were acquired at 40x magnification. Scale bars represents 50µm. (Diii) Quantification of the area covered by vWF per image (N=50-100 images per experiment) and a representative experiment from N=3 independent experiments is shown. Statistical significance was assessed with Student's T-test (A-B, D) or two-way ANOVA with Sidak's multiple comparison test (C). * P<0.05, ** P≤0.01, **** P≤0.0001, ns = not significant.
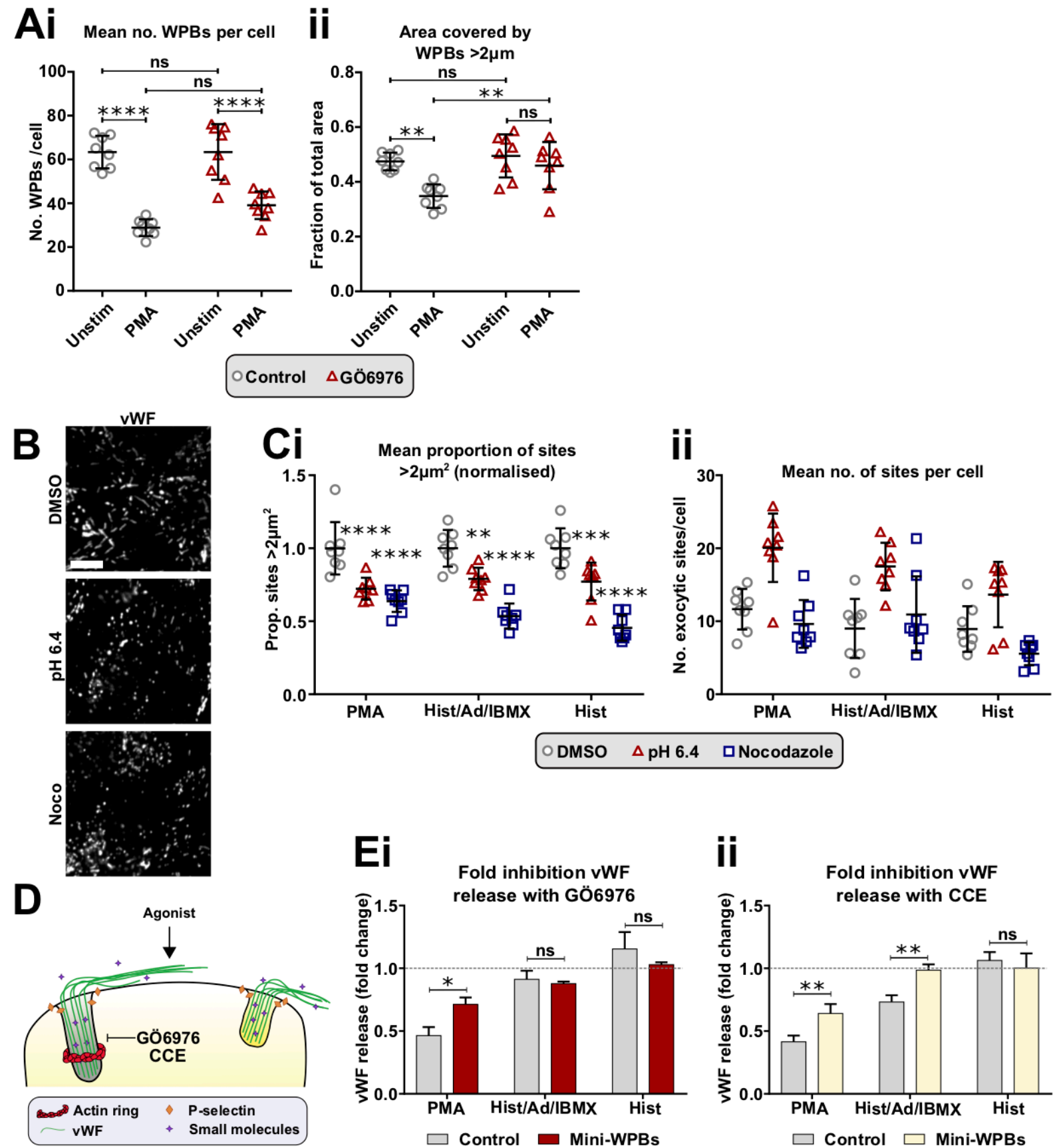
Figure 4: Internal and external control points that influence endothelial output

Schematic summarising the proposed control points that can influence the magnitude of the haemostatic response. Endothelial cells can drive the biogenesis of shorter (yellow) or larger (grey) organelles at the Golgi (blue), therefore controlling the availability of longer pro-thrombotic WPBs. External stimuli influence the response via selection of organelles based on size. Finally, the exocytic machinery selected controls the amount of vWF released. Kiss and run exocytosis results in the release of small molecules (purple stars) with vWF (green) failing to be exocytosed. This mode of exocytosis can presumably occur from WPBs of all lengths (left). Full fusion of WPBs releases small molecules, membrane proteins (orange diamonds) and vWF, but strings produced from cells with populations of shorter WPBs are fewer and shorter, whilst the strings produced from cells in which the actin ring is inhibited are shorter, (centre). The recruitment of an actin ring to the largest WPBs confers the most pro-thrombotic response (right).
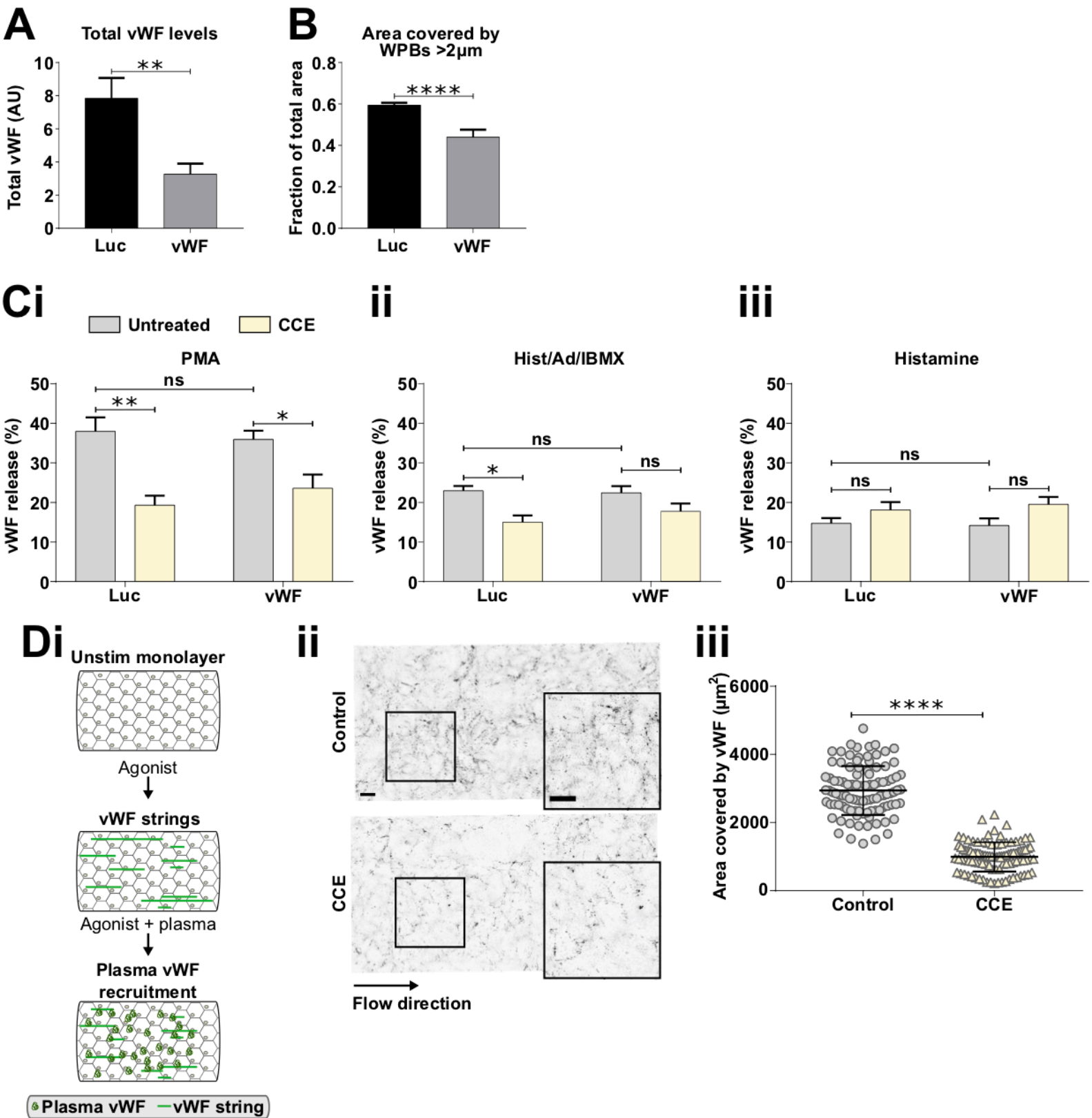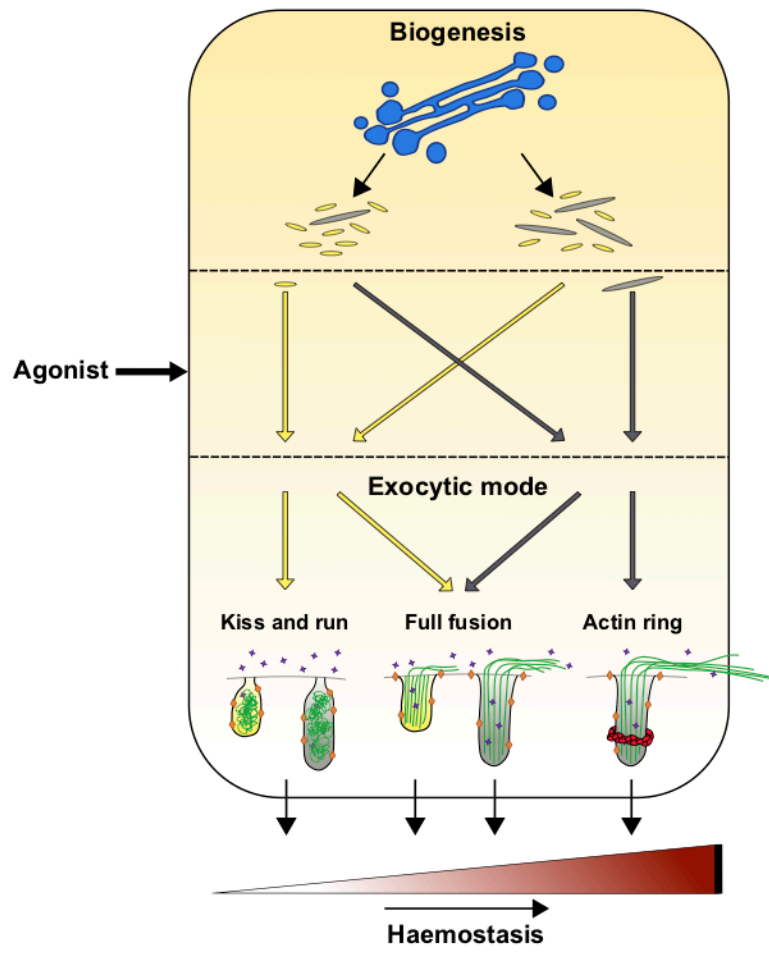
Figure 1

Figure 2

Ai Mean no. WPBs per cell

ii Area covered by WPBs >2µm

B vWF

Ci Mean proportion of sites >2µm² (normalised)

ii Mean no. of sites per cell

D Agonist

GÖ6976 CCE

Actin ring — P-selectin
vWF — Small molecules

Ei Fold inhibition vWF release with GÖ6976

ii Fold inhibition vWF release with CCE

Figure 3

A Total vWF levels

B Area covered by WPBs >2μm

Ci PMA

ii Hist/Ad/IBMX

iii Histamine

Untreated / CCE

Di Unstim monolayer → Agonist → vWF strings → Agonist + plasma → Plasma vWF recruitment

Plasma vWF — vWF string

ii Control / CCE / Flow direction

iii Area covered by vWF (μm²) / Control / CCE

Figure 4

Supplementary Figure 1:

HUVECs were grown in 96 well plates and stimulated with increasing concentrations of agonists (units are µM for histamine and histamine/adrenaline/IBMX and in ng/ml for PMA) as indicated in either the presence of anti-vWF to visualise exocytic events (Ai) or fixed and subsequently stained for pro-vWF to visualise WPBs remaining in cells (Aii). Ai) The number of exocytic events per image was quantified and the mean number of sites per cell per well plotted with bars representing SEM (N=8 wells, a representative experiment shown from N=3 independent experiments). Aii) The fraction of the area covered by long WPBs (length greater than 2µm) was calculated per image and the mean fraction of the area per well plotted from N=8 wells with bars representing SEM. A representative experiment is shown from N=3 experiments. Statistical significance was assessed using ANOVA with Dunnet's multiple comparison test. * P<0.05, ** P≤0.01, **** P≤0.0001.

Supplementary Figure 2:

HUVECs were grown in 96 well plates and either untreated or treated with the PKC inhibitor GÖ6976 to inhibit formation of the actin ring. Subsequently cells were either unstimulated or stimulated with histamine/adrenaline/IBMX (A) or histamine alone (B) for 30 minutes. Cells were fixed and stained for vWF and 72 images acquired from 8 wells per condition. WPBs were segmented and the total number of WPBs segmented per cell (Ai, Bi) and the fraction of the area covered by long WPBs (length greater than 2µm) (Aii, Bii) calculated per image. The mean of all images per well is plotted with bars representing SEM. A representative experiment shown from N=3-4 experiments. Statistical significance was assessed with two-way ANOVA with Sidak's multiple comparison test on N=8 wells (Ai-ii, Bi-ii). ** P≤0.01, *** P≤0.001, **** P≤0.0001, ns = not significant.
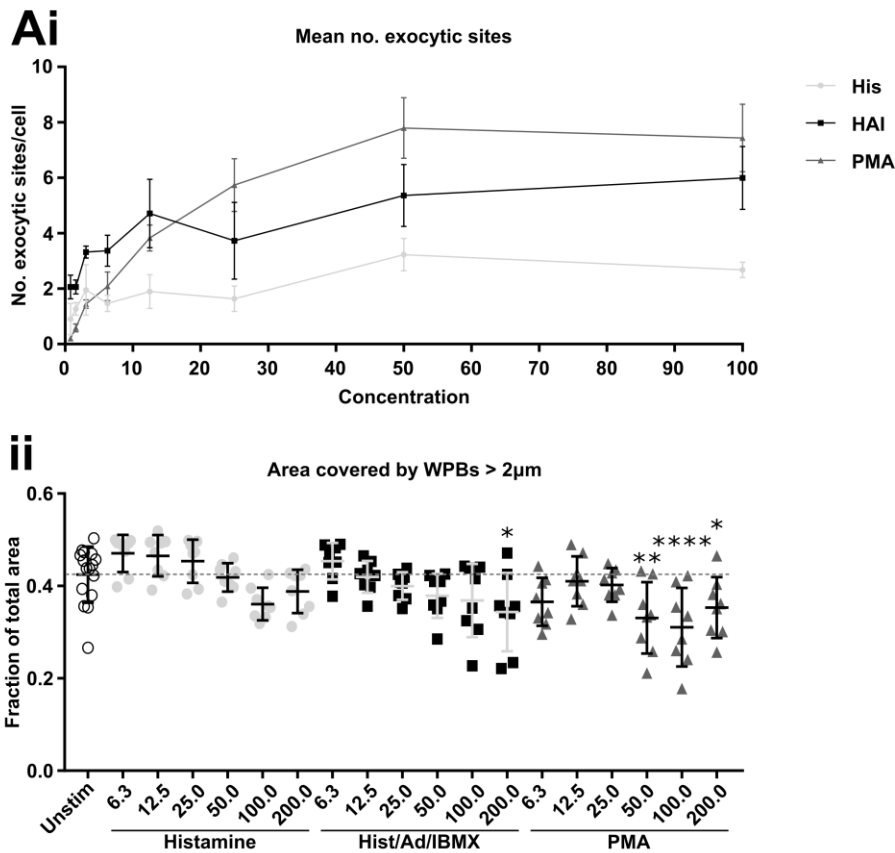
Supplementary Figure 3:

HUVECs grown in 96 well plates were incubated with either DMSO, nocodazole (Noco) or pH 6.4 media overnight. Cells were fixed and stained for pro-vWF and 72 images acquired across 8 wells per condition. WPBs were segmented and the mean number of WPBs per cell (Ai) and area covered by long WPBs (length >2µm) (Aii) plotted per well. The length distributions were also plotted as a histogram (binning at 0.5µm) (Aiii). Statistical significance was assessed with one-way ANOVA with Dunnet's multiple comparison test. **** P≤0.0001.

Supplementary Figure 4:

The amount of vWF released from control cells and cells incubated for 24 hours with pH 6.4 medium to generate mini-WPBs in the presence and absence of GÖ6976 (A) or CCE (B) was assessed by ELISA following stimulation with histamine, histamine in combination with adrenaline and IBMX and PMA. The mean amount of vWF released from n=3-4 independent experiments is shown. Normalised data are shown in Figure 2C-D. Statistical significance was assessed with two-way ANOVA with Sidak's multiple comparison test. ** P≤0.01, ns = not significant.
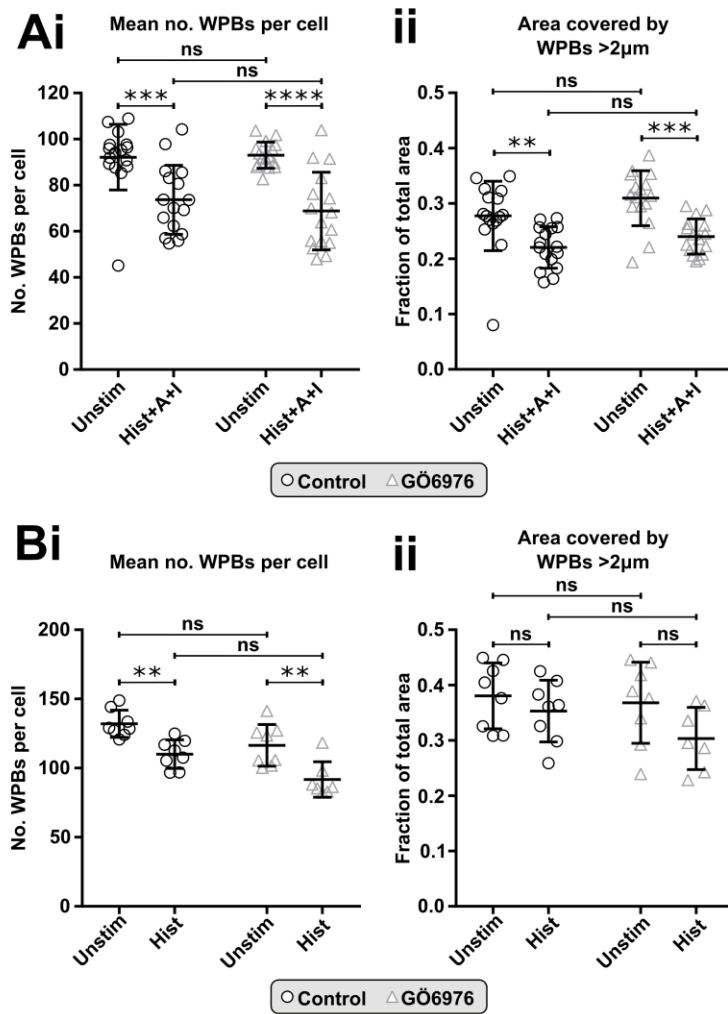
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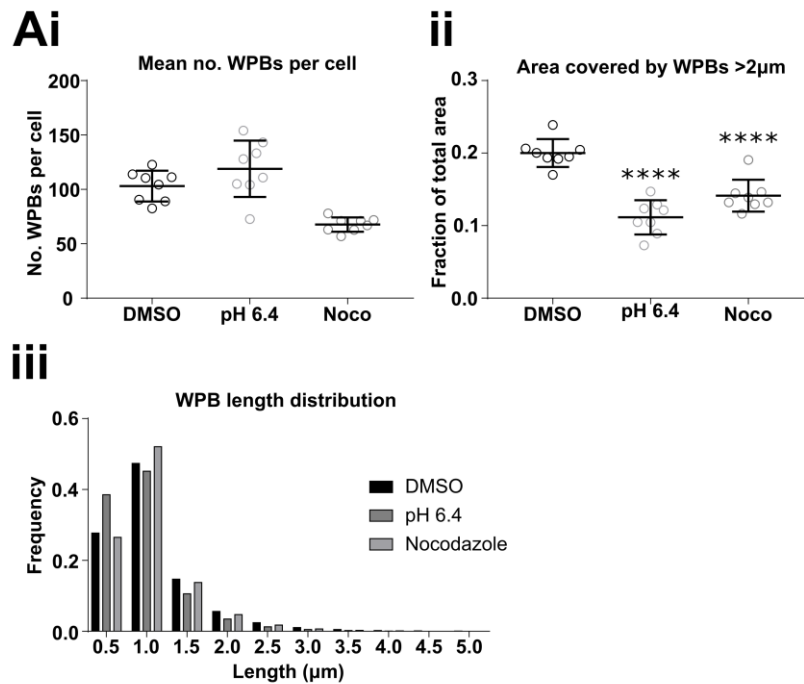# Supplementary Figure 2

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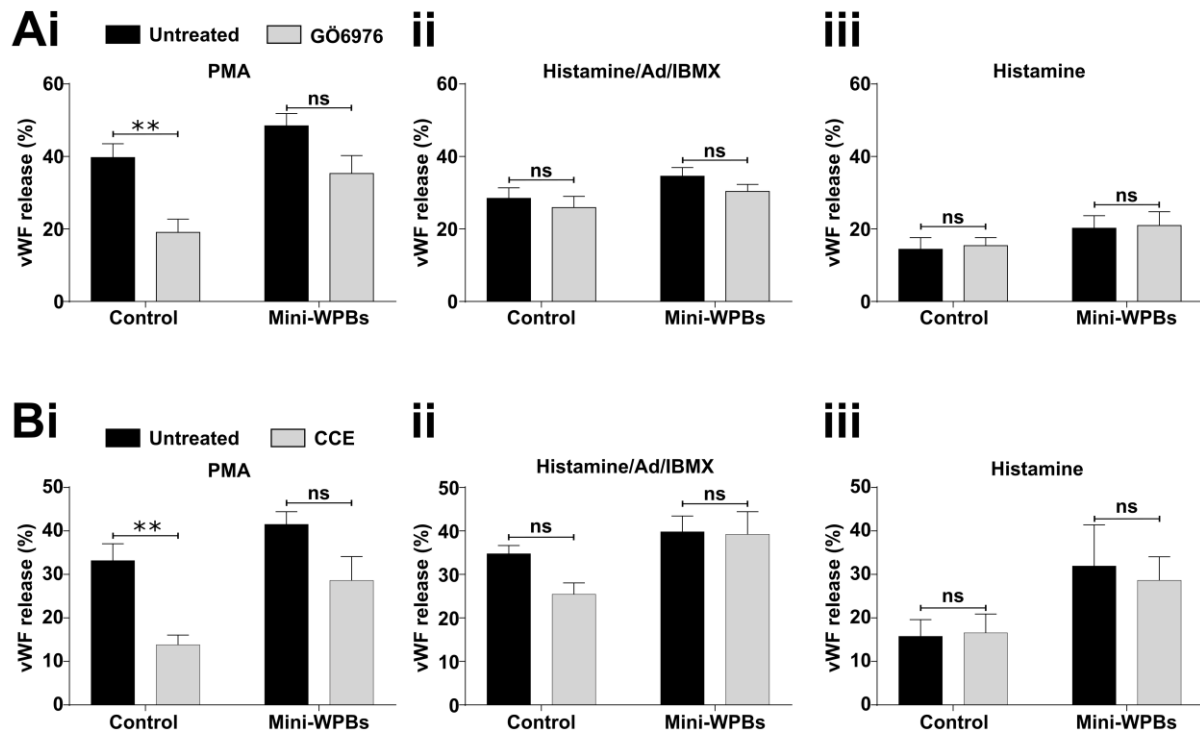
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Supplementary table 1: Summary of agonist effects on endothelium

Agonist	Induces release of long WPBs?	vWF release inhibited by blocking actin ring	Functional consequences
Histamine	Yes*	No	
Histamine/Adrenaline/IBMX	Yes	Yes	Reduction in plasma vWF recruitment
Thrombin	No		
Thrombin/Adrenaline/IBMX	Yes		
PMA	Yes	Yes	

*Very minimally