

AKT2-NADPH oxidase 2 signaling in thromboinflammation

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Abstract: 181 words

Total word count: 2,656 words

ABSTRACT

Purpose of review

Interactions between neutrophils and platelets contribute to the progression of thromboinflammatory disease. However, the regulatory mechanism governing these interactions is poorly understood. This review focuses on the crucial role of AKT2-NADPH oxidase 2 (NOX2) signaling in regulating neutrophil and platelet activation and their heterotypic interactions under thromboinflammatory conditions.

Recent findings

Growing evidence has shown that platelets, leukocytes, and blood coagulation need to be considered to treat thromboinflammatory disease in which inflammation and thrombosis occur concurrently. In addition to plasma proteins and intracellular signaling molecules, extracellular reactive oxygen species (ROS) produced from activated leukocytes could be an important factor in the pathophysiology of thromboinflammatory disease. Recent studies reveal that AKT2-NOX2 signaling has critical roles in Ca^{2+} mobilization, ROS generation, degranulation, and control of the ligand-binding function of cell surface molecules, thereby promoting heterotypic cell-cell interactions in thromboinflammation. These findings have provided novel insights into attractive therapeutic targets for the prevention and treatment of thromboinflammatory disease.

Summary

Recent discoveries concerning molecular mechanisms regulating neutrophil-platelet interactions have bridged some gaps in our knowledge of the complicated signaling pathways exacerbating thromboinflammatory conditions.

Keywords: Neutrophil, Platelet, AKT2, NOX2, ROS, thromboinflammation

INTRODUCTION

Neutrophils are mainly responsible for innate immunity, whereas platelets are critical for hemostasis. Studies using real-time *in vivo* imaging techniques have demonstrated that neutrophil-platelet interactions on the activated endothelium result in microvascular occlusion and tissue damage under thromboinflammatory conditions, including ischemia/reperfusion injury (1, 2), transfusion-related acute lung injury (3, 4), and sickle cell disease (4-6). Despite high mortality rates in patients with thromboinflammatory disease, only a limited number of therapies are currently available. Thus, understanding the underlying mechanisms mediating neutrophil-platelet association might be of considerable importance for developing novel therapies. In this review, we will discuss the emerging understanding of the role for neutrophil and platelet AKT2-NOX2 signaling in the pathophysiology of thromboinflammation.

NEUTROPHIL-PLATELET INTERACTIONS IN THROMBOINFLAMMATION

Neutrophils are the first blood cells to be recruited to sites of inflammation. Initial neutrophil rolling over the inflamed endothelium is mediated by the interaction between selectins and their ligands (7). Subsequently, activated neutrophil integrins, mainly α L β 2 and α M β 2, bind to their ligands such as intercellular adhesion molecule 1 and result in neutrophil adhesion and crawling. α 4 β 1 in mouse neutrophils and α 9 β 1 in human neutrophils also participate in neutrophil adhesion and transmigration through the interaction with vascular cell adhesion molecule 1 (8-10). In the presence of chemoattractants or cytokines, crawling neutrophils transmigrate across the inflamed endothelium. Under sterile inflammatory conditions, adherent and crawling neutrophils exacerbate disease progression by releasing proinflammatory cytokines, interacting with platelets, and/or obstructing the vascular lumen. Unlike neutrophil-mediated inflammation occurring under low shear stress, platelet-mediated arterial thrombosis occurs at sites of endothelial cell (EC) disruption or activation under high shear stress. Following arterial injury, platelets rapidly adhere to collagen and von Willebrand factor through the interaction with their receptors, leading to platelet activation and platelet-platelet aggregation. In addition to homotypic cell-cell interaction, adherent platelets also support neutrophil rolling and adhesion (11).

Regardless of blood shear, the receptors and ligands required for neutrophil-platelet association are similar. As key surface molecules, neutrophil P-selectin glycoprotein ligand-1 (PSGL-1) and α M β 2 integrin interact with platelet P-selectin and glycoprotein Iba (GPIba), respectively. Furthermore, neutrophil α M β 2 and α L β 2 integrins bind to platelet junctional adhesion molecule 3 and intercellular adhesion molecule-2, respectively. Readers are referred to recent reviews on this subject (12, 13). Neutrophil-

platelet interactions in vessels participate in a myriad of pathophysiological events such as neutrophil transmigration (4), EC permeability (14), vascular occlusion (2, 5), sterile tissue injury (2), and thrombosis (15).

AKT2 AND NOX2 SIGNALING IN NEUTROPHIL FUNCTION

Stimuli, such as bacteria-derived N-formyl peptides (G-protein coupled receptor ligands) and Fc γ receptor ligation, activate neutrophil AKT (Ser/Thr protein kinase B) which regulates numerous cellular processes (16). Full activation of AKT requires phosphorylation of two critical residues, Thr308 in the activation loop by phosphoinositide-dependent kinase-1 and Ser473 in the C-terminal hydrophobic region by the mammalian target of rapamycin complex 2. In addition, Ser477 and Thr479 were identified as novel phosphorylation sites which promote AKT activation (17). Activated AKT phosphorylates Ser/Thr residues on numerous substrates such as glycogen synthase kinase 3 β (16). Among three AKT isoforms, human and mouse neutrophils express AKT1 and AKT2. A previous study revealed that AKT2, but not AKT1, translocates to the leading edge of migrating neutrophils and phosphorylates p47^{phox}, a key cytosolic component of the NOX2 complex (18). Another study showed that AKT1 deletion enhances phosphorylation of signal transducer and transcription activator 1 in lipopolysaccharide-stimulated neutrophils and promotes bactericidal activity and acute lung injury, suggesting its negative role in neutrophil function (19). Using intravital microscopy in mice deficient in each AKT isoform, we demonstrated that AKT2, but not AKT1 and AKT3, is important for neutrophil-EC and neutrophil-platelet interactions during TNF- α -induced vascular inflammation (5). Neutrophil AKT2 had no effect on

PSGL-1 shedding and neutrophil rolling during inflammation but controlled the membrane translocation (degranulation) and activation of $\alpha M\beta 2$ integrin required for the interaction of neutrophils with both ECs and platelets (Figure 1). Current investigations suggest that AKT2-mediated Ca^{2+} mobilization may be involved in regulatory functions (5).

Several NOXs including NOX1, NOX2, NOX4, and NOX5 are expressed in human intravascular cells, and NOX-produced ROS have both homeostatic and pathological functions (20). Among the isoforms, NOX2 is dominant in neutrophils, as shown in patients with chronic granulomatous disease (21) and NOX2-deficient mice (22). The NOX2 complex has two membrane molecules, gp91^{phox} and p22^{phox}, and cytosolic regulatory components, p47^{phox}, p67^{phox}, p40^{phox}, and Rac1/2 (Figure 1). During neutrophil activation, p47^{phox} is phosphorylated at 8 to 9 Ser residues by several protein kinases, including AKT2 and protein kinase C (18, 23). Phosphorylation and membrane translocation of p47^{phox} is required for the recruitment of other cytosolic components and the enzymatic activity of NOX2. In addition to the phagocytic function, ROS produced from neutrophil and monocyte/macrophage NOX2 contribute to the pathophysiology of cardiovascular diseases, such as atherosclerosis, restenosis, and hypertension (20). Furthermore, NOX2-produced ROS modulate a variety of cellular functions including endoplasmic reticulum stress, apoptosis, and autophagy (20). The differential roles of ROS in cellular functions are likely to depend on the spatiotemporal dynamics of NOX-derived ROS generation.

Our recent studies using mice deficient in NOX2 and adoptive cell transfer demonstrated that NOX2 deletion does not affect neutrophil adhesion to TNF- α -

inflamed endothelium but abrogates neutrophil-platelet association on the vessel wall (2). Interestingly, NOX2-produced ROS are important for binding of talin1 to the cytoplasmic domain of the $\beta 2$ integrin subunit and the ligand-binding activity of $\alpha M\beta 2$ integrin following neutrophil activation, but do not alter $\alpha M\beta 2$ membrane translocation (2). NOX2 deletion has no effect on intracellular Ca^{2+} release but impaired Ca^{2+} influx through store-operated Ca^{2+} entry in stimulated neutrophils. Although it has been controversial whether neutrophils isolated from patients with chronic granulomatous disease exhibit a defect in cytosolic Ca^{2+} levels during cell activation or phagocytosis (24, 25), our mouse studies provide evidence that AKT2-mediated intracellular Ca^{2+} release contributes to the exocytosis-mediated membrane translocation of $\alpha M\beta 2$ integrin during neutrophil activation, whereas Ca^{2+} influx promoted by ROS produced from AKT2-activated NOX2 is involved in integrin activation. Furthermore, consistent with previous reports showing that H_2O_2 can oxidize Cys residues on phosphatases and impair their activities (26), we found that NOX2-generated ROS attenuate the activity of phosphatase and tensin homolog and conversely enhance the activity of AKT and its downstream kinases upon agonist stimulation (2). Therefore, NOX2-produced ROS are required to activate a feed-forward mechanism of the AKT2 signaling pathway by inhibiting the activity of phosphatases. A recent report showed that neutrophil-platelet interactions can induce neutrophil extracellular trap (NET) formation under non-septic conditions (27). Since NOX2-produced ROS are critical for NETosis (28), it would be of interest to study the role of AKT2-NOX2 signaling in NETosis.

AKT2 AND NOX2 SIGNALING IN PLATELET FUNCTION

All three AKT isoforms are present in human and mouse platelets and have overlapping but distinct roles during platelet activation (29-31). Although it is not a dominant isoform in platelets, AKT1 is crucial for Ca^{2+} mobilization, granule secretion, and platelet aggregation following stimulation with low concentrations of thrombin and participates in hemostasis (29). We and others showed that AKT2 deletion impairs granule secretion, Ca^{2+} release from intracellular stores but not Ca^{2+} influx, fibrinogen binding, and platelet aggregation in response to low concentrations of thrombin and thromboxane A2 and reduces arterial thrombosis without affecting the bleeding time (5, 30). Although earlier work reported the absence of AKT3 in platelets (32), a later study identified AKT3 as a major AKT isoform that promotes granule secretion and platelet aggregation after low concentrations of thrombin and thromboxane A2 and participates in *in vivo* thrombus formation (31). Using a platelet-neutrophil aggregation assay under conditions mimicking blood shear, we reported that all AKT isoforms in platelets regulate the interaction with neutrophils by promoting the surface exposure of P-selectin which binds to neutrophil PSGL-1 (5). Nevertheless, this study using isolated cells could not explain our findings that AKT2, but not AKT1 or AKT3, knockout mice exhibited a remarkable reduction in neutrophil-platelet interactions during vascular inflammation (5). The cell aggregation assay using AKT1- or AKT2-null neutrophils further showed that neutrophil AKT2 enhances the ligand-binding activity of $\alpha\text{M}\beta 2$ integrin, a counter receptor for platelet GPIb α , and promotes neutrophil-platelet aggregation (5). Therefore, these results suggest the contribution of both platelet and neutrophil AKT2 to the cell-cell interaction. Since *AKT2* gene is associated with platelet hyperreactivity in genome-

wide meta-analyses (33), these studies strongly suggest that AKT2 could be an attractive therapeutic target to treat thromboinflammatory disease.

Compared to neutrophil NOX2, platelet NOX1 and NOX2 produce low amounts of ROS. A study using a NOX1 inhibitor and NOX2 knockout mice suggested that platelet NOX1, but not NOX2, is key for ROS production following stimulation with collagen-related peptide (CRP), a GPVI ligand and that neither NOX1 nor NOX2 regulates CRP-induced granule secretion and platelet aggregation (34). However, using mice deficient in NOX1 or NOX2, Delaney et al. demonstrated a distinct role for NOX1 and NOX2 in platelet function: both NOX1 and NOX2 promote platelet activation and aggregation induced by a low concentration of thrombin, whereas only NOX2 is crucial for platelet functions following stimulation with CRP (35). The discrepancy between the two studies may result from the concentration of agonists used and off-target effects of the NOX1 inhibitor. Interestingly, only platelet NOX2 has an important role in laser-induced arteriolar thrombus formation without prolonging tail bleeding times (35). Using intravital microscopic studies in NOX2 knockout mice combined with adoptive cell transfer, we reported that platelet NOX2 is also important for neutrophil-platelet interactions during TNF- α -induced vascular inflammation by promoting P-selectin exposure and the ligand-binding function of GPIIb α (Figure 1) (2). These results indicate that the low amount of platelet NOX2-mediated ROS is still important for the progression of thromboinflammation, and suggest that the local redox environment is crucial for neutrophil-platelet interactions.

CLINICAL IMPLICATIONS

1. Ischemia reperfusion (I/R) injury

Animal models of stroke induced by middle cerebral artery occlusion have been utilized to understand the pathophysiological mechanism of ischemic stroke. During ischemia, blood cells, such as platelets and leukocytes, adhere to the activated endothelium. Reperfusion injury following ischemia induces leukocyte transmigration into the brain parenchyma, resulting in tissue damage due to the release of granular molecules and the production of ROS and cytokines (36). Inhibition or deletion of GPIIb/IIIa and α M β 2 integrin, but not α IIb β 3, reduces infarct volumes and improves neurological status following I/R injury in animals (37, 38), suggesting that both platelets and leukocytes, but not platelet aggregation per se, have a pathological role in ischemic stroke. Because ROS-mediated reperfusion injury is crucial for disease progression, the role of NOXs in stroke has been evaluated (39). A study using NOX2 bone marrow chimeric mice suggested that ROS generated from blood cell NOX2 are responsible for tissue damage during ischemic stroke (40). Our study showed that platelet and neutrophil NOX2-produced ROS control the function of cell surface molecules essential for neutrophil-platelet interactions, thereby participating in the pathophysiology of hepatic I/R injury, another model of thromboinflammatory disease (2). NET initiates inflammatory responses and induces organ damage during hepatic I/R injury (41). Since ROS generated from neutrophil NOX2 are required for NET formation (28), these results support the long-term premise that NOX2 could be a therapeutic target for thromboinflammatory disease (21). However, it remains unknown whether patients with chronic granulomatous disease are protected from ischemic stroke and other thrombotic diseases.

Although AKT has been speculated as a signaling molecule in the pathogenesis of ischemic stroke, little information is available to define a direct role for AKT. A study using AKT1 knockout mice revealed that AKT1 does not affect the infarct induced by middle cerebral artery occlusion, presumably due to the compensatory effect of other AKT isoforms in the knockout mice (42). However, lentivirus-mediated overexpression of constitutively active AKT1 or AKT3 diminished neuronal cell death after ischemia-induced stroke in rats (43). Future studies are needed to determine the precise role for each AKT isoform in the pathophysiology of stroke.

2. Sickle cell disease (SCD)

SCD is an inherited blood disorder which results in hemolysis of red blood cells, oxidative stress, EC activation, and chronic inflammation (44). Vaso-occlusive pain crisis (VOC), a hallmark of SCD, is mediated by intravascular cell-cell aggregation and increases mortality in SCD patients. Although stem cell therapy is a potential cure for the patients, there are major obstacles such as the lack of suitable donors and graft-versus-host disease (45). A recent preclinical study using CRISPR/Cas9 demonstrated efficient correction of Glu6Val mutation in CD34⁺ hematopoietic stem/progenitor cells derived from SCD patients (46), which requires future clinical studies to evaluate the benefit and risk of the genome editing therapies. To treat acute VOC, many drugs blocking platelet and neutrophil functions are in clinical trials (44). Although hydroxyurea is currently the only FDA-approved drug treatment for SCD, the patients undergoing hydroxyurea therapy still suffer from VOC. Recent clinical studies with rivipansel (a pan selectin inhibitor) or crizanlizumab (an antibody against P-selectin) demonstrated that

inhibition of the leukocyte-EC interaction attenuates VOC in SCD patients (47, 48). We reported that the basal level of AKT phosphorylation is enhanced in neutrophils and platelets isolated from SCD patients, compared with those from healthy donors and that treatment with an AKT2 specific inhibitor attenuates aggregation of platelets and neutrophils of SCD patients *in vitro* and blocks platelet-neutrophil aggregation in microvessels of TNF- α -challenged SCD mice, improving blood flow rates (5). Importantly, co-administration of hydroxyurea and an AKT2 specific inhibitor increases the plasma level of nitric oxide, which is protective against oxidative stress, and inhibits AKT2 phosphorylation in neutrophils and platelets, thereby efficiently reducing cell-cell aggregation in vessels and improving survival in TNF- α -challenged SCD mice (6). Similar beneficial effects were also observed after oral administration of hydroxyurea and ARQ 092, a highly-selective, orally-available AKT inhibitor in SCD mice (49). Since heme derived from the hemolysis of red blood cells induces AKT phosphorylation in neutrophils (50), our recent finding warrants further clinical studies of this drug to determine whether AKT specific inhibitors could provide novel therapies for the treatment of VOC in SCD patients or a supplement to hydroxyurea therapy. Cell-free hemoglobin binds and consumes nitric oxide (51). Further, extracellular heme induces NETosis (52) and activates the inflammasome, presumably through NOX2-generated ROS in leukocytes (53). Although apocynin, a non-specific NOX inhibitor, reduces ROS-mediated oxidative stress in SCD mice (54), future studies are needed to determine whether NOX2 is the major source of ROS generation in SCD patients and participates in VOC.

3. Transfusion-related acute lung injury (TRALI)

TRALI is the major cause of mortality related to blood transfusion, but there is no effective therapy to treat these patients (55). Disruption of the lung endothelial barrier by activated neutrophils and inflammatory responses are critical for initiating TRALI. In addition, growing evidence shows that neutrophil-platelet interactions may contribute to disease progression. Studies using mice challenged with both lipopolysaccharide and an anti-major histocompatibility complex I antibody revealed that deficiency in $\alpha M\beta 2$ integrin or inhibition of PSGL-1 as well as depletion of platelets or neutrophils improved survival (4). Treatment of mice with aspirin reduced TRALI and mortality, but inhibition of $\alpha M\beta 2$ integrin had no beneficial effect (3, 56). Furthermore, NETs were observed in the plasma and lungs of humans and mice with TRALI (57), suggesting that targeting NETosis may be a novel therapy for the treatment of TRALI. Consistent with the finding that ROS are critical for NETosis (28), NOX2-generated ROS contribute to the progression of TRALI (58). It would be of interest to investigate the role for AKT2-NOX2 signaling in the pathophysiology of TRALI.

CONCLUSION

Multiple groups have studied the mechanisms regulating neutrophil-platelet interaction under different disease conditions. In this review, we summarized the emerging understanding of AKT2-NOX2 signaling in neutrophil and platelet activation and the cell-cell interaction under thromboinflammatory conditions. Recent studies have raised several questions: 1) Do EC AKT and NOX-generated ROS have a role in neutrophil-platelet interactions in thromboinflammatory disease? 2) Are other NOX

isoforms in intravascular cells important for neutrophil-platelet interactions? 3) Do ROS act as signaling molecules or directly oxidize neutrophil and platelet receptors? Understanding the underlying mechanisms of neutrophil-platelet interaction would help identify potential therapeutic targets for the prevention and treatment of thromboinflammatory disease.

Key points

- Neutrophil-platelet interactions participate in the progression of thromboinflammatory disease.
- AKT2 and NOX2-generated ROS control the function of neutrophil and platelet surface molecules required for the cell-cell interaction.
- Specific inhibitors of AKT and NOX2 could be potential therapies for the treatment of thromboinflammatory disease.

Acknowledgement

None.

Financial support and sponsorship

This work was supported by grants from the National Institutes of Health, American Society of Hematology, and American Heart Association.

Conflicts of interest

There are no conflicts of interest.

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Figure legends

Figure 1. AKT2-NOX2 signaling regulates neutrophil-platelet interactions in thromboinflammation. During neutrophil activation, phosphorylated and activated AKT2 (AKT2-p) promotes Ca^{2+} release and the membrane translocation and activation of $\alpha\text{M}\beta 2$ integrin. Furthermore, AKT2 phosphorylates p47^{phox} and activates the NOX2 complex, producing ROS. Neutrophil NOX2-generated ROS affect Ca^{2+} influx, and H_2O_2 ,

a cell-diffusible ROS, triggers neutrophil NET formation and inhibits the activity of phosphatases, activating a feed-forward mechanism to amplify AKT2 signaling. During platelet activation, activated AKT2, along with other AKT isoforms, increases P-selectin exposure by degranulation. Platelet NOX2 generates ROS upon glycoprotein VI (GPVI) or G-protein coupled receptor (GPCR) agonist stimulation and regulates intracellular Ca^{2+} release and the ligand-binding function of GPIb α , thereby enhancing neutrophil-platelet interactions.