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INORGANIC POLYPHOSPHATE: A KEY MODULATOR OF INFLAMMATION

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Running title: Proinflammatory Functions of PolyP

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Abstract

Inorganic polyphosphate (PolyP) is a molecule with prothrombotic and proinflammatory properties in blood. PolyP activates the NF-κB signaling pathway, increases the expression of cell surface adhesion molecules and disrupts the vascular barrier integrity of endothelial cells. PolyP-induced NF-κB activation and vascular hyperpermeability are regulated by the mTORC1 and mTORC2 pathways, respectively. Through interaction with

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RAGE and $P2Y_1$ receptors, PolyP dramatically amplifies the proinflammatory responses of nuclear proteins. Moreover, PolyP-mediated activation of the contact pathway results in activation of the kallikrein-kinin system, which either directly or in cross-talk with the complement system induces inflammation in both cellular and animal systems. Thus, polyP is a novel therapeutic target for the treatment of metabolic and acute/chronic proinflammatory diseases, including severe sepsis, diabetes, cardiovascular disease and cancer. In this review, we discuss recent findings on the inflammatory properties of polyP and propose a model to explain the molecular mechanism of proinflammatory effects of this molecule in different systems.

Keywords: polyphosphates, inflammation, blood coagulation, platelets, thrombosis

Introduction

Inorganic polyphosphates (polyP) are linear polymers of from 3 to over 1,000 orthophosphate (p_i) residues that are linked together by ATP-like bonds [1]. Synthesis of phosphoanhydride bonds between phosphate residues in bacteria is catalyzed by polyP kinases (PPK). Most bacterial species have both PPK1 and PPK2. PolyP Kinase 1 (PPK1) is the principal enzyme in polyP synthesis in many bacterial species, which converts the terminal phosphate of ATP to polyP [2-4]. There is an ATP-binding pocket in the active site of the enzyme that probably accommodates the translocation of newly synthesized PolyP [5]. PPK1 catalyzes synthesis of polyP from ATP, but PPK2 favors synthesis of the nucleoside triphosphate, particularly GTP, by using polyP as phosphate reservoir [3]. The reverse reaction (synthesis of polyP from GTP) is >75-fold less favorable for the PPK2 enzyme [4]. PPK2 is also implicated in the virulence of some pathogens [3]. PolyP kinases 1 and 2 are absent in yeast and animals, and are new therapeutic targets for treatment of microbial diseases [3]. Degradation of polyP is mediated through the activity of exopolyphosphatases and endopolyphosphatases in cells. Exopolyphosphatases remove the terminal phosphate from polyP chain ends, whereas endopolyphosphatases hydrolyze polyP within the chain to partially digest the molecule [6-9].

PolyP in Bacteria

PolyP exerts different biological functions in bacteria. *E. coli* uses polyP to respond to nutrient deficiencies and environmental stresses. Amino acid starvation and low level of nitrogen are signals for regulation of polyP accumulation in *E. coli* [10-12]. Accordingly, *E. coli* cells deficient in polyP survive only for a few days after exposure to nutrient stress, oxidative damage and UV irradiation [13]. As a stable reservoir of P_i, polyP modulates phosphate metabolism in *E. coli* cells [14]. Furthermore, polyP is a polyanionic polymer and is a strong chelator of metal ions [15]. Chelation of metal ions with polyP may render those ions inactive in generating toxic radicals. Mn^{2+} is not an enzyme, but Mn^{2+}-bound to polyP is able to catalyze the dismutation of superoxide anion in *Lactobacillus plantarum* [15]. Complexes of Ca^{2+} with polyP in the membranes of competent cells induce physical changes in the membrane that facilitate DNA uptake in bacteria [16-18]. Due to its negative charge, PolyP also binds to cationic DNA-binding proteins (e.g., histones and nonhistone nuclear proteins) [19]. Such interactions could modulate gene expression in bacteria.

PolyP in eukaryotes

PolyP is found in eukaryotes from protists to mammalian cells [20]. In mammals, polyP has been found in nuclei, mitochondria, lysosomes and dense granules of platelets [21-23]. PolyP is also detected in granules of mast cells and basophils [24]. These granules are similar to acidocalcisomes, acidic organelles rich in PolyP and cations, first described in protists [25]. Recent results have established an important role for polyP in bone mineralization [26, 27], cell proliferation [28], apoptosis [29, 30], tumor metastasis [31, 32], blood clotting [33-40] and, of special interest in this review, in regulating inflammation [24, 30, 33, 41-45]. Some of the principles of polyP functions in blood coagulation will be covered in this review, but for detailed information, readers are referred to the reviews of J. H. Morrissey and colleagues [46-50].

PolyP in blood coagulation

Human platelets store high concentrations of polyP in dense granules, which can be released to circulation upon platelet activation [23]. Deficiencies in these granules significantly reduce the concentration of polyP in platelets, which leads to bleeding [51-53]. PolyP modulates the blood clotting cascade at different steps. It activates the contact pathway [33, 35, 46], accelerates the activation of factor V by factor Xa, thrombin or factor XIa [34-36]. It also acts as a cofactor for thrombin in factor XI activation and also enhances factor XI autoactivation [37]. Moreover, polyP incorporates into fibrin and stabilizes fibrin clot structure. Incorporation of polyP with the fibrin clot alters clot turbidity and generates the clots that are firmer and more resistant to fibrinolysis [35, 38, 39]. The other modulatory role of polyP in blood clotting is mediated through the inhibitory effect of this polymer on the anticoagulant function of tissue factor pathway inhibitor (TFPI) [34, 35]. TFPI is constitutively synthesized by endothelial cells and is a potent inhibitor of tissue factor-mediated coagulation. Consistent with the procoagulant functions of polyP, it has been shown that polyP inhibitors are new anticoagulant agents with fewer side effects than conventional counterparts and could be therapeutic drugs in treating arterial thrombosis [54-56].

PolyP in inflammation

Blood coagulation and inflammation are closely intertwined pathways [57]. Although the procoagulant function of polyP has been intensively investigated, the mechanisms of proinflammatory responses of polyP are poorly understood. In this section, we explore recently reported functions of polyP in inflammation and the molecular mechanisms underlying this process.

PolyP elicits proinflammatory responses through activation of NF-κB signaling

Recent studies on the proinflammatory functions of inorganic polyP show that polyP-70 (platelet-sized polyP) up-regulates the expression of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and E-selectin by activating the NFκB signaling pathway, enhancing the adhesion of monocytic THP-1 cells to polyP-stimulated endothelial cells [30]. These effects of polyP cannot be recapitulated by another anionic polymer, unfractionated heparin, suggesting that the proinflammatory effect of polyP on treated endothelial cells is specific and possibly is mediated through activation of a receptor-dependent signaling mechanism.

PolyP amplifies the proinflammatory effects of histone and HMGB-1

It has been shown that nuclear proteins including HMGB1 and histones, when secreted from activated immune cells, function as late-acting proinflammatory cytokines [58-60]. Elevated histone H4 levels in plasma correlates with poor prognosis and high mortality in severe sepsis and cancer [60, 61]. Consistent with its role in the pathogenesis of severe sepsis, pharmacologic inhibition of H4 improves survival in experimental models of endotoxemia, whereas infusion of H4 into mice is highly cytotoxic, causing death from multiple organ failure [60].

Proinflammatory functions of extracellular histones could in part be attributed to platelet activation and thrombin generation. Semeraro et al. showed that histone activated platelets display a procoagulant phenotype driving thrombin generation in a TLR2 and TLR4-dependent manner [43]. The effect of histones on thrombin generation is abrogated in the presence of polyP-targeted phosphatases, suggesting that thrombin generation in histone-treated platelet-rich plasma is driven by polyP. Moreover, it has been shown that polyP-70 (platelet-sized polyP) or polyP-700 (similar to size in bacteria) binds to both H4 and HMGB1 with high affinity and potently amplifies their proinflammatory signaling effects in cellular and *in vivo* models [41]. PolyP synergistically potentiates H4- and HMGB1-mediated vascular permeability, cell surface adhesion molecules expression, leukocyte migration and apoptosis.

These results clearly suggest that polyP potentiates the inflammatory functions of histones through at least three different mechanisms: 1) enhancing extracellular histone activities, 2) increasing histone-mediated thrombin generation and 3) amplifying proinflammatory responses of nuclear proteins through interaction with RAGE and P2Y1 purinergic receptors.

PolyP elicits proinflammatory responses through activation of mTOR

The mammalian target of rapamycin (mTOR) is a serine-threonine kinase that nucleates at least two distinct multi-protein complexes, mTOR complex-1 (mTORC1) and mTOR complex-2 (mTORC2) [62]. mTORC1 regulates cell growth and metabolism [63] whereas mTORC2 plays a critical role in reorganization of cytoskeletal structure and cell morphology [64, 65]. Both platelet- and bacterial-sized polyP (P-70 and -700 respectively), activate mTORC1 and 2 through activation of the PI3K/AKT and PLC/PKC/Ca^{2+} signaling pathways in endothelial cells [42]. The polyP-mediated NF-κB activation is regulated by mTORC1, whereas siRNA knockdown of rictor (mTORC2-specific component) but not raptor (mTORC1-specific component) abolishes the vascular barrier-disruptive effect of polyP, suggesting that the hyper-permeability function of polyP is mediated through mTORC2 activation [42]. These results can be recapitulated by boiled platelet releasate in the absence, but not in the presence, of the specific polyP inhibitor EcPPXc or alkaline phosphatase.

PolyP triggers inflammation through activation of the contact pathway

Bradykinin is a potent proinflammatory mediator that disrupts vascular barrier integrity and increases vascular leakage [66]. It has been shown that polyP activates the contact pathway, but that its efficacy depends on the polyP chain length, with bacterial-sized polyP (≥ 500mers) being several orders of magnitude more efficient than platelet-derived polyP (~60-100mers) [35]. PolyP-driven contact pathway activation (kallikrein-kinin system) potently triggers bradykinin generation and significantly increases vascular permeability in skin microvessels of mice that is abrogated in factor XII- or bradykinin B2 receptor ($B2R^{-/-}$)-deficient mice [33]. Contact system-mediated kinin release is a critical component of *E.coli*-induced sepsis and septic shock [67]. In agreement with these animal studies, Moreno-Sanchez et al. demonstrated that polyP is also present in acidocalcisomes-like granules in mast cells and basophils [24], which stimulates more bradykinin generation in plasma.

Bradykinin regulates vessel permeability and is a key agent in the swelling disorder hereditary angioedema (HAE) [68]. Although the molecular mechanism accounting for vascular hyperpermeability and swelling in HAE is not yet known, increased generation of bradykinin due to over-activation of the FXII-driven plasma contact system is observed in patients during the acute phase of the disease [68]. Binding of vasoactive peptide bradykinin to its receptors on endothelial cells induces the release of inflammatory and vasodilatory factors, including prostacyclin and nitric oxide, resulting in vasodilation and vascular leakage [69].

polyP regulates the inflammatory complement system

PolyP-mediated contact pathway activation could initiate the classical complement system, which enhances inflammatory responses, induces migration of phagocytic cells to infected areas and stimulates the adaptive immune response. Interestingly, polyP inhibits complement via the terminal pathway by destabilizing C5b, 6, thereby reducing the lytic capacity of the membrane attack complex in erythrocyte lysis assays [44]. Consistent with these findings, Wijeyewickrema et al. showed that polyP is a physiologic cofactor for the interaction between the serpin, C1 inhibitor (C1-INH), and the C1s, the initiating serine proteases of the classical pathway of complement system [45]. PolyP-induced C1-INH:C1s interaction suppresses C1s-mediated activation of the classical pathway in a polymer length- and concentration-dependent manner [45].

Conclusions

Recent studies suggest that in addition to modulation of coagulation, polyP can elicit potent proinflammatory responses in cellular and animal models. The proinflammatory signaling effect of polyP increases release of the proinflammatory mediator bradykinin, triggers vascular permeability, promotes leukocytes migration, activates the NF-κB pathway, induces expression of CAMs, amplifies proinflammatory signaling of nuclear cytokines (H4 and HMGB1) and links inflammation to activation of the metabolic regulatory mTOR signaling pathway. Mechanisms of polyP-mediated proinflammatory responses are presented in Fig 1.

This review summarizes the proinflammatory functions of polyP, but the detailed mechanisms in this process have yet to be defined. Recent studies clearly demonstrate that polyP is a key modulator of inflammation and polyP inhibitors might consequently have future utility as potent antiinflammatory agents for treating/preventing polyP-mediated inflammatory responses during infection, injury, and/or various other proinflammatory conditions.

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Disclosure of Conflict of Interests

The authors declare no competing financial interest.

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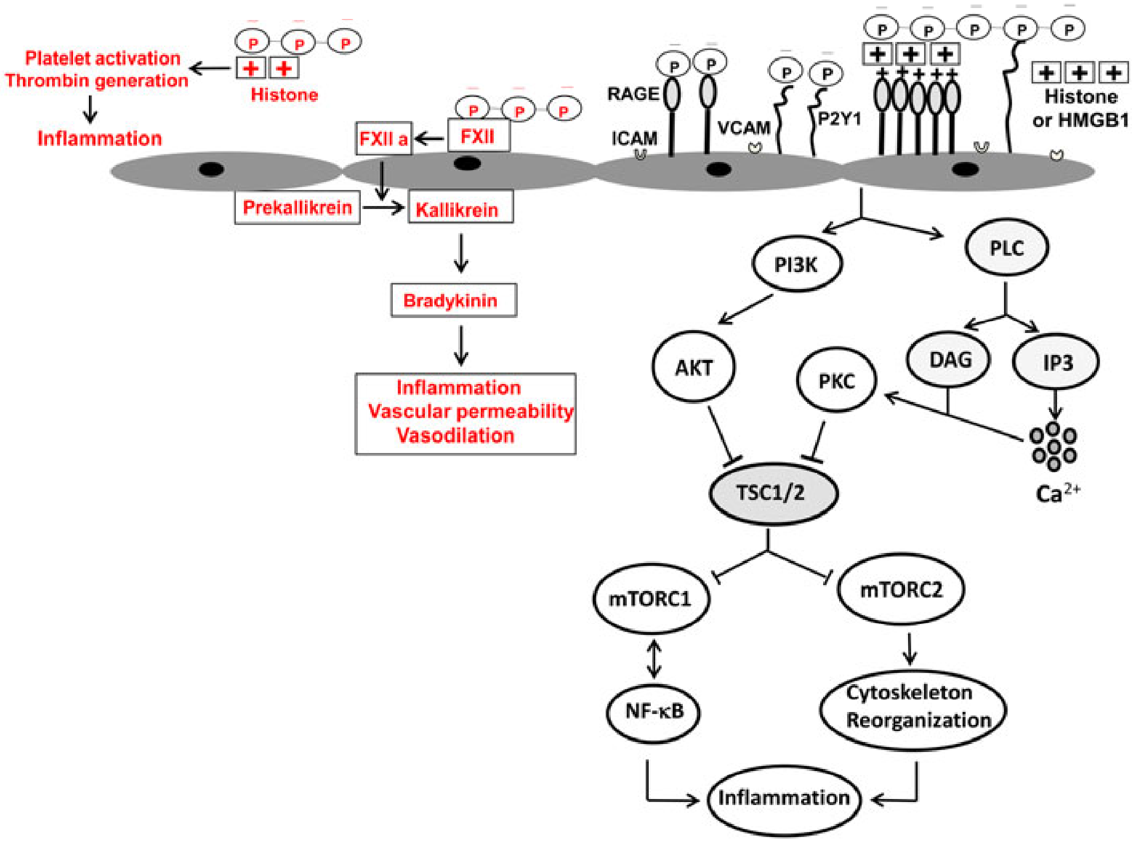
Figure Legend

Figure 1. Schematic representation of the mechanism of polyP-mediated proinflammatory signaling responses. PolyP elicits inflammatory responses through different mechanisms. 1) The interaction of the highly negatively charged polyP polymers with the positively charged residues of nuclear proteins neutralizes the basic charges of these residues, thus enhancing their affinity for the receptor. The polyP-loaded ligand interaction with RAGE, plus the interaction of polyP with P2Y1, results in the clustering of the oligomeric forms of RAGE that potentiates the inflammatory signaling of the nuclear proteins. 2) Moreover, binding of polyP to RAGE and $P2Y_1$ receptors triggers the PI3K/AKT and PLC/PKC/Ca^{+2} signaling pathways, thereby activating mTORC1 and mTORC2 through inhibition of the tumor suppressor TSC1/2 complex. PolyP mediates the phosphorylation-dependent activation of IKK-α/β, thereby activating NF-κB whereas the effect of polyP on cytoskeleton reorganization is mediated through mTORC2 activation. 3) PolyP-driven contact pathway activation (kallikrein-kinin system) potently triggers bradykinin generation and significantly increases vascular permeability and inflammation. 4) polyP potentiates inflammatory functions of histones by enhancing extracellular histone activities and increasing histone-mediated thrombin generation.



Platelet activation
Thrombin generation
Inflammation
Histone
FXII a
FXII
Prekallikrein
Kallikrein
Bradykinin
Inflammation
Vascular permeability
Vasodilation
RAGE
ICAM
VCAM
P2Y1
Histone
or HMGB1
PI3K
PLC
AKT
PKC
DAG
IP3
Ca2+
TSC1/2
mTORC1
mTORC2
NF-κB
Cytoskeleton
Reorganization
Inflammation