



was then unknown, it was postulated that the purpose of these novel long, cigar-shaped ‘cytoplasmic inclusions’ (later termed Weibel–Palade bodies; WPBs) as seen by electron microscopy (EM) (see poster), is associated with vascular or blood physiology. However, the first functional cargo of WPBs was not identified until 1982 when the pro-haemostatic protein von Willebrand factor (VWF) was shown to localise within these organelles (Wagner et al., 1982). The second WPB cargo to be identified was the leukocyte receptor P-selectin (Bonfanti et al., 1989; McEver et al., 1989), which demonstrated that WPBs are also involved in the inflammatory response. New bioactive cargos of WPBs continue to be identified and a current list of WPB cargos is summarised on the poster (albeit the location of some cargos is complex; see, e.g. Knipe et al., 2010). Another crucial advance was the discovery that these cytoplasmic inclusions act as a store, thereby facilitating the regulated secretion of VWF, when thrombin was shown to stimulate the release of VWF from cultured endothelial cells (Levine et al., 1982). Subsequently, both phorbol myristate acetate (PMA) and ionophores were found to stimulate the release of VWF; this established what is still an expanding list of agonists (Lowenstein et al., 2005) (see Table 1), reflecting the range of physiological stimuli to which the endothelium responds.

However, far from simply being an inert store for cargo, the active interplay between VWF (during its biosynthesis) and its carrier organelle (during its biogenesis) is being increasingly recognised. Although the presence of VWF clearly drives the formation of WPBs, the cellular machinery that controls the formation of this organelle also influences the structural arrangement of VWF and, thus, affects its functioning (Ferraro et al., 2014, 2016; Lui-Roberts et al., 2005; Michaux et al., 2006; Wagner et al., 1991). This article and the poster aim to show the essential features of WPB formation and function, with a particular focus on the emerging role of endothelial cells in actively controlling the amount and architecture of VWF, which is released through their three different secretory pathways (see below).

**Table 1. Endothelial cell agonists**

Agonist	Reference
ATP, ATP	(Palmer et al., 1994)
Ca <sup>2+</sup> ionophore	(Loesberg et al., 1983)
Ceramide	(Bhatia et al., 2004)
Complement components (C5b, C6, C7, C8, C9)	(Foreman et al., 1994; Hattori et al., 1989)
Epinephrine (adrenaline)	(Vischer and Wollheim, 1997)
Fibrin	(Ribes et al., 1987)
Histamine	(Hamilton and Sims, 1987)
Hydrogen peroxide*	(Matsushita et al., 2005a)
Hypoxia	(Pinsky et al., 1996)
IL-1, IL-6, IL-8, TNF $\alpha$	(Bernardo et al., 2004; Giddings and Shall, 1987; Paleolog et al., 1990)
Leukotrienes	(Datta et al., 1995)
NO*	(Matsushita et al., 2003)
Ox-LDL	(Vora et al., 1997)
PMA	(Giddings and Shall, 1987)
Radiation	(Hallahan et al., 1998)
Reactive oxygen species	(Vischer et al., 1995)
Serotonin	(Palmer et al., 1994)
Shear stress	(Galbusera et al., 1997)
Shiga toxin 1B and 2B	(Nolasco et al., 2005)
Sphingosine-1 phosphate	(Matsushita et al., 2004)
Thrombin	(Levine et al., 1982)
Trauma	(Reidy et al., 1989)
VEGF	(Matsushita et al., 2005b)

\*Inhibitor of WPB exocytosis.

### Biosynthesis of VWF – the cargo that drives formation of WPB

VWF is a large multidomain molecule containing different binding sites that mediate the crucial interactions needed to administer vascular ‘first aid’. Some of these binding sites are only exposed when the molecule is stretched under flow, and VWF is, thus, best thought of as a mechanosensitive binding platform (Sadler, 2009; Schneider et al., 2007; Springer, 2014). Following co-translational translocation into the ER, VWF dimerises through its cysteine knot domain located at its C-terminus, and is then trafficked to the Golgi complex (see poster). Triggered by changes in the luminal milieu of the Golgi (acidic pH and Ca<sup>2+</sup> ions), the dimers rearrange themselves into so-called ‘dimeric bouquets’, in which their central to carboxyl domains closely pair into a rod-like ‘stalk’, with the amino-terminal D1–D3 domains forming a more-globular shape to provide the ‘flower’ (Springer, 2014; Zhou et al., 2011) (see poster). The trans-Golgi luminal conditions also allow the stacking of the dimers into a right-handed coil (Huang et al., 2008). Here, the D1–D3 domains from juxtaposed dimers interact, thereby forming the core of a tubule, with the stalks sticking out akin to a bottlebrush. In this configuration, the dimers are correctly lined up to allow for further disulphide-bond formation between D3 domains that then generate multimers consisting of up to 50 dimers as measured by multimer gel analysis. At the same time, still in the Golgi lumen, the pro-peptide of VWF (encompassing D1–D2 domains) is proteolytically cleaved from the mature molecule by furin (Wise et al., 1990). Cleaved mature and pro-peptide VWF remain associated while they are kept within an environment of acidic pH present in the Golgi and also later in WPBs. Importantly, as a complete turn along the axis of a tubule accommodates on average 4.2 dimers (with an axial mean length of 11 nm) and the extended VWF dimer spans 120 nm (Fowler et al., 1985), tubulation allows for an ~45-fold compaction of VWF inside WPBs.

In a cell, there are different populations of WPB that vary in size from ~0.5–5  $\mu$ m in their longest axis (i.e. length); these will be packed with VWF tubules that can extend along the entire length of the organelle. Because a single VWF multimer consisting of 50 dimers can only generate a 130-nm-long tubule, a tubule of 5  $\mu$ m in length must consist of many such multimers packed together by a pH-sensitive mechanism (Sadler, 2009; Springer, 2014). The tubules formed of coiled VWF inside WPBs can be seen by conventional, cryo-EM and high-pressure freezing EM as bundles of parallel electron-dense tubules surrounded by a limiting membrane (Zenner et al., 2007) (see poster). Heterologous expression of VWF can lead to formation of tubules that drive the formation of pseudo-WPBs, which are indistinguishable from the endothelial organelles as seen by EM (Wagner et al., 1991).

### WPB size is controlled at the Golgi complex

While VWF biogenesis gives rise to VWF dimers that subsequently assemble into tubules, another process runs in parallel (Ferraro et al., 2014). During their transport from cis- to trans-Golgi mini-stacks (the functional subunits of the Golgi, that are linked into a ribbon in vertebrates), VWF dimers coalesce into a so-called ‘quantum’. These quanta can be seen by super-resolution microscopy as peaks of VWF immunoreactivity (see poster), or by transmission EM as a periodic distribution of density (Ferraro et al., 2014). The size of the quantum is determined by the dimensions of the Golgi mini-stack (~500–1000 nm in diameter) with the average quantum size measured by super-resolution microscopy being around 500 nm. On their final transfer to the trans-Golgi network (TGN), which provides a continuous luminal space, VWF quanta can be co-packaged into nascent WPBs in a process that is dependent on the

endocytic adaptor AP-1 and clathrin (Lui-Roberts et al., 2005). How VWF tubules and their assembly at the TGN relate to the quanta is not yet understood but, because tubules can be as long as an entire WPB, they must be capable of extending beyond the size of a single quantum.

The number of quanta that are co-packaged as a linear assembly to form a WPB will control the ultimate size (i.e. length) of the organelle. This results in a range of WPB sizes that show step-size increases of ~500 nm (the average size of a quantum). The balance between short and long WPBs within a cell can be modulated. siRNA-mediated ablation of Rab6a isoforms, which increases the cisternal diameter of the mini-stacks in endothelial cells by ~200 nm, gives rise to a corresponding increase in the size of the VWF quanta and the resulting WPBs (Storrie et al. 2012; Ferraro et al., 2014). Knockdown of the transcription factor KLF2 also increases the size of WPBs by an as yet unknown cellular mechanism (Ferraro et al., 2014, 2016). By contrast, unlinking of the Golgi into mini-stacks – each one with their own small TGN – by using genetic, physico-chemical or pharmacological approaches, shifts the balance towards a production of shorter WPBs (or ‘mini-WPBs’) (Ferraro et al., 2014, 2016). Alternatively, lowering the expression level of VWF reduces the number of quanta being delivered into the TGN; this decreases the probability of their co-packaging, thereby also resulting in the predominant generation of mini-WPBs (Ferraro et al., 2014) (see poster). The final size of WPBs is set during their formation at the TGN, rather than by post-Golgi homotypic fusion, as is the case for many granules (Hammel et al., 2010). The size of WPBs has a major impact on VWF function (Ferraro et al., 2014, 2016). By regulating the size of the organelle, the cellular machinery is, thus, able to influence the acute endothelial response.

#### Pathways of secretion—polarity and multimeric state

After formation and separation from the Golgi, WPBs move on microtubules to the cell periphery where they anchor to actin fibres. VWF multimerisation continues to occur within maturing WPBs after they have left the TGN, together with compaction, which results in organelles that appear opaque and thinner by EM (Nightingale et al., 2009) (see poster). The multimerisation state of VWF is of great physiological importance. For instance, the ability of plasma VWF to recruit platelets disproportionately increases with its multimeric state, and mutations in VWF that affect its multimerisation cause the serious bleeding disorder von Willebrand disease (Sadler, 2005). VWF multimerisation can be assayed by using gel electrophoresis on SDS-agarose gels, where multimers appear as a protein ladder on western blots (Ledford-Kraemer, 2010).

VWF is secreted via three pathways (Giblin et al., 2008). Regulated and basal secretion both occur from WPBs and deliver highly multimerised VWF. Regulated secretion is via an acute pathway that is triggered by agonist-mediated activation of the endothelium. In contrast, basal secretion occurs continuously. How basal release is controlled is still largely unknown, but it probably occurs in response to background levels of agonists and, possibly, also to mechanical stimulation provided by circulating blood. Finally, the third pathway occurs by constitutive release of VWF and releases VWF that has not been sorted into WPBs and, thus, has not undergone high levels of multimerisation (Lopes da Silva and Cutler, 2016) (see poster).

The release of VWF is also polarised, in that most of the highly multimerised cargo that is delivered from WPBs through basal or regulated secretion, is released from the apical surface of the

endothelium into the vessel lumen where it can engage with platelets (Lopes da Silva and Cutler, 2016). However, constitutive release of low-molecular-weight VWF occurs from the basolateral surface of the endothelium, from which it incorporates into the subendothelial matrix, and is likely to bind to collagen through its A3 domain. The continuous basal release pathway is likely to be the source of circulating plasma VWF, whereas the acute regulated pathway provides a highly localised bolus of so-called ultra-large (UL)-VWF in order to initiate primary haemostasis by recruiting both plasma VWF and platelets.

#### Functional consequences of WPB size on VWF function

After its exocytosis, the agonist-stimulated secreted UL-VWF is cleaved by the circulating protease ADAMTS13 into the less-multimerised form that is found in plasma (Turner et al., 2012). However, VWF that is released by regulated secretion first transiently associates with the endothelial surface where it unfurls under flow into long (up to millimetres in length) strings (Dong et al., 2002). Since the largest observed VWF multimers (~50 dimers, see above) can only extend for ~6 µm, VWF strings must, therefore, be formed by several multimers. These VWF strings are highly efficient in recruiting not only platelets but also plasma VWF (Ferraro et al., 2016). However, string formation is also dependent on the initial coiling of VWF into tubules, as disruption of coiling impairs an orderly unfurling and generates tangles of multimers that fail to recruit platelets (Michaux et al., 2006).

Although the length of WPBs does not affect the size of VWF multimers (Ferraro et al., 2016), it constrains the length of VWF strings through a mechanism that is at present unclear. Shorter WPBs produce shorter strings, and, as shorter strings have a reduced capacity to recruit platelets and plasma VWF, these inevitably have a reduced pro-haemostatic potential (Ferraro et al., 2014, 2016) (see poster). WPBs are involved in the initial response to inflammation (the rolling adhesion of leukocytes), as they carry the integral membrane protein and leukocyte receptor P-selectin to the endothelial surface to initiate the recruitment of leukocytes (Ley et al., 2007). Leukocytes start adhering and rolling in proximity to the inflamed site, until the adhesion becomes tight, and they finally cross the endothelial monolayer and migrate by chemoattraction into the underlying inflamed tissue. Interestingly, WPB size does not affect rolling adhesion of leukocytes (Ferraro et al., 2014, 2016). However, secreted VWF has been shown to promote leukocyte extravasation (Petri et al., 2010), yet the impact of WPB size on this process has not been investigated. Modulation of WPB size, thus has the potential to uncouple the haemostatic from the inflammatory functions mediated by WPBs. This might solve the long-standing question of how a single endothelial secretory granule can generate different responses to inflammatory or haemostatic cues.

#### Modes and mechanisms of exocytosis

At steady state, WPBs are decorated with different Rab proteins and anchored to actin stress fibres by the small GTPase Rab27A in complex with the Rab effector MyRIP which, in turn, is bound to myosin Va (Nightingale and Cutler, 2013; Nightingale et al., 2009; Rojo Pulido et al., 2011) (see poster). Several other Rabs have been localised to WPBs, including Rab3 (isoforms A, B and D), Rab15, Rab33 and Rab37 (Knop et al., 2004; Zografou et al., 2012). Whilst the functions of these Rab proteins are not entirely clear, both Rab3 and Rab15 have been shown to be important for VWF release from stimulated cells (Bierings et al., 2012; Knop et al., 2004; Zografou et al., 2012). Interestingly, some overlapping binding partners have been identified, potentially offering clues as to the function of these

Rab proteins. For example, both Rab27a and Rab15 bind Munc 13-4 (also known as UNC13D) (Zografou et al., 2012), which is required for agonist-stimulated release of VWF (Chehab et al., 2017; Zografou et al., 2012). Furthermore, Rab27a and Rab3 (isoforms B and D) also share a binding partner in synaptotagmin-like protein 4 (Slp4-a; also known as SYTL4) (Bierings et al., 2012). Slp4-a is recruited to WPBs by Rab3 and acts as a positive regulator of VWF release (Bierings et al., 2012). The balance between Rab27a binding to its effectors MyRIP or Slp4-a can control exocytosis. Binding of Rab27a to Slp4-a acts as a negative regulator of the interaction between Rab27a and MyRIP, thereby disrupting actin cytoskeleton anchoring and promotes exocytosis (Bierings et al., 2012; Nightingale et al., 2009) (see poster).

Exactly how the process of WPBs docking to and fusing with the plasma membrane proceeds is not fully understood. One relatively well-characterised component is the small GTPase RalA (de Leeuw et al., 2001, 1999; Rondaj et al., 2004) (see poster). Several different agonists are able to bring about the release of the RalA dissociation stimulator RalGDS from a complex it forms with  $\beta$ -arrestin, allowing it to activate RalA at the plasma membrane (Rondaj et al., 2008). Depletion of RalGDS inhibits the release of VWF (Rondaj et al., 2008), whereas expression of constitutively active RalA promotes release of WPBs (de Leeuw et al., 2001). One way RalA might operate is through activation of phospholipase D1 (PLD1). PLD1 is required for VWF release (Disse et al., 2009; Huang et al., 2012) and RalA can activate PLD1 in some cell types (Vitale et al., 2005) – although this has not been demonstrated in endothelial cells. Alternatively, in other cells types, RalA can interact with components of the exocyst complex, which then targets vesicles to the plasma membrane (Moskalenko et al., 2002). Further details of the machinery involved in WPB exocytosis are discussed in Box 1.

Unravelling the molecular mechanisms underlying WPB exocytosis is complicated by the fact that endothelial cells can be activated by such a range of agonists that, in turn, activate numerous downstream signalling pathways (Table 1) (Rondaj et al., 2006). It is generally considered that agonists work via two main pathways that raise the intracellular levels of either  $\text{Ca}^{2+}$  or cyclic adenosine monophosphate (cAMP) (Rondaj et al., 2006); examples are thrombin – which acts via  $\text{Ca}^{2+}$ , and epinephrine (also known as adrenaline) – which signals through cAMP. However, this is a simplified view. First, *in vivo* activation will most likely occur as a result of stimulation by multiple agonists; epinephrine is always present in plasma (Cryer, 1976). Similarly, many scenarios will result in the generation or release of multiple endothelial agonists, for example both thrombin and serotonin are likely to be encountered by endothelial cells at sites of injury, as thrombin is generated as part of the coagulation cascade and serotonin released from activated platelets (Ivanciu and Stalker, 2015; Palmer et al., 1994). However, very little is known about how stimulation with combinations of different agonists influences WPB exocytosis. Second, even when considering agonists that work through the same pathway, the functional consequences that occur following stimulation can be quite distinct. For example, both thrombin and histamine act by raising  $\text{Ca}^{2+}$  levels, but result in distinct alterations to the actin cytoskeleton (Vischer et al., 2000) and, consequently, elicit different functional outcomes; thrombin promotes barrier permeabilisation to a greater extent than histamine (Stolwijk et al., 2016; Vischer et al., 2000). Different pathways and most likely different machinery must, therefore, be activated following stimulation with these agonists. Indeed, whilst agonists that raise  $\text{Ca}^{2+}$  levels can promote the activation of protein kinase C (PKC)

### Box 1. Molecular machinery involved in WPB exocytosis

Fusion of WPBs with the plasma membrane is facilitated by soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptors (SNAREs), residing on both WPB and the plasma membrane, both of which interact to bring these membranes into close contact to trigger fusion. A limited number of SNAREs and SNARE-binding proteins have been found to play a role in WPB exocytosis. Two proteins of the vesicle-associated membrane protein (VAMP) family – VAMP3 and VAMP8 – were found localising to WPBs (Matsushita et al., 2003; Pulido et al., 2011). However, only VAMP3 appears to have a function in WPB exocytosis; incubation of human aortic endothelial cells with anti-VAMP3 antibodies or expression of dominant-negative VAMP3 in human umbilical vein endothelial cells (HUVECs) significantly inhibits VWF release (Matsushita et al., 2003; Pulido et al., 2011). Both VAMP3 and VAMP8 can form a complex with two plasma membrane SNAREs, syntaxin-4 and SNAP23 (Pulido et al., 2011; Zhu et al., 2014). These SNAREs both appear to be necessary for WPB exocytosis. Depletion of SNAP23 causes a significant fall in the stimulated release of VWF from HUVECs and human dermal microvascular endothelial cells (HDMVEC) (Han et al., 2017); although previous experiments in depleting SNAP23 failed to show any effect on VWF release in response to histamine, potentially due to less-effective depletion of protein (Pulido et al., 2011). Depletion of syntaxin-4 reduces the amount of P-selectin present on the plasma membrane and also perturbs neutrophil adhesion, indicating an inhibition of WPB exocytosis (Fu et al., 2005). The assembly of this complex can be regulated to control WPB exocytosis: Munc18c (also known as STXBP3) binds syntaxin-4, thus preventing its association with SNAP23 and VAMP3 (Fu et al., 2005). Disruption of the interaction between Munc18 and syntaxin-4 occurs following thrombin activation, which leads to PKC-dependent phosphorylation of both proteins and WPB exocytosis, evidenced by an increase in P-selectin levels on the plasma membrane (Fu et al., 2005). This interaction might, in part, be regulated through the activities of protein phosphatase 2B, which interacts with Munc18c and is itself a positive regulator of VWF release (Nolasco et al., 2009).

Syntaxin-binding proteins that might regulate WPB exocytosis have also been identified. Syntaxin-binding protein 5 (STXBP5) binds syntaxin-4, but not SNAP23, and is expressed in several endothelial cell types where it acts as a negative regulator of WPB exocytosis (Zhu et al., 2014). Depletion of STXBP5 increases stimulated secretion of VWF in endothelial cells, and STXBP5 knock-out mice display increased levels of VWF in plasma (Zhu et al., 2014). By contrast, STXBP1 appears to be a positive regulator of WPB exocytosis; depletion of this protein reduces stimulated secretion in HUVECs, and cells grown from individuals carrying mutations in this gene are also defective in agonist-induced secretion (van Breevoort et al., 2014). STXBP1 interacts with the syntaxin-1 and -2 – but not with syntaxin-4 – as well as with the Rab27a effector Slp4-a (see main text).

isoforms (through  $\text{Ca}^{2+}$ - or diacylglycerol-mediated activation) not all of these agonists dependent on PKC to release VWF (Carew et al., 1992; Lorenzi et al., 2008). By contrast, both histamine (which signals through  $\text{Ca}^{2+}$ ) and forskolin (which signals through cAMP) require the complex between annexin A2 and S100A10 for the release of VWF (Brandherm et al., 2013; Knop et al., 2004).

Another interesting aspect of WPB exocytosis is that multiple modes of exocytosis can be utilised, which can result in different types and amounts of cargo being released. First, a ‘lingering kiss’ is the endothelial slow-motion equivalent of the ‘kiss-and-run’ mode that occurs during exocytosis of synaptic vesicles and neuroendocrine granules; here, a partial fusion event leads to the release of small molecules but not VWF (Babich et al., 2008). Second, a full fusion of a single WPB with the plasma membrane can also occur, with or without the formation of an actin–myosin ring that helps to ‘squeeze out’ VWF cargo (Han et al., 2017; Nightingale et al., 2009, 2011). Last, there are also circumstances

where multiple WPBs can be present at a single fusion site (Kiskin et al., 2014; Stevenson et al., 2017; Valentijn et al., 2010). Such cumulative exocytic events occur when WPBs fuse with a pre-fused WPB, rather than with the plasma membrane directly. It is unclear how these different modes of exocytosis influence different WPB cargo and the ultimate functional response. However, VWF, with its large multimers and complex quaternary structure is probably the cargo that is most affected by the specific mechanism of exocytosis, rather than smaller WPB cargos, such as cytokines. This is the case for cumulative exocytosis; promotion of cumulative exocytosis (by blocking compensatory endocytosis) impairs the release of VWF and, consequently, the formation of VWF strings (Stevenson et al., 2017). An exception is the lingering kiss, which only permits the secretion of molecules less than ~40 kDa. With regard to WPB membrane cargos, the membrane protein CD63 can transfer to the plasma membrane, but this mode of exocytosis also precludes the transfer of P-selectin (Babich et al., 2008). Taken together, it is thus likely that the different modes of exocytosis employed primarily modulate the extent of the haemostatic function of WPBs. A key challenge will be in identifying machinery that is specific for the different modes of exocytosis. Whilst the focal adhesion proteins Zyxin and  $\alpha$ -actinin have recently been identified on the actin–myosin ring (Han et al., 2017), very little is known about other proteins that are involved in any of these exocytic modes.

## Perspectives

WPBs, the secretory granules of endothelial cells, store bioactive molecules that act as first-response mediators to re-establish vasculature homeostasis. Although the function of WPBs in haemostasis and inflammation is firmly established and the roles of some of its cargos, particularly VWF, have been extensively studied, there are many aspects that are not yet fully understood. First, to date we do not have a definitive and complete list of WPB cargoes, mainly owing to technical hindrances that include the fragility of these organelles – making their purification difficult – and the predominance of VWF as a WPB cargo, hindering the detection of less-abundant cargos. Second, even though we have identified some of the machinery involved in exocytosis, we still have very little idea how it operates in concert, nor do we have outlines of all the signalling pathways that are involved in its regulation. Third, the emerging plasticity of WPBs, especially with regard to how it is linked to the control of their size, awaits further exploration – especially *in vivo*. Last, the combined effects of all the factors noted above on WPB physiology need to be established. Given their role in thrombosis and inflammation alone, learning how to control WPB functioning will, inevitably, have important consequences for clinical interventions.

## Funding

Work in this laboratory was funded by the MRC (MC\_UU\_12018/2) and the BHF (PG/14/76/31087).

## Cell science at a glance

A high-resolution version of the poster and individual poster panels are available for downloading at <http://jcs.biologists.org/lookup/doi/10.1242/jcs.208033.supplemental>.

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