ROLE OF REACTIVE OXYGEN SPECIES IN PLATELET FUNCTION IN NORMAL STATES AND CHRONIC MYELOPROLIFERATIVE DISORDERS

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Platelets play a crucial role in physiological and pathophysiological processes such as primary hemostasis and arterial thrombosis. Growing evidence indicates that reactive oxygen species, mainly superoxide generated by transmembrane NAD(P)H oxidase, have a key regulatory role in platelets. They mediate the intracellular signaling pathways activated by growth factors through tyrosine kinase receptors and the bidirectional signaling through integrins, influencing thus platelet activation and subsequent aggregation. In chronic myeloid leukemia, the BCR-ABL tyrosine kinase receptor is constitutively active, kinase inhibitors being currently used as anti-mieloproliferative drugs.

Key words: reactive oxygen species, platelet, NAD(P)H-oxidase, signaling, chronic myeloid leukemia.

INTRODUCTION

Platelets (thrombocytes) are circulating cells in the blood that have a major role in primary hemostasis, a process that maintains the integrity of the circulatory system after vascular damage. Circulating platelets are recruited to the site of injury and the platelet activation cascade is initiated (1). It includes receptor-mediated tethering to the endothelium, rolling, adhesion and aggregation, and results in the formation of thrombus. In the repair process, the initial adhesion of platelets to the extracellular matrix of the vessel wall is amplified by action of the agonists thrombin, ADP and thromboxane A_2, which activate platelets, resulting in increased Ca^{2+} level intracellularly and platelet aggregation. When the regulatory mechanisms of hemostasis are overwhelmed, a pathologic process termed thrombosis occurs (2). Platelets play a key role in thrombotic events associated to acute coronary syndromes and cerebrovascular events (3–5).

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Reactive oxygen species (ROS) are diffusible and short-lived molecules that have a major role in the regulation of platelet activation (6). In turn, activated platelets have the capability to synthesize ROS, which are able to modify platelet function (7). Intracellular ROS can also contribute to the initiation and promotion of carcinogenesis. Many studies in the latest decade have focused on the deciphering the mechanisms by which exogenous stimuli induce intracellular ROS generation and on the roles of ROS in mediating specific signaling pathways.

REACTIVE OXYGEN SPECIES FORMATION

ROS are generated in biological systems by oxygen reduction in electron transfer processes. Some of ROS are oxygen-derived free radicals, *i.e.*, chemical species possessing an impaired electron in the outer shell of the molecule. They are either positively or negatively charged or electrically neutral. The main ROS generated in the body are the superoxide anion radical ($O_2^{\bullet -}$), the hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\bullet}$); they are successively formed in the following reactions:

$$O_2 + e^- \rightarrow O_2^{\bullet -}$$

$$O_2^{\bullet -} + e^- + 2H^+ \rightarrow H_2O_2$$

$$H_2O_2 + e^- \rightarrow OH^- + OH^{\bullet}$$

$O_2^{\bullet -}$ is central to ROS chemistry because it may be converted into other physiologically relevant ROS by enzymatic or non-enzymatic reactions. It generates very toxic ROS through the following reactions (8):

a) Superoxide dismutation in the presence of superoxide dismutase (SOD):

$$2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2$$

b) Haber-Weiss reaction:

$$O_2^{\bullet -} + H_2O_2 \rightarrow OH^{\bullet} + OH^- + O_2$$

$O_2^{\bullet -}$ can react with nitric oxide radical ($NO^{\bullet}$) to form peroxinitrite anion ($ONOO^-$), a powerful oxidant and also a nitrating agent:

$$O_2^{\bullet -} + NO^{\bullet} \rightarrow ONOO^-$$

Theoretically, both SOD and $NO^{\bullet}$ compete for $O_2^{\bullet -}$ scavenging, but the rate of SOD-catalysed conversion is only one-third of the reaction rate of $O_2^{\bullet -}$ with $NO^{\bullet}$.

H_2O_2 also serves for production of more detrimental ROS, such as $OH^{\bullet}$ formed according to the Fenton reaction (the iron-catalysed Haber-Weiss reaction):

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + OH^{\bullet}$$

Hypochlorous acid (HOCl) is generated by the action of neutrophil myeloperoxidase on H_2O_2. The plasma level of myeloperoxidase has a predictive value for coronary thrombotic events. In the presence of catalase, H_2O_2 is degraded to H_2O. Glutathione peroxidase (GPx) also exerts an antioxidant function; it catalyzes a reaction that degrades H_2O_2 by oxidizing reduced glutathione (GSH) to its disulfide form (GSSG).

$OH^{\bullet}$ is the most reactive and destructive oxidising radical. It reacts with most biomolecules at controlled diffusion rates, so the reactions with biomolecules occur immediately. $OH^{\bullet}$ is several orders of magnitude more reactive towards cell constituents than $O_2^{\bullet-}$ and H_2O_2.

ROS formation occurs both in normal physiological processes and in abnormal conditions of the body. In normal states, action of ROS is physiological and protective. When ROS are produced in excess amounts and overwhelm the antioxidant defence mechanisms of the body, they are dangerous. This condition is called "oxidative stress" and can cause cell injury by DNA fragmentation, lipid peroxidation of cell membranes and cross-linking of proteins (2, 9).

REACTIVE OXYGEN SPECIES AND PLATELET PHYSIOLOGY

ROS play different roles in platelet physiology. The redox-sensitive pathways control platelet activation in response to thrombin or collagen stimulation and mediate various cell functions including growth, migration, differentiation and apoptosis. The exogenously produced ROS, *i.e.*, ROS released by endothelial cells, vascular smooth muscle cells and fibroblasts within the vessel wall, can also affect platelet activation (10, 11).

GENERATION OF REACTIVE OXYGEN SPECIES IN PLATELETS

Nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) oxidase is the predominant source of ROS in platelets (12). It is responsible for both basal and stimulated ROS production, by catalyzing oxygen reduction using NADH or NADPH as the electron donor:

$$NAD(P)H + 2O_2 \rightarrow NAD(P)^+ + H^+ + 2O_2^{\bullet-}$$

$O_2^{\bullet-}$ is then metabolized to H_2O_2, and both of these ROS serve as second messengers to activate multiple intracellular signaling pathways.

NAD(P)H oxidases are a broad family of enzymes, originally described in phagocytes, and found in many cell types. They are multiprotein complexes of various compositions, depending on the cell type, and consist of two membrane-bound subunits (the catalytic subunit Nox and the small subunit p22phox) and

potentially three cytosolic subunits: Rac1 (Rac2 in phagocytes), p47phox and p67phox. All NAD(P)H oxidases require p22phox for activity. The Rac subunit possesses guanosine triphosphatase (GTPase) activity that converts GTP to GDP. The cytosolic subunits are recruited to the membrane-bound Nox/p22phox complex upon activation (Figure 1).

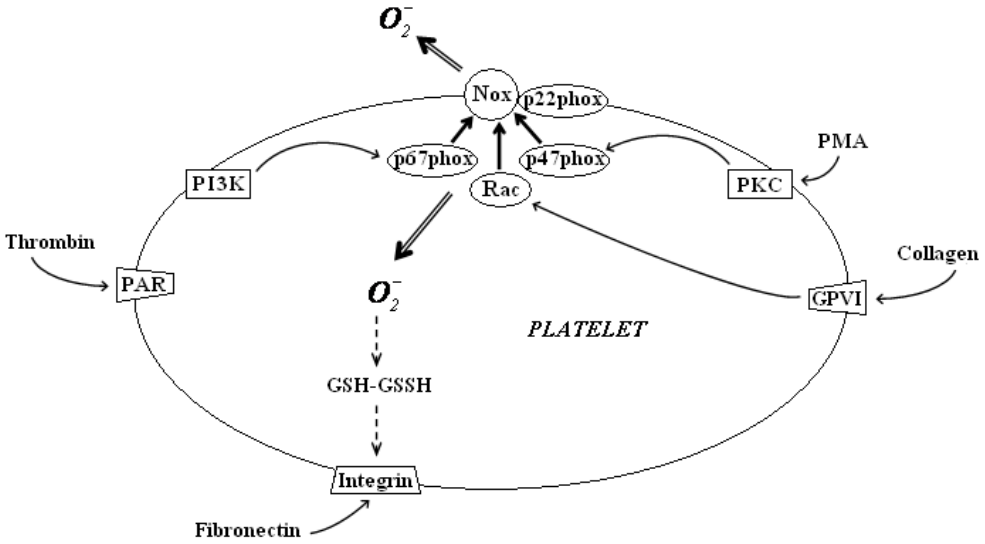

Fig. 1. – Generation of reactive oxygen species in platelets by NAD(P)H oxidase (Nox). *Abbreviations*: PAR=protease-activated receptor, PI3K=phosphatidylinositol 3-kinase, GPVI=glycoprotein VI, PKC=protein kinase C, and PMA=phorbol 12-myristate 13-acetate.

Agonists such as thrombin and collagen stimulate NAD(P)H oxidase and induce platelet activation. They generate ROS both acutely (minutes) for signaling events and chronically (hours) to enhance ROS production. The mechanism of NAD(P)H oxidase activation involves stimulus-induced activation and translocation of p47phox, p67phox and Rac1 subunits to the membrane, followed by interaction with p22phox and Nox subunits (13). The NAD(P)H oxidase activity is regulated by protein disulfide isomerase (PDI), a thiol-disulfide oxidoreductase that associates with Nox subunit. PDI inhibitors diminish basal and agonist-stimulated activity of NAD(P)H oxidase.

Thrombin is generated at the sites of vascular injury from circulating prothrombin. It is a serine protease that converts soluble fibrinogen into insoluble strains of fibrin and represents the most potent platelet activator. Thrombin promotes platelet activation by activating the protease-activated receptor (PAR1 in humans, PAR4 in mice), a G-protein coupled receptor that activates in turn phosphatidylinositol 3-kinase (PI3K), generating translocation of p67phox subunit

and regulating thus NAD(P)H oxidase-dependent release of $O_2^{\bullet-}$ (14). Thrombin induces both intracellular and extracellular ROS production. Intracellular thrombin-induced ROS generation is inhibited by NAD(P)H oxidase inhibitors diphenylene iodonium (DPI) and apocynin, by cyclooxygenase inhibitor acetylsalicylic acid and by $O_2^{\bullet-}$ scavengers.

Thrombin-activated platelets release potent platelet agonists such as ADP, serotonin and thromboxane A_2 (TXA_2). ADP strongly activates platelets in an autocrine and paracrine fashion (15). Platelet activation by ADP is mediated by the G protein-coupled receptors $P2Y_1$ and $P2Y_{12}$ (16). TXA_2 is synthesized by activated platelets from arachidonic acid through the cyclooxygenase pathway and it can diffuse across the membrane and activates other platelets. By its action, the platelet membrane releases the second messengers inositol triphosphate (IP_3) and diacylglycerol (DAG). DAG activates intracellular protein kinase C (PKC) and IP_3 increases the release of Ca^{2+} from the endoplasmic reticulum. By activating other platelets, these agonists amplify the signals for thrombus formation (17).

Collagen stimulation of platelets occurs through the glycoprotein VI, the major collagen receptor on platelets. This glycoprotein belongs to the immunoglobulin superfamily and possesses tethering function (18). Collagen binding induces only intracellular ROS production. Platelet $O_2^{\bullet-}$ production following collagen stimulation occurs with a delay of 3 to 5 min, so the involvement of platelet $O_2^{\bullet-}$ in the initial steps of aggregation may be minimal. After binding to the collagen component of the extracellular matrix in the vessel wall, platelets are activated and recruit other circulating platelets, contributing to thrombus growth.

When the phorbol ester phorbol 12-myristate 13-acetate (PMA) is used to stimulate platelets, activation of the protein kinase C (PKC) receptor occurs, resulting in p47phox recruitment to the plasma membrane and a rapid release of ROS. The released ROS activate the non-receptor protein tyrosine kinase c-Src, which subsequently orchestrates the signaling in lipid rafts of the plasma membrane.

$O_2^{\bullet-}$ is able to decrease the ratio between GSH and GSSG, thus modifying the thiol groups in proteins. As the GSH/GSSG ratio is high intracellularly and these thiols exist in a reduced form, a decreased GSH/GSSG ratio increases platelet sensitivity to activating agents (19).

$O_2^{\bullet-}$ can activate platelets directly or by decreasing the threshold for platelet activation. It can react with platelet or endothelium-derived $NO^{\bullet}$, which is a potent inhibitor of platelet activation. The resulted $ONOO^-$ nitrates the aromatic residues in proteins, most notably tyrosine. Nitrotyrosine could serve as a signaling molecule and was detected in many pathological conditions. By decreasing the bioavailability of $NO^{\bullet}$, $ONOO^-$ has a major role in thrombus formation. It is also a highly reactive molecule that exerts additional effects on platelets. Direct administration of $ONOO^-$ to platelets inhibits the aggregation induced by ADP, thrombin and collagen.

There are also other potential sources for ROS from platelets: xanthine oxidase, lipooxygenase, arachidonic acid-dependent phospholipase, uncoupled endothelial $NO^{\bullet}$ synthase and mitochondrial respiration. Plasma membrane depolarization also stimulates $O_2^{\bullet -}$ production in platelets and endothelial cells, while hyperpolarisation prevents platelet ROS release and reduces the *in vitro* adhesion of platelets to endothelial cells (7, 20).

Inhibitors of xanthine oxidase, of PI3K and PKC enzymes, and of the glutathione pathway facilitate reduction of the ROS level. Drugs including dexamethasone, prednisolone, captopril, quinapril, vitamin C and transresveratrol (phenolic antioxidant from red wine) also reduce the platelet $O_2^{\bullet -}$ generation (21, 22).

REACTIVE OXYGEN SPECIES AND SIGNAL TRANSDUCTION

Activation of NAD(P)H oxidase enzyme contributes to the stimulation of signaling pathways that modulate cell proliferation and thrombosis (23, 24). For ROS to serve as signaling molecules, both their production and removal must be regulated by agonists. But the mechanisms by which ROS modify and activate signaling proteins are not fully elucidated. These proteins are redox-regulated by S-glutathiolation, S-nitrosylation and disulfide modifications (25, 26).

ROS-dependent intracellular signaling includes modulation of protein kinases, protein phosphatases, transcription factors and genes (27, 28). ROS and Ca^{2+} cooperate in the signaling pathways that keep the balance between cell proliferation and apoptosis (19).

Signaling Through Protein Kinases

Phosphorylation of proteins by kinases (phosphorylases) is an important mechanism for the regulation of enzyme activity in signal transduction. Successive phosphorylations of specific kinases in a signaling pathway result in phosphorylation of mitochondrial and nuclear proteins, followed by biological responses such as growth, survival, apoptosis and migration. Platelet-generated ROS, particularly $O_2^{\bullet -}$ and H_2O_2, act as secondary messengers in these pathways and activate many serine/threonine and tyrosine protein kinases (29, 30)

Human platelets release a potent mitogenic stimulus, the platelet-derived growth factor (PDGF), during activation. By PDGF receptor (PDGFR), which is a transmembrane tyrosine kinase receptor, PDGF acts as a negative feedback regulator during platelet activation and inhibits thrombin and collagen-induced activation. When PDGFR is activated by the PDGF stimulus, a signaling pathway triggering ROS production by NAD(P)H oxidase is initiated. Thus, PDGF binding induces receptor dimerisation and activation of the intrinsic tyrosine kinase activity of the receptor. The activated receptor recruits, phosphorylates and activates the target proteins c-Src and Rac1, leading to NAD(P)H oxidase release of ROS (31).

The tyrosine kinase receptors can be transactivated by G protein-coupled receptors, in a common pathway, for further transmission of ROS-sensitive signals. In this context, the Ras GTPase can be activated through tyrosine kinase receptor, particularly PDGFR, to induce ROS generation. Ras stimulates the membrane NAD(P)H oxidase complex through the extracellular signal-regulated kinase 1/2 (ERK1/2), which is a ROS-sensitive mitogen-activated protein kinase (MAPK). MAPKs phosphorylate proteins at serine/threonine residues and regulate cell proliferation, differentiation and apoptosis. Activation of the ERK, c-Jun N-terminal kinase (JNK) and p38 members of the MAPK family was observed in response to changes in the cell redox balance. The balance between ERK and JNK activation is a key determinant for cell survival, a decrease of ERK and an increase of JNK being required for induction of apoptosis. MAPK activation directly determines the increase of transcription factor activator protein-1 (AP-1) activity, resulting in increased cell proliferation. Activation of the transcription factor nuclear factor-kappa B (NF-κB) has a role in inflammation, differentiation and cell growth, and is involved in carcinogenesis.

The growth factor-stimulated ROS generation mediates diverse intracellular signaling pathways by activating the non-receptor protein tyrosine kinases JAK, PYK and Src, by inhibiting protein tyrosine phosphatases and by regulating redox-sensitive gene expression. The signaling factor redox factor-1 (Ref-1) responds to PDGF stimulation. Its C-terminal domain has DNA repair activity and the N-terminus contains the redox regulatory domain that reduces the transcription factors AP-1 and NF-kB (32).

Most tyrosine kinases have an associated protein tyrosine phosphatase (PTP), which restricts the activity of kinase. PTPs are redox-sensitive proteins that are directly inhibited by ROS through modification of cysteine residues in their catalytic sites (33). The inhibiting potential of ROS is similar to that of the PTP-ase inhibitor pervanadate. The modification is reversed by intracellular reducing agents. The reversible oxidation of PTPs was demonstrated during PDGF stimulation, when their inhibition by ROS helped the propagation of tyrosine kinase receptor signal mediated by protein tyrosine phosphorylation. Inactivation of a specific phosphatase by oxidative stress results in prolonged activity of the kinases that it controls and an increased activation of the specific intracellular signaling pathways (10). Oxidative inhibition of the focal adhesion kinase (FAK) tyrosine phosphatase is necessary for cell adhesion (34).

Signaling Through Integrins

The cell surface receptors belonging to the integrin family interact with the extracellular matrix components fibronectin and collagen to mediate various intracellular signaling pathways. Signaling through integrins and integrin activation

play a central role in the hemostasis process by controlling both platelet aggregation (fibronectin stimulation) and adhesion (collagen stimulation) at the site of vessel-wall injury.

ROS have an important role in both signal transduction associated with integrin stimulation (outside-in signaling) and modulation of integrin coagulation function (inside-out signaling). Thus, fibronectin binding induces activation of the Ras/MAPK, NF-kB, PI3K and Rho GTPase pathways. In turn, the intracellular-generated ROS induce conformational changes in integrins, to change their binding affinity and coagulation function. Under the action of agonists, platelet integrin acquires the ability to bind soluble adhesive proteins such as fibrinogen and von Willebrand factor (35).

Structurally, platelet integrins consist in an α_{2b} chain (platelet glycoprotein IIb) and a β_3 chain (platelet glycoprotein IIIa), forming the $\alpha_{2b}\beta_3$ integrin (gp IIb/IIIa) that acts as a fibronectin receptor. The extracellular domains of $\alpha_{2b}\beta_3$ integrin are rich in disulfide bonds, which, on reduction, activate it and stabilize the ligand–integrin interactions (36). The number of thiol groups within $\alpha_{2b}\beta_3$ is regulated by the extracellular GSH/GSSG ratio. Following ligand binding, the intracellular tail of β_3 chain is tyrosine phosphorylated. This β_3 tyrosine phosphorylation can also be induced by H_2O_2, resulting in spontaneous platelet aggregation (37).

Activated $\alpha_{2b}\beta_3$ integrin mediates recruitment of platelets to the thrombus and platelet–platelet interactions. Activation requires PDI that catalyzes cleavage or formation of disulfide bonds between cysteine residues (38). This enzyme is also required for fibrin generation (39). Activation of platelets bound to the injured vessel causes a conformational change in $\alpha_{2b}\beta_3$ integrin that increases the integrin affinity for its ligand fibrinogen. Activation of $\alpha_{2b}\beta_3$ integrin is inhibited by NAD(P)H oxidase inhibitors and $O_2^{\bullet-}$ scavengers, denoting a direct role of platelet NAD(P)H oxidase-generated ROS in integrin activation (40).

ROS, mainly $O_2^{\bullet-}$, generated and released by endothelial and smooth muscle cells in response to injury can indirectly affect platelet function by scavenging $NO^{\bullet}$ and neutralizing the anti-platelet activity of $NO^{\bullet}$. It is known that intact endothelium prevents adhesion and activation of platelets by producing prostacyclin and $NO^{\bullet}$. $NO^{\bullet}$ diffuses into platelets and regulates several protein kinases, suppressing the conformational change in $\alpha_{2b}\beta_3$ integrin that is required for integrin binding to fibrinogen during thrombus formation (41).

REACTIVE OXYGEN SPECIES AND CHRONIC MYELOID LEUKEMIA PATHOGENESIS

Chronic myeloid leukemia (CML) is a myeloproliferative disorder that affects all lineages of hematopoiesis (erythrocytes, leukocytes and platelets), and is characterized by increased and unregulated growth of myeloid cells in the bone

marrow and their accumulation in the blood (42). It accounts for 15–20% of adult leukemias. CML is associated with a specific chromosomal translocation consisting in genetic exchange between region q34 of chromosome 9 and region q11 of chromosome 22, designated as t(9;22)(q34;q11). The resulting shortened chromosome 22 is known as the Philadelphia chromosome (Ph). The chimeric *BCR-ABL* gene that results by fusion of the *ABL* ("Abelson" leukemia virus) oncogene from chromosome 9 to the breakpoint cluster region (*BCR*) from chromosome 22 encodes the BCR-ABL protein. This protein has a constitutive tyrosine kinase activity (43). In the so-called Ph-negative CML, complex chromosomal abnormalities exist, which mask the specific translocation. It has been shown that the hyperactive tyrosine kinase receptors confer a ligand-independent, non-regulated growth stimulus to the cells and perturbation of cell signaling through these receptors can result in malignant transformation. By activating different intracellular signaling pathways, the constitutively active tyrosine kinase leads to alterations in the proliferative, adhesive and survival properties of CML cells (44). Competitive inhibitors such as imatinib (brand names Gleevec and Glivec) are used therapeutically to bind the catalytic cleft of the BCR-ABL tyrosine kinase, inhibiting its activity (45).

Many other fusion tyrosine kinases such as BCR-FGFR1, TEL-ABL, TEL-JAK2, or TEL-PDGFR can be generated by chromosomal translocation, and they may also appear under the action of free radicals, chemicals or ionising radiation. Recently, the V617F point mutation in the *JAK2* gene has been identified in BCL-ABL-negative chronic myeloproliferative diseases, the mutation resulting in alteration of several tyrosine kinase signaling pathways (46).

The Ph-positive cell lines show an increased level of ROS, which correlates with the presence of *BCR-ABL* oncogene. Imatinib inhibits specifically the tyrosine kinase activity of this oncogene and also reduces the ROS level. Furthermore, drugs that affect the ROS metabolism or induce activation of PTP-ases may antagonize the *BCR-ABL* fusion (47). Cells with JAK2V617F mutation also have an increased ROS level, and has been demonstrated that ROS play an important signaling role in these cells. It has been also demonstrated that ROS alter the DNA structure especially in the cells having a reduced function of the tumor suppressor p53, and this is the case for about 25% of the patients having Ph-positive CML (48).

A better understanding of the mechanisms that generate ROS in Ph-positive cells may supply useful information on the new targets for the treatment of CML.

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