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The contribution of zinc to platelet behaviour during haemostasis and thrombosis

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Platelets are the primary cellular determinants of haemostasis and pathological thrombus formation leading to myocardial infarction and stroke. Following vascular injury or atherosclerotic plaque rupture, platelets are recruited to sites of damage and undergo activation induced by a variety of soluble and/or insoluble agonists. Platelet activation is a multi-step process culminating in the formation of thrombi, which contribute to the haemostatic process. Zinc (Zn^{2+}) is acknowledged as an important signalling molecule in a diverse range of cellular systems, however there is limited understanding of the influence of Zn^{2+} on platelet behaviour during thrombus formation. This review evaluates the contributions of exogenous and intracellular Zn^{2+} to platelet function and assesses the potential pathophysiological implications of Zn^{2+} signalling. We also provide a speculative assessment of the mechanisms by which platelets could respond to changes in extracellular and intracellular Zn^{2+} concentration.

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Introduction

Zn^{2+} is the second most abundant trace metal in the human body and is a biologically important cation. Approximately 10–15% of genes within the human genome encode proteins that utilise Zn^{2+} as a cofactor. These include transcription factors, enzymes, structural and signalling proteins.[1,2] Vesicular release of neuronal Zn^{2+} from presynaptic terminals has been implicated in signal transduction, suggestive of a role for Zn^{2+} as a neurotransmitter.[3–6] Additionally, elevation of the intracellular Zn^{2+} concentration ($[Zn^{2+}]_i$), as a result of influx or release from intracellular stores, may serve as a second messenger. The activities of a number of cytosolic proteins such as protein kinase C (PKC), Ca^{2+}/calmodulin dependent kinase II (CaMKII), insulin receptor activated kinase (IRAK), adenylate cyclase, calcineurin, protein tyrosine phosphatases (PTPs), caspase 3 and phosphodiesterases (PDEs) are affected by Zn^{2+}, supporting a role for this cation in the modulation of intracellular signalling pathways.[7–20] Although the involvement of Zn^{2+} in haemostatic processes has been the subject of recent reviews,[21,22] there is limited understanding of the contributions by Zn^{2+} to platelet function during haemostasis and thrombosis. Here we consider the current understanding of Zn^{2+} as an extracellular agonist and an intracellular signalling modulator in platelets. Given the limited information on this subject, we draw on Zn^{2+}-responsive signalling pathways and cellular processes described in other cell types, and assess the mechanism(s) and/or machinery available for use by Zn^{2+} in the context of platelet activation.

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Platelet physiology

Platelets play a central role in haemostasis and inappropriate platelet activation is a major precipitating factor in cardiovascular disease.[23] Engagement of platelet surface receptors with thrombogenic extracellular matrix molecules (*e.g.* collagen and von Willebrand factor) or by soluble mediators (including ADP, ATP, thrombin and thromboxane A_2) leads to platelet activation, culminating in stable adhesion to the subendothelium and formation of thrombi. This is a highly dynamic process involving cytoskeletal rearrangements, granule release and increased affinity of integrins for their cognate ligands.[24] Activation is mediated by platelet membrane receptors, including tyrosine kinase-associated receptors (such as GpVI and CLEC-2, reviewed in ref. 25) and G-protein coupled receptors (GPCRs, reviewed in ref. 26). Elevation of intracellular Ca^{2+} ($[Ca^{2+}]_i$), mediated by release from intracellular stores and influx through membrane ion channels is a hallmark of platelet activation (reviewed in ref. 27). The primary endpoint of platelet activation is high affinity binding of integrin $\alpha_{IIb}\beta_3$ to fibrinogen, leading to thrombus formation.[28] Conversion of fibrinogen to insoluble fibrin by thrombin, generated by the coagulation pathway, stabilises the thrombus leading to the cessation of bleeding. Chronic cardiovascular diseases narrow the arterial lumen, reducing blood flow and elevating local shear rates. Subsequent atherosclerotic plaque rupture exposes collagens which, in conjunction with elevated shear rates, increase

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the propensity for platelet activation.[23,29,30] Thrombus formation at these sites may lead to vessel occlusion or generate emboli, elevating the risk of myocardial infarctions and stroke.

Zn^{2+} as a mediator of haemostasis

The first observations of a functional relationship between Zn^{2+} and platelet activation were made in rats undergoing experimental Zn^{2+} deficiency. These studies demonstrated that Zn^{2+}-deficient rodents have an increased bleeding tendency, more difficult parturition and prolonged tail bleeding times.[31–34] Consistent with a link between Zn^{2+} status and platelet function, this bleeding diathesis has been reported in both male and female animals[31–33] and is phenotypically similar to that of aspirin treatment.[32] Interestingly, this phenomenon has also been reported in human subjects. Stefanini *et al.* described two cancer patients, one with squamous cell carcinoma and one with non-Hodgkin's lymphoma, with concurrent nutritional Zn^{2+} deficiency whom presented with ecchymoses, prolonged bleeding times and abnormal platelet aggregation responses.[35] In both cases, the bleeding phenotype was normalised following oral administration of zinc sulphate, but returned as a result of discontinued therapy. In addition, healthy human volunteers subjected to experimental Zn^{2+} deficiency display defective platelet aggregation responses to ADP and arachidonate.[34] Again, normal platelet function was returned following dietary zinc supplementation. It is also worth noting that nutritional Zn^{2+} supplementation promotes platelet reactivity. Marx *et al.* demonstrated that increased dietary Zn^{2+} in rats correlates with enhanced platelet responses to sub-maximal doses of collagen, ADP, thrombin and adrenaline.[36] Given that dietary Zn^{2+} intake is linked to plasma Zn^{2+} levels,[38] these studies provide evidence for a direct association between Zn^{2+} status and platelet reactivity. Thus, these data suggest that maintenance of normal dietary Zn^{2+} is required for physiological platelet function.

Zn^{2+}-deficiency also impairs rodent platelet aggregation in response to multiple agonists.[37,38] For example, ADP-stimulated rat platelets displayed normal primary aggregation responses, but failed to undergo secondary activation.[33] Zn^{2+}-deficiency has also been shown to reduce platelet reactivity to ADP, thrombin and the thromboxane A2 analogue U46619.[38–40] These studies suggest that secondary platelet activation is, in part, mediated by Zn^{2+}-dependent signalling pathways. Interestingly, aggregation in response to fluoride and the Ca^{2+} ionophore, A23187 are unaltered in Zn^{2+}-deficient animals; indicating that aggregatory mechanisms are not intrinsically Zn^{2+}-dependent.[37,40] However, platelet responses to the PKC-dependent agonist, phorbol-myristate acetate (PMA) were impaired as a result of Zn^{2+} deficiency, suggesting that Zn^{2+}-dependent modulation of platelet reactivity converges on PKC activation.[40] Furthermore, this effect was only apparent when exogenous Ca^{2+} was included in the extracellular medium, implying a functional link between Ca^{2+} entry and Zn^{2+}-dependent activation.

Labile Zn^{2+} is increased at sites of injury

Plasma Zn^{2+} concentration ($[Zn^{2+}]_o$) ranges from 10 to 20 μM, much of which is bound to plasma proteins such as albumin (forming a labile, freely-exchangeable pool) and α2 microglobulin (a non-exchangeable pool), leading to a free Zn^{2+} concentration of approximately 0.5 μM.[1,21,41–43] However, given that Zn^{2+} freely moves between albumin and plasma, $[Zn^{2+}]_o$ may be considerably higher within microenvironments of the vascular network. Zn^{2+} is also located in the extracellular matrix of dermal and epidermal tissues, thus vascular injury gives rise to localised increases of $[Zn^{2+}]_o$.[44,45] In this model, Zn^{2+} released from epithelial cells activates Zn^{2+}-sensing receptors on adjacent cells, promoting wound healing.[46] $[Zn^{2+}]_o$ increases in the early inflammatory phase of experimental wounds.[44,47,48] For example, within 20 hours of injury in a rat skin wound model, Zn^{2+} levels were found to increase in the wound margin by 15–20%.[44] Cells involved in haemostasis (*e.g.* neutrophils, lymphocytes, platelets and erythrocytes) have a high Zn^{2+} content, and their recruitment into the wound margin may represent an additional mechanism for Zn^{2+} delivery to sites of vascular damage.[49] Within the platelet, micromolar Zn^{2+} concentrations are stored in the cytosol and α-granules.[1,50–52] Given that platelets release Zn^{2+} upon activation,[53,54] labile $[Zn^{2+}]_o$ is likely to be significantly higher within and in the proximity of a growing thrombus. Furthermore, in relation to healthy tissues, Zn^{2+} concentration is approximately six times higher within atherosclerotic plaques.[55] Combined with the exposure of other thrombogenic molecules during plaque rupture, elevation of $[Zn^{2+}]_o$ may help to explain platelet hyper-reactivity at these sites.

Zn^{2+} modulates platelet function

Zn^{2+} may modulate platelet behaviour in a number of ways, for example by acting as an intracellular second messenger, a transmembrane signalling agonist or by interacting with and influencing membrane receptor activity. Given the number of proteins whose activities are sensitive to cation concentration, platelet function is likely to be modulated following changes of $[Zn^{2+}]_i$. Intracellular Zn^{2+} is mostly associated with Zn^{2+}-binding proteins (*i.e.* metallothioneins), but is liberated in numerous cell systems following changes of redox state.[56] Although present in platelets, the function of endogenous metallothioneins is not fully understood.[57] Experiments using $[Zn^{2+}]_i$ chelators demonstrate a clear role for $[Zn^{2+}]_i$ during platelet activation. The membrane-permeant heavy metal chelator, *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), has a high affinity for Zn^{2+} ($K_d = 2.6 \times 10^{-16}$ M) and low affinities for Ca^{2+} and Mg^{2+} ($K_d = 4 \times 10^{-5}$ M and 2×10^{-2} M, respectively).[58,59] TPEN inhibits platelet aggregation induced by conventional agonists including ADP, thrombin and PMA, although the effect seen with thrombin is restricted to low agonist concentrations.[60–62] TPEN also reduces thrombin-evoked $[Ca^{2+}]_i$ mobilisation, phosphatidylserine externalisation and phosphorylation of eIF2a.[63] In a physiological blood flow model, TPEN-treated platelets were unable to activate to a degree sufficient for generation of full thrombi.[62] Whether this effect is due to chelation of labile

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Zn^{2+} or removal of Zn^{2+} from their interacting proteins is unclear. Regardless of the underlying mechanism, this work suggests an important role for $[Zn^{2+}]_i$ in platelet activation.

As discussed above, local plasma $[Zn^{2+}]_o$ likely increases significantly within the vicinity of a growing thrombus. Exogenous Zn^{2+} has been shown to activate platelets directly. Incubating washed platelets or heparin anti-coagulated PRP with exogenous Zn^{2+} causes aggregation in a concentration-dependent manner.[60,62,64] In calcified (2 mM $CaCl_2$) washed platelet suspensions, maximal aggregation occurred at a concentration of around 0.5 mM. Increasing Zn^{2+} concentrations above this value gave a sub-optimal response.[64] A magnitude increase in Zn^{2+} concentration is required to aggregate platelets in PRP;[64] however, this likely reflects the buffering effect of plasma Zn^{2+}-binding proteins and anticoagulants. Zinc-induced platelet aggregation occurs within a physiologically-relevant timeframe, comparable to that of the conventional agonists ADP, collagen and thrombin.[60–62] Aggregation is biphasic and accompanied by a discernible shape change,[60,64] indicating a role for secondary activation by soluble agonists and cytoskeletal rearrangements, respectively. Taken together, these features of Zn^{2+}-induced aggregation are reminiscent of those observed by others in response to other agonists, suggesting that Zn^{2+} is a *bona fide* platelet agonist.

Zn^{2+}-induced platelet activation is integrin $\alpha_{IIb}\beta_3$-dependent

Whether Zn^{2+} acts as a transmembrane signaller in a similar manner to that reported for synaptic transmission and insulin release,[3,65] or modulates extracellular receptor activity directly has not yet been fully resolved. Platelet aggregation is mediated by integrin $\alpha_{IIb}\beta_3$ which, upon switching to its high affinity state, is able to bind to arginine–glycine–aspartate (RGD) motifs in proteins such as fibrinogen and von Willebrand factor facilitating platelet crosslinking.[23] Conversely, platelet agglutination occurs as a result of direct activation of adhesive receptors, without a requirement for intracellular signalling.[23] Integrins transduce signals in response to extracellular and intracellular cues *via* outside-in and inside-out signalling, respectively. Inside-out activation concerns cytosolic events that increase the affinity of the receptor for its ligand, whereas outside-in signalling coordinates cellular responses to ligand binding.[23] A number of cation binding sites are present on both the α and β subunits of integrin $\alpha_{IIb}\beta_3$, with Ca^{2+} or Mg^{2+} association being required for activity. In addition, direct activation has been shown in response to exogenous Mn^{2+}.[66–69] Several studies using antibodies and small molecule inhibitors of integrin $\alpha_{IIb}\beta_3$ have demonstrated that Zn^{2+}-induced aggregation requires inside-out activation of these receptors.[60,62,64] Furthermore, in contrast to Zn^{2+}, Mn^{2+} does not induce platelet aggregation and direct integrin $\alpha_{IIb}\beta_3$ activation by Zn^{2+}, independently of intracellular signalling, has yet to be demonstrated.[62,70,71] Thus, it is unlikely that Zn^{2+}-induced activation is mediated by cellular agglutination.

Potential routes for elevation of $[Zn^{2+}]_i$

Recent work suggests that exogenous Zn^{2+} is able to gain access to the platelet cytosol.[62] Platelets stained with the fluorescent Zn^{2+} indicator, Fluozin-3, gave a rapid and sustained increase in fluorescence upon application of exogenous Zn^{2+}.[62] However, the mechanism(s) of platelet Zn^{2+} entry are unknown. Studies of Zn^{2+} homeostasis by other cell types have identified several distinct Zn^{2+} entry pathways, which include Zn^{2+}-permeable transporters, exchangers and ion channels. At present, it is unclear whether Zn^{2+}-selective ion channels or exchangers exist within the human genome. Although, evidence suggests that platelets express Zn^{2+} transporters and several non-selective cation channels that may facilitate Zn^{2+} transit.

In many cell types, elevation of $[Zn^{2+}]_i$, in response to exogenous Zn^{2+}, occurs *via* members of the ZIP (Zrt/Irt-like, Slc39A) family of Zn^{2+} transporters, of which fourteen family members have been reported.[72] Within the platelet proteome, ZIP7 (SLC29A7) and ZIP3 (SLC39A3) have been identified;[73] although their expression is yet to be verified by Western blotting and functional assays. ZIP3 is a plasma membrane Zn^{2+} uptake transporter that is required for survival of mammary gland epithelial cells.[74,75] In a knockout mouse model there were no phenotypic defects when fed normal diets, but maternal Zn^{2+}-deficiency caused abnormal embryonic development.[76] Haemostatic defects were not reported in this study.

A number of membrane-localised non-selective cation channels, including voltage-gated Ca^{2+} channels, transient receptor potential (TRP) channels and ionotropic glutamate receptors have been reported to permeate Zn^{2+} in a variety of cellular models.[77,78] The nature of ion channels expressed on the platelet surface has been the subject of a recent review.[27] Here, we limit our discussion to ion channels/exchangers known to be expressed by platelets, which have shown Zn^{2+}-permeability in other cell types and may therefore contribute to Zn^{2+} entry.

Glutamate receptor subunits 1–6 are expressed on platelet membranes and glutamate has previously been shown to mediate platelet activation through kainate and AMPA receptor activities.[79,80] Platelets and megakaryocytes (platelet precursor cells) also express NMDA receptors.[80–84] Although unable to aggregate platelets directly, glutamate potentiates platelet responses to agonists including thrombin and thromboxane A_2, indicating that these channels are functional during platelet activation.[80] NMDA and AMPA/kainite receptors are both implicated in Zn^{2+} movement in other cell types.[77,85] Co-treatment of neocortical neurones with Zn^{2+} and NMDA results in measurable increases of $[Zn^{2+}]_i$ that are sensitive to NMDA channel blockers.[85] Furthermore, addition of AMPA and Zn^{2+} causes elevations of $[Zn^{2+}]_i$ and potentiates Zn^{2+}-induced neurotoxicity of cortical neurones.[85–87] Whilst glutamate receptors provide a means by which Zn^{2+} can enter cells, further work is required to investigate whether this constitutes a genuine route for platelet Zn^{2+} entry. Platelets also express α7-nicotinic acetylcholine subunits, which form functional Ca^{2+} channels.[88] Additionally, γ- and ε-acetylcholine receptors are permeable to Zn^{2+}, although acetylcholine-induced whole-cell currents are reduced by millimolar Zn^{2+}.[89] However, nicotinic cholinergic-evoked currents have not been reported in platelets, indicating that these are not likely to be involved in Zn^{2+} entry.[27]

TRP channels are a superfamily of approximately 30 non-selective cation channels, broadly expressed at the plasma

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membrane of mammalian cells.[27] TRP channels known to be expressed on megakaryocytes include, TRPC1, TRPC6, TRPM2 and TRPM7.[90] Of these, TRPC6 and TRPM7 have been shown to be Zn^{2+}-permeable. TRPM7 is a ubiquitously expressed non-specific cation channel that possesses a C-terminal α-kinase domain. These channels are constitutively active and four times more permeable to Zn^{2+} than Ca^{2+}.[78] TRPM7 contributes to Zn^{2+}-induced cytotoxicity in cultured mouse cortical neurones.[91,92] Functionality of these channels was determined electrophysiologically using primary rat megakaryocytes, and could be blocked by elevating intracellular Mg^{2+}.[90] Interestingly, TRPM7 expression on the surface of transfected HEK293 cells increases following exposure to shear forces.[93] To date, attempts to detect TRPM7 on platelets using commercially available antibodies have been unsuccessful (Taylor, unpublished observation). However, should suitable tools become available it would be interesting to assess changes of platelet TRPM7 expression/function in response to mechanical stimulation, which may reflect the effect of elevated shear stress at sites of vessel stenosis. TRPC6 channels open in response to diacylglycerol (DAG) analogues, decreased membrane PIP_2 concentration, protons and hyperforin.[27,94,95] Overexpression of TRPC6 by HEK293 cells coincides with an accumulation of $[Zn^{2+}]_i$ in response to the DAG analogue (SAG) and hyperforin.[95,96] Electrophysiological recordings of these cells reported channels that were permeable to both Zn^{2+} and Ca^{2+}.[95] Interestingly, a brief report has shown that TRPC6-deficient mice have a mild phenotype with increased bleeding times, associated blood loss and reduced numbers of occlusive thrombi in an $FeCl_3$-induced thrombosis model.[97] It is possible that deletion of TRPC6 may affect platelet Zn^{2+} handling, contributing to the observed bleeding phenotype.

The Na^+/Ca^{2+} exchanger (NCX) represents an additional candidate for Zn^{2+} entry into platelets. Although conventionally thought to exchange one Ca^{2+} for three Na^+ in an electronic manner, NCX exchangers have also been shown to contribute to Zn^{2+} movement.[98] Sensi *et al.*, demonstrated slow benzamil–amiloride-sensitive increases of $[Zn^{2+}]_i$ in response to exogenous Zn^{2+} in cortical neurones, indicating NCX involvement.[85] Additionally, NCX has been shown to be involved in transepithelial uptake of dietary Zn^{2+}.[98] Platelets express three NCX isoforms; NCX1.3, NCX3.2 and NCX3.4.[99] These are known to contribute to platelet Ca^{2+} homeostasis (forward-mode) in quiescent cells and promote Ca^{2+} influx (reverse-mode) in activated cells.[100] Thus, these exchangers are an attractive candidate for the regulation and influx of Zn^{2+} against physiological gradients.

Zinc entry and storage

Zn^{2+} release from intracellular stores

Zn^{2+} has been shown to act as a second messenger, being released from intracellular stores into the cytosol in a manner analogous to that for Ca^{2+}. For example, transmembrane signalling *via* $Fc\varepsilon_1$ receptors gives rise to elevations of $[Zn^{2+}]_i$ from the perinuclear area of mast cells.[101] Pathways leading to the elevation of $[Zn^{2+}]_i$ *via* release from stores may represent an alternative mechanism for the regulation of platelet activity. However, this has yet to be studied in platelets. Intracellular Zn^{2+} homeostasis is principally regulated by members of the SLC30A family (ZnTs, reviewed in ref. 102). Of these, three members exist in the platelet proteome; ZnT1, ZnT5 and ZnT6.[73] ZnT1 functions as a Zn^{2+} exporter in cultured neurones.[103] ZnT5 and ZnT6 are predominantly located on the membranes of intracellular organelles,[104,105] although, splice variants of ZnT5 have been shown to be present on the plasma membrane where they may regulate Zn^{2+} transit.[106] ZnT5 is important in the sequestration of Zn^{2+} into vesicles, including insulin-containing secretory vesicles, where Zn^{2+} is co-released with insulin. ZnT5 activity is dependent upon an electrochemical proton gradient and protects cells from glucose-induced apoptosis by sequestering Zn^{2+} into the Golgi apparatus of peritoneal mesothelial cells.[107,108] Knockout of ZnT5 results in osteopenia muscle weakness and male-specific cardiac death.[109]

ZIP7 is reportedly associated with the membranes of $[Ca^{2+}]_i$ stores of MCF-7 breast cancer cells.[110,111] These transporters mediate release of Zn^{2+} into the cytoplasm in response to membrane signalling, akin to PLC-mediated Ca^{2+} release. Given that ZIP7 is present in platelets, it is feasible that these transporters may perform similar functions. The existence of a Zn^{2+} store in platelets will also require verification. It is plausible that the platelet dense tubular system, which acts as a Ca^{2+} store, is also a Zn^{2+} pool, in which case, co-release of Ca^{2+} *via* the IP3-receptor and Zn^{2+} *via* ZIP7 may occur. Indeed, the gating of structurally related ryanodine receptors on the sarcoplasmic reticulum of cardiac myocytes has recently been shown to be modulated by $[Zn^{2+}]_i$.[112] At present, the relative expression of Zn^{2+} transporters has not been determined using biochemical techniques, and given the paucity of pharmacological tools available for targeting these proteins, further research may warrant the use of genetically modified organisms or cell lines. However, in spite of these technical considerations, it remains important to account for their potential contributions to platelet physiology.

Zn^{2+} ionophores have been used to examine changes in $[Zn^{2+}]_i$ levels from a number of cell types. Low concentrations of the Zn^{2+} ionophore pyrithione (5 μM) do not induce aggregation of washed platelets, however, this additional stimulus appears to potentiate ADP-induced aggregation.[61] In our laboratory we find that higher concentrations of an alternative Zn^{2+} ionophore, clioquinol (50 μM), induce sub-maximal aggregation responses (unpublished observation). Given that our experiments were conducted in the absence of exogenous Zn^{2+}, these point to a role for the release of intracellular Zn^{2+} in the propagation of platelet activation.

Liberation of Zn^{2+} from interacting proteins (*i.e.* metallothioneins), which occurs in response to changes of the intracellular redox state, has been shown to elevate $[Zn^{2+}]_i$ in neurones.[113] Oxidation of thiols removes potential binding sites for Zn^{2+} in proteins, liberating them for use in active processes. Thus, the redox state of platelets may influence the free Zn^{2+} concentration,

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consequently affecting platelet activation. The redox environment is an important modulator of platelet function affecting platelet receptor activity including integrins, GPVI and P2Y12.[114–117] Platelet membranes from Zn^{2+}-deficient rats have a reduced protein sulfhydryl level,[118] indicating Zn^{2+}-dependent regulation of membrane protein redox state. The aggregation defect associated with Zn^{2+}-deficiency is rectified by treatment with the reducing agent glutathione-*S*-transferase (GST), which had no effect on platelets from rats fed control diets. Addition of GST to Zn^{2+}-deficient platelets enhances Ca^{2+} uptake following ADP activation, supporting a role for Zn^{2+} in Ca^{2+} entry. Zn^{2+} may act by regulating multimerisation-mediated control of protein disulphide isomerase enzyme activity.[119]

The influence of Zn^{2+} on platelet processes

Zn^{2+}-mediated potentiation of platelet activation

In isolation, Zn^{2+}-induced platelet activation requires supra-physiological concentrations.[60,62,64] However, it is plausible that release of Zn^{2+} from the endothelium and other blood cells at sites of vascular injury may sufficiently elevate $[Zn^{2+}]_o$ to directly activate platelets; particularly within the self-contained environment of a growing thrombus protected from rheological conditions. $[Zn^{2+}]_o$ is reliant upon the cellular context. For example, Zn^{2+} release from cortical neurones has been suggested to result in local Zn^{2+} concentrations of approximately 300 μM.[3] If similar levels of labile Zn^{2+} were achieved within a thrombus, it would be sufficient to induce full platelet activation.

Low, sub-activatory Zn^{2+} concentrations potentiate platelet activation in response to other agonists. Zn^{2+} potentiates collagen- and ADP-induced platelet aggregation.[60,61,64] Heyns Adu *et al.*, demonstrated that whilst stimulation of platelets by 2.8 μg ml^{-1} collagen gave a partial aggregation, inclusion of 100 μM Zn^{2+} in the suspension was potentiatory and resulted in full aggregation.[64] Similar results were observed using ADP as an agonist. The potentiating effect on ADP signalling is blocked following TPEN treatment. Low concentrations of Zn^{2+} also potentiate ADP-mediated α-granule release, measured using platelet-released β thromboglobulin.[60] More recently, we have demonstrated that this potentiating effect is evident when platelets are pre-incubated with 30 μM Zn^{2+} and challenged with CRP-XL (collagen-related peptide, a GpVI ligand), thrombin, U46619 or adrenaline.[62] Thus, low $[Zn^{2+}]_o$ potentiates agonist-evoked platelet activation, supporting the concept of a physiological role for Zn^{2+} in platelet thrombus formation.

Mechanism of action of Zn^{2+} in platelet activation

The mechanism by which Zn^{2+} induces platelet aggregation has been studied by pharmacological targeting of various aspects of platelet signalling. Unlike agglutination, platelet aggregation occurs as a result of PKC activation which is dependent on $[Ca^{2+}]_i$ increases following mobilisation from stores and also from extracellular sources *via* gated Ca^{2+} channels, exchangers and transporters (reviewed in ref. 27). Zn^{2+}-induced aggregation is inhibited following treatment with $[Ca^{2+}]_i$ chelators such as TMB8 and BAPTA-AM, further indicating that Zn^{2+} initiates an intracellular signalling response leading to Ca^{2+} release.[62,64] These observations are complicated by the fact that commonly used $[Ca^{2+}]_i$ chelators often have a higher affinity for Zn^{2+} than Ca^{2+}. For example, the K_ds of BAPTA for Zn^{2+} and Ca^{2+} are 8 nM and 160 nM respectively.[120] Hitherto, the impact of Ca^{2+} chelators on Zn^{2+}-dependent processes has not been considered in platelet research.

In rat platelets, basal $[Ca^{2+}]_i$ levels are unaffected by dietary Zn^{2+} deficiency,[61] but agonist-evoked Ca^{2+} rises are impaired following stimulation by ADP, thrombin or fluoride.[40,121,122] This effect was not apparent in the absence of extracellular Ca^{2+}, indicating that it is due to entry of external Ca^{2+}, and not an effect on Ca^{2+} store release. Such an effect has been reported in epithelial monolayers, where exogenously applied Zn^{2+} induced sustained $[Ca^{2+}]_i$ increases that were consistent with P2X channel conductances.[123,124] Conversely, Zn^{2+} has been shown to block Ca^{2+} release-activated channel currents (I_{CRAC}) in mast cells.[125] I_{CRAC}, which is attributable to the channel Orai1, is a major Ca^{2+} pathway in platelets following $[Ca^{2+}]_i$ release.[27] Thus, Zn^{2+} may modulate the activity of platelet calcium channels, such as P2X1, and Orai1 to influence platelet responses.

Influence of $[Zn^{2+}]_i$ on cytosolic kinases

Zn^{2+}-induced aggregation is blocked by PKC inhibitors, such as staurosporine or Ro31.[60–62] PKCs are a family of serine/threonine kinases that are central to platelet processes, including granule release, integrin activation and cytoskeletal rearrangements (reviewed in ref. 126). PKC isoforms are metalloenzymes containing cysteine-rich Zn^{2+}-binding domains that are essential for structural integrity.[8,9,127] Exogenously applied Zn^{2+} in the milimolar range increases PKC activity in thymocytes in a concentration-dependent manner.[7] This action appears to be specific to Zn^{2+}, as incubation of cells with either Cl^-, SO_4^{2-}, Ca^{2+} or Mg^{2+} had no effect.[7,40,60] In platelets from Zn^{2+}-deficient rats, membrane association of PKC is reduced, indicating that Zn^{2+} contributes to PKC mobility during platelet activation.[40] Thrombin-induced PKC activation leads to increased integrin $\alpha_{IIb}\beta_3$ activation.[128] This mechanism also holds true for Zn^{2+}-induced activation, as 50 μM exogenous Zn^{2+} caused a 2-fold increase in the number of active fibrinogen receptors on the platelet surface upon ADP-stimulation.[60]

Dense granules contain molecules such as serotonin, ADP, ATP and polyphosphates, whilst α-granules contain haemostatic and angiogenic factors.[129] Thromboxane A_2, but not serotonin release, has been detected in Zn^{2+}-activated platelets.[61] Although granule release was not observed in platelets by electron microscopy, low levels of β-thromboglobulin were shown to be released, indicating a role for α-granules downstream of Zn^{2+}-induced activation.[60] Platelet δ-granule release is mediated by PKC in a Ca^{2+}- and DAG-dependent manner.[130,131] Zn^{2+}-induced PKC activation might be expected to expedite this process. However, as Zn^{2+} induces α-granule, but not δ-granule release,[60] PKC

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activation likely potentiates platelet activation *via* an alternative pathway.

Autoradiography of platelet proteins following Zn^{2+} treatment revealed phosphorylation of a 47 kDa protein, that may correlate with the PKC-specific substrate pleckstrin.[60] This protein was also phosphorylated following PMA or thrombin treatment. In further support of a role for Zn^{2+}, these phosphorylation events were inhibited by TPEN and potentiated by pyrithione.

In other cell types, Zn^{2+}-induced PKC activation is blocked by staurosporine, TPEN and PGE_1 (a prostanoid receptor agonist).[7,132] Csermely and colleagues reported that the tyrosine kinase inhibitor H-7 blocks Zn^{2+}-induced PKC activation in T cells, suggesting that changes in tyrosine phosphorylation are required for Zn^{2+}-induced intracellular signalling.[7] Tyrosine phosphorylation is a key regulatory step during signal transduction in platelets. Phosphorylation occurs downstream of platelet receptor engagement (*i.e.* GpVI and CLEC-2) and subsequent phosphorylation *via* Src family kinases and Syk.[133] Exogenously applied Zn^{2+} is known to promote tyrosine phosphorylation of a number of important signalling proteins in a variety of cell systems.[134–137] Although not directly affected by Zn^{2+}, Mitogen-activated protein kinase family member (MAPK) activation in response to Zn^{2+} has been observed in a number of different cell types. For example, ERK becomes activated by Zn^{2+} treatment of fibroblasts, neurones and neuroblastoma cells.[137–140] Transient MAPK activation has been demonstrated in platelets in response to agonist stimulation and is thought to be involved in regulation of integrin activation in flowing blood.[141] In our laboratory, we have utilised tyrosine phosphorylation-specific antibodies to assess Zn^{2+}-dependent changes of platelet protein phosphorylation.[62] Our data demonstrate time-dependent increases in tyrosine phosphorylation of a panel of platelet proteins. Interestingly, the pattern of phosphorylated proteins differs to that induced by GpVI- and thrombin-dependent signalling, suggestive of a novel signalling pathway. Further experiments are required to identify the cohort of platelet proteins phosphorylated in response to stimulation by Zn^{2+}.

Influence of $[Zn^{2+}]_i$ on cytosolic phosphatases

Platelet activation is positively and negatively regulated by a number of protein tyrosine phosphatases (reviewed in ref. 142), some of which are strongly inhibited by Zn^{2+}.[136] The IC_{50} values for many of these phosphatases closely correlate with cellular labile $[Zn^{2+}]_i$ (in the nM range), thus minor increases in $[Zn^{2+}]_i$ may inhibit PTPs and support phosphorylation. For example, PTP1b, with an IC_{50} for Zn^{2+} of 17 nM, is a positive regulator of outside-in integrin signalling in platelets.[134,142] It dephosphorylates inhibitory tyrosines of the β3 integrin-associated Srk Family Kinase (SFK), thereby promoting downstream signalling.[142–145] SHP-1 and SHP-2 have IC_{50} values for Zn^{2+} of 93 nM and 1–2 μM, respectively.[134,135] SHP-1 is a positive regulator of platelet activation *via* GpVI-mediated integrin $\alpha_{IIb}\beta_3$ activation, whereas SHP-2 negatively regulates platelet activation initiated by GpVI or CLEC-2.[146–150] PTEN is another Zn^{2+}-sensitive phosphatase with an IC_{50} of 0.59 nM. PTEN dephosphorylates PIP_3, reducing AKT activation resulting in negative regulation of collagen-induced platelet aggregation.[151] AKT phosphorylation in T-cells is abrogated by Zn^{2+} chelation using TPEN, but induced by pyrithione, an effect that was ineffective following siRNA knockout of PTEN.[152] Thus, the activation of PTPs by discrete rises of $[Zn^{2+}]_i$ may play a significant role in Zn^{2+}-mediated platelet activation.

Influence of $[Zn^{2+}]_i$ on the modulation of cyclic nucleotide levels

Intraplatelet concentrations of the cyclic nucleotides cAMP and cGMP are central to negative regulation of platelet activation.[153] Zn^{2+}-induced platelet aggregation is inhibited following treatment with the G_s-coupled prostanoid receptor agonists PGE_1 and PGI_2.[62,64] This suggests that Zn^{2+} modulates cAMP-mediated inhibition of platelet activation. Zn^{2+} has been shown to regulate both adenylate cyclase and phosphodiesterase (PDE) activity. For example, in PC12 cells, forskolin-induced rises of cytosolic cAMP were abolished by addition of 300 μM Zn^{2+} independently of PDE activity.[19] Furthermore, a greater reduction was observed in the presence of pyrithione, suggesting that this effect requires elevation of $[Zn^{2+}]_i$. Other studies suggest that Zn^{2+} alters the conformation of adenylate cyclase and impedes cAMP synthesis.[154] Zn^{2+} also modulates cGMP levels *via* interactions with PDEs.[155,156] Thus, Zn^{2+}-induced platelet activation may, in part, be regulated by interactions with PDEs and adenylate and guanylate cyclases.

Perspectives

Research into the role of Zn^{2+} in platelet behaviour has been limited. This is surprising, given the involvement of Zn^{2+} in platelet behaviour as evidenced by work on rodent and human platelets following dietary Zn^{2+} deficiency. There is clear evidence using both *in vivo* and *ex vivo* assays that dietary Zn^{2+} intake is inversely correlated with a bleeding diathesis. The work discussed herein indicates that Zn^{2+} is a modulator of platelet function and may contribute to pathophysiological thrombus formation.

Our working hypothesis suggests a model whereby vascular injury leads to localised release of Zn^{2+} from damaged and inflammatory cells (Fig. 1). This Zn^{2+} potentiates platelet activation in response to other agonists, such as exposed subendothelial collagen, ADP or thrombin. Autocrine platelet activation and adhesion promotes Zn^{2+} release from granules, further increasing $[Zn^{2+}]_o$. Intracellular Zn^{2+} modulates the activity of a variety of different enzymes, leading to integrin $\alpha_{IIb}\beta_3$ activation and granule release. Whether Zn^{2+} is also released from intracellular stores, analogous to that of Ca^{2+}, remains to be seen. Future studies are required to further elucidate platelet Zn^{2+} entry pathways, and the underlying signalling pathways that contribute to Zn^{2+}-induced platelet activation.

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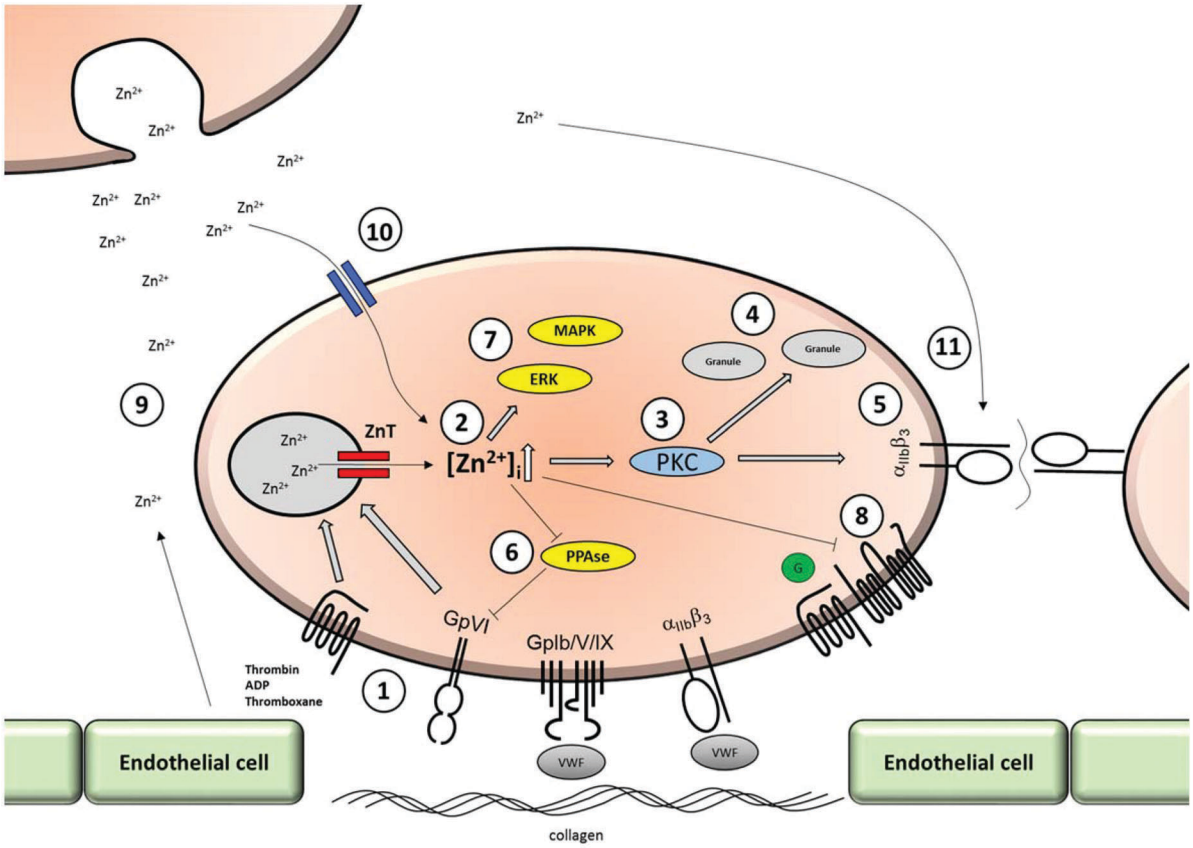

Fig. 1 Speculative model of the mechanisms and machinery that are influenced by Zn^{2+} during platelet activation. (1) Platelets respond to vascular injury by interaction with sub-endothelial matrix proteins (*i.e.* collagen and VWF), and by activation *via* soluble agonists (*i.e.* ADP, thrombin and thromboxane A_2). (2) Activation leads to Zn^{2+} release from intracellular stores into the platelet cytosol, *via* channels or ZnT transporters, leading to an increased $[Zn^{2+}]_i$. (3) $[Zn^{2+}]_i$ interacts with PKC, upregulating enzyme activity. PKC-mediated phosphorylation promotes granule release (4) and activation of integrin $\alpha_{IIb}\beta_3$ (5), which cross-links platelets *via* binding of fibrinogen, mediating platelet aggregation. (6) Inhibition of cytosolic protein phosphatases by $[Zn^{2+}]_i$ enhances tyrosine phosphorylation of platelet signalling proteins. (7) $[Zn^{2+}]_i$ activates protein tyrosine kinases (*e.g.* ERK), which regulate tyrosine phosphorylation events downstream of platelet activation. (8) $[Zn^{2+}]_i$ inhibits adenylate cyclase, thereby reducing cAMP levels and promoting platelet activation. (9) Zn^{2+} is released from damaged endothelial cells, sub-endothelial matrix and platelet granules, contributing to a localised increase in labile $[Zn^{2+}]_o$. (10) Extracellular Zn^{2+} gains access the cytosol *via* non-selective transporters and cation channels. (11) Zn^{2+} may also interact directly with integrin $\alpha_{IIb}\beta_3$ altering the activity and regulating platelet/platelet interactions leading to thrombus formation.

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