NO/cGMP and ROS Pathways in Regulation of Platelet Function and Megakaryocyte Maturation

Dissertation

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SUMMARY

In physiological conditions platelets have a major role in maintaining haemostasis. Platelets prevent bleeding from wounds by distinguishing normal endothelial cells in vasculature from areas with lesions to which they adhere. Interaction of platelet agonists and their receptors is controlled by intracellular signaling molecules that regulate the activation state of platelets. Very important intracellular signaling molecules are cyclic nucleotides (cGMP and cAMP), both involved in inhibition of platelet activation. Formation of cGMP and cAMP in platelets is stimulated by endothelial-derived NO and prostacyclin (PGI_2), which then mediate inhibition of platelets by activating protein kinase G (PKG) and protein kinase A (PKA). Recently, it has been suggested that reactive oxygen species (ROS) represent new modulators of cell signaling within different cell types. The work summarized here describes the involvement of platelet ROS production in platelet activation, the relation of NO/cGMP/PKG I pathway to ROS and to mitogen-activated protein kinases (MAP kinase) signaling, and the involvement of cyclic nucleotides in megakaryocyte and platelet development.

Platelets activated with different agonists produce intracellular but not extracellular ROS by activation of NAD(P)H oxidase. In addition, ROS produced in platelets significantly affects αIIbβ3 integrin activation but not alpha/dense granule secretion and platelet shape change. Thrombin induced integrin αIIbβ3 activation is significantly decreased after pretreatment of platelets with NAD(P)H oxidase inhibitors and superoxide scavengers. These inhibitors also reduce platelet aggregation and thrombus formation on collagen under high shear and achieve their effects independently of the NO/cGMP pathway.

ADP secreted from platelet dense granules with subsequent activation of $P2Y_{12}$ receptors as well as thromboxane A_2 release are found to be important upstream mediators of p38 MAP kinase activation by thrombin. However, p38 MAP kinase activation does not significantly contribute to calcium mobilization, P-selectin expression, αIIbβ3 integrin activation and aggregation of human platelets in response to thrombin. Finally, PKG activation does not stimulate, but rather inhibit, p38 and ERK MAP kinases in human platelets.

Further study revealed that cyclic nucleotides not only inhibit platelet activation, but are also involved, albeit differentially, in megakaryocyte and platelet development. cAMP is engaged in haematopoietic stem cell differentiation to megakaryocytes, and cGMP has no impact on this process. While PKA is already present in stem cells, expression of proteins involved in cGMP signaling (soluble guanylyl cyclase, sGC; PKG) increases with maturation of megakaryocytes. In the final step of megakaryocyte maturation that includes release of platelets, cGMP and cAMP have mild but opposing effects: cGMP increases platelet production while cAMP decreases it indicating a finely regulated process that could depend on stimulus coming from adjacent endothelial cells of sinusoids in bone marrow.

The results of this thesis contribute to a better understanding of platelet regulation and of the possible molecular mechanisms involved in megakaryocyte maturation in bone marrow vascular microenvironment.

ZUSAMMENFASSUNG

Blutplättchen spielen unter physiologischen Bedingungen eine wichtige Rolle bei der Erhaltung der Hämostase. So verhindern sie ein andauerndes Bluten von Wunden, indem sie in Blutgefässen zwischen normalen Zellen des Endothels und beschädigten Bereichen unterscheiden und sich dort gezielt anheften können. Das Zusammenspiel der Plättchenagonisten und den dazugehörigen Rezeptoren wird durch intrazelluläre Signalmoleküle kontrolliert, die die Aktivierung der Blutplättchen regulieren. Äusserst wichtige intrazellulare Signalmoleküle stellen dabei die zyklischen Nukleotide cGMP und cAMP dar, die bei der Hemmung der Plättchen beteiligt sind. Die Bildung von cGMP und cAMP in den Blutplättchen wird durch die aus dem Endothel freigesetzten Moleküle NO und Prostacyclin (PGI_2) stimuliert, die ihrerseits Blutplättchen hemmen, indem sie Proteinkinase G (PKG) und Proteinkinase A (PKA) aktivieren. Neuerdings wird vorgeschlagen, dass es sich bei ROS („reactive oxygen species“) um einen neuen Modulator bei der Signaltransduktion zwischen verschiedenen Zelltypen handelt. Die hier zusammengefasste Arbeit beschreibt die Rolle der ROS-Produktion bei der Aktivierung von Blutplättchen, die Beziehung zwischen dem NO/cGMP/PKG I Signalweg und der ROS bzw. MAP-Kinase Signaltransduktion, und die Rolle von zyklischen Nukleotiden bei der Entwicklung von Megakaryozyten und Blutplättchen. Werden Blutplättchen durch unterschiedliche Einflüsse aktiviert, so produzieren sie über die Aktivierung von NAD(P)H-Oxidase nur intrazelluläres aber nicht extrazelluläres ROS. Dabei beinflusst das in den Blutplättchen produzierte ROS signifikant die Aktivierung von αIIbβ3 Integrin, nicht jedoch die Sekretion von alpha- bzw. dichten Granula oder die Gestalt der Blutplättchen. Die Thrombin-induzierte Integrin αIIbβ3-Aktivierung ist nach Behandlung der Blutplättchen mit Hemmstoffen der NAD(P)H-Oxidase oder Superoxid-Fängern signifikant reduziert. Diese Inhibitoren reduzieren auch die Aggregation der Blutplättchen bzw. die Thrombusbildung auf Kollagen, wobei diese Effekte unabhängig vom NO/cGMP Signalweg vermittelt werden.

Sowohl ADP, das von dichten Granula der Blutplättchen sezerniert wird und zur Aktivierung von $P2Y_{12}$-Rezeptoren führt, als auch die Freigabe von Thromboxan A_2

stellen wichtige, vorgeschaltete Vermittler bei der p38 MAP Kinase-Aktivierung durch Thrombin dar. Jedoch spielt die p38 MAP-Kinase-Aktivierung keine signifikante Rolle bei der Thrombin-induzierten Kalzium-Mobilisierung, P-Selektin Exprimierung, αIIbβ3 Integrin Aktivierung oder Aggregation der Blutplättchen. Abschliessend kann festgestellt werden, dass sich die Aktivierung der PKG insgesamt klar hemmend auf die p38 and ERK MAP-Kinasen in menschlichen Blutplättchen auswirkt.

Desweiteren zeigt diese Studie, dass zyklische Nukleotide nicht nur die Blutplättchen hemmen, sondern auch einen Einfluss auf die Entwicklung der Megakaryozyten und Blutplättchen haben, aber auf unterschiedliche Weise. cAMP ist an der Differenzierung von embryonalen hämatopoietischen Zellen zu Megakaryozyten beteiligt, wobei cGMP keine Rolle bei diesem Prozess spielt. Während PKA in embryonalen Zellen schon vertreten ist, steigt beim Reifungsprozess der Megakaryozyten die Expression von Proteinen, die bei der cGMP Signalverbreitung („soluble guanylyl cyclase“, sGC; PKG) mitwirken, stetig an. In der letzten Phase der Reifung von Megakaryozyten, die durch die Freisetzung der Blutplättchen charakterisiert ist, zeigen cGMP und cAMP leicht divergierende Effekte: cGMP verstärkt die Bildung von Blutplättchen, während cAMP dieselbe reduziert. Dies deutet auf einen fein abgestimmten Prozess hin, abhängig von einem Stimulus, der von den benachbarten Zellen des Sinusoid-Endothels stammen könnte.

Die Ergebnisse dieser Dissertation tragen zu einen besseren Verständnis der Regulation von Blutplättchen sowie der möglichen molekularen Mechanismen bei, die eine Rolle bei der Reifung von Megakaryozyten im vaskularen Mikroumfeld des Knochenmarks innehaben.

INTRODUCTION

“Humans have developed complex hemostatic system designed to maintain blood in a fluid state under physiologic conditions but arranged to react to vascular injury in a rapid manner to prevent blood loss by sealing damaged vessel wall. Thrombosis may occur as an outcome of unregulated hemostatic stimulus, either as a result of impaired inhibitory pathways or natural anticoagulant mechanisms are overwhelmed by the strength of the stimulus” (Colman RW 2001).

Blood fluidity is kept by the vascular endothelium that inhibits blood coagulation and platelet aggregation and promotes fibrinolysis. Endothelium represents a protective barrier that separates blood cells and plasma factors from highly reactive components in deeper layers of the vessel wall. These components include adhesive proteins such as collagen, fibronectin, laminin, vitronectin, and von Willebrand factor (vWF), which promote platelet adhesion, and tissue factor, a membrane protein located in smooth muscle, fibroblasts, and macrophages that triggers blood coagulation. Vessel wall constricts when injured and thereby diverts blood from the site of injury. Shed blood is exposed to the subendothelial structures that stimulate hemostatic plug formation by platelet activation and aggregation and by activation of blood coagulation. This process results in thrombus formation that amplifies its own production by further stimulation of platelets. Although, activation of platelets and plug formation are very rapid, these processes are precisely controlled and modulated by inhibitory pathways and anticoagulant mechanisms that lead to the clot dissolution, formation of fibrous tissue, and wound healing (Colman RW 2001; Ruggeri 2002).

In 1841, William Addison made the first observation of platelets:

“I observed that the fluid, i.e. liquor sanguinis, contained a great number of extremely minute molecules or granules, varying in size, the largest being at least eight or ten times less than the colourless corpuscles and they were in much greater abundance. Whilst examining these minute bodies, I observed the coagulation of the fibrin commence.” (Addison 1841).

Addison was observing the interactions of platelets, leukocytes, and fibrin in the formation of clot. However, only 40 years later these “minute molecules or

granules" were named platelets by Bizzozero, who described platelet adherence to a point of injury (Bizzozero 1882).

1. PLATELETS

Platelets are the smallest corpuscular components of human blood (diameter 2-4 µm). Their physiological number varies from 1.5 $x10^5$ to 3 $x10^5$ per µl in humans, and in mice it is about 1 $x10^6$ per µl of blood. Mammalian platelets are not provided with a nucleus, in contrast to thrombocytes in birds, fish and reptiles. The origin of platelets is the bone marrow, where megakaryocytes - as the results of mitotic proliferation of a committed progenitor cell - liberate platelets. Platelets typically circulate for 10 days before they are removed from blood by macrophages (George 2000). The typical shape of resting platelets is discoid, but upon activation they undergo a shape change to a spherical form with pseudopodia, up to 5 µm long (Fig. 1). Multifunctional platelets are involved in many physiological and pathophysiological processes of which the most important are hemostasis and thrombosis. They also take part in clot retraction, vessel constriction, inflammation including promotion of atherosclerosis, tumor growth, metastasis, and angiogenesis (Harrison 2005).

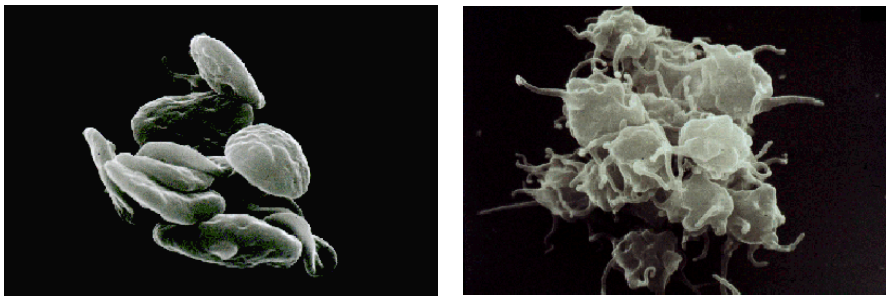

Figure 1. Typical smooth discoid shape of resting platelets, and spiny spherical shape of activated platelets. www.perfusion.com/perfusion/articles/general/9905-platelet-anatomy

1.1. Morphology

The ultrastructure of platelets can be divided into four morphological regions: the peripheral zone, the structural zone, the zone of organelles, and the membrane system.

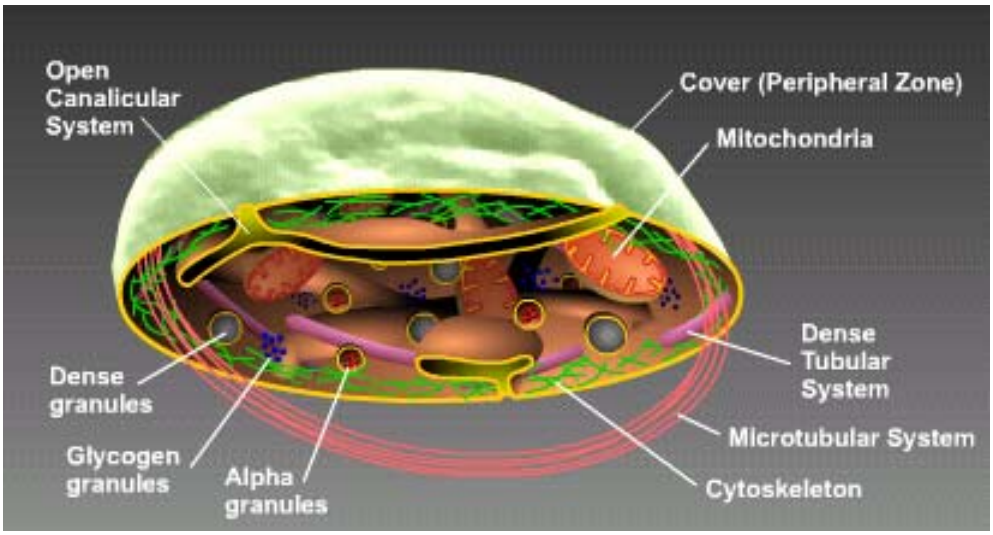

Figure 2. Platelet morphology. http://www.platelet-research.org/1/intro.htm

The Peripheral Zone – Platelet Surface

Plasma membrane is made up of a polarized phospholipid bilayer containing the membrane proteins. Asymmetric distribution of the phospholipids in resting platelets is an important factor for platelet function (Bevers, Comfurius et al. 1982), including neutral phospholipids pointing outside, and negatively charged aminophospholipids, phosphatdylserine (PS) and phosphatidylethanolamine (PE) almost exclusively present in the inner leaflet. Such organization of phospholipids changes during activation of the platelets, and anionic phospholipids are able to accelerate several steps in plasma coagulation (Zwaal and Schroit 1997).

Glycocalix is a thin layer of glycoproteins, glycolipids, adsorbed plasma proteins, and mucoploysaccharides that coats the plasma membrane on the extracellular side, making a negatively charged net-surface mainly due to sialic acid residues attached to proteins and lipids (Coller 1983). This electrostatic repulsion is likely to prevent resting platelets from attaching to each other as well as to negatively charged endothelium.

Structural Zone - Cytoskeleton

The components of the structural zone serve to maintain discoid shape of resting platelets and actively participate in changing the shape of activated platelets. It is consisted of *microtubuli* (tubulin threads) that are surrounded by *structural proteins* that form cytoskeleton (Hartwig, Barkalow et al. 1999). Cytoskeleton mainly consists of actin that is associated with myosin in cytoplasm and of actin binding protein. Upon platelet activation and rise of intracellular calcium, soluble monomeric actin (G-actin) polymerizes into filaments (F-actin) at the platelet periphery, and bundles of new filaments fill the developing filopodia. Binding of myosin to actin provides the tension required for granule centralization and retraction of filopodia.

Membrane System

The surface-connected open canalicular system (SCCS) represents canals that reach from their connections with plasma membrane far inside the platelet and are accessible from extracellular space by pores. It acts as internal membrane reservoir to facilitate platelet spreading and filopodia formation after activation. *The dense tubular system* is derived from rough endoplasmic reticulum of megakaryocytes and is one of the main storage sites of free calcium ions (Ca^{2+}). When the cytoplasmic free Ca^{2+} concentration exceeds a certain threshold the platelet undergoes a shape change with formation of filopodia and lamellipodia and degranulates.

Zone of Organelles

Cytoplasm consists of mitochondria, glycogen stores and three different storage granules: dense granules, α-granules, and lysosomes. These secretory granules posses molecules that affect platelet function, coagulation, and fibrinolysis, vascular tone, inflammation, and wound healing. Some of the components are synthesized by megakaryocytes, other are taken up from plasma and incorporated into the granules (Reed, Fitzgerald et al. 2000). The main components of platelet storage granules are listed in Table 1. Upon activation, granules fuse with platelet

surface membrane and extrude their content. It is a graded process depending on the number, nature, and concentration of the original stimuli.

Table 1. Components of platelet storage granules.

Dense Granules	ATP ADP Ca^{2+} serotonin
α-Granules	• **Adhesion proteins** e. g. fibrinogen, fibronectin, vWF, αIIbβ3, P-Selectin • **Mitogenic and agiogenic factors** e. g. PDGF, VEGF, TGF β • **Cytokine-like proteins** e. g. PF4, CD40L, β-thromboglobulin • **Coagulation factors** e. g. PAI-1, plasminogen, factor V, factor XI, protein S • **Others** e. g. α1-antitrypsin, α2-antiplasmin, albumin
Lysosomes	• **Acid hydrolases** e. g. elastase, collagenase, cathepsin

1.2. Adhesive Platelet Receptors

Platelets possess a number of adhesive receptors coupled to intracellular signaling systems leading to platelet activation. Most important of these include glycoprotein Ib/IX/V (for vWF), collagen receptors glycoprotein VI and integrin α2β1, and fibrinogen receptor integrin αIIbβ3 that are in details described below. Other adhesive receptors expressed on platelets specific for intracellular matrix components are receptors for fibronectin (α5β1), laminin (α6β1), and vitronectin (αvβ3).

Glycoprotein Ib/IX/V (GPIb/IX/V)

GPIb/IX/V (also named GPIb complex) contains four distinct subunits that each spans the plasma lipid bilayer once: GPIb (GPIbα (CD42b) and GPIbβ (CD42c)), GPIX, and GPV. These polypeptides are expressed on the plasma membrane in a molar ratio of 2:2:2:1, with approximately 25 000 copies per platelet of each of the subunits except GPV (half) (Lopez, Weisman et al. 1994). Activation of platelets by several agonists leads to a decrease in surface levels of all components of the complex by a cytoskeletal-mediated redistribution to the membranes of the SCCS (Michelson, Benoit et al. 1996) and by calpain cleavage of GPV and GPIbα (a calcium-dependent protease released from platelet stores after platelet activation) (McGowan, Yeo et al. 1983).

After vessel wall injury in the presence of high shear rates, GPIb/IX/V mediates initial contact adhesion of platelets to the von Willebrand factor (vWF) bound to the collagen within exposed vascular subendothelium. vWF is derived by basolateral secretion from the endothelial cells or is rapidly bound to collagen from the blood. Studies of the adhesion platelets and transfected cells to surfaces of purified vWF showed that GPIb complex-vWF interaction is not sufficient to adhere platelets firmly to the matrix, but rather allows them to roll at much slower velocities over thrombogenic surfaces (Fredrickson, Dong et al. 1998). Increasing shear stress increases adhesion of platelets to a surface through GPIb complex–vWF interaction most probably by shear induced changes in the conformation of both GPIb complex and vWF (Siedlecki, Lestini et al. 1996). GPIbα-vWF interactions result in αIIbβ3 activation that allows platelets to stop and firmly adhere to the matrix. Activated integrins then bind to vWF and fibrinogen, thus contributing to thrombus formation (Kasirer-Friede, Cozzi et al. 2004). *In vivo*, platelet activation and firm adhesion is facilitated by collagen receptors GPVI and α2β1.

Deficiency of GPIbα, GPIbβ, or GPIX results in Bernard-Soulier syndrome, characterized by variable bleeding symptoms, thrombocytopenia, and giant platelets (Lopez, Andrews et al. 1998).

Except vWF, it has been shown that GPIb complex can bind other ligands as well. Since platelets adhere and roll on activated endothelium, in process dependent of expressed endothelial P-selectin, GPIb complex has been proposed as receptor for endothelial P-selectin (Romo, Dong et al. 1999), playing potential role in

inflammation. Adherent platelets are able to capture leukocytes by high levels of P-selectin expression, which then transmigrate through thrombus in process dependent of leukocyte activated Mac-1 and GPIb on platelets (Rogers, Edelman et al. 1998). GPIb complex possess also a cleavage site for thrombin within GPIbα, and GPV component, having a role in thrombin signaling (De Candia, Hall et al. 2001).

Collagen Receptors

Glycoprotein VI (GPVI)

GPVI is a collagen receptor of the immunoglobulin superfamily. It is comprised of two extracellular immunoglobulin C2-like domains, mucin-like core, transmembrane region, and a short cytoplasmic tail. It is exclusively expressed on megakaryocytes, and platelets. The transmembrane domain is associated through a salt bridge to FcR γ-chain, which serves as transducing part of the receptor complex (Nieswandt and Watson 2003). The strength of intracellular signals arising from ligand binding to GPVI depend on its ability to cross-link and induce clustering of GPVI (Farndale, Sixma et al. 2004). A number of snake venom peptides that mediate their action through GPVI have been identified, such as C-type lectin convulxin (Polgar, Clemetson et al. 1997). The snake venom toxins are multimeric, and can cluster the receptors. Crosslinking of GPVI leads to tyrosin phosphorylation of ITAM (immunoreceptor tyrosine-based activation motif) domain in FcR γ by Src kinases Fyn and Lyn. This causes binding and activation of Syk tyrosine kinase, resulting in downstream activation of PLCγ, PI3-K.

Collagen binds directly or indirectly to platelet activated integrins α2β1, or αIIbβ3, and such interaction is sufficient to turn rolling of platelets to stable adhesion. GPVI here plays important role, since it is responsible for integrin activation, and therefore, firm adhesion on collagen under high shear requests GPVI intracellular signaling, potentated by ADP, TxA_2, and thrombin generation. This was shown by defective adhesion of GPVI/FcR γ-deficient platelets to collagen under static and flow conditions that was restored in the presence of Mn2+ that directly activates integrins or by ADP, by inside-out signaling (Nieswandt, Brakebusch et al. 2001).

Integrin α2β1

α2β1 (GPIaIIa, or lymphocyte VLA-2) was first collagen receptor identified on platelets, and mediates adhesion in Mg^{2+}-dependent manner (Santoro 1986). For a long time, α2β1 was considered to be the major receptor on the platelets supporting adhesion and activation and considered to play a key role in hemostasis. Since α2β1 does not stimulate tyrosine kinase activity required for collagen activated platelets, a "two-step, two-site" model was proposed in which platelets first bind to collagen by α2β1 and are then activated by second receptor, thought to be GPVI (Santoro, Walsh et al. 1991). However, recent studies showed that changes in the affinity of α2β1 for collagen occur after platelet activation, supporting a model in which the platelets are first activated by interactions between specific sites on collagen and GPVI (Moroi, Onitsuka et al. 2000). This was further supported by studies in mouse deficient for GPVI (caused by injection of anti-mouse GPVI antibody) and FcR γ, where no activation or adhesion to collagen under static or flow conditions was observed (Nieswandt, Brakebusch et al. 2001). Under high shear conditions neither α2β1 nor GPVI is adequate to initiate adhesion and both GPIb on platelets and vWF in the plasma are essential for platelet interactions with collagen.

Integrin αIIbβ3

αIIbβ3 (GPIIbIIIa) is the only integrin expressed uniquely on platelets. It is the major platelet integrin with 50 000-80 000 copies per platelet. Its absence or deficiency leads to Glanzmann thrombasthenia, the most common bleeding disorder caused by a platelet receptor defect. Integrin αIIbβ3 is essential for platelet aggregation. 70% of the receptor is expressed constitutively on the surface, and rest is only released upon platelet activation from intracellular stores (Gawaz 2001). αIIbβ3 is a heterodimer, type I transmembrane receptor, and αIIb is composed of two polypeptide chains (heavy and light), and β3 is a single polypeptide. Each subunit typically contains a relatively large extracellular domain, a single-pass transmembrane domain, and a short cytoplasmic tail. The main task of this receptor is binding of soluble fibrinogen to the activated platelets that cross-link the receptors on adjacent platelets, and subsequent platelet aggregation. This binding

requires the presence of Ca^{2+} or Mg^{2+}. αIIbβ3 recognizes a stretch of amino-acids at C-terminal end (KQAGDV) of the fibrinogen γ-chain, and RGD sequence of fibrinogen α-chain (Moroi and Jung 1998).

Specificity of αIIbβ3 for ligand is very broad, and it can bind many adhesive proteins containing RGD sequence, such as fibrinogen, fibronectin, vWF, and collagen. However, considering plasma concentrations, fibrinogen is a major ligand.

Platelets adhere to immobilized fibrinogen under static and flow conditions with low shear through αIIbβ3 and do not require previous activation. αIIbβ3 was suggested to bind to vWF after initial fast interaction of vWF with GPIb complex and supports the firm adhesion of platelets (Savage, Shattil et al. 1992).

Under physiological conditions integrin on resting platelets has very low affinity to fibrinogen. When platelets are activated, αIIbβ3 rapidly changes conformation into the form that can bind ligands with high affinity. The binding of fibrinogen to the activated integrin induces further change in the conformation with exposure of cryptic epitopes (LIBS, ligand induced binding site). Integrins receive signals from inside the cell that cause increase of integrin affinity and avidity for the ligand, so this process is referred as inside-out signaling. Following ligand binding, integrins transmit extracellular information into the cell, a process called outside-in signaling (Shattil and Newman 2004).

The activation mechanism of αIIbβ3 is still not fully explained. Many proteins are shown to interact with cytoplasmic tails of integrin β3, including cytoskeletal proteins (α-actinin, talin, filamin, praxillin), protein kinases (c-Src, FAK, ILK1), potential regulatory proteins (β3-endonexin, cytohesin1) (Moroi and Jung 1998).

Signaling of αIIbβ3

Binding of adhesive ligands to αIIbβ3 in inside-out signaling can be triggered by soluble agonists (ADP, thrombin, TxA_2), as well as by adhesion receptors (GPIb complex, GPVI). Signals are promoted by second messengers such as Ca^{2+}, tyrosine kinases and products of phospholipases, and are mediated by protein kinase C, PI3-K, and Rap1b (reviewed in Jackson, Nesbitt et al. 2003; Shattil and Newman 2004).

Maximal platelet secretory, procoagulant, and clot retraction responses demand ligand binding, and close platelet-platelet contact, a process named “contact-dependent signaling”. Platelet adhesion to fibrinogen and vWF triggers morphological changes (filopodia, lamellipodia to full spreading) that are mediated by small GTPases Rho A, Rac1, Cdc42. Initiation of the outside-in signaling after fibrinogen binding and integrin clustering starts with Src kinase activation, that is constitutively bound to β3 chain (Arias-Salgado, Lizano et al. 2003). In unstimulated platelets, phosphorylation of Tyr529 in c-Src is mediated by Csk, a protein kinase selective for this residue. Upon clustering of integrins, c-Src also clusters and autophosphorylates Tyr418. This leads to dissociation of Csk from c-Src, and recruitment of PTP-1B that dephosphorylates c-Src at Tyr529 (Arias-Salgado, Haj et al. 2005). After c-Src activation, Syk is recruited to the β3 tail and activated by c-Src. After Src/Syk phosphorylate adaptor molecules (SLP-76, ADAP), Vav, FAK, PLC γ and α-actinin, that influence actin dynamics and reorganization (Buensuceso, Arias-Salgado et al. 2004; Shattil 2005).

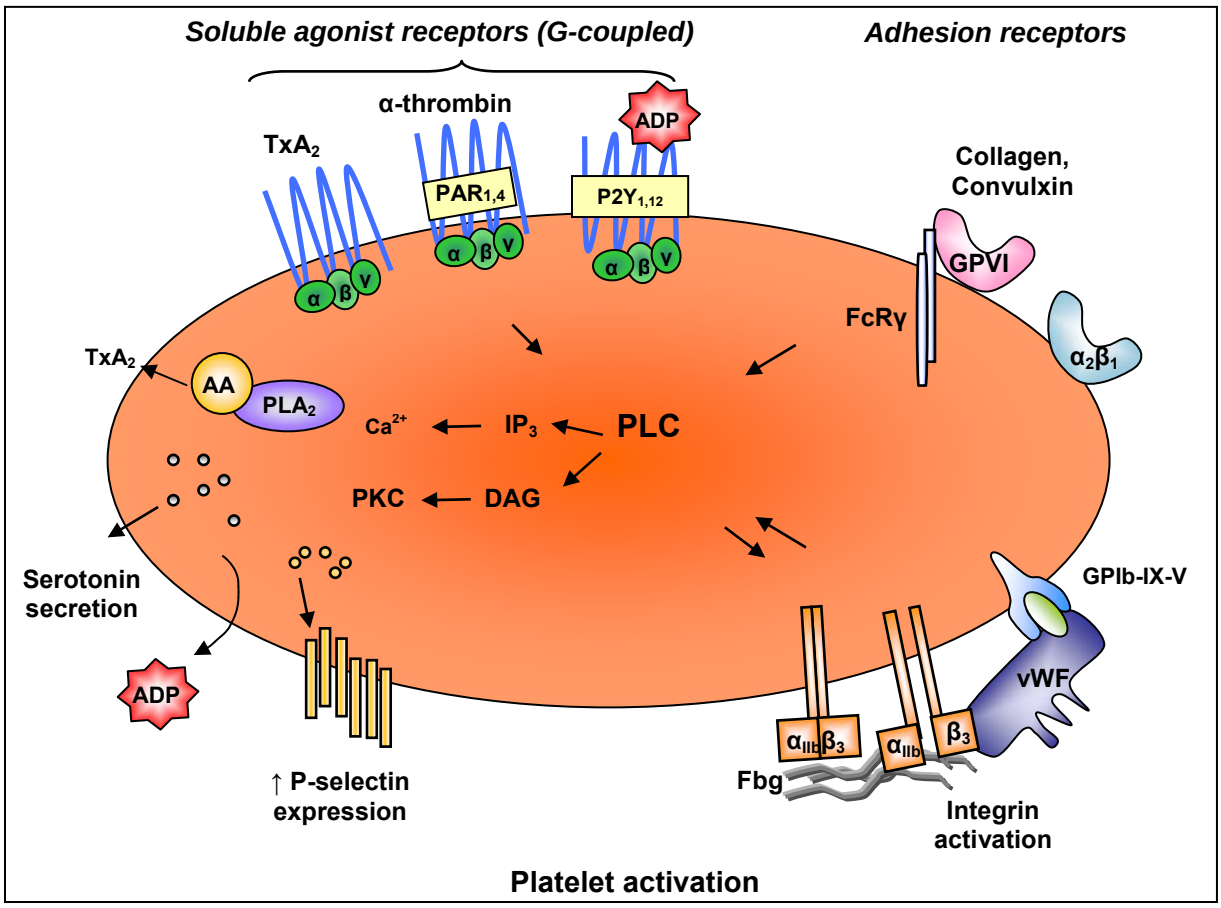

Figure 3. Platelet receptors and signaling. The first step is adhesion to thrombogenic substances, among which the main role have collagen and vWF. Signals from adhesion receptors, GPVI, α2β1 bound to collagen and GPIbα-complex bound to vWF, act in synergy with signals from soluble agonists to induce platelet activation. PLA_2, phospholipase A_2; AA, arachidonic acid; Fbg, fibrinogen, vWF, von Willebrand factor; PLC, phospholipase; PKC, protein kinase C. Modified from Ruggeri Z 2002 Nat Med.

1.3. Platelet Receptors for Soluble Agonists

A number of soluble agonists may be generated in the vicinity of vessel wall damage, including thrombin, ADP, epinephrine, TxA_2. Thrombin is the most potent platelet agonist, and ADP is present in large amounts in platelets. Receptors for these two are described in details below. TxA_2 is produced from arachidonic acid by cyclooxigenase-1 after stimulation with different agonists and amplifies primary stimulation of platelets through G-coupled thromboxane receptors (Roth and Calverley 1994). Epinephrine acts through α_2 adrenergic receptors, inducing aggregation and secretion, but not shape change (Mustonen, van Willigen et al.

2001). In addition, platelets have G-coupled receptors for serotonin, platelet-activating-factor, vassopresin, adenosin.

Thrombin Receptors

Thrombin is the main effector serine protease in coagulation cascade, derived from prothrombin by coagulation factor Xa and factor Va. In addition to its key role in coagulation, it modulates effects of different cell types (neurons, endothelial cells, leukocytes, SMC), and is the most effective activator of platelets *in vivo* and *in vitro* (Davey and Luscher 1967). Thrombin induces platelet shape change, promotes secretion, synthesis and release of TxA_2, stimulates expression of P-selectin, and leads to integrins activation.

Thrombin mediates its action through the cell-surface protease-activated receptors (PARs) that are members of large family of G-protein coupled seven transmembrane domain receptors. PARs are activated by unique proteolitic cleavage within the first extracellular loop. This cleavage releases a new N-terminus of the receptor which than serves as a ligand intramolecularly binding to the body of receptor to effect transmembrane signaling (Vu, Hung et al. 1991). Therefore, PARs carry their own ligand that is active only after receptor cleavage. Four PARs have been identified (PAR1, PAR2, PAR3, PAR4), and only PAR2 cannot be activated by thrombin, but can by trypsin.

Certain differences exist between human and mouse platelets concerning PARs. Human platelets express PAR1, and PAR4, and the later has higher affinity for thrombin. Mouse platelets express PAR3 and PAR4, and appears that PAR3 does not mediate signaling by itself, but rather functions as a cofactor that localizes thrombin to platelet surface and promotes cleavage and activation of PAR4 (Nakanishi-Matsui, Zheng et al. 2000). PAR3 knock out mice are, indeed unresponsive to thrombin, and have prolonged bleeding times when challenged (Sambrano, Weiss et al. 2001).

Signaling by PARs

PAR receptors are coupled to members of the $G\alpha_{12/13}$, $G\alpha_q$, and $G\alpha_i$ protein families. $G\alpha_i$ inhibits adenylyl cyclase (AC), resulting in decreased levels of cAMP

and enhanced platelet responsiveness. The $G\alpha_q$ subunit activates PLC β, resulting in increase of 1,4,5-inositol triphosphates (IP3), and increase of cytosolic calcium. In the same time there is increase of diacyglycerol (DAG) that activates PKC, and finally calcium regulated kinases, tyrosine kinases, mitogen-activated protein kinases (MAP kinases), and integrin activation (Brass and Molino 1997). The $G\alpha_{12/13}$ subunits are connected to guanine-nucleotide exchange factor (GEF) that activates small GTPases Rac, Cdc42. This leads, with a help of activated PI3-K, to cytoskeleton rearrangements, and it is responsible for shape change, adhesion, and platelet aggregation (Klages, Brandt et al. 1999).

ADP Receptors

ADP is a critical autocrine agonist that is secreted from dense granules. ADP receptors belong to the P2 receptor family, which contains two distinct classes: P2Y G-coupled receptors, and P2X ligand-gated cation channels. Platelets express $P2Y_1$ and $P2Y_{12}$, activated by ADP, and $P2X_1$, activated by ATP (reviewed in Gachet 2006). $P2Y_1$ is coupled to $G\alpha_q$, and, therefore, triggers calcium mobilization from internal stores, resulting in platelet shape change and transient aggregation after ADP stimulation (Hechler, Leon et al. 1998). It plays a crucial role in collagen-induced platelet shape change when TxA_2 formation is blocked. The $P2Y_{12}$ receptor is coupled to $G\alpha_i$, and is responsible for full aggregation. It plays a central role in amplification of aggregation and secretion induced by all known platelet agonist. The mechanism by which it acts is inhibition of cAMP production, activation of PI3-K and Rap1b. Coactivation of both $P2Y_1$ and $P2Y_{12}$ receptors is necessary for normal ADP induced aggregation; however, $P2Y_1$ has a minor role in amplification when platelets are activated by agonist other then collagen (Jin and Kunapuli 1998). $P2X_1$ stimulation by ATP induces fast calcium influx associated with transient shape change, and participates in collagen and shear induced aggregation (Hechler, Lenain et al. 2003).

1.3. The Role of Platelets in Haemostasis and Thrombosis

Platelet Tethering, Adhesion, Activation and Aggregation

In physiological conditions platelets monitor integrity of vascular wall, and prevent bleeding after tissue trauma. After vascular injury the loss of endothelial cell layer exposes extracellular matrix components (EMC) to flowing blood and leads to a hemostatic thrombus (plug) formation. This event, regarding to platelets, develops in three successive and closely integrated stages: adhesion, activation, and aggregation (reviewed in Ruggeri 2002; Abrams 2005). Collagen, vWF bound to collagen, laminin and fibronectin are the main components of EMC that can bind platelets. Adhesion begins with tethering and rolling of platelets to the collagen-vWF complex in EMC, event that is at high shear conditions dependent on GPIbα-vWF binding. Several lines of evidences suggest that adhesion to collagen is mediated by α2β1 with GPVI as a central receptor for activation of adherent platelets (Nieswandt, Brakebusch et al. 2001). This results in platelet shape change, spreading along collagen fibrils, and secretion of ADP and TxA_2 recruiting additional platelets to the site of injury. Therefore, activation begins already with binding of adhesive ligands (collagen, vWF) and excitatory agonists (ADP secreted from vascular cells and platelets, TxA_2 synthesized in platelets, α-thrombin generated on the membrane of stimulated platelets) to platelet receptors, and is propagated by intracellular signaling reactions. Activation of platelets leads to actin polymerization and cytoskeleton reorganization, secretion of storage granules (calcium, vWF, ADP, P-selectin) that amplify activation and aggregation, and exposure of phosphatidylserins that enhance procoagulant activity and contribute to thrombus stability. Aggregation is amplification step that leads to accumulation of platelets into the hemostatic thrombus. It is mediated by activated αIIbβ3 integrin, an important factor for stable adhesion and binding of soluble adhesive proteins, namely, fibrinogen, vWF, and fibronectin. Therefore, αIIbβ3 integrin is a crucial player for platelet-platelet interactions, and stable platelet aggregation.

Physiological Inhibition of Platelet Activation

Maintaining a balance between blood fluidity and rapid thrombus formation in response to injury is a task of endothelial cells by controlling vascular tone and synthesizing inhibitors and activators of platelet function. Physiological endothelial derived platelet antagonist prostacyclin (PGI_2) and nitric oxide (NO) inhibit platelet function by increasing intracellular levels of cAMP, and cGMP, respectively; other inhibitory systems include CD39 (ectoADPase) that degrades ADP, thrombomodulin that changes substrate specificity of thrombin, heparin sulphate participates in the inactivation of thrombin.

Many platelet agonists (thrombin, ADP, epinephrine) inhibit cAMP synthesis by coupling to G_i protein. Very small increases in the basal cAMP will impair platelet activation, and for complete platelet aggregation activation of both G_q and G_i-coupled receptors is mandatory (Jin and Kunapuli 1998). cAMP is synthesized by membrane adenylyl cyclase (AC) that is activated by Gs-coupled prostaglandin receptors. cGMP is produced by soluble guanylyl cyclase (sGC) in cytoplasm. Protein kinase A (PKA) and protein kinase G (PKG) are the major effectors of inhibitory pathway (Fig. 4), activated by cAMP, and cGMP, respectively (Geiger, Nolte et al. 1992).

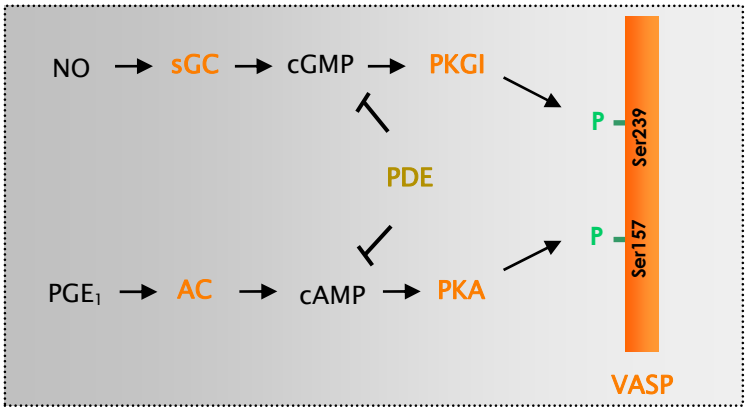

Figure 4. cAMP/cGMP inhibitory pathway in platelets. Nitric oxide (NO) synthesized mainly by endothelial nitric oxide synthase 3 (eNOS, NOS3), stimulates soluble guanyly cyclase (sGC), increasing cGMP and thus stimulating protein kinase G (PKG). Prostaglandin E_1 (PGE_1) stimulates adenylyl cyclase (AC), increasing cAMP and stimulating protein kinase A (PKA). PKG and PKA phosphorylate VASP among other proteins in platelets (Hsp27, LASP, Rap1b, IP_3R, IRAG)

preferentially at different sites and mediate inhibition of platelet activation. Degradation of cyclic nucleotides is regulated by PDEs.

Beside PKG, phosphodiesterases (PDEs) are another important target of cGMP in platelets thus regulating the level of cyclic nucleotides (Haslam, Dickinson et al. 1999). Platelets contain PDE2 (cGMP-stimulated) that hydrolyzes both cAMP and cGMP, PDE3 (cGMP-inhibited) that preferentially hydrolyzes cAMP, and PDE5 (cGMP-binding) that is specific for cGMP degradation (Schwarz, Walter et al. 2001).

PKA and PKG are serine/threonin kinases, they phosphorylate number of proteins, and some of the proteins are substrates for both kinases (Schwarz, Walter et al. 2001). It has been shown that PKA and PKG inhibit agonist induced calcium increase from intracellular stores as well as secondary store-mediated cacium influx (Geiger, Nolte et al. 1994). PKG I deficiency causes increased adhesion and aggregation of platelets (Massberg, Sausbier et al. 1999).

One of the most prominent substrates for both PKA and PKG is vasodilator-stimulated phosphoprotein (VASP), a cytoskeleton- associated protein found to be localized in focal adhesions, stress fibers and areas of cell-cell contacts (Reinhard, Jarchau et al. 2001). VASP has three phosphorylation sites, Ser157 preferentially phosphorylated by PKA, Ser239, preferentially phosphorylated by PKG, and Thr278, suggested to be target of AMP kinase. Studies in different systems implicate VASP phosphorylation in inhibition of actin polymerization (Harbeck, Huttelmaier et al. 2000; Reinhard, Jarchau et al. 2001). VASP-deficient mice showed reduced aggregation time in response to collagen, increased activation of αIIbβ3, and P-selectin expression upon thrombin stimulation (Aszodi, Pfeifer et al. 1999; Hauser, Knobeloch et al. 1999). Other PKA and/or PKG substrates have been suggested to be involved in cytoskeleton and integrin regulation including GPIbβ, Hsp27, Rap1b, ARB, MLCK, IRAG (Schwarz, Walter et al. 2001; Antl, ML et al. 2006).

2. REACTIVE OXYGEN SPECIES

Reactive oxygen species (ROS) formation has been implicated in various disease-related oxidative stress conditions such as diabetes mellitus, cancer, atherosclerosis, neurodegenerative diseases, and aging. However, recent findings indicate ROS as second messengers to control a variety of physiological responses like monitoring of oxygen concentration in the regulation of respiratory ventilation and erythropoietin production, effects on signaling cascades, cell growth, gene transcription, and apoptosis (Droge 2002).

ROS includes oxygen free radicals ($O_2^{-\cdot}$, superoxide anion), hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^{-\cdot}$) and hydroxyl radical ($OH^{\cdot}$) (Li and Shah 2004). Superoxide anion is the most important since other physiologically important ROS are derived from it. Superoxide anion is formed by addition of one electron to molecular oxygen:

$$\mathbf{O_2 + e^- \longrightarrow O_2^{-\cdot}}$$

Superoxide anion is unstable in aqueous solution ($t_{1/2}$ few seconds), it is poorly membrane permeable, and it is generally restricted to the cell compartment where it is produced. Superoxide anion is converted rapidly into the hydrogen peroxide in reaction that is catalyzed by superoxide dismutase (SOD):

$$\mathbf{O_2^{-\cdot} + O_2^{-\cdot} + 2H^+ \xrightarrow[SOD]{} H_2O_2 + O_2}$$

Hydrogen peroxide is more stable and cell membrane permeable and most biological effects are likely to be due to H_2O_2 production since the measured production of $O_2^{-\cdot}$ is relatively low. If NO is present at high levels, it can outcompete SOD and react with $O_2^{-\cdot}$ to produce $ONOO^{-\cdot}$**.** High $O_2^{-\cdot}$ levels lead to the iron (Fe^{2+}) release so that H_2O_2 can break down in the presence of metal ions very easily and produce hydroxyl radical (Fenton reaction):

$$\mathbf{H_2O_2 + Fe^{2+} \longrightarrow OH^{\cdot} + Fe^{3+} + OH^-}$$

Hydroxyl radical is the most reactive species; it has a strong oxidizing ability to react with various organic structures, and it is often involved in oxidative stress tissue damage (Phillis 1994).

Many potential sources of ROS exist, such as mitochondria, xanthine oxidase, cyclooxigenase, cythocrome P-450, uncoupled NOS, NAD(P)H oxidase (NOX). However, except NOX, most other sources produce ROS accidentally, as by-products in certain conditions (Li and Shah 2004). Cells also possess very efficient antioxidant defence mechanism, such as catalyse and peroxidase to decompose peroxides, and large reserves of reducing compounds in the form of glutathione.

2.1. NAD(P)H Oxidases

NAD(P)H oxidase (NOX) is the only enzyme that deliberately generates ROS. Primarily it was described in neutrophils and macrophages where it is important for bactericidal activity in respiratory burst (Babior, Lambeth et al. 2002). NAD(P)H oxidase is multimeric enzyme comprised of several subunits: gp91phox (also termed as NOX2) that is a catalytic subunit, and forms with p22phox integral membrane complex (named cytochrome b558); p67phox, p47phox, p40phox comprise regulatory cytoplasmic complex. gp91phox, p22phox, p67phox, and p47phox are required for activity since mutation in any of them causes chronic granulomatous disease, an immune deficiency resulting from impaired phagocyte function. Additional subunits are also required and include p40phox, and small GTPases Rac and Rap1. After activation of cells, cytoplasmic complex transfers to the membrane and associates with membrane cytochrome forming a functional enzyme (Fig. 5, Lassegue and Clempus 2003)).

Recently it has been shown that cells other then phagocytes produce ROS, such as cells within vascular wall. Furthermore, new homologue proteins of NOX2 were identified, namely NOX1, NOX3, NOX4, NOX5, DUOX1, and DUOX2 (Suh, Arnold et al. 1999; Geiszt, Kopp et al. 2000). gp91phox (NOX2) is structurally similar to NOX1, NOX3, NOX4, while NOX5 has additional calcium binding domain, and DUOX1 and DUOX2 in addition to calcium-binding have also peroxidase domain.

New homologues are expressed in various tissues (within vessel wall, spleen, kidney, thyroid, colon) and are characterized with low activity as compared to phagocyte NOX, inducible activity, intracellular ROS production (probably inside vesicles), role in signaling by activation of transcription factors and inhibition of tyrosine kinases (Irani 2000).

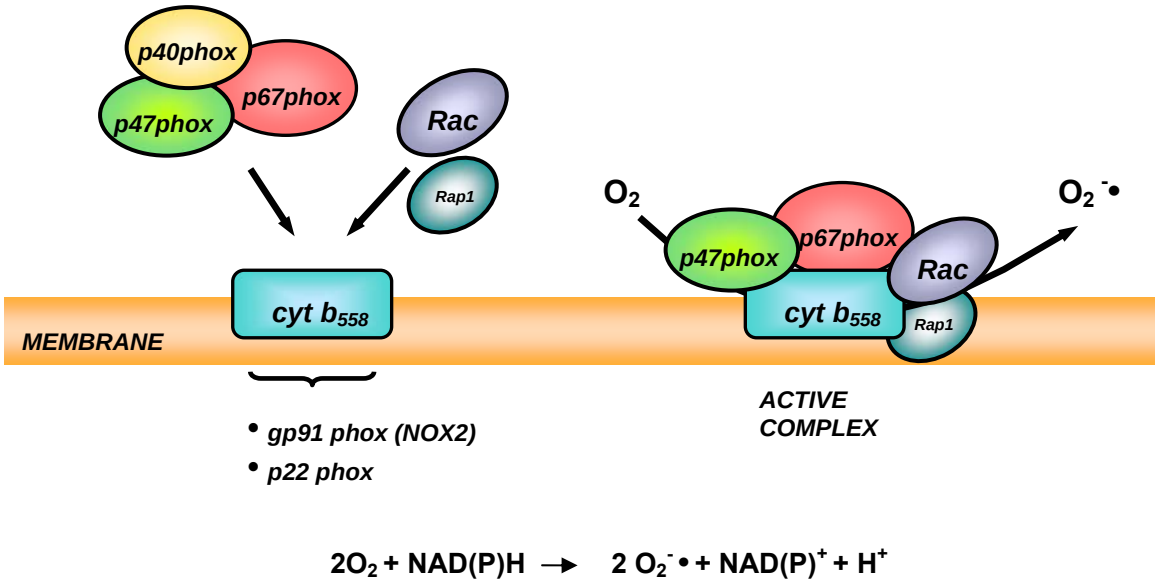

Figure 5. The structure of NAD(P)H oxidase. Active enzyme complex is assembled after translocation of cytoplasmic subunits p47phox, p40phox, p67phox and small GTPases Rac and Rap1 to the membrane and binding to cytochrome b558 that is comprised of gp91phox and p22phox. Assembled enzyme catalyzes production of superoxide anion ($O_2^{\cdot-}$) from molecular oxygen (O_2). NAD(P)H is a cofactor in the reaction.

2.2. Regulation of NOX

Activation of NOX2 system occurs by at least three events to form assembled enzyme: protein kinases PKC and Akt phosphorylate p47phox allowing its binding to p22phox, and lipids; PI3-K and PLD produce 3-phosphorylated phosphatidylinositols (PtdInsP) and phosphatidic acid, respectively, providing lipids to which plextrin domains of p47phox and p40phox bind; activation of Rac that causes conformational change promoting its binding to p67phox, and finally assembling active complex (reviewed in Lambeth 2004). The key step of assembly is to juxtapose activation domain of p67phox to contact region on cytochrome since

the activation domain activates electron transfer from NAD(P)H (cofactor) to FAD of the enzyme (Nisimoto, Motalebi et al. 1999).

Homologues of p47phox and p67phox have been also found and termed as NOXO1, and NOXA1, respectively, and they regulate NOX1 activity (Banfi, Clark et al. 2003). NOX3 is activated by NOXO1, but does not require NOXA1. NOX5, and DUOX1, DUOX2 are regulated by calcium, and NOX4 is constitutively active and might not need other subunits for further activation (Lambeth 2004).

2.3. ROS and Platelets

Recently it has been suggested that ROS take part in platelet activation (Krotz, Sohn et al. 2004). In platelets the presence of NAD(P)H oxidase subunits has been shown by several groups (Seno, Inoue et al. 2001; Krotz, Sohn et al. 2002; Pignatelli, Sanguigni et al. 2004). Early studies report constitutive release of $O_2^{-\cdot}$ from non-activated platelets (Finazzi-Agro, Menichelli et al. 1982) or H_2O_2 after addition of zymosan or latex particles to platelets (Krotz, Sohn et al. 2002). However in later studies, the production of ROS was mainly observed by collagen stimulation, but not with thrombin or ADP stimulated platelets (Del Principe, Menichelli et al. 1991; Pignatelli, Pulcinelli et al. 1998; Caccese, Pratico et al. 2000; Pignatelli, Sanguigni et al. 2004). ROS may regulate platelet function by decreasing NO bioavailability as ROS scavenges platelet or endothelium-derived NO (Tajima and Sakagami 2000; Chakrabarti, Clutton et al. 2004; Clutton, Miermont et al. 2004). Nonetheless, it is still not clear whether the only possible mechanism for the regulation of platelet activation by ROS is due to decreased NO bioavailability or perhaps due to a direct role of ROS in the control of platelet functions.

3. BIOGENESIS OF PLATELETS

3.1. Development of Megakaryocytes from Haematopoietic Stem Cells

Platelets derive from cytoplasm of megakaryocytes, large cells that reside in bone marrow. Megakaryopoiesis and platelet formation in mammals is unique process in animal world, since in other species thrombocytes differentiate directly from progenitor cells. Megakaryocytes derive from pluripotent haematopoietic stem cells (HSC) in bone marrow. During mammalian development stem cells also successively populate the embryonic yolk sac, fetal liver, spleen. Stem cells either self-renew or commit to a specific cellular linage that ultimately gives rise to mature blood cells.

Megakaryopoiesis can be divided into three sequences of events. It starts with commitment of pluripotent HSC in response to various stimuli to develop myeloid progenitor cell from which erythroid-megakaryocyte progenitor develops. These cells then give rise to more differentiated progenitors, like megakaryocyte precursor cells that proliferate producing a pool of promegakaryocytes (Kaluzhny and Ravid 2004). Thrombopoiesis is resembled more in details in figure 6.

At the end of proliferation phase mononuclear megakaryocyte precursors exit diploid state to differentiate and undergo endomitosis. They become polyploid through repeated cycles of DNA replication without cell division resulting in cells containing up to 64N. Endomitosis probably results in prematurely terminated mitosis that does not go to anaphase B, telophase and cytokineses (Ravid, Lu et al. 2002). After endomitosis is completed, megakaryocytes begin a maturation stage in which cytoplasm rapidly fills with platelet-specific proteins, organelles, and membrane systems that will finally be transferred into platelets (Patel, Hartwig et al. 2005). During the final stage of development mature, polyploid megakaryocytes fragment into the platelets.

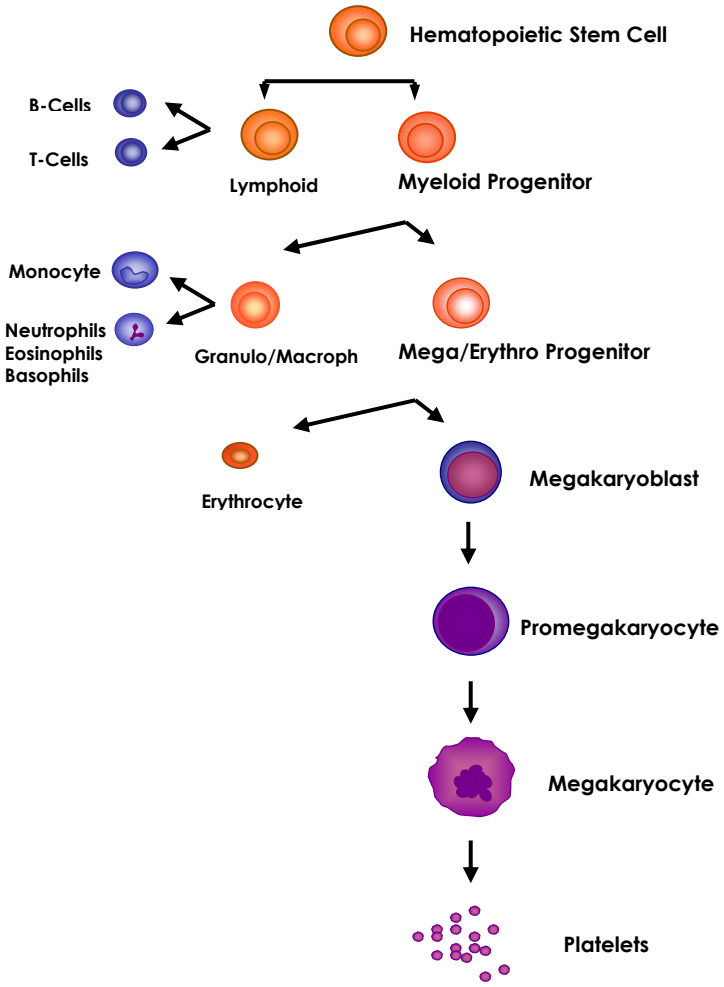

Figure 6. A model of thrombopoiesis. A haematopoietic stem cell differentiate into committed progenitor cells: lymphoid or myeloid progenitors. Myeloid progenitors develop to more differentiated cells: granulocyte/ macrophage or erythrocyte/ megakaryocyte progenitors. Erythrocyte/ megakaryocyte progenitors can give rise to megakaryoblasts that matures to promegakaryocyte and finally to megakaryocyte that releases platelets. Development of other blood cell lineages are also shown in simplified way.

3.2. Platelet Formation

The proplatelet formation or flow model has been proposed to explain how platelets are released from megakaryocytes in last stage of their maturation (Italiano, Lecine et al. 1999; Patel, Hartwig et al. 2005). According to this model mammalian platelets assemble through long cytoplasmic extensions, called proplatelets that contain platelet organelles and consist of multiple platelet-size swellings along their length. Proplatelets represent intermediate structures of megakaryocyte to platelets

transformation. Such model has been supported since proplatelets has been captured to extend from megakaryocytes in bone marrow through junctions in the endothelial lining into the sinusoidal lumen, where they subsequently fragment into platelets (Tavassoli and Aoki 1989). After platelet release, megakaryocyte nucleus enveloped in thin cytoplasm remains in the marrow where it is rapidly phagocytosed by macrophages.

Cytoplasmic reorganization is crucial for proplatelet formation; therefore cytoplasmic proteins (actin, tubulin) are driving force for all aspects of megakaryocyte fragmentation into the platelets.

3.3. Thrombopoietin and Signals Involved in Thrombopoiesis

Thrombopoietin (TPO) is the key physiological regulator of steady-state megakaryopoiesis, with actions in proliferation of megakaryocyte progenitor cells, as well as the maturation of megakaryocytes, and is the most potent *in vivo* stimulus of platelet production of all known cytokines (Kaushansky 1995). TPO is primarily produced in liver; however it is also detectable in kidneys, stromal cells of bone marrow and spleen (Nagahisa, Nagata et al. 1996). Circulating concentrations are regulated by receptor-mediated internalization and degradation by megakaryocytes and platelets (Stefanich, Senn et al. 1997). TPO consists of two distinct regions, the amino portion that is markedly homolog to erythropoietin, it is a binding domain and is sufficient for biological function, and highly glycosylated carboxyl-domain that plays role in stability and secretion (Bartley, Bogenberger et al. 1994). Receptor for TPO is c-Mpl protein, a member of the haematopoietic receptor family. TPO binding to c-Mpl causes receptor dimerisation and activation of non-receptor tyrosine kinases Jak2 and Tyk2. Jak2 seems to be the major initiator of TPO signaling, since mice lacking Jak2 fail to develop megakaryocytes (Parganas, Wang et al. 1998). Active Jak then phosphorylates tyrosine residues within the receptor, molecules that promote cell survival and proliferation (STAT1, STAT3, STAT5, Ras, MAPKs, PI3-K) and those that inhibit signaling, such as SHP1, SHIP1 phosphatases (reviewed in Fishley and Alexander 2004; Kaushansky 2005). Src family kinases were also shown to play role in TPO-stimulated

responses, with Lyn as negative regulator of proliferation and maturation (Lannutti and Drachman 2004).

3.4. Other Cytokines and Transcription Factors

Although TPO is a potent stimulus of platelet production, it appears to have a little role in final megakaryocyte maturation and platelet release, even inhibiting this last event. Mice deficient in TPO and c-Mpl had low number of progenitor cells of all lineages, implying a role of TPO in stem cell compartment regulation (Carver-Moore, Broxmeyer et al. 1996). TPO and c-Mpl deficient mice have profound but not absolute thrombocytopenia, indicating TPO-independent thrombopoiesis mechanisms. Stromal-derived factor-1 (SDF-1, ligand for CXCR4 receptor) and fibroblast growth factor-4 (FGF-4) can restore platelet number in TPO and c-Mpl deficient mice, by enhancing the movement of megakaryocyte progenitors to vascular niche in the bone marrow (Avecilla, Hattori et al. 2004). Many other cytokines have influence at different aspects of megakaryocyte biology including GM-CSF, IL-3, IL-6, IL-11, IL-12, and erythropoietin that stimulate proliferation of progenitors, or IL-1α that modulate maturation and platelet release (Gordon and Hoffman 1992). However, they have broad effects on all haematopoietic lineages.

Several transcription factors have been shown to have a role in a control of megakaryocyte-specific gene expression and subsequent platelet formation, including GATA-1, FOG, NF-E2, Fli1, SCL (Pang, Weiss et al. 2005). GATA-1 is involved in lineage commitment of megakaryocytes, as well as later in controlling proliferation (Shivdasani, Fujiwara et al. 1997). NF-E2 has been identified as a major regulator of platelets biosynthesis since NF-E2 null-mice experience lethal thrombocytopenia, and die from haemorrhage (Shivdasani, Rosenblatt et al. 1995). These mice also lack β1-tubulin (Lecine, Italiano et al. 2000), and small GTPase Rab27 (Tiwari, Italiano et al. 2003), and have decreased levels of thromboxane synthase and caspase-12 (Kerrigan, Gaur et al. 2004).

Apoptotic events have been associated with changes in megakaryocytes during platelet assembly, such as cytoskeletal reorganization, membrane condensation and ruffling. Apoptosis inhibitory proteins Bcl-2 and Bcl-X_L when expressed in megakaryocytes inhibit proplatelet formation (Kaluzhny, Yu et al. 2002). Inhibition of caspases has been shown to block proplatelet formation (De Botton, Sabri et al.

2002). Furthermore, NO has been shown to induce apoptosis of megakaryocytes that was connected with increased platelet release (Battinelli, Willoughby et al. 2001).

Haematopoietic stem and progenitor cells are not randomly distributed in the bone marrow, but rather localize close to endosteum (osteoblastic niche) and around blood vessels (vascular niche). Osteoblastic niche is quiescent microenvironment responsible for the maintenance of cells in the undifferentiated state (Calvi, Adams et al. 2003), from where cells can be mobilized to the vascular niche to undergo differentiation and transfer to the peripheral circulation (Abkowitz, Robinson et al. 2003). In bone marrow it was found that mature megakaryocytes localize near the thin-walled sinusoids (microvasculature whose endothelial cells are not covered with connective tissue), and transmigrate through intact endothelial cells (Tavassoli and Aoki 1989). *In vitro* it has been shown that bone marrow endothelial cells support proliferation and differentiation of haematopoietic progenitors dependently of cytokines production as well as physical contact (Rafii, Shapiro et al. 1995), and the presence of bone marrow endothelial cells was obligatory for chemokine SDF-1 induction of proplatelet formation (Avecilla, Hattori et al. 2004), suggesting that cellular contact of megakaryocytes with bone marrow endothelial cells is necessary for the thrombopoiesis. Translocation of megakaryocyte progenitors to the vicinity of vascular sinusoids was sufficient to induce their differentiation and platelet production in the TPO deficient mice (Avecilla, Hattori et al. 2004), dependent on chemokines SDF-1 (stromal-derived factor-1) and FGF-4 (fibroblast growth factor-4), and adhesion molecules. However, molecular mechanisms by which these processes are conducted are unknown.

AIMS OF THE STUDY

Interaction of platelet agonists and their receptors is controlled by intracellular signaling molecules that regulate activation state of platelets. Among these intracellular signaling molecules cyclic nucleotides (cGMP and cAMP) are involved in inhibition of platelets activation. *In vivo* endothelial NO and PGI_2 stimulate formation of cGMP and cAMP in platelets which then mediate inhibition through PKG and PKA. Recently, it has been suggested that ROS represent a new modulator of cell signaling within different cell types.

The goals of the presented work can be divided into three parts:

- Investigation of intracellular and extracellular ROS production in platelets by different agonists, determination of sources of ROS formation in platelets, and its significance for the platelet function as well as its relation to NO/cGMP pathway

- Analysis of signaling cascade and molecules involved (e.g. MAP kinase) in the activation of NO/cGMP/PKG I pathway and its functional role for platelets biology. Furthermore, determination of the signaling cascade that leads to the activation of p38 MAP kinase, and its role in regulation of platelets

- Determination of the physiological role of cyclic nucleotides and their effector molecules in the development of megakaryocytes and platelets in *in vitro* system using mouse fetal liver stem cells

MATERIALS AND METHODS

4. MATERIALS

4.1. Cell culture

Cell Line

HL-60

Cell Culture Media

RPMI 1640 with L-Glutamine (**Gibco**)
Dulbecco´s modified Eagle medium (DMEM, 1000 mg/l glucose, **Gibco**)
Fetal Calf Serum (FCS, **Gibco**)
Penicillin / Streptomycin 100x stock; 10000U /10mg/ml (**Sigma**)

4.2. Animals

NOX1 knock out mice were produced in Department of Rehabilitation and Geriatrics, Geneva, Switzerland (Gavazzi, Banfi et al. 2006), and provided to us by kindness of prof. Harald H. Schmidt.
Timed pregnant C57BL/6 mice were from **Charles River**.

4.3. Plasmids

GST-PBD (Rac1 binding domain)
GST-RalGDS (Rap1 binding domain)
GST-Rothekin (RhoA binding domain)

4.4. Markers

- Protein Standard (**MBI Fermentas**)

4.5. Kits

- BCA Protein Assay (**Pierce**)
- cAMP and cGMP ELISA (**R&D**)

4.6. Antibodies

Primary Antibodies

ANTIBODY	EPITOPE	SPECIES	SOURCE/Class	SUPPLIER
eNOS	NOS3 (1185-1205)	H, M, R	Rabbit, IgG	**Sigma**
ERK-P	Phospho-Thr^{202}/Tyr^{204}	H, M, R	Rabbit, IgG	**Cell Signaling**
gp91phox		H, M	Rabbit	**Upstate**
GPDH		H, M, R	Mouse, IgG1	**Chemicon**
Hsp27	Phospho-Ser^{82}	H, Mk	Rabbit	**Cell Signaling**
NOX1		H, M, R	Rabbit	**H. Schmidt, Giessen**
NOX4		H, M, R	Rabbit	**H. Schmidt, Giessen**
p22phox		H, M, R	Rabbit	**Santa Cruz**
P38-P	Phospho-Thr^{180}-Gly-Tyr^{182}	H, M, R	Mouse, IgG	**Sigma**
p47phox		H, M, R	Rabbit	**Santa Cruz**
p67phox		H, M, R	Rabbit	**Santa Cruz**
PKA C-α	α Catalitic subunit	H, M, R	Rabbit	**Cell Signaling**
PKG I		H, M	Rabbit	**S. Gambaryan**
PKG I α		H, M	Mouse	**S. Gambaryan**
PKG I β		H, M	Mouse	**S. Gambaryan**
PKG II		H, M	Rabbit	**S. Gambaryan**
Rac1		H, M, R	Mouse, IgG2b	**BD**
Rap1		H, M, R	Mouse, IgG1	**BD**
RhoA		H, M, R	Rabbit, IgG	**Santa Cruz**
VASP-16C2	Phospho-Ser^{239}-VASP	H, M, R	Mouse, IgG1κ	**Nanotools**
VASP-5C6	Phospho-Ser^{157}-VASP	H, M	Mouse, IgG1κ	**Nanotools**
VASP-M4		H, M, R	Rabbit	**Cell Signaling**
β sGC		H, M	Rabbit	**M. Kuhn, Würzburg**
CD62P^{PE}	P-Selectin	H	Mouse	**Dako**
PAC1^{FITC}	activated αIIbβ3	H	Mouse, IgM κ	**BD**
CD40L^{PE}	CD154	H	Mouse	**Dako**
JON/A^{PE}	activated αIIbβ3	M	Rat, IgG2b	**Emfret**
Wug.E6 ^{FITC}	P-Selectin	M	Rat, IgG1	**Emfret**
CD41 ^{FITC}	Integrin αIIb	M	Rat, IgG1 κ	**BD**
CD61 ^{FITC}	Integrin β3	M	Rat, IgG2a	**Emfret**

Secondary Antibodies

ANTIBODY	SPECIES	SOURCE	SUPPLIER
Anti-Mouse-HRP	M	Goat	**BioRad**
Anti-Rabbit-HRP	R	Goat	**BioRad**
Anti-Mouse-Cy3	M	Goat	**Dianova**
Anti-Rabbit-Cy3	R	Goat	**Dianova**

Isotype Controls

ANTIBODY	SOURCE	SUPPLIER
Polyclonal IgG, FITC labeled	Rat	**Emfret**
IgG1 κ, FITC labeled	Rat	**BD**
Polyclonal IgG, PE labeled	Rat	**Emfret**

4.7. Chemicals

2´, 7´-dichlorodihydrofluorescein diacetate (H_2DCF-DA)	**Molecular Probes**
8-Br-cGMP	**Biolog**
8-pCPT-cGMP	**Biolog**
acetylsalicylic acid (ASA)	**Sigma**
ADP	**Sigma**
apocynin	**Sigma**
apyrase	**Sigma**
AR-C69931MX	**a gift from Astra-Zeneca**
Complete® Mini EDTA Free Proteinase Inhibitor Cocktail	**Roche**
Convulxin	**Alexa**
DEA/NONOate	**Alexis**
diphenyleniodoniumchloride (DPI)	**Sigma**
Forskolin	**Sigma**
gp91ds-tat, RKKRRQRRRCSTRIRRQL	**PANATecs**
H89	**Calbiochem**
Indomethacin	**Sigma**
LIBS6	**M. Ginsberg, USA**
Mn (III) tetrakis (1-methyl-4-pyridyl) porphyrin (MnTMPyP)	**Calbiochem**

MRS2179	**Sigma**
nitro-L-arginine methyl ester (L-NAME)	**Sigma**
ODQ	**Calbiochem**
oxypurinol	**Sigma**
PGE_1	**Sigma**
Phaloidin Oregon Green 488	**Molecular Probes**
phorbol-12-myristate 13-acetate (PMA)	**Sigma**
ReoPro®	**Eli Lilly**
RGDS	**Sigma**
RGES	**Sigma**
rotenone	**Sigma**
SB202190	**Calbiochem**
SB202474	**Calbiochem**
SB203580	**Calbiochem**
SCRAMB-tat, RKKRRQRRRCLRITRQSR	**PANATecs**
SNP	**Sigma**
thrombin	**Sigma, Roche**
Thrombopoietin (TPO)	**R&D**
tiron	**Sigma**
Trap-6	**Bachem**
TruCOUNT Tubes	**BD**
U46619 (Thromboxane A2 analog)	**Sigma**

4.8. Special Materials and Equipment

Centrifuges:

Eppendorf 5415C

Hettich ROTIXA/K

Eppendorf Thermomixer 5436

Rocking Platform DUOMAX 1030, **Heidolph**

Spectrophotometer: Ultrospec 2000, **Amersham Pharmacia Biotech**

Aggregometer: Platelet Aggregation Profiler Model PAP-4, **BioData Corporation**

Flow Cytometer: Calibur BD

Power Supplies:

Model 200/2.0 Power Supply, **BioRad**

PowerPac 200, **BioRad**

pH-Meter: PHM 92 LAB pH METER, **Radiometer**

Vortex Genie 2, **Scientific Industries**

Developer: X-OMAT M35, **Kodak**

Medical X-Ray Films, **FUJI Photo Film**

Transfer Device: **BioRad**

Protran (nitrocellulose membrane), **Whatman Schleicher & Schuell**

Microscope:

Axiovert 50, **Zeiss**

Fliters for fluorescent microscopy:
No. 37 (green) excitation 450/50 emission 510/50
No. 15 (red) excitation 546/12 emission 590
No. 37 (blue) excitation 365 emission 420

Microtiter Plates Counter: Wallac Victor 1420 MultiLabel Counter, **Wallac**

Cell Counters:

Casy® Cell Counter, **Schäfer System**

Coulter cell counter

Cell culture incubator Labotect, **Labor-Technik-Göttingen**

4.9. Software

Imaging

MetaMorph Version 4.6

Cell Counter

Casy® Stat Version 2.1; © Schärfe System GmbH

Coulter Micro Diff 18, Krefeld

Microtiterplate Reader

Wallac 1420 Version 2.0; © Wallac Oy

Graphs & Statistics

GraphPad PRISM Version 2.01

5. METHODS

5.1. Cell Culture

Maintaining Cell Lines

HL-60 cells were cultivated in RPMI 1640 supplemented with 5% FCS, penicillin/ streptomycin solution (100 U/0.1mg/ml) and with 1.25% DMSO for differentiation. Cells were grown at 37 °C in the atmosphere with 5% of CO_2. Cells were passaged according to ATCC recommendations. In order to passage or use cells for the experiments (i.e. split cells) cells were centrifuged for 5 min at 280g, growth medium was removed and cells were washed with adequate volume of PBS. Afterwards cells were resuspended in growth medium, counted with a CASY®1 cell counter and seeded at density of 0.5 x 10^6/ml. For experiments, cells were resuspended in HBSS (Hank´s balanced salt solution) in order to measure ROS production.

HBSS

5.33 mM KCl
0.441 mM KH_2PO_4
4.17 mM $NaHCO_3$
137.9 mM NaCl
0.338 mM Na_2HPO_4

Cell Freezing and De-freezing

Cells were pelleted (200g, 5 min), washed with PBS and counted. The pellet was resuspended in freezing medium at a final concentration of 4-5 x10^6/ml, aliquoted into 1 ml cryotubes. The aliquots were stored over night at -80˚C and then in liquid N_2.

Freezing Medium

RPMI 1640
20% FCS
Penicillin/Streptomycin
10% DMSO

Frozen cells were thawed by putting the vials into a water-bath at 37 °C and immediately transferred to 15 ml tubes with 10 ml of growth medium. Cells were pelleted by centrifugation, medium was removed, and cells were resuspended in fresh growth medium and seeded in a small dish T-75.

5.2. Blood Preparations

Preparation of Human Washed Platelets

Blood from healthy volunteers was collected into ACD solution. Platelet rich plasma (PRP) was obtained by 15 min centrifugation at 330g. Platelets were pelleted by 5 min centrifugation at 400g, washed once in CGS buffer, and resuspended in HEPES buffer at a final concentration of $3x10^8$ platelets/ml. After 1 h rest in a 37 °C water bath, washed platelets were used for experiments.

ACD buffer

12 mM citric acid, pH 6.5
15 mM tri-Na-citrate
25 mM D-glucose

CGS buffer, pH 6.5

120 mM NaCl
12.9 mM tri-Na-citrate
30 mM D-glucose

HEPES buffer, pH 7.4

150 mM NaCl
5 mM KCl
1 mM $MgCl_2$
10 mM D-glucose
10 mM HEPES

Blood Sampling (mice)

Adult mice were narcotized with ether, and blood from retroorbital veins was taken with a heparinezed capillary into a tube containing 1/10 volume of ACD buffer (see

before preparation of human platelets). With this method a maximum of 800 µl of blood can be taken per mouse. The animal survives and blood can be taken again one week later (4 to 5 times from the same animal).

Preparation of Mouse Washed Platelets

Blood was centrifuged for 5 min at 300g and PRP was removed with the white platelet phase and some erythrocytes into a new tube. PRP was centrifuged for 8 min at 200g, and PRP without any erythrocytes was collected into a new tube. To wash platelets 0.5 µM of PGI_2 was added and PRP was centrifuged for 5 min at 1000g. The pelleted platelets were resuspended in 1 ml modified Tyrode buffer, and 0.02 U/ml apyrase and 0.5 µM PGI_2 was added. Platelets were then incubated for 5 min at 37°C, and centrifuged for 5 min at 1000g. Finally, the pelleted platelets were resuspended in modified Tyrode at a density of approximately $1x10^9$ platelets/ml containing 1 mM Ca^{2+} and 0.02 U/ml apyrase. Platelets were incubated for 30 min at 37 °C.

Modified Tyrode buffer, pH 7.4

137 mM NaCl
2 mM KCl
12 mM $NaHCO_3$
0.3 mM Na_2HPO_4 x $2H_2O$
5.5 mM Glucose
5 mM HEPES
0.35% BSA

Preparation of Human Neutrophils

Polymorphonuclear neutrophils were isolated from the rest of the blood. Erythrocyte and leukocyte samples were mixed with 6% dextran/0.9% NaCl solution (1:1) and allowed to sediment for 90 minutes at room temperature. After sedimentation of erythrocytes, the upper fraction was used for Ficoll-gradient isolation of neutrophils. Residual erythrocytes were removed by hypoosmotic lysis with 1 ml of ice-cold deionized water, and lysis was stopped with 5 ml of PBS with 0.1% BSA. At the end, $2x10^6$ leukocytes were resuspended in Ca^{2+}-free PBS

containing 0.1% BSA. More then 95% of the isolated leucocytes were neutrophils as measured by Coulter cell counter.

5.3. Culturing and Differentiation of Mouse Megakaryocytes

Preparing Single-cell Suspensions from Fetal Mouse Liver Cells

Fetal liver isolates contain a large proportion of haematopoietic stem cells. To achieve high megakaryocyte (MK) cell yield, livers from 14.5 days postcoital (dpc) were used for experiments.

Pregnant mice were anesthetized and sacrificed by CO_2 asphyxiation. A small vertical abdominal incision was made to expose the peritoneal and pelvic cavities. Uterus was placed in 10-cm Petri dish containing sterile HBSS, and was dissected to isolate individual fetuses. Using fine-tip forceps, the liver (a large, deep red-lobed organ) was isolated from any adhering connective tissue or gut segments and transferred to a new dish with DMEM/10% FCS/penicillin-streptomycin. Six to 10 livers were collected in 10 ml per 10-cm Petri dish. Single-cell suspension was prepared by aspirating and expelling each tissue 8-10 times by sterile syringe with an 18-G needle, then with 21-G and finally if cell clumps are still visible under the microscope with 23-G needle. Cell suspension was pipetted into a sterile conical 15- or 50-ml tube and any contaminating connective tissue or residual large clumps were allowed to sediment for 3-5 min. Then the suspension was transferred into a new tube and centrifuged at 200g for 5 min at RT. The cells were resuspended in DMEM/10% FCS/penicillin-streptomycin and with 10 ng/ml recombinant TPO at cell concentration of $5x10^6$/ml.

Preparation of a Megakaryocyte Suspension from Mouse Fetal Liver Cells

Mouse fetal liver cells were cultured 2 to 5 days with TPO. To prepare an unsynchronized mouse megakaryocytes suspension culture, a two-step BSA density gradient was used. Prewaremed sterile 3% BSA in PBS (1.5 ml) was pipetted in 15-ml conical tube and gently overlayed with 1.5% BSA in PBS (1.5 ml). Cell suspensions from culture days 2 to 5 were centrifuged at 200g for 5 min and resuspended in 1 ml of DMEM/ 10% FCS. This solution was overlayed on the 1.5%

to 3% BSA gradient and incubated 30 to 45 min at room temperature. The upper phase was removed and the sedimented cells (0.5 to 0.8 ml) were resuspended in DMEM/10% FCS and centrifuged for 5 min at 200g. Cells were then used for analysis (FACS or Western) or were cultured for additional 1 day in the presence of TPO to enrich for proplatelet formation and released nascent platelets.

5.4. Cell Count

A volume of 10 µl of platelets´ or neutrophils´ suspension was diluted in 100 µl PBS and counted with the Coulter haematological cell counter as instructed by the manufacturer. For CASY®1 cell counter, 2 µl of the cell suspension was diluted in 10 ml Casyton® solution and counted as instructed by manufacturer.

5.5. ROS Measurement

Intracellular ROS Production (H_2DCF-DA)

For detection of intracellular ROS, washed platelets and neutrophils were preloaded with 50 or 10 µM H_2DCF-DA, respectively, for 30 min at 37 °C in PBS/5.5 mM glucose/1 mM EDTA. Platelets and neutrophils were then centrifuged and resuspended in HEPES buffer or PBS/5.5 mM glucose, respectively. H_2DCF-DA is a cell permeable nonfluorescent dye converted intracellularly to a cell impermeable DCFH by intracellular esterases. The dye then can be oxidised by ROS to the fluorescent species that were measured by flow cytometry (FACS Calibur, Becton Dickinson).
Incubation of H_2DCF-DA loaded platelets and neutrophils was performed at 37 °C in a water bath.

Extracellular ROS Production (Chemiluminescent Assay)

For extracellular ROS production, 100 µM L-012 was added to platelets in HEPES buffer or neutrophils and HL-60 cells in PBS/5 mM glucose or HBSS, respectively, cells were aliquoted on microtiter plate and incubated for 10 min at 37 °C. The L-012 chemiluminescence signal was measured at room temperature after

stimulation with agonists in the presence or absence of DPI and tiron. Photon emission was recorded for 20 min in multilabel counter Wallac Victor 1420. Results were expressed in arbitrary units as indicated on figures.

5.6. Flow Cytometry

Human Washed Platelets: P-Selectin, CD40L Expression and aIIbβ3 Activation

For P-Selectin expression after stimulation, platelets were fixed with 1% formaldehyde for 10 min, pelleted by 5 min centrifugation at 2700 g, resuspended in PBS/5.5 mM glucose/0.5% BSA, and stained with RPE-conjugated anti-CD62P antibody. For αIIbβ3 activation, platelets were prestained with FITC-PAC-1 antibody for 15 min, after stimulation reaction was stopped by diluting platelets (1:10) in PBS/5.5 mM glucose/0.5% BSA.

Mouse Megakaryocytes: CD41, CD61, P-Selectin, aIIbβ3 Activation

Mouse fetal liver cells or differentiated megakaryocytes were resuspended in PBS /BSA 0.3% and 5 µl of corresponding antibody (or isotype control) was added to 50 µl of cells (10^5 cells). Cells were incubated for 20 min at room temperature in dark, and finally staining was terminated by adding 300 µl of PBS. Fluorescence was measured on corresponding channel on FACS Calibur (FL1 channel for FITC labelled, and FL2 channel for PE labelled antibodies).

Megakaryocytes Ploidity Assay

Fetal liver cell suspension day 2 to 5, or megakaryocyte suspension after BSA gradient was centrifuged for 5 min 200g. Cells were resuspended in citrate buffer (100 µl for one condition, app. 10^5 cells) and staining buffer was added (400 µl for one condition). Cells were mixed and incubated on ice for 15 min. Because of high ploidity in megakaryocytes, fluorescence was measured on FL2 log scale and 2N cells were adjusted at 10^1.

Citrate buffer

40 mM	Na-Citrat, pH 7.4
0.25 M	Sucrose

Staining buffer

20 µg/ml	Propidium Iodide
0.5 %	NP-40
0.5 mM	EDTA
0.5 mg/ml	RNase, freshly added

5.7. Serotonin Secretion

Before platelet stimulation, 2 µmol/L fluoxetine, a selective serotonin reuptake inhibitor, was added to the platelet suspension. An aliquot (350 µL) of washed platelets was preincubated with various inhibitors and stimulated with thrombin. The reaction was stopped with an ice-cold mixture of 35 µL EDTA (50 mmol/L, pH 7.4) and 100 µL silicon oil. Samples (amount of secreted serotonin after stimulation of platelet suspension with agonist) and totals (total amount of serotonin present in 350 µL platelet suspension) were mixed for 15 seconds, and only the samples were additionally centrifuged for 30 seconds at 5000*g*. Supernatants (270 µL) were mixed with 60 µL of 100% trichloroacetic acid and then centrifuged at 5000*g* for 2 minutes. An aliquot (250 µL) of these supernatants was added to 1 ml of *ortho*-phthalaldehyde reagent (0.5% wt/wt) in ethanol, then mixed with 10 volumes of 8N HCl and heated at 95 °C for 10 minutes. Samples and totals (each 200 µL) were washed twice with 5 ml of chloroform, and fluorescence emission of the upper water phase was measured at an excitation wavelength of 355 nm and an emission wavelength of 475 nm (Wallac Victor 1420).

5.8. Thromboxane Synthase Activity

Thromboxane synthase activity was determined by fluorometric quantification of derivatized malondialdehyde. Briefly, aliquots of washed human platelets resuspended in HEPES buffer were incubated with the inhibitors and/or agonists at 37 °C in a thermomixer and stopped by addition of ice-cold trichloroacetic acid

(20% wt/vol). After 10 min on ice, samples were centrifuged and equal volumes of the derivatization reagent thiobarbituric acid (0.53% wt/vol) and the supernatant were mixed and incubated for 30 minutes at 70°C. Fluorescence was measured at an excitation wavelength of 533 nm with a slit width of 2.5 nm and at an emission wavelength of 550 nm with a slit width of 15.0 nm (Wallac Victor 1420).

5.9. Aggregometry

Aggregation was carried out using a Biodata PAP-4 aggregometer with PRP (0.3 ml of citrated plasma). PRP was preincubated for 5 min with DPI (100 µM) and apocynin (1.2 mM) or SNP (10 µM), RGDS (1mM), P38 inhibitors (20 µM) and platelet aggregation was induced with 30 µM trap6 or thrombin (0.005 U/ml). Aggregation was measured under continuous stirring at 1000 rpm at 37°C.

5.10. Adhesion Under Flow Conditions (Flow Chamber)

Transparent flow chamber experiments were carried out with cover slips coated with Horm-type collagen which were connected to a syringe filled with the anticoagulated whole blood. Perfusion was performed using pulse-free pump under high shear conditions (1000 s-1, 4 min). Chambers were rinsed by a 4-min perfusion with HEPES buffer at the same shear conditions, and phase-contrast figures were recorded from at least five different microscopic fields (63x objectives). These figures were further processed to obtain the area coverage as an indicator of platelet adhesion by MetaMorph software.

5.11. Protein Analysis

Determination of Protein Concentration

Protein concentrations were determinated using the BCA Protein Assay. This assay is based on the reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium (Biuret reaction). Cu^{1+} is then chelated with two molecules of bicinchoninic acid (BCA) producing coloured complex. BSA standard solutions and samples (0, 0.025,

0.125, 0.25, 0.5, 0.75, 1 µg/µl) were prepared in 0.1 M HCl or 0.1 NaOH and mixed with the reagent as follows:

Standard or Sample:	10 µl
Reagent C	200µl

Reagent C → Reagent A + Reagent B (50:1)

Samples were mixed and after 30 min incubation at 37 °C the absorbance at 526 nm were measured. Absorbance obtained was correlated to the protein concentration according to the standard values.

SDS-Polyacrylamid Gel Electrophoresis (PAGE) and Western blot

Sodium dodecyl sulphate polyacrilamide gel electrophoresis (SDS-PAGE) was performed using 9% or 13% (w/v) polyacrilamide separating gels. Proteins were loaded into the slots of the gel and separated with a starting current of 70 V (stacking gel) that was increased to 130 V after samples reached separation gel. Then, proteins in the gel were transferred onto the nitrocellulose membranes by blotting at 2 A for 1 h at 4 ˚C.

After the transfer, membranes containing the proteins were stained with Ponceau S, washed with TBS-T buffer and subsequently blocked with 3% milk in TBS-T buffer for 30 min. Membranes were incubated 2 hours at RT or over night at 4˚C with the first antibody freshly diluted in TBS-T 3% milk. Then, membranes were washed several times with TBS-T and incubated with the secondary antibody diluted in TBS-T 3% milk for 30 min. After washing several times in TBS-T, Western blots were visualized using enhanced chemiluminescence detection kit (ECL, Amersham), Röntgen films and Kodak developer.

Gels

	Stacking gel	**Separating gel**		
	4%	**8%**	**9%**	**13%**
Acrylamide/Bisacrylamide 30%/0.8% (w/v) 29:1	2 ml	10.7 ml	12 ml	17.3 ml
Tris-HCl (pH 8.9) 3 M	-	5 ml	5 ml	5 ml
Tris-HCl (pH 6.7) 0.5 M	2.5 ml	-	-	-
SDS 10% (w/v)	0.2 ml	0.4 ml	0.4 ml	0.4
H_2O	15 ml	23.5 ml	22.43 ml	17.5 ml
Ammonium Persulphate (APS) 10% (w/v)	10 µl	20 µl	20 µl	20 µl
TEMED 99%	0.8 ml	0.4 ml	0.4 ml	0.4 ml
Total volume	20 ml	40 ml	40 ml	40 ml

Sample Loading buffer (3x)

200 mM Tris-HCl, pH 6.7
6% (w/v) SDS
0.03% (w/v) Bromphenol Blue
15% Glycerol
10% (v/v) β-Merkaptoethanol

Electrophoresis buffer

25 mM Tris, pH 8.9
192 mM Glycin
0.1% (w/v) SDS

Transfer buffer, pH 10

25 mM Tris
192 mM Glycin
20% (v/v) methanol

TBS-T

10 mM Tris, pH 7.5
150 mM NaCl
0.1% Tween 20

5.12. Pull Down Assays

Preparation of GST-PBD, GST-RBD, GST-RalGDS Sepharose Beads

Bacteria containing the expression plasmid for GST fused to Rac/Cdc42 binding domain of Pak (GST-PBD) or to RhoA binding domain of Rhotekin (GST-RBD) or to Rap binding domain of RalGDS (GST-RalGDS) were cultured over night in LB-Ampicillin medium at 37 ˚C with an agitation of 200 rpm until they reached an OD_{600} = 1 (log phase). The expression of the fusion protein was induced by addition of 0.5 mM IPTG, 3 h incubation at 37 ˚C and for GST-RBD at 30 °C since at higher temperature most of the protein becomes insoluble. Bacteria were pelleted by centrifugation at 5000g for 15 min at 4 ˚C and resuspended in PBS. Lysozyme was added to a final concentration of 1 mg/ml and lysis was carried out for 30 min on ice. Then 10 ml of 0.2% (w/v) Triton X-100 in PBS and 5 µg/ml each of DNase and RNase were added per 100 ml of original culture medium. For GST-PBD, bacteria pellet was lysed with cold lysis buffer with protease inhibitors, 0.5% (w/v) Triton X-100, lysozyme, Dnase and Rnase. After 10 min incubation at 4 ˚C the suspension was centrifuged at 3000g for 30 min at 4 ˚C in order to pellet intact cells membranes rest and chromosomal DNA. The supernatant containing the expressed GST-biding domain was collected and 1 mM DTT was added. A 50% (v/v) slurry of glutathione sepharose was added. The GST-biding domain was allowed to bind to the glutathione sepharose for 30 min at room temperature with gentle agitation. For the GST-RBD, the supernatant was diluted 1:2 with lysis buffer w/o Triton X-100 to reach a final Triton X-100 concentration of 0.25% before adding sepharose beads. The sample was then centrifuged at 500g, the supernatant was removed and the GST-biding domain sepharose beads were washed three times with PBS (washing buffer with 0.25% Triton X-100 and 1 mM DTT in the case of GST-RBD). Finally the beads were resuspended in one volume of PBS (washing buffer) and stored in 50 µl aliquots at -80 ˚C.

LB-medium

1% (w/v) Bacto-tryptone
0.5% (w/v) Bacto-yeast extract
1% (w/v) NaCl
50 µg/ml Ampicillin

Lysis buffer for GST-RBD, pH 8

50 mM Tris, pH 8
5 mM EDTA
100 mM NaCl
10 mM $MgCl_2$
spoon tip $MnCl_2$
Protease inhibitors (Complete Mini™ EDTA free, Roche)

Washing buffer for GST-RBD

50 mM Tris pH 8
150 mM NaCl
5 mM $MgCl_2$

Pull Down Assays for Rac, Rap1 and RhoA

The amount of activated Rac1, RhoA and Rap1 was determined by pull-down assays with corresponding binding proteins (Rac-binding domain of PAK1 for Rac, the Rho-binding domain of Rhotekin for RhoA or the Rap1 binding domain of RalGDS for Rap1 as described Benard, Bohl et al. 1999; Ren, Kiosses et al. 1999). Platelets were lysed in 2x lysis buffer for 15 min at 4 °C. After centrifugation for 10 min at full speed in microcentrifuge at 4 °C, an aliquot of clarified lysates were used to estimate the total amount of GTPases. The rest of lysates were incubated with approximately 20 µg of corresponding GST fusion protein-agarose beads at 4 °C for 45 min. The beads were washed three times with lysis buffer and were finally resuspended in 30 µl of SDS gel Laemmli sample buffer and analyzed by 12% SDS-PAGE.

Platelet Lysis & Washing buffer (2x)

100 mM Tris pH 7.5
5 mM MgCl2
400 mM NaCl
2% NP40
20% Glycerol
Protease inhibitors (Complete Mini™ EDTA free, Roche) 2/10 ml

5.13. Immunofluorescence of Megakaryocytes

Chamber slides were coated with 100 µg/ml fibrinogen in PBS at 37 °C for 30 min. Megakaryocytes (10^5) were inreached from fetal liver cells by BSA gradient on day 3 (just before forming proplatelets) and cultured (1 ml) for 1 additional day with TPO to from proplatelets. Medium was carefully removed and 4% paraformaldehyde was added for 15 min. Then cells were washed 3 times with PBS, and 50 mM NH_4Cl in PBS was added and incubated for 15 min. Cells were permeabilized with 0.2% Triton X-100 for 15 min, then 5% goat serum in 0.1% Triton X-100 was added for 15 min. Cells were incubated over night at 4 °C with first antibody diluted in 5% goat serum in 0.1% Triton X-100. Next morning slides were washed 3 times with PBS and were incubated with secondary antibody for 30 min at RT. Slides were washed in PBS, last washing in H_2O and mounted with glass coverslides with Moviol. Slides were kept in dark at 4°C.

Dilutions of antibodies used for IF:

Primary antibodies	source	dilution
PKG I	R	1:100
VASP M4	R	1:50
Secondary antibodies		
Anti R Cy3	G	1:500
Anti M Cy3	G	1:500

5.14. Platelet Formation Assay

At early day 3 megakaryocytes were separated by BSA gradient and seated in DMEM/FCS with TPO at concentration $1x10^5$/ml in 24-well plate (500 µl per condition) with or without different compounds where indicated. After 24 h megakaryocytes and developed platelets were transferred to Eppendorf tubes, and wells were washed with 500 µl PBS to collected remaining cells. Then cells were centrifuged at 330g, resuspended in 100 µl PBS/0.5% BSA, and anti-CD61 antibody or isotype control was added (4 µl). Cells were stained 20 min in dark, at room temperature, and then added to the TruCOUNT tubes with beads in 400 µl PBS. Platelet sized particles were counted with help of beads and analyzed for

CD61 marker in the gate adjusted for mouse platelets (Plt). Equation down was used to calculate absolute number of platelets:

$$\frac{\text{Number of events in Plt region}}{\text{Number of events in absolute count bead region}} \times \frac{\text{Number of beads per test}}{\text{Test volume}} \times \%\ CD61^{+} \text{ events in Plt region}$$

5.15. Data Analysis

All experiments were performed at least in triplicate and data shown are means ± SD. Data were analyzed using Analysis of variance (ANOVA) followed by Bonferroni test or Student t test. Differences were considered significant when the significant level was $p<0.05$.

RESULTS

6. Role of ROS in Platelets

6.1. Platelets Produce Intracellular ROS after Stimulation with Different Agonists

In order to assess oxidative processes inside activated platelets, a cell permeable nonfluorescent dye H_2DCF-DA (50 µM) was used that was trapped within the cells by deacetylation and oxidised by ROS to fluorescent dichlorofluorescein (DCF). After 1 min stimulation of platelets with thrombin (0.2 U/ml), Trap6 (30 µM), U46619 (1 µM) or convulxin (100 ng/ml), ROS formation significantly increased compared to control (n=6, $p<0.05$, Fig. 6). This increase was highest after thrombin and convulxin stimulation (around 16 fold increase for both), lower for Trap6 (9 fold increase) and smallest for the thromboxane analogue U46619 (4 fold increase) while ADP (20-50 µM) did not cause any ROS formation.

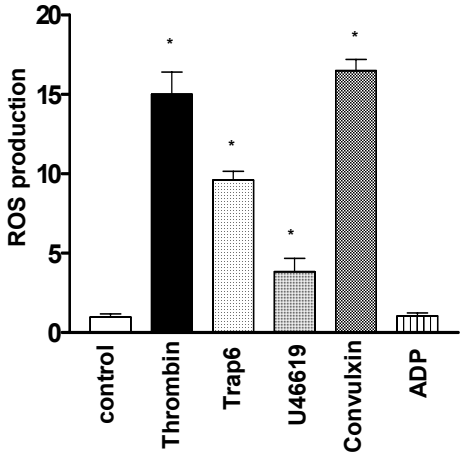

Figure 6. Intracellular ROS production in platelets. Different platelet agonists induce ROS production in platelets. Platelets were stimulated with thrombin (0.2 U/ml), Trap 6 (30 µM), TxA2 analogue U46619 (1 µM), convulxin (100 ng/ml) and ADP (50 µM) and ROS was measured by DCF-DA oxidation by FACS. Data are expressed as fold increase, control taken as 1, mean of 3 different experiments ± SD; * $p< 0.05$.

Extracellular ROS production was measured by a chemiluminescence assay with the luminol derivate L-012. None of the used platelet agonists increased extracellular ROS production under these conditions (data not shown). As a positive control for platelet experiments, intracellular and extracellular ROS production was measured in neutrophils, which normally produce ROS for their bactericidal activity (Fig. 7). Neutrophils stimulated with PMA (0.1 µM) after 20 min produced high amounts of extracellular ROS measured by L-012 chemiluminescence (Fig. 7 B, 420 fold increase vs. control) and weak intracellular increase (Fig. 7 A, 6 fold increase vs. control). This extracellular and intracellular ROS increases in neutrophils were inhibited by NAD(P)H oxidase inhibitor DPI and superoxide scavenger tiron (73±3% inhibition with DPI, 50±5% inhibition with tiron of intracellular ROS production, and 97±1% inhibition with DPI, 96±1% inhibition with tiron of extracellular ROS production).

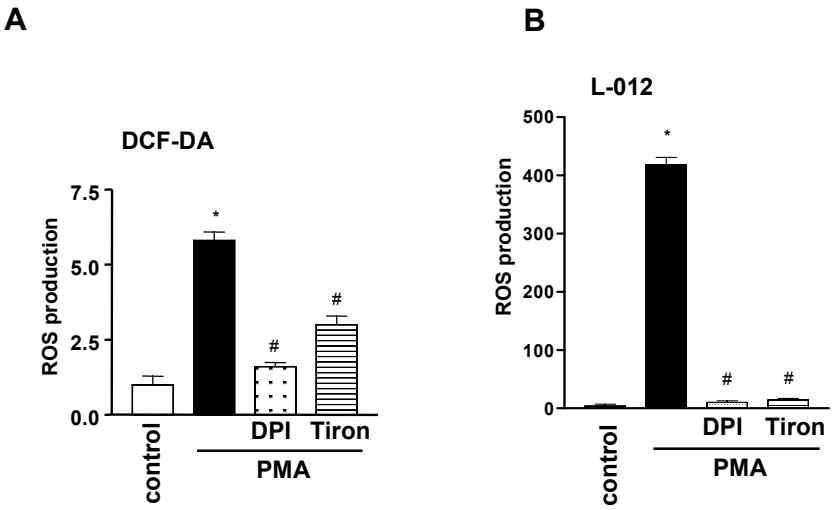

Figure 7. Intracellular and extracellular ROS production in neutrophils. Intracellular ROS production was measured by oxidation of DCF-DA in flow cytometry, and extracellular in chemiluminescence assay with L-012. Stimulation of neutrophils was performed by PMA (0.1 µM) and production of ROS was inhibited by NAD(P)H oxidase inhibitor DPI (10 µM) and ROS scavenger tiron (3 mM). Data are expressed as fold increase, control taken as 1, mean of 3 different experiments ± SD; *significantly different as compared to control, # compared to PMA stimulation, $p < 0.05$.

6.2. NAD(P)H Oxidase and COX are the Source of ROS Production in Platelets

To further investigate the possible source and type of measured intracellular ROS production several different inhibitors and ROS scavengers were used. Two different NAD(P)H oxidase inhibitors, DPI (10 μM) and apocynin (600 μM) inhibited thrombin induced ROS production for 70±8% and 83±7%, respectively (n=8, $p<0.05$, Fig. 8 A). The inhibitor of cyclooxigenase (COX), ASA (100 μM) also caused inhibition of ROS production by 70±15%. To rule out other possible sources of ROS, platelets were preincubated with an inhibitor of mitochondrial respiration, rotenone (100 μM), a xanthine oxidase inhibitor, oxypurinol (100 μM) and a NOS inhibitor, L-NAME (100 μM). None of these inhibitors had a significant effect on the increase of ROS production in stimulated platelets indicating that NAD(P)H oxidase and COX are the major sources of ROS production in platelets (Fig. 8). To further confirm the specificity of DPI and apocynin inhibition of NAD(P)H oxidase, the intracellular superoxide scavenger tiron (3 mM) and the intracellular SOD mimetic MnTMPyP (100 μM) were used. After 15 min of preincubation, both tiron and MnTMPyP inhibited the thrombin-stimulated increase in DCF fluorescence (75±11% and 70±15% inhibition vs. control, respectively, n=6, $p<0.05$, Fig. 8 B). In order to find out whether other types of radicals are produced, the platelets were treated with hydroxyl radical scavenger, mannitol. There was no effect on ROS production after preincubation with mannitol (100 μM - 5 mM, data not shown) showing that superoxide radicals are the main ROS produced in platelets.

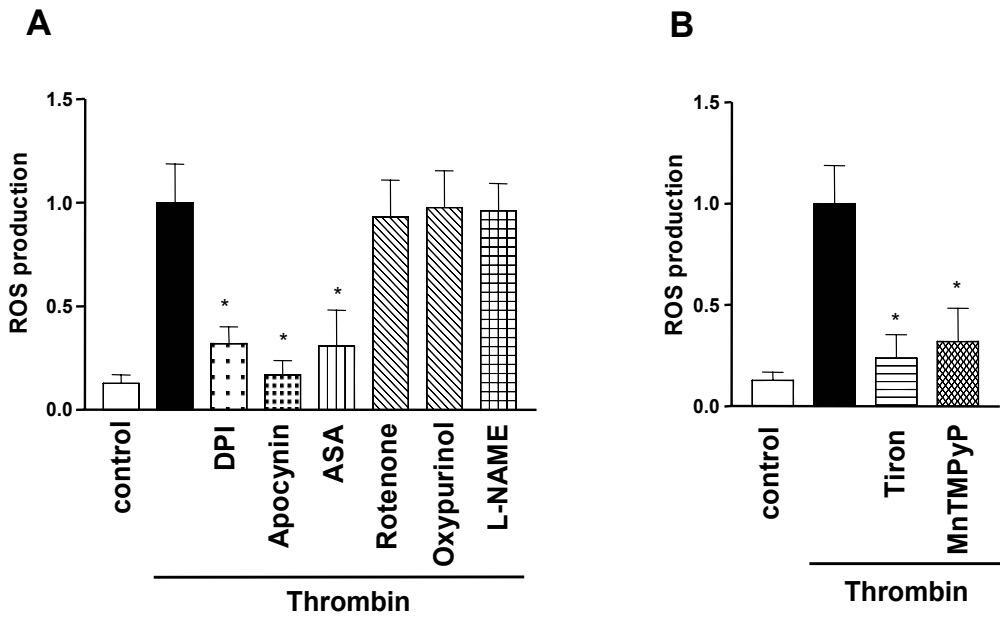

Figure 8. Platelet ROS production derives from NAD(P)H oxidase and cyclooxigenase. (A) Washed human platelets were preincubated with NOX inhibitors DPI (10 µM) and apocynin (600 µM), inhibitor of mitochondria metabolism rotenone (100 µM), xanthine oxidase inhibitor oxypurinol (100 µM), cyclooxigenase inhibitor ASA (100 µM) and NOS inhibitor L-NAME for 5 min and activated by thrombin (0.2 U/ml) for 1 min. **(B)** Inhibition of ROS production with superoxide scavengers tiron (3 mM) and MnTMPyP (100 µM) after thrombin stimulation. Data are shown as arbitrary units, mean ± SD, thrombin induced ROS production taken as 1, * compared to thrombin, $p<0.05$, n=6.

To further find out if NOX and COX cause independently production of ROS and represent therefore different sources and probably different types of ROS in platelets upon their stimulation, experiments were performed in which platelets were preincubated with DPI, tiron and ASA alone and ASA in combination with DPI or tiron. As shown on figure 9, additive inhibition of ROS was obtained when combination of COX and NOX inhibitor or ROS scavenger were used. These results confirmed two different sources of ROS in platelets measured by DCF.

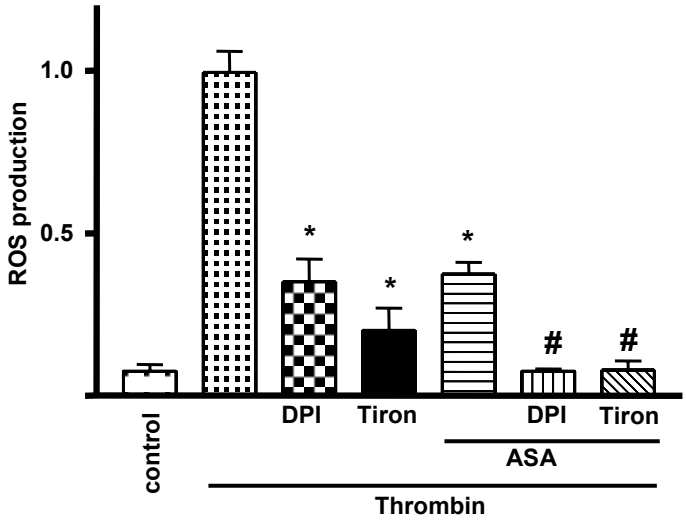

Figure 9. Additive inhibition of ROS by DPI and ASA or tiron and ASA. Platelets were preincubated with DPI (10 M), tiron (3 mM), ASA (100 µM), DPI and ASA or tiron and ASA and then stimulated by thrombin (0.2 U/ml). DPI and ASA or tiron and ASA inhibited ROS production stronger than DPI, tiron and ASA alone. Additive inhibition of ROS indicates two different sources but also a different type of radicals. Data are shown as arbitrary units, mean ± SD, thrombin induced ROS production taken as 1, * significantly different compared to thrombin, # significantly different compared to ASA with thrombin, $p<0.05$, n=3.

NOX inhibitors are not effective in targeting only NOX enzymes since they also can inhibit other flavin-dependent enzymes. Therefore, to study more specifically the role of NOX in platelets, chimeric peptide (gp91ds-tat) that inhibits p47phox association with NOX2 was used (Rey, Cifuentes et al. 2001). Since corresponding sequences that bind p47phox exist also in NOX1, and NOX4, such peptide is also predicted to block assembly of these NOX isoforms. This peptide is linked to the 9-amino-acid peptide of HIV viral coats (HIV-tat) that is internalized by all cells (Fawell, Seery et al. 1994). gp91ds-tat was used with its negative control, scrambled sequence of the same peptide (scramb-tat) in platelet experiments. However, gp91ds-tat peptide showed not to be specific since both gp91ds-tat and negative control (Fig. 10) in the same way inhibited ROS production and integrin activation at low and high concentrations (10 and 100 µM) and therefore cannot be used in platelet experiments.

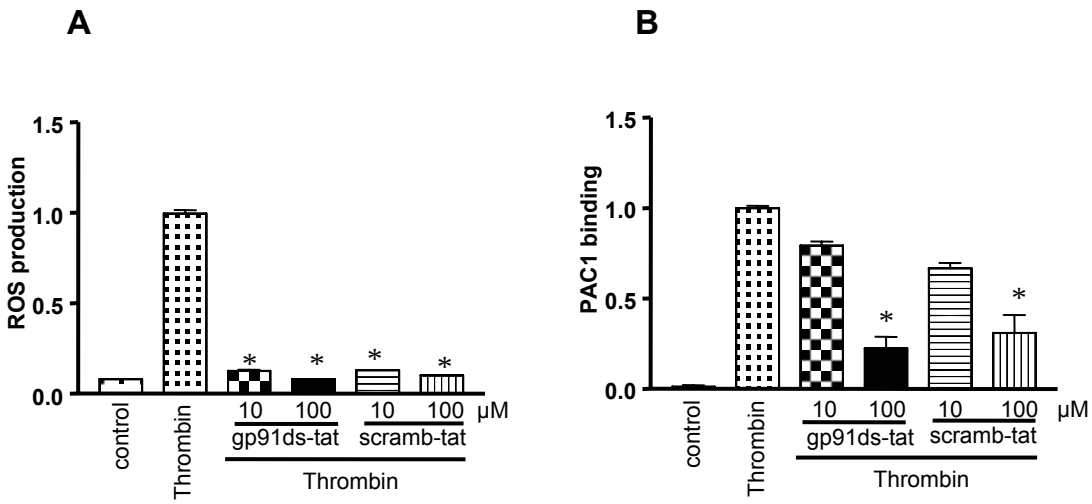

Figure 10. Peptide gp91ds-tat and negative control scramb-tat cannot be used to study NOX in platelets. Platelets were preincubated with 10 or 100 μM of NOX inhibitory peptide and negative control, and stimulated by thrombin. ROS production **(A)** and integrin activation **(B)** were analyzed. NOX inhibitory peptide and negative control have the same effect on ROS and integrin activation.

6.3. NAD(P)H Oxidase Subunits in Platelets

Several groups showed the presence of classical NOX subunits in platelets (Seno, Inoue et al. 2001; Krotz, Sohn et al. 2002; Pignatelli, Sanguigni et al. 2004). In order to determine whether some of the newly found isoforms are also expressed in platelets, highly purified platelets were run on Western blots for NOX1 and NOX4. For the first time NOX1 was shown to be expressed in platelets (Fig. 11), but not NOX4 that was present only in control cells (data not shown). As shown on figure 11, and in agreement with previous findings all 4 classical subunits were found in platelets. Leukocytes and HL-60 were used as control cells for classical NOX2, p22phox, p47phox, p67phox; Caco2 cells were used as a positive control for NOX1 and endothelial cells (EVC) for NOX4.

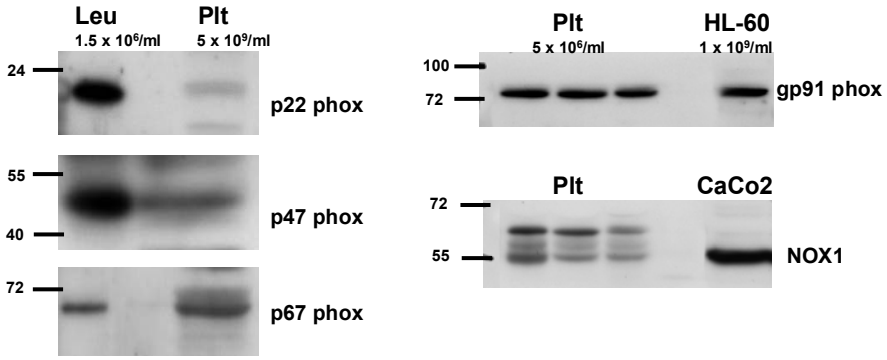

Figure 11. NOX subunits in platelets. Purified platelets were lysed and analyzed by Western blot for p22phox, p47phox, p67phox, gp91phox, and NOX1. leukocytes, HL-60 and CaCo2 cells were used as positive controls. Plt, platelets; Leu, leukocytes.

6.4. ROS Production Affects αIIbβ3 Activation

To address the role of ROS in platelet regulation, the NAD(P)H oxidase inhibitors DPI (50 μM) and apocynin (1.2 mM) were used in aggregation experiments. In accordance with previous studies (Salvemini, Radziszewski et al. 1991; Chlopicki, Olszanecki et al. 2004), platelet aggregation induced by Trap6 (30 μM) was significantly reduced in the presence of DPI and apocynin (45% and 35%, respectively, Fig. 12 A). Aggregation traces showed no loss of shape change even when high concentrations of DPI (300 μM) and apocynin (4.8 mM) were used (data not shown). This was further confirmed by unaltered Rho A and MLC phosphorylation activation (not shown), one of the mediators of platelet shape change in signaling cascade RhoA/Rho kinase/MLC (Bauer, Retzer et al. 1999). Since αIIbβ3 integrin activation plays a major role in the regulation of platelet adhesion and aggregation, the PAC-1 antibody was used that binds to the active conformation of this integrin to determine the possible role of ROS in integrin activation. DPI (10 μM) and apocynin (600 μM) inhibited thrombin-induced PAC-1 binding 45±9% and 43±11% ($n=5$, $p<0.05$, Fig. 12 B), respectively. In addition superoxide scavengers tiron and MnTMPyP (100 μM) also inhibited 60±9% and 70±6% ($n=5$, $p<0.05$), respectively αIIbβ3 activation while ASA, in agreement with previous studies (Santos, Moscardo et al. 2000; Valles, Santos et al. 2002), had no

effect on integrin activation indicating that peroxy compounds do not play a significant role in integrin activation.

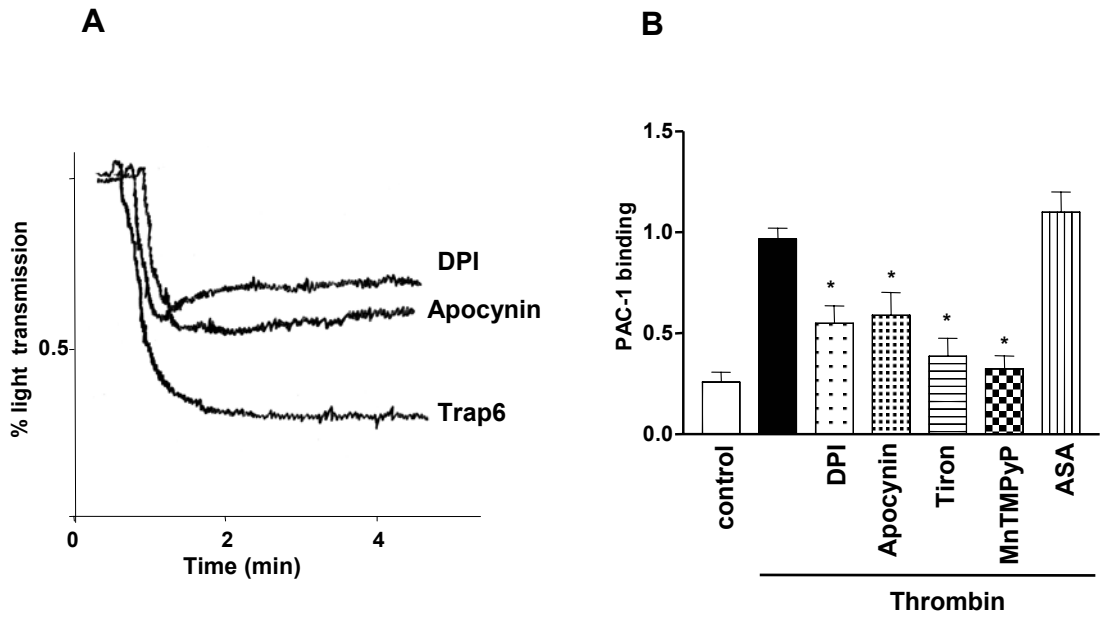

Figure 12. NAD(P)H oxidase inhibitors and superoxide scavenger reduce platelet aggregation and αIIbβ3 activation. (A) Platelet rich plasma was preincubated with DPI (50 µM) and apocynin (1.2 mM), then stimulated with Trap6 (30 µM) and allowed to aggregate for 5 min, with stirring at 1000 rpm. A representative aggregation tracing from 3 independent experiments is shown. (B) Washed platelets were preincubated with DPI (10 µM), apocynin (600 µM), tiron (3 mM), MnTMPyP (100 µM), ASA (100 µM), and stimulated with thrombin (0.2 U/ml) for 1 min. DPI, apocynin, tiron and MnTMPyP, but not ASA, inhibited αIIbβ3 activation (measured with FITC-PAC-1 mAb that binds to the activated form of the integrin). Data are shown as arbitrary units, mean ± SD, thrombin induced PAC-1 binding taken as 1, * significantly different at $p<0.05$ compared to thrombin, n=5.

At high shear, activated integrins are a key step in platelet-platelet interactions and thrombus formation. In here presented experiments, convulxin (a GPVI agonist) caused strong ROS production in platelets. To further confirm involvement of ROS produced by activated platelets in thrombus formation under blood flow conditions, whole blood was perfused in flow chamber over a collagen-coated surface under high shear conditions (1.000 s^{-1}). Platelets adhered to the collagen surface and formed thrombi in the presence and absence of DPI and apocynin. However, the formation of thrombi under high shear was significantly reduced by DPI and

apocynin (Fig. 13, i-iii). As measured by area coverage of thrombi (Fig. 13, iv), DPI and apocynin caused an 18±5% and 20±6% (n=3, $p<0.05$) inhibition of thrombus formation, respectively. Furthermore, platelet stimulation by thrombin, Trap6, U46619, convulxin and ADP showed a similar pattern of αIIbβ3 activation as for ROS production (Fig. 14). These results provide evidence that NAD(P)H oxidase-generated ROS is involved in integrin regulation.

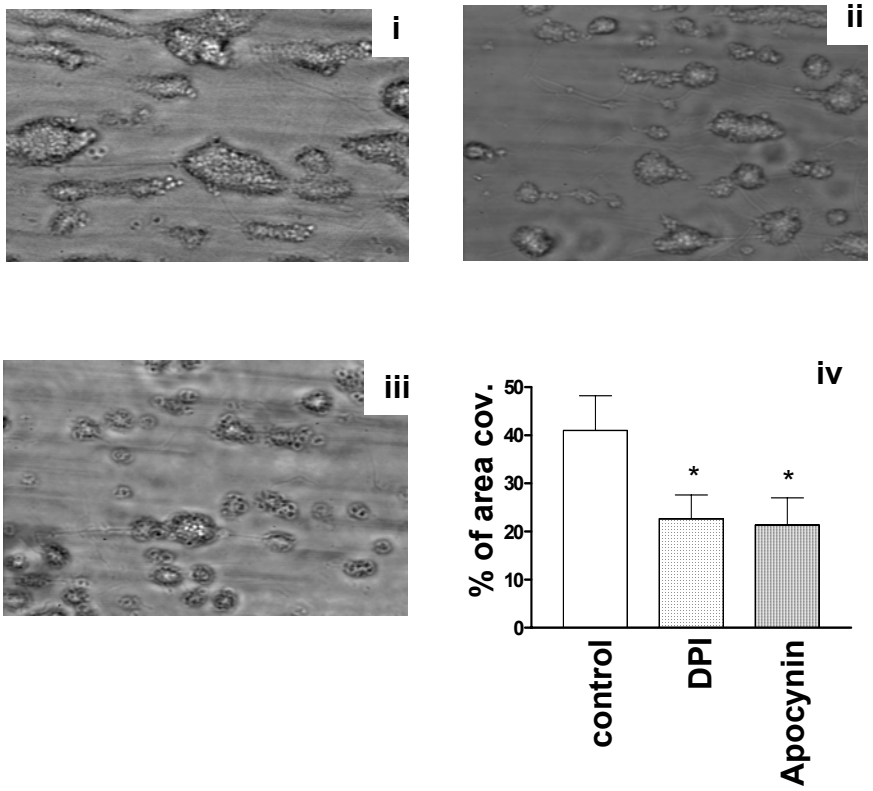

Figure 13. Inhibition of thrombus formation after treatment of platelets with DPI and apocynin. Whole blood from different individuals was treated with DMSO (i, control), DPI (ii, 100 µM) or apocynin (iii, 1.2 mM) and was perfused over a collagen-coated surface for 4 min under high shear conditions (1.000 s^{-1}). The results of the experiments are summarized as bar graphs showing % of measured area coverage (iv). DPI and apocynin caused 18±5% and 20±6 % inhibition of platelet adhesion, respectively, * significantly different at $p<0.05$ compared to control, n=3. The images shown are representative of three individual experiments.

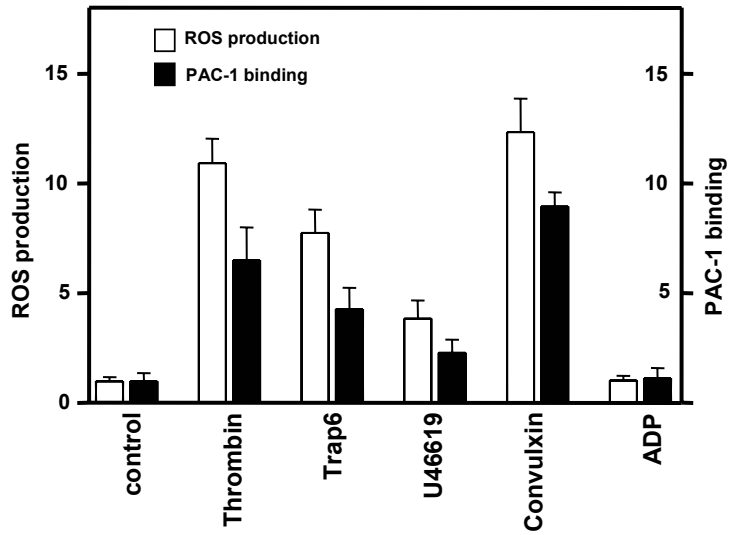

Figure 14. Platelet stimulation with different agonists showed similar pattern of αIIbβ3 activation as for ROS production. ROS production and αIIbβ3 activation measured by FITC-PAC-1 mAb after stimulation with different platelet agonists, thrombin (0.2 U/ml), trap6 (30 μM), U46619 (1 μM), convulxin (100 ng/ml) and ADP (50 μM). Data are shown as arbitrary units, mean ± SD, thrombin induced ROS production taken as 1, n=3.

6.5. P-Selectin, CD40L Expression, Serotonin Secretion and TxA_2 Production

To investigate whether inhibition of ROS production affects granule secretion, P-selectin expression (as a marker of alpha granule release) and serotonin secretion (as a marker of dense granule release) were analyzed.

Preincubation of platelets with NAD(P)H oxidase inhibitors and superoxide scavengers had no significant effect on alpha and dense granule secretion (n=4, $p>0.05$, Fig. 15 A, C). CD40L as inflammatory marker was reported to be expressed upon ROS production (Pignatelli, Sanguigni et al. 2004), however, in presented experiments decrease of ROS was not accompanied with decrease of CD40L expression (Fig. 15 B). Thromboxane synthase activity also was not affected by inhibition of ROS showing that these inhibitors had no effect on COX as possible source of ROS (Fig. 15 D).

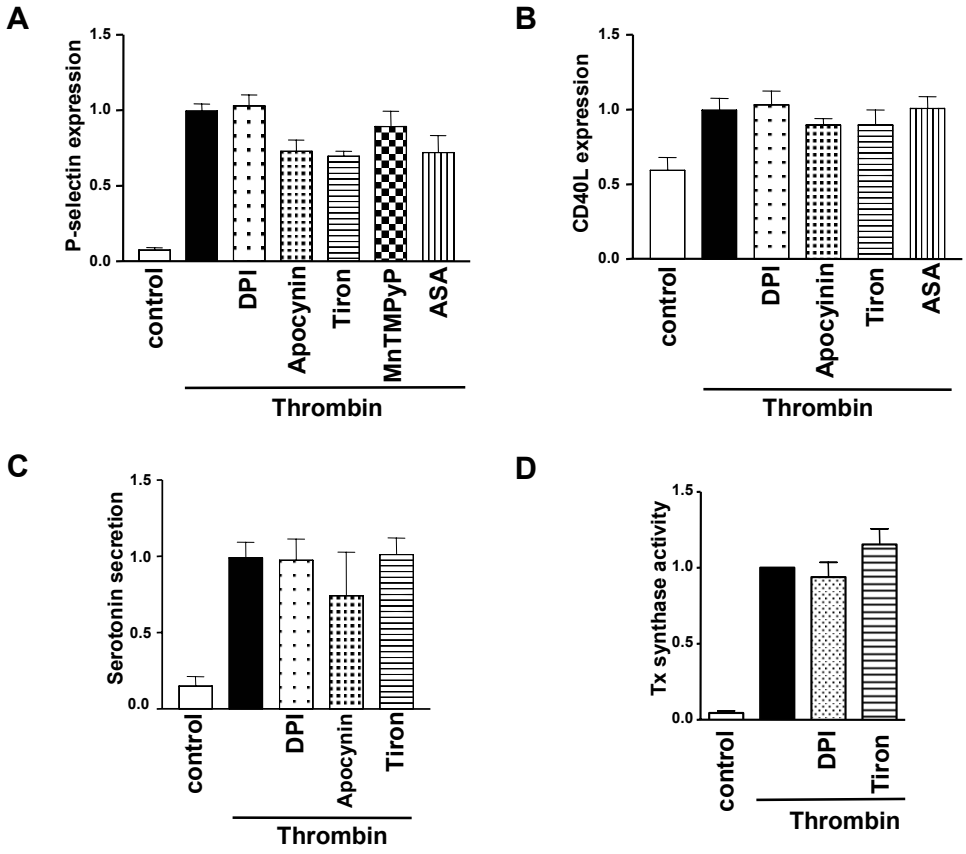

Figure 15. NAD(P)H oxidase, COX inhibitors and ROS scavengers do not inhibit P-selectin, CD40L expression, serotonin secretion and throboxane synthase activity. Surface expression of P-selectin **(A)**, CD40L **(B)**, serotonin secretion **(C)** and thromboxane synthase **(D)** were determined in washed human platelets preincubated with DPI (10 µM), apocynin (600 µM), tiron (3 mM), ASA (100 µM) after stimulation with thrombin (0.2 U/ml). Data are shown as arbitrary units, mean ± SD from 3 independent experiments, with thrombin-activated platelets defined as 1.0 ($p>0.05$ compared to thrombin).

6.6. ROS and NO Interaction

Several studies suggested that intracellularly produced ROS scavenges endothelial or platelet derived NO in a fast reaction forming peroxynitrite ($ONOO^-$) as end product (Bauersachs, Bouloumie et al. 1999; Chakrabarti, Clutton et al. 2004; Clutton, Miermont et al. 2004). This reaction may contribute to the inhibition of vasodilatation or promotion of platelet activation. Therefore, it was hypothesized that inhibition of ROS production in platelets might lead to the increase of cGMP levels and VASP phosphorylation as more NO should be present. VASP

phosphorylation (as a marker of NO/cGMP pathway) closely correlates with the inhibition of fibrinogen binding to the integrin αIIbβ3 of human platelets (Horstrup, Jablonka et al. 1994). However, as can be seen from Figure 16, there were no changes in VASP phosphorylation (Fig.16 A) or cGMP (Fig. 16 B) or cAMP (not shown) that would imply involvement of NO/cGMP/cGK Iβ pathway in the inhibitory effects of NAD(P)H oxidase inhibitors and superoxide scavengers on αIIbβ3 activation. SNP, a known NO donor, was used in experiments as a positive control for VASP phosphorylation and cGMP accumulation. These data suggest that ROS act in platelets by other mechanisms than scavenging NO with respect to integrin activation.

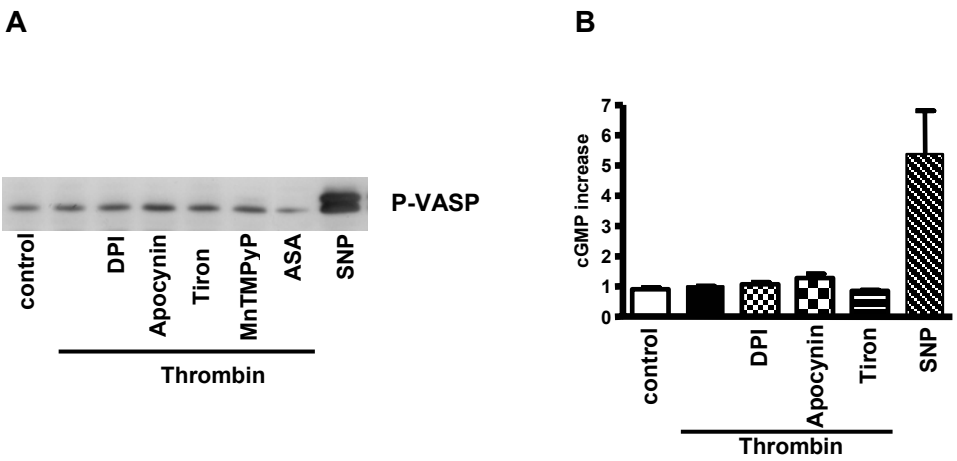

Figure 16. NAD(P)H oxidase inhibitors do not change VASP phosphorylation and cGMP content. Washed human platelets ($3x10^8$/ml) were preincubated with DPI (10 μM), apocynin (600 μM), tiron (3 mM), MnTMPyP (100 μM), or ASA (100 μM), and stimulated with thrombin (0.2 U/ml) for 1 min. Platelets were analysed for VASP phosphorylation **(A)** (Smolenski, Bachmann et al. 1998) by Western blot and for cGMP levels **(B)**. SNP (1 μM) was used as a positive control for P-VASP and cGMP. Data for cGMP are shown as fold increase, mean±SD, thrombin taken as 1, * significantly different at $p<0.05$ compared to thrombin, n=3.

6.7. Small GTPases Rap1 and Rac1

Rac can be activated by different agonists (Gratacap, Payrastre et al. 2001)and regulates actin assembly in activated platelets (Hartwig, Bokoch et al. 1995). Rap1 has been shown to be involved in control of αIIbβ3 mediated cell adhesion in

megakaryocytes and platelet aggregation (Bertoni, Tadokoro et al. 2002; Schultess, Danielewski et al. 2005). Both Rac1 and Rap1 participate in control of the superoxide production by NAD(P)H (Abo, Pick et al. 1991; Gabig, Crean et al. 1995). To test whether the activation of small GTPases was altered after ROS inhibition, the effect of DPI, apocynin, tiron and ASA was analyzed on thrombin induced activation of Rac1 and Rap1. Although tiron caused some partial inhibition of Rac1 activation, no significant changes in activation of Rac1 and Rap1 by thrombin was observed in platelets (Fig. 17).

These results show that integrin αIIbβ3 inhibition by NAD(P)H oxidase inhibitors and ROS scavengers is not due to the inhibition of small GTPases Rac1 and Rap1b that are probably upstream of NAD(P)H oxidase and ROS production.

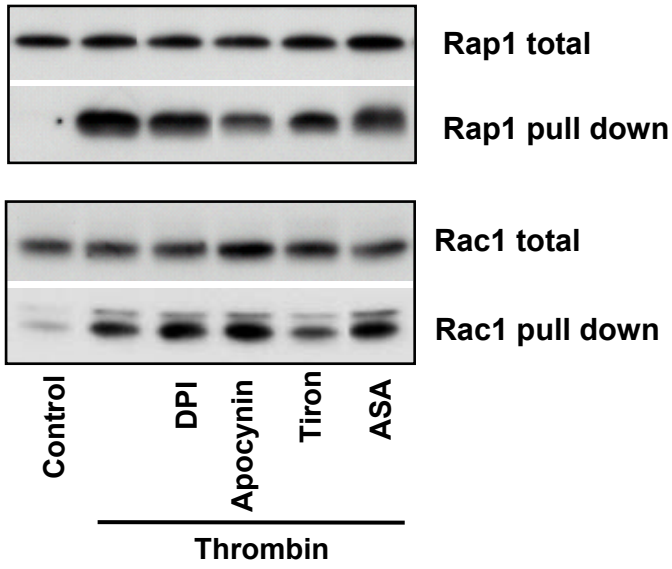

Figure 17. Small GTPases Rap1 and Rac1 in platelets after ROS inhibition. Washed platelets were preincubated with stimulated with thrombin (0.2 U/ml) for 1 min after preincubation with DPI (10 μM), apocynin (600 μM), ASA (100 μM), tiron (3 mM). The reaction was stopped by lysing the platelets with 2x lysis buffer. Aliquots for total amount of protein of interest were taken and the rest of lysed platelets were incubated with fused GST- protein binding domain beads (Rap1 or Rac1) for 1 hour on 4 ºC to have the activated form of the GTPases. Representative figures of 3 experiments are shown.

6.8. Platelet Activation and Thrombus Formation in NOX1 Knockout Mice

Since NOX1 isoform was found to be present in platelets NOX1 knockout mice were used to study the role of the ROS produced by NOX1 in platelets. These animals were healthy and did not show any spontaneous phenotype. It has been reported that NOX1-deficient mice have a moderately decreased basal blood pressure and a defect in sustained blood pressure in response to angiotensin II (Gavazzi, Banfi et al. 2006).

ROS production was measured in platelets of knock out (KO) and wild type (WT) mice after thrombin stimulation. However, no significant difference was observed between two groups of mice, although in WT platelets increase in ROS was 2 fold and in KO platelets 1.5 fold in response to thrombin when compared to control (Fig. 18 A).

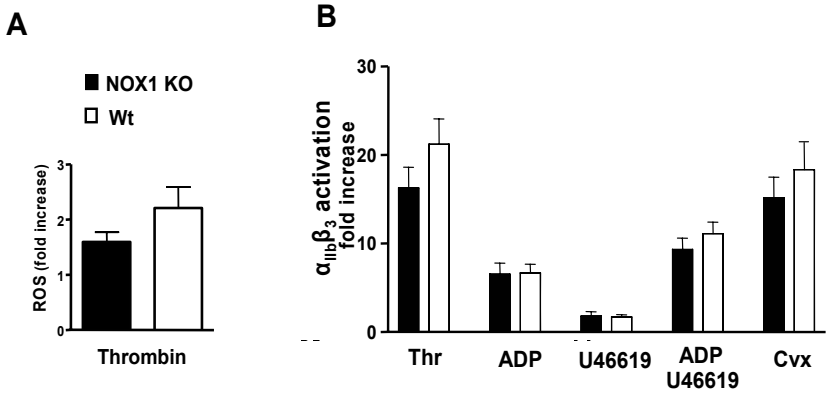

Figure 18. ROS production and integrin activation in NOX1 KO mice. Washed mouse platelets were stimulated with thrombin (0.01 U/ml), ADP (10 μM), U46619 (3 μM), ADP and U46619 together, and convulxin (100 ng/ml) for 1 min. Platelet activation was monitored by ROS production (A) and integrin αIIbβ3 activation (B). Results are presented as fold increase from control, in at least 3 experiments, as mean±SEM. There was no significant difference in the activation of ROS and integrin αIIbβ3 between WT and KO mice.

Since NOX inhibitors and scavengers decreased integrin activation in human platelets, knock out and wild type platelets were analysed for αIIbβ3 integrin activation after stimulation. Platelets were activated with thrombin (0.01 U/ml), ADP (10 μM), thromboxane analogue U46619 (3 μM), ADP/U46119, and convulxin (100 ng/ml) for 1 min (Fig 18 B), stained with JON/A antibody and analyzed by FACS.

Increase in integrin activation was slightly different for thrombin (16 vs. 21 fold in KO and WT, respectively), and for convulxin (15 vs. 19 fold in KO and WT, respectively). However, these differences were not significant.

Thrombus formation was as well evaluated during perfusion of whole blood from mice over collagen covered strips under high shear in flow chamber as described in Methods.

Wild type and NOX1-deficient platelets adhered to collagen fibers and adherent platelets initiated the formation of platelet aggregates. However, thrombus formations of NOX1 KO mice were slightly reduced in size then of WT mice. After analysis of area covered by thrombi, in the case of WT area coverage was 64%, while in the case of KO it was 53% (Fig. 19), however, this was not significant.

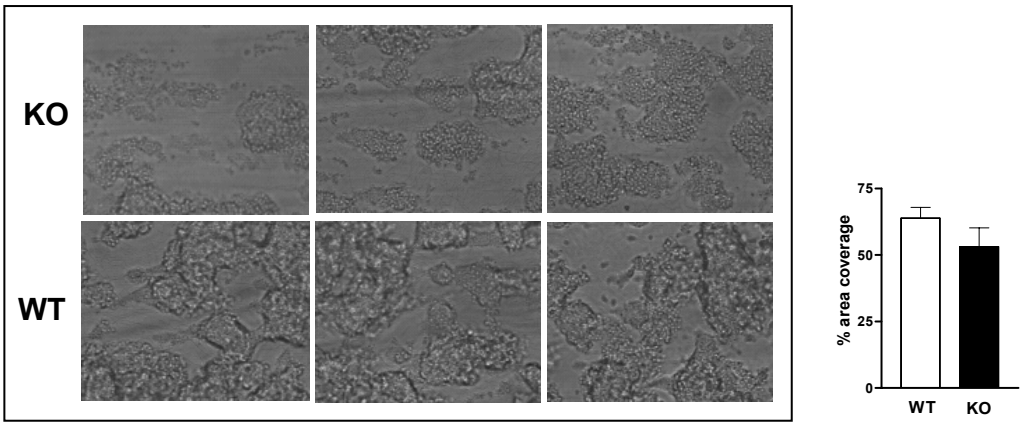

Figure 19. Flow chamber analysis of NOX1 KO and WT mice. Whole blood from KO and WT mice was perfused over a collagen-coated surface for 4 min under high shear conditions (1.000 s^{-1}). The results of the experiments are summarized in histogram showing % of measured area coverage. The images shown are representative of three individual experiments.

Although the differences were not significant, platelets from NOX1 KO mice showed tendency to form less ROS after thrombin activation, and their integrin activation in response to strong agonists was slightly decreased, as well as thrombus formation over collagen, suggesting at least a partial contribution of NOX1 in platelet function. One of the possible reasons of minor differences could be because of the presence of other subunit, NOX2 that could compensate for the deficient NOX1 in ROS production. Therefore, a double knock out NOX1/NOX2 might be an approach to study the role of ROS in platelets.

7. Role of Protein Kinase G I in Platelets

7.1. Stimulation of sGC and PKG I Does not Activate ERK or p38 Kinases

NO/cGMP and prostacycline inhibit platelet activation and adhesion to the injured endothelial cells. NO/cGMP mediates its effects through PKG I, and in conditions of ischemia/reperfusion platelet adhesion and aggregation in arterioles, capillaries, and venules are drastically enhanced in PKG I deficient mice (Massberg, Sausbier et al. 1999). Recently, a stimulatory role for NO/cGMP at low concentrations has been proposed in response to platelet agonists that is dependent on PKG stimulation. Mitogen-activated protein kinases (MAPK) have been shown to be downstream effectors of PKG which then modulate platelet secretion and integrin activation (Li, Xi et al. 2003; Li, Zhang et al. 2006; Stojanovic, Marjanovic et al. 2006). In order to study in detail this newly proposed pathways, experiments were performed to answer questions whether increase of NO/cGMP will induce stimulatory pathways (like MAP kinases ERK and p38) that will result in activated platelets.

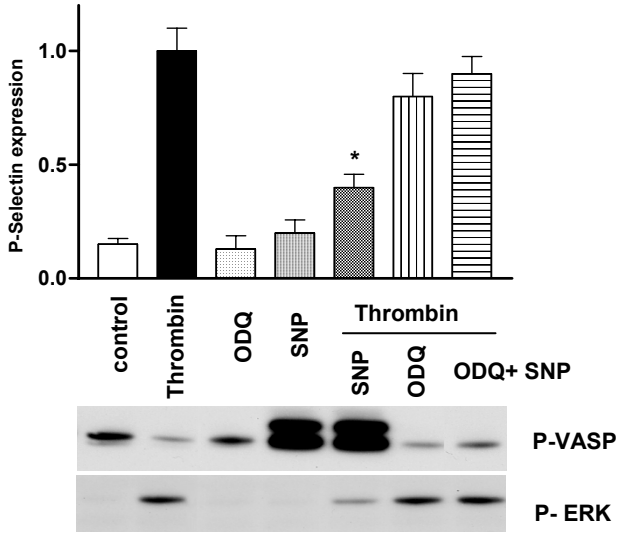

Figure 20. NO plays inhibitory role in platelets via sGC. Platelets were stimulated with thrombin (0.005 U/ml), SNP (10 μM), ODQ (10 μM) alone, or in combination with thrombin. SNP and ODQ alone had no effect on platelet activation, while SNP inhibited P-selectin expression and ERK activation stimulated by thrombin. ODQ had no effect on stimulation of platelets, while applied

together with SNP, ODQ prevented inhibitory effect of SNP on platelet activation, * significantly different as compared to thrombin, $p<0.05$, n=3.

Platelets treated alone with NO donor SNP, and soluble guanylyl cyclase (sGC) inhibitor ODQ showed no increase in ERK activation, while SNP increased VASP phosphorylation, but no P-selectin expression. When platelets were stimulated with thrombin, SNP inhibited thrombin induced ERK phosphorylation and P-selectin expression, while ODQ had no effect. However, ODQ by inhibition of cGMP production blocked the SNP inhibited ERK activation and P-selectin expression after thrombin stimulation, and also reduced VASP phosphorylation (Fig. 20). These results indicate that NO has an inhibitory effects in platelets that are mediated through sGC.

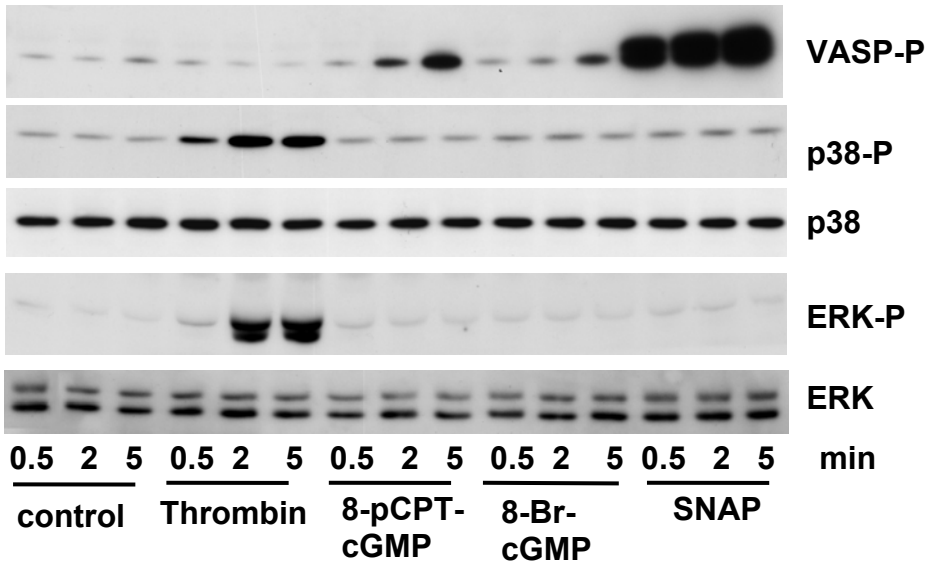

Figure 21. p38 and ERK are not activated by cGMP-dependent protein kinase (PKG). Washed human platelets were incubated with thrombin (0.01 U/ml), 8-pCPT-cGMP (200 µM), 8-Br-cGMP (200 µM), or SNAP (100 µM) for 0.5, 2 or 5 min, and analyzed for VASP (VASP-P Ser239), p38 (p38-P) and ERK (ERK-P) phosphorylation by Western blotting. p38 (p38) and ERK (ERK) proteins served as loading control. The blots shown are representative of three individual experiments.

Further to investigate whether PKG, that is activated by cGMP, has a role in ERK and p38 phosphorylation and therefore platelet activation, experiments were done

to directly stimulate this kinase by cGMP analogues. Incubation of platelets with 8-pCPT-cGMP (200 µM) and 8-Br-cGMP (200 µM) time-dependently increased VASP serine239 phosphorylation, a validated marker of PKG activation (Gambaryan, Geiger et al. 2004; Lohmann and Walter 2005). SNAP (100 µM) as NO donor caused strong VASP phosphorylation. However, neither cGMP analogues nor cGMP-elevating agents increased p38 or ERK phosphorylation (Fig. 21) which was clearly observed with thrombin (0.01 U/ml) used as a positive control.

7.2. p38 Has no Effect on Platelet Aggregation and P-selectin Expression

Platelet p38 MAP kinase has been found to be phosphorylated and activated in response to collagen, thromboxane analogue, thrombin, vWF and other platelet activating agents (Kramer, Roberts et al. 1995; Saklatvala, Rawlinson et al. 1996; Canobbio, Reineri et al. 2004). However, both the upstream mechanisms of p38 activation, and their downstream effects, are presently controversial and unclear. Therefore, signals leading to p38 activation were studied and the role of p38 in platelets was investigated by using p38 inhibitors and control compound.

In low dose (0.005 U/ml) thrombin-stimulated platelets, both p38 inhibitors (SB202190, SB203580) did not prevent phosphorylation of p38 itself but strongly inhibited phosphorylation of Hsp27 (a distal target downstream of p38) whereas the control compound (SB202474) had no effect on Hsp27 phosphorylation (Fig. 22 B). Surprisingly, p38 inhibitors under these conditions did not significantly inhibit P-selectin expression (Fig. 22 B) and integrin activation (data not shown). Furthermore, the cGMP elevating agent SNP inhibited P-selectin expression and p38 phosphorylation (Fig. 22 B). In contrast to some published data (Li Z 2006 Blood), p38 inhibitors caused only moderate, unspecific effects in these experiments, since both p38 inhibitors as well as the inactive analog had very little if any effect even on low (0.005 U/ml) thrombin-induced platelet aggregation which was inhibited by the αIIbβ3 integrin antagonist RGDS (1 mM), and SNP (10 µM) (Fig. 22 A). Similar p38 inhibitor effects were observed not only with respect to

thrombin-stimulated P-selectin expression and platelet aggregation. Therefore, it can be concluded that p38 does not play a significant role in thrombin-induced activation and aggregation of human platelets.

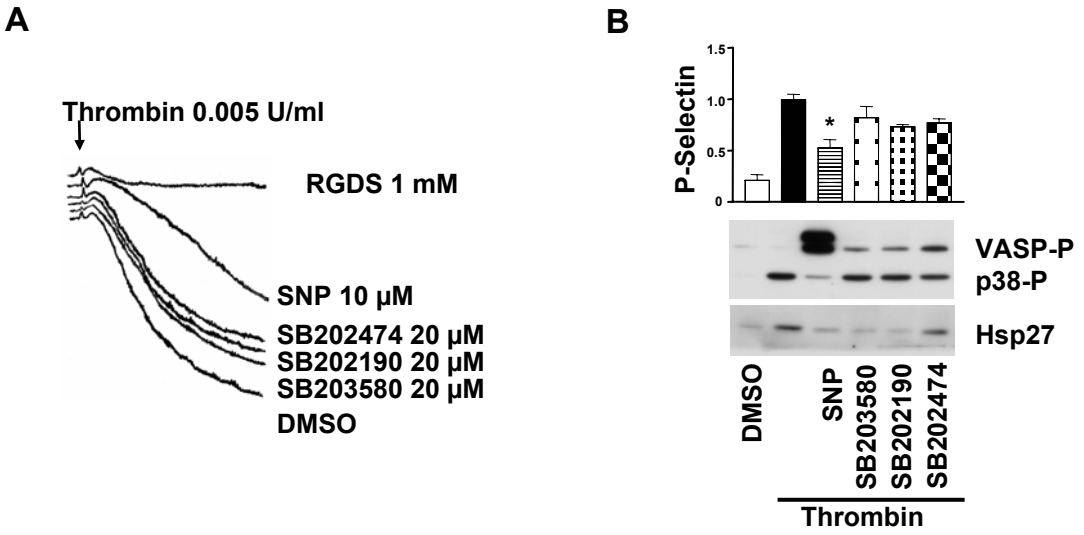

Figure 22. p38 MAP kinase inhibitors had no significant effect on thrombin-induced aggregation and P-selectin expression. (A) Washed platelets were assayed for thrombin (0.005 U/ml)-induced aggregation in a turbidometric aggregometer. Platelets were preincubated for 5 min with RGDS (1 mM), SNP (10 µM), p38 inhibitors (SB203580, SB202190, 20 µM), a control (inactive) compound (SB202474, 20 µM), or DMSO, and then stimulated with thrombin. **(B)** In the same type of experiments washed human platelets were analyzed for P-selectin. Data, expressed in arbitrary units for P-selectin, represent means ± SEM for 4 independent experiments, with thrombin-stimulated samples designated as 1, * significantly different at $p<0.05$ as compared to thrombin. Also shown are effects on VASP-P (Ser239), p38-P, and Hsp27-P obtained in at least 3 independent experiments.

7.3. Activation of p38 MAP Kinase by ADP and TxA_2 Secretion

A central role among platelet activating factors is played by ADP which induces multiple platelet responses and potentates platelet activation and aggregation caused by other agonists (Kunapuli, Dorsam et al. 2003; Hardy, Conley et al. 2005). Thrombin, TxA_2, ADP are well known to induce p38 activation (Dangelmaier, Jin et al. 2000) but their interdependence for producing this effect has not been established. Therefore, the role of ADP in thrombin-stimulated

platelet p38 activation, P-selectin expression and integrin activation was investigated.

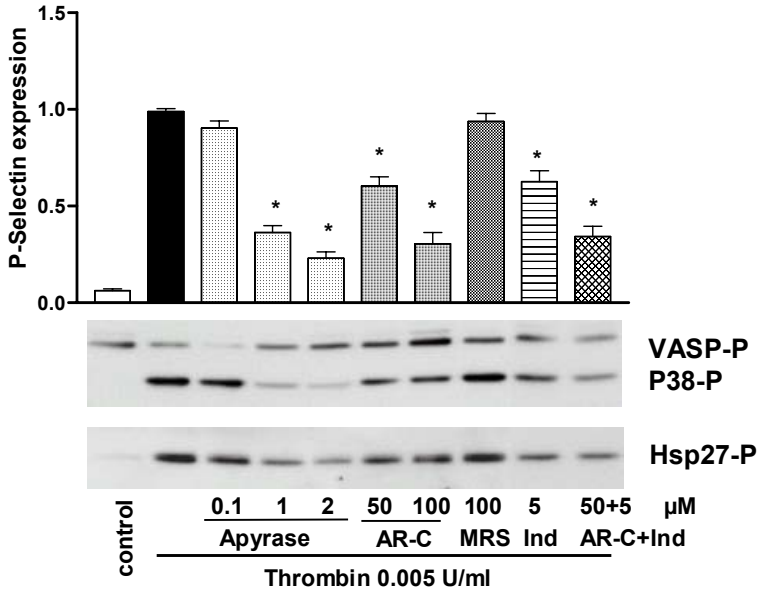

Figure 23. ADP and TxA_2 mediated activation of p38 in low dose thrombin concentration. Washed human platelets ($4x10^8$/ml) were preincubated 5 min with either buffer alone, apyrase (0.1, 1 and 2 U/ml), AR-C (50 and 100 nM), MRS (100 µM), indomethacin (Ind, 5 µM), or AR-C (50 µM) and indomethacin (5 µM) together, and then stimulated with thrombin (0.005 U/ml) for 1 min. Samples were then analyzed for P-selectin expression (FACS), and phosphorylation of VASP (serine 239), p38, and Hsp27 (VASP-P (Ser239), p38-P, Hsp27-P) on Western blots using phosphospecific antibodies. Data for P-Selectin are shown as fold increase, mean ± SEM, thrombin taken as 1, * significantly different at $p<0.05$ compared to thrombin, n=5.

Apyrase dose-dependently inhibited thrombin-induced effects such as p38 activation, phosphorylation of Hsp27, P-selectin expression (Fig. 23), as well as integrin αIIbβ3 activation (data not shown). Therefore, secreted ADP is, at least partially, the mediator of these thrombin effects which were also inhibited by a $P2Y_{12}$ receptor antagonist (AR-C69931MX) but not by a $P2Y_1$ receptor antagonist (MRS21799). These data support the mediating role of secreted ADP and its $P2Y_{12}$ receptor in these thrombin effects. Consistent with this conclusion, as well as with previously published data (Oury, Toth-Zsamboki et al. 2002), a selective $P2X_1$ receptor agonist (α, β-MeATP, 2.5 µM), failed to cause any p38 phosphorylation (data not shown).

Preincubation of platelets with indomethacin also inhibited thrombin-stimulated p38 activation, Hsp27 phosphorylation and P-selectin expression to a similar extent as that observed with AR-C69931MX (Fig. 23). Indomethacin and AR-C69931MX had additive inhibitory effects on thrombin-stimulated p38 activation, Hsp27 phosphorylation and P-selectin expression. p38 was slightly activated by either ADP (20 µM) or a thromboxane receptor agonist U46619 (1 µM) used alone (data not shown). These results indicate that activation of $P2Y_{12}$ (but not $P2Y_1$ or $P2X_1$) and thromboxane receptors by secreted ADP and TxA_2, respectively, plays a significant amplifying role in p38 activation by low dose thrombin. However, potent platelet agonists (i.e. high thrombin concentration, 0.01 U/ml) did not require the amplifying mechanisms of secondary ADP/ TxA_2 secretion for p38 activation, since AR-C69931MX or indomethacin then had no inhibitory effects (data not shown). Similar observations have been made in the case of ERK activation (Falker, Lange et al. 2004).

8. Role of NO/cGMP and cAMP Pathways in Megakaryocytes

8.1. *In vitro* Development of Megakaryocytes

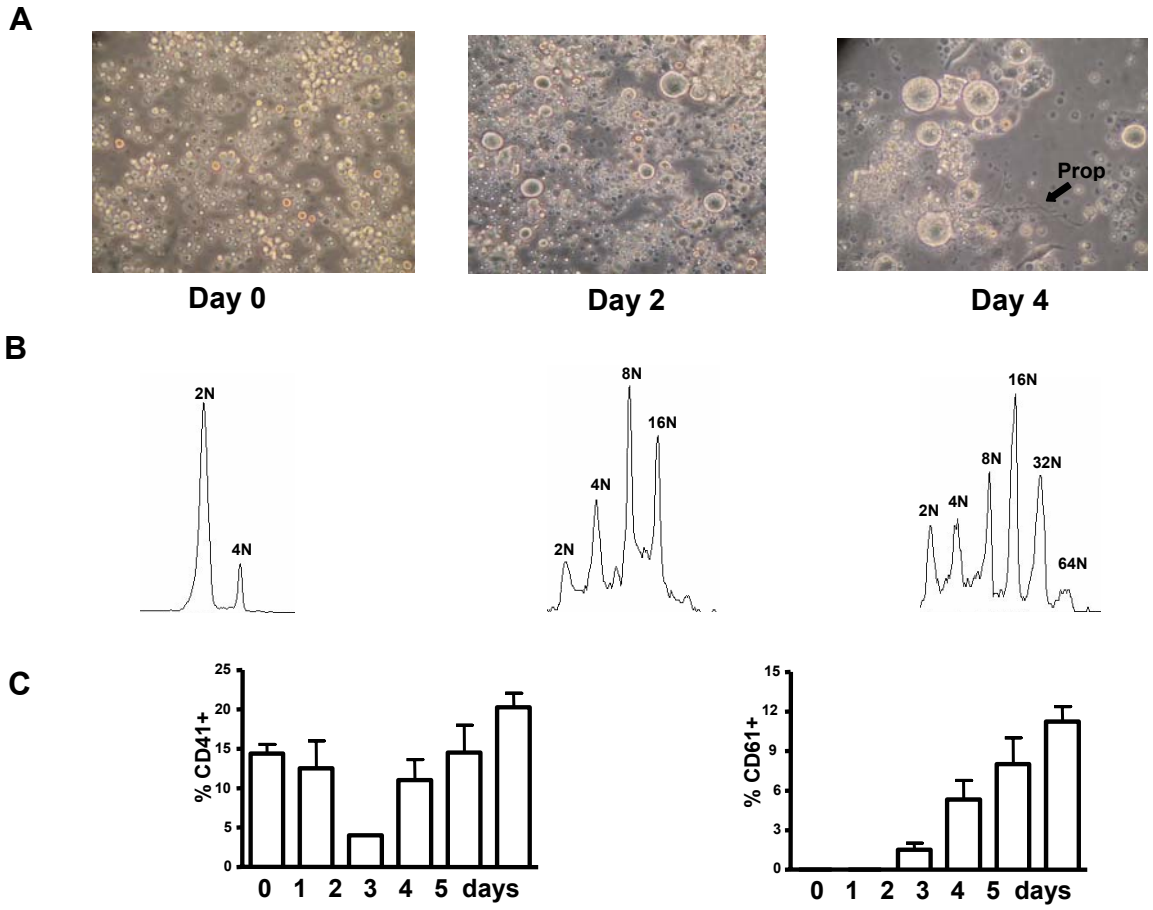

Figure 24. Culturing of *in vitro* derived mouse megakaryocytes. (A) Phase contrast images of mouse fetal liver cells on staring day of culture (day 0), and increasing number of megakaryocytes and proplatelets on day 2 and 4. **(B)** Ploidity analysis of starting cells and megakaryocytes. Notice increase of DNA content in megakaryocytes up to 64N at day 4. **(C)** MK markers CD41 and CD61 were monitored in whole culture during 5 days.

In order to study the role of cyclic nucleotides (cGMP, cAMP) in megakaryocyte and platelet development, *in vitro* derived megakaryocytes were established from mouse fetal liver cells.

Preparation of megakaryocyte cultures is described in details under Methods (page 39). Mouse fetal liver cells derived from 13.5 or 14.5 dpc were cultured with thrombopoietin (TPO) for 4 to 5 days. As seen on fig. 24, starting cultures (Day 0) comprised of small, homogenous cells that were characterised as diploid and tetraploid cells (2N, 4N, Fig. 24 B) typical for dividing cells. With increased time of culturing, big cells with multilobular nuclei appeared, resembling megakaryocytes. At day 2 ploidity of cells increased to 16 N, while majority of cells were in 8N. At day 3 proplatelet structures started to appear, and reached maximum at day 4-5 (Fig. 24 A). Also ploidity in these cells reached maximum by having 64N, with a peak of 16N. In the culture of fetal liver cells, under treatment of TPO megakaryocytes develop, however also other haematopoietic cell lineages are present. Therefore, cells were also analyzed for megakaryocytic markers CD41 and CD61. Interestingly, while starting cultures were negative for CD61, in the same time they comprised around 10-15% CD41 positive cells. At day 1 and 2 this amount dropped to 5%, and then again started to increase with culturing till day 5. These findings are in agreement with recent reports that show presence of CD41 marker on certain pluripotent stem cells, they lose CD41 through differentiation and again gain it at some point of maturation to megakaryocytes (Pang, Weiss et al. 2005). Therefore, in fallowing experiments CD61 was used as a secure marker of megakaryocytic maturation.

8.2. Increased PKG, PKA, VASP and β sGC Expression During Megakaryocyte Differentiation

Expression levels of proteins involved in cGMP and cAMP pathways (eNOS, PKG, VASP, β sGC, PKA catalytic subunit) were analyzed by Western blot in starting fetal liver cells (FLC), as well as in there from developed megakaryocytes after day 2 and day 4 in the culture. Since megakaryocytes were obtained by enrichment over BSA gradient, also the non-sedimented small cells (non-megakaryocytes, negative for megakaryocyte markers, data not shown) were analyzed in parallel (Rest).

Interestingly, PKG I and VASP were expressed at a very low level or not at all in starting fetal liver cells at day 0 and were highly increased in megakaryocytes at day 2 and at least doubled at day 4. At the same time there were decreased levels of these proteins in the “rest” fraction of the culture. Increase in the expression level was also observed for β sGC that was not primarily expressed in FLC. However, once expressed the level did not change during further differentiation of megakaryocytes (Fig. 25 A). The same results were observed for α sGC (data not shown). PKA C (catalytic subunit) was expressed in starting FLC, and significant enrichment of this protein was observed during the maturation of megakaryocytes. Phosphorylation of VASP at the position Ser157 was observed already in megakaryocytes at day 2 when no proplatelets were observed and highly increased at day 4 while phosphorylation of Ser239 was observed late, just at day 4 when culture was full of proplatelet structures. These results indicated potential role of PKA in the process of megakaryocytes´ differentiation from haematopoietic stem cells of fetal liver while later appearance in phosphorylation of more PKG specific site in VASP (phospho Ser239) could implicate importance of this kinase in proplatelet formation.

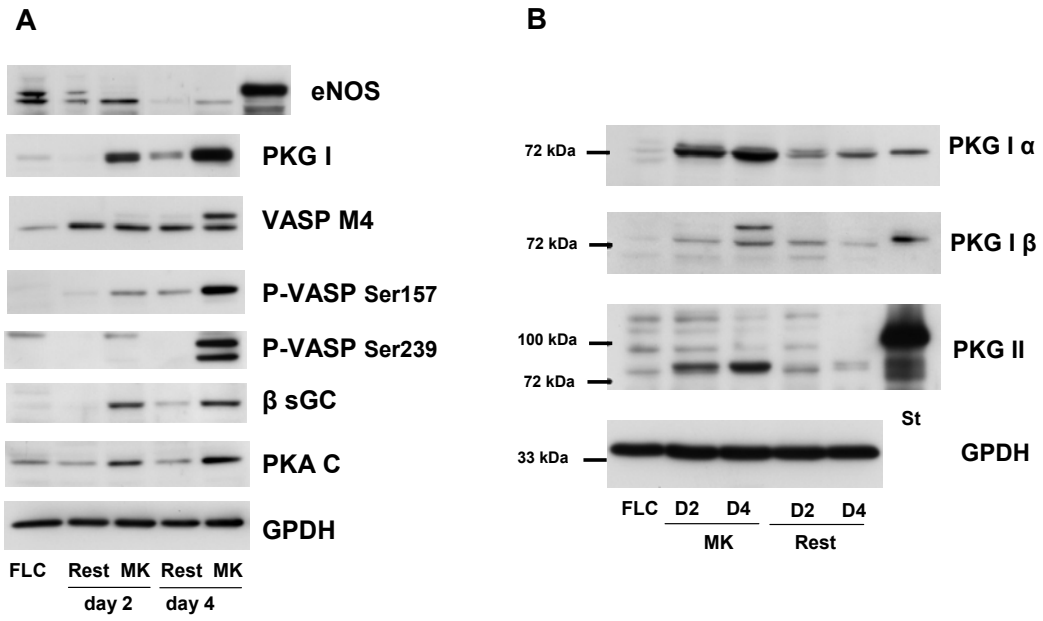

Figure 25. Increased expression of PKG I, VASP, β sGC and PKA C during differentiation of mouse fetal liver cells into megakaryocytes. (A) Megakaryocytes were collected by BSA gradient on days 2 and 4, and were analyzed together with the rest of “non” MK cells (Rest,

negative for CD61) for eNOS, PKG I, VASP, phospho-VASP (Ser159, Ser239), β sGC, PKA C subunit. Huvec cells were used as control for eNOS. **(B)** Megakaryocytes showed to contain both isoforms of PKG I (PKG I α, PKG I β), however PKG II was not expressed. GPDH was used as a loading control.

eNOS was present in the fetal liver cells, however later in megakaryocytes it disappeared and a band of lower size then in control Huvec cells was observed. Because of the possibility of a new isoform present in platelets, and functional proofs of platelet NO production that has been so far shown (Freedman, Li et al. 2000), the presence of eNOS should be analyzed on the level of mRNA. Megakaryocytes did not contain any iNOS or nNOS (data not shown, as positive control for iNOS a lysate of LPS treated mouse macrophages was used).
Cultured megakaryocytes showed to contain both isoforms of PKG I α and β, while PKG II was not observed (Fig. 25 B).

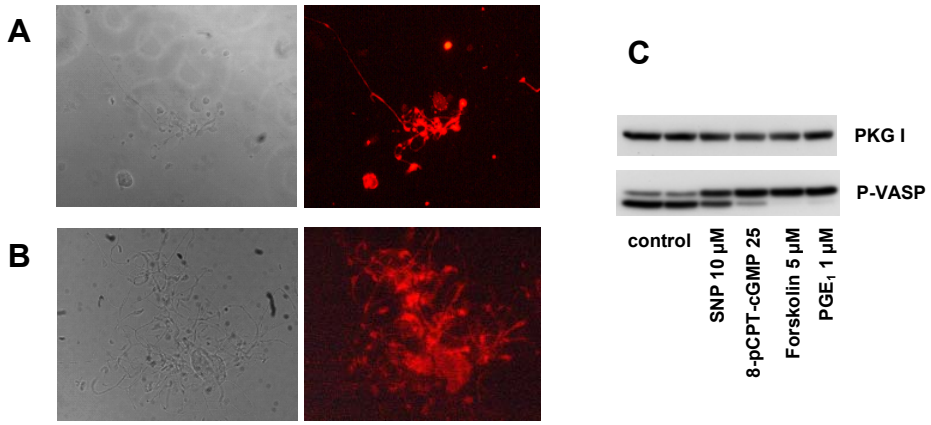

Figure 26. Proplatelets contain PKG and VASP. Phase contrast and immunofluorescence images of megakaryocytes and proplatelets for PKG I **(A)** and VASP **(B)**. Magnification 1000x. **(C) PKG and PKA can be stimulated in *in vitro* derived megakaryocytes.** Megakaryocytes were rested for 1 h after BSA gradient in DMEM without FCS or TPO, and stimulated for 5 min with SNP (10 µM), 8-pCPT-cGMP (25 µM), forskolin (5 µM), and PGE_1 (1 µM). Western blot of phospho-VASP 239Ser (P-VASP) and PKG is shown.

Immunofluorescence staining was made for PKG I and VASP at day 4 when megakaryocytes culture is rich in proplatelet structures. The staining for PKG and

VASP was present in proplatelet structures, thus further confirming the expression of these proteins in *in vitro* system (Fig. 26 A, B).

To show *in vitro* generated megakaryocytes have functional PKA and PKG, activation assay was done. After BSA gradient, enriched megakaryocytes were resuspended in DMEM without FCS or TPO and rested for one hour. Afterwards cells were stimulated with SNP (10 μM), 8-pCPT-cGMP (25 μM), forskolin (5 μM) or PGE_1 (1 μM) for 5 min and analyzed for phospho-Ser239 VASP (Fig. 26 C). As seen on the blot, although in control cells VASP was already phosphorylated, further increase of VASP phosphorylation was observed after cGMP increase and a typical shift of phosphorylated VASP after cAMP increase was observed.

8.3. Modulation of Megakaryocyte Differentiation by cGMP and cAMP

Several reports suggested a role for NO/cGMP and cAMP in differentiation of stem cells, and megakaryocytes. In order to investigate the role of cyclic nucleotide pathways in haematopoietic stem cell differentiation, mouse fetal liver cells were treated throughout culturing with substances that modulate cGMP levels, namely, PKG activator (8-pCPT-cGMP, 100 μM), NO donor (DEA/NO, 5 μM), and β sGC inhibitor (ODQ, 10μM), or modulate cAMP levels, namely, adenylate cyclase activator (forskolin, 1 and 5 μM, and PGE_1, 1μM), and PKA inhibitor (H89, 1 and 5 μM). On day 5 cell cultures were analyzed for the presence of megakaryocytes by determination percentage of CD61 positive cells, for proliferation (count) and for the expression levels of proteins involved in cGMP/cAMP pathway.

Every day treatment with 8-pCPT-cGMP lead to the downregulation of PKG expression (Fig. 27 B), however, there was no influence on megakaryocytes production or cell proliferation. In the same time, increase in VASP phosphorylation (expression level was unchanged) at both sites (Ser157 and Ser239) was observed, indicating high PKA and PKG activity. ODQ caused decrease in β sGC expression, however no significant change in megakaryocyte production or cell proliferation was observed.

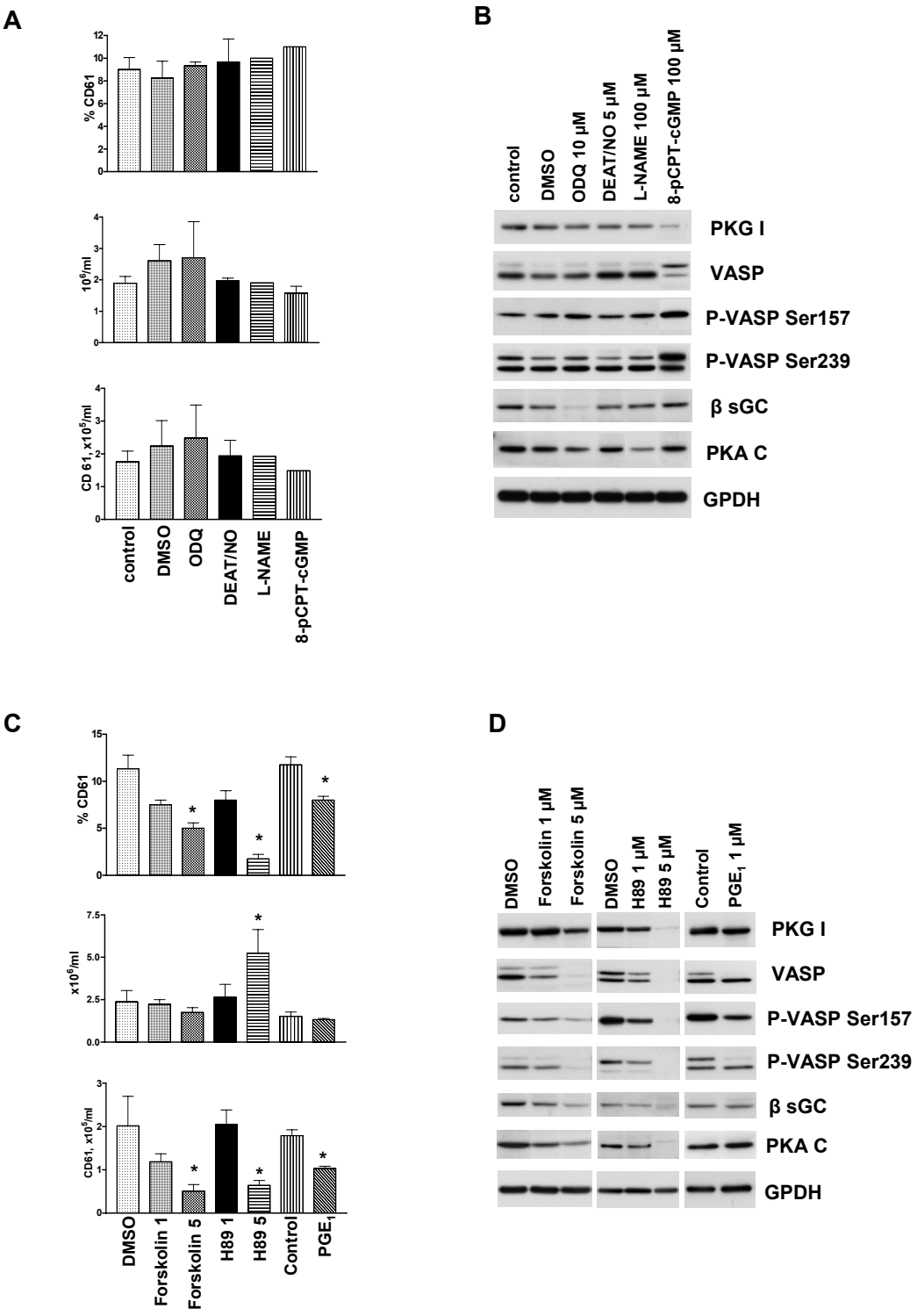

Figure 27. PKA but not PKG plays role in megakaryocytes generation. For modulation of cGMP pathway fetal liver cells were cultured with TPO and stimulated every day with DMSO, ODQ (10 µM), DEA/NO (5 µM), L-NAME (100 µM), and 8-pCPT-cGMP (100 M). On day 5 cells were analyzed for CD61 (expressed as %, and as an absolute count), cell count **(A)** and Western blot **(B).** For modulation of cAMP pathway cells were treated every day with DMSO, forskolin (1, and 5

μM), H89 (1, and 5 μM) and PGE_1 (1 μM) and analyzed on day 5 for CD61 (expressed as %, and as an absolute count), cell count **(C)** and Western blot **(D),** * significantly different as compared to DMSO, or control, $p<0.05$, n=6.

On the other hand, forskolin dose dependently, as well as PGE_1 and H89 inhibited the generation of megakaryocytes, as decrease in percentage of CD61 positive cells was observed (Fig. 27 C). Decrease in megakaryocytes was not due to decrease in cell number, as proliferation was not changed as compared to control (Fig. 27 C, 2nd graph) and there was a decrease in absolute number of CD61 positive cells (Fig. 27 C, 3rd graph). Extensive treatment with lower concentration of forskolin and H89 decreased the expression of PKA, VASP as well as β sGC (Fig. 27 D). Phosphorylation was also decreased, probably due to the lower expression of VASP. PGE_1 only caused decrease in total and phosphorylated VASP. Higher concentration of forskolin and H89 (5 μM) downregulated almost completely VASP, PKA, β sGC (Fig. 27 D) and inhibited in the same way the production of megakaryocytes (Fig. 27 C). GPDH, a cytoplasmic protein, was used as loading control. H89 5 μM increased the proliferation of cells, however when CD61 was expressed as absolute number, it was obvious that it also caused the inhibition of megakaryocyte generation (Fig. 27 C). Viability of cells was tested by tripan blue staining, that showed no significant change in cells treated with high concentration of substances as compared to control.

Downregulating effect of substances due to extensive treatment of cells allowed to evaluate whether PKG, PKA, VASP or β sGC have impact on megakaryocyte differentiation when their expression is very low or completely absent. Therefore, obtained results suggest an important role of PKA and cAMP, but not PKG in the process of megakaryocyte development from stem cells.

To further study the role of cAMP and cGMP on endomitosis and maturation of megakaryocytes, ploidity as well as size and granularity of megakaryocytes was studied in the same settings of experiments.

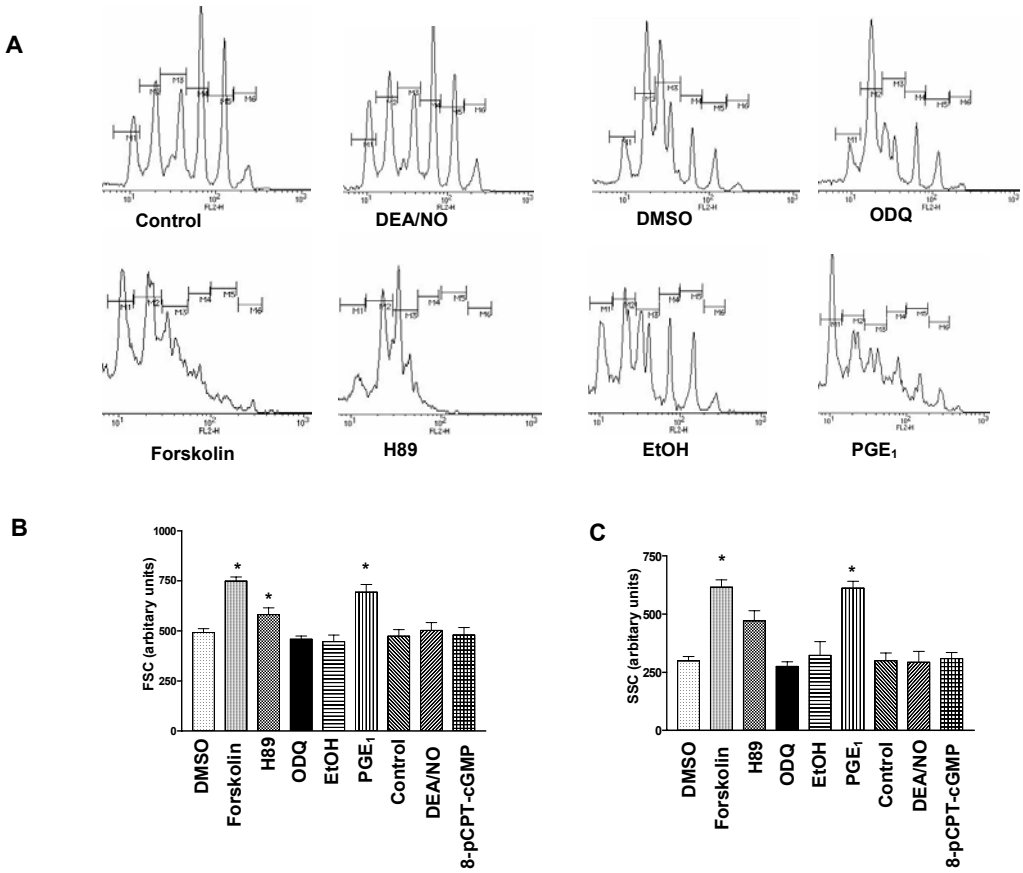

Figure 28. PKA, but not PKG pathway inhibits ploidity and increase size and granularity in megakaryocytes. Fetal liver cells were cultured with TPO and stimulated every day with DMSO, ODQ (10 μM), DEA/NO (5 μM), forskolin (5 μM), H89 (5 μM) and PGE_1 (1 μM). On day 5 cells were analyzed for ploidity **(A)** by propidium iodide staining, size **(B)** by forward scatter (FCS) characteristics and granularity **(C)** by side scatter (SSC) characteristics of megakaryocytes on flow cytometry. For ploidity representative histograms of 3 experiments are presented; size and granularity are expressed as arbitrary units, mean±SEM of 4 experiments, * significantly different as compared to DMSO, or control, $p<0.05$, n=6.

When cells were treated with DEA/NO, ODQ, cells showed to have normal distribution of ploidity to 64N, and a similar percentage of cells were present in every stage of different DNA content when compared to control or DMSO treated cells (Fig. 28 A). However, in the case of cAMP pathway when PKA was downregulated (as shown in previous experiments, Fig. 28 D), there was less cells with higher ploidity, and the most of the cells were in 2N-4N stage for forskolin and PGE_1, while H89 completely blocked ploidity higher then 16N, and most of the cells were in 4N-8N stage of ploidity.

Interestingly, when CD61 positive cells were analyzed for size (Fig. 28 B) by flow cytometry, forskolin and PGE_1 treated cell cultures had around 50% and H89 treated cell cultures had around 20% bigger megakaryocytes when compared megakaryocytes in control cultures. When these megakaryocytes were analyzed for granularity (Fig. 28 C), forskolin and PGE_1 (that leads to downregulation or increased activity of PKA, Fig. 28 D), showed to cause increase in cytoplasmic structures from 90 to 100%, and H89 around 60% in megakaryocytes as compared to control megakaryocytes. DEA/NO, ODQ, 8-pCPT-cGMP had no effect on these cell features. These results indicate a role of cAMP and PKA pathway in process of endomitosis in megakaryocytes as well as in cell growth and maturation of their cytoplasm.

8.4. Platelet Formation

Increased VASP phosphorylation at Ser 239 was observed only at day 4, when megakaryocytes with many proplatelet protrusions were present in the culture. Since this site is preferentially phosphorylated by PKG I, involvement of this kinase in the final step of megakaryocyte differentiation was investigated, when producing platelets. For this reason, megakaryocytes were separated from other cells at day 3, when they express higher levels of PKG I and still do not reach the maximum of platelet production. Megakaryocytes were seated always at the same concentration ($1x10^5$/ml) and incubated for 24 h with DEA/NO, 8-pCPT-cGMP, sildenafil (PDE5 inhibitor), ODQ, forskolin, PGE_1 or H89. Then platelet sized particles that are positive for platelet marker (CD61) were counted. As seen on Fig. 29, 8-pCPT-cGMP increased platelet production around 36% ($p<.0.05$, $n=3$), and DEA/NO and sildenafil 13% and 10%, respectively, that was not significant ($p>0.05$). cGMP analogue allows constant stimulation of PKG I that might lead to the discrete but significant increase of platelet number. However, DEA/NO releases NO that is short lived, and inhibition of PDE5 by sildenafil probably is not sufficient to accumulate enough cGMP if constitutive activity of sGC is low, therefore the effect of these compounds was mild. Interestingly, physiologically more relevant stimulator of adenilate cyclase, PGE_1 reduced platelet production around 30% ($p>0.05$), while forskolin induced inhibition was around 15% ($p>0.05$).

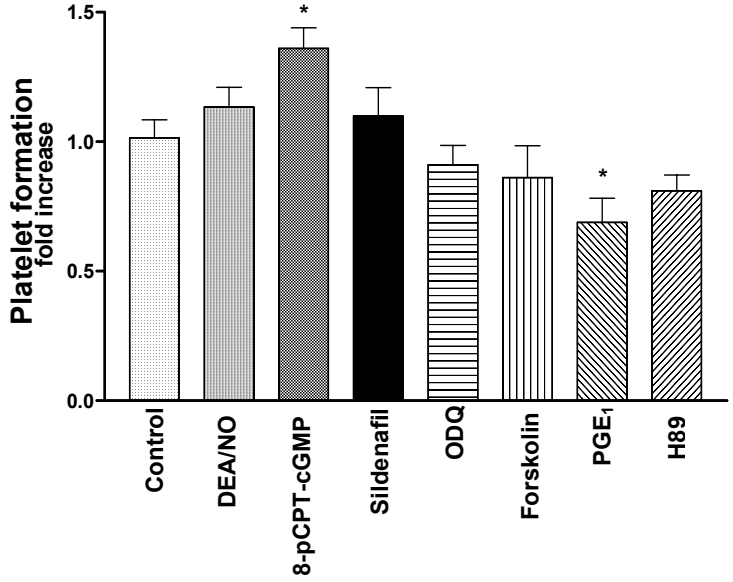

Figure 29. PKG stimulation increased, while PKA stimulation decreased platelet formation. At day 3 megakaryocytes were separated by BSA gradient, and cultured over night with TPO (10 ng/ml) and DEA/NO (5 µM), 8-pCPT-cGMP (100 µM), sildenafil (5 µM), ODQ (10 µM), forskolin (5 µM), PGE_1 (1 µM) or H89 (5 µM). Then platelet production was analyzed by counting platelet sized particles that are CD61 positive. Data are expressed as fold increase of platelet production, control taken as 1, * significantly different as compared to control, $p<0.05$, n=3.

These data indicate differential role of cyclic nucleotides at different stages of megakaryocyte maturation. Megakaryocytes have been shown to be located in the vicinity of bone marrow endothelial cells in vascular sinusoids (Tavassoli and Aoki 1989), and it was suggested that direct contact of endothelial cells and megakaryocytes is necessary for SDF-1 induced thrombopoiesis (Hamada, Mohle et al. 1998; Avecilla, Hattori et al. 2004). Therefore, endothelial derived NO and prostacyclin could finely regulate megakaryocytic fate dependently of their developmental stage.

DISCUSSION

9. Intracellular ROS Production from NOX in Platelets

The understanding of the physiological role and influence of ROS on platelets is scarce in comparison to other cells such as leucocytes, fibroblasts, vascular smooth muscle or endothelial cells.

In previous studies, the production of ROS was mainly observed with collagen- (Del Principe, Menichelli et al. 1991; Pignatelli, Pulcinelli et al. 1998; Caccese, Pratico et al. 2000; Krotz, Sohn et al. 2002) and thrombin- (Chlopicki, Olszanecki et al. 2004; Clutton, Miermont et al. 2004) stimulated platelets. However, presented study (Begonja, Gambaryan et al. 2005) shows that intracellular ROS is produced in platelets stimulated with Trap6 and the stable thromboxane A2 analogue U46619 (Fig. 6), while no extracellular signals for ROS were observed. Thrombin, stimulating protease-activated receptors (PAR) and GPIb, and convulxin stimulating GPVI, induced the strongest signal for ROS production. Trap6 that stimulates only PAR1 receptor induced ROS production to lesser extent. The lowest signal for ROS production was obtained with thromboxane analogue U46619 (Fig. 6), while ADP did not cause any changes in ROS production, as has been previously reported (Pignatelli, Pulcinelli et al. 1998; Caccese, Pratico et al. 2000; Krotz, Sohn et al. 2002).

Discrepancies between observations in different studies could be explained by different methodological approaches and experimental conditions used in detecting ROS. Lucigenin, the most frequently used dye in previous studies has been reported to undergo redox cycling even at low concentrations, leading to false positive signals (Liochev and Fridovich 1997). L-012 is a highly sensitive luminol derivative that detects superoxide production outside of the cells. Although, L-012 has been reported as a ROS indicator in platelets in one study (Krotz, Sohn et al. 2002), it was not possible to detect any signal in platelets with L-012 in presented experimental conditions. Whereas, in the control experiments in neutrophils the L-012 signal increased more than 400 times after PMA stimulation (Fig. 7 B). Intracellular ROS production was measured by FACS analysis of H_2DCF-DA

loaded platelets. This method also has been criticized for difficulties in discrimination between different free radicals. However, this assay shows generalized oxidative condition of the cells (Patel, Hallett et al. 1987) and thereby provides a more physiological representation of processes inside activated platelets.

To investigate the possible source and type of intracellular ROS production, several different inhibitors and superoxide scavengers were used. Inhibitors of mitochondrial respiration, xanthine oxidase, and nitric oxide synthase (NOS) had no effect on ROS production, while NAD(P)H oxidase inhibitors, DPI and apocynin, and the COX inhibitor ASA significantly inhibited thrombin-induced ROS production (Fig. 8 A). These results indicate that NAD(P)H oxidase and COX are major sources of ROS in platelets. However, COX is a part of prostaglandin endoperoxide H synthase (PGHS) which produces peroxy compound 12(R)-hydroxyperoxy-eicosatetraenoic acid (15(R)-HPETE) in arachidonic metabolism during TxA_2 production. This compound could in turn oxidize H_2DCF-DA and give false positive signals. Furthermore, non-ROS related enzymatic oxidation of H_2DCF-DA could occur as shown by Larsen et al (Larsen, Dahl et al. 1996). The lack of effect of NAD(P)H oxidase inhibitors and superoxide scavengers on thromboxane synthase activity (Fig. 15 D) which is downstream of COX and the additive inhibition of ROS by DPI and ASA or tiron and ASA (Fig. 9) indicates that NAD(P)H oxidase-generated ROS are independent from COX. Inhibition of ROS by COX could be also due to secondary secreted TxA_2 that amplifies thrombin activation of platelets and additionally increases ROS production. This is further supported by ROS generation in platelets after stimulation with TxA_2 analog U46619 (Fig. 6), further complicating the understanding of signals that lead to ROS production in platelets. The intracellular superoxide scavengers tiron and SOD mimetic MnTMPyP, but not hydroxyl scavenger mannitol inhibited ROS production suggesting superoxide as a major radical in platelets.

9.1. NOX is Involved in Integrin Activation

To address a role of ROS in platelet regulation, the major markers of platelet activation were assessed, namely P-selectin expression and dense granule secretion (serotonin), shape change, platelet aggregation, thrombus formation, integrin activation, and expression of inflammatory marker CD40L. Platelets aggregation induced by Trap6 was significantly reduced in the presence of DPI and apocynin (Fig. 12 A). Aggregation traces showed no loss of shape change even when high concentrations of DPI (300 μM) and apocynin (4.8 mM) were used (not shown). This was further confirmed by unaltered MLC phosphorylation and Rho A activation (not shown). Integrin αIIbβ3 activation plays a major role in the regulation of platelet adhesion and aggregation. Integrin αIIbβ3 activation was inhibited by NAD(P)H oxidase inhibitors and superoxide scavengers. In agreement with previous studies, (Santos, Moscardo et al. 2000; Valles, Santos et al. 2002), ASA had no effect on integrin activation indicating that COX derived peroxy compounds do not play a significant role in integrin activation.

At a high shear, activation of integrins is a key step in platelet-platelet interactions and thrombus formation. Experiments in a flow chamber over a collagen-coated surface showed significantly reduced thrombus formation under high shear flow conditions (1.000 s^{-1}) in the presence of DPI or apocynin (Fig. 13). Furthermore, platelet stimulation by thrombin, Trap6, U46619, convulxin and ADP showed a similar pattern of αIIbβ3 activation and ROS production (Fig. 14). Integrin inhibition by NOX inhibitors and ROS scavengers was not due to Rap1b and Rac1 inhibition since no changes in activation of these two small GTPases were observed (Fig. 17). This results indicate a role of this GTPases upstream of NAD(P)H oxidase. NAD(P)H oxidase inhibitors and superoxide scavengers had no significant effect on alpha (P-selectin), dense granule secretion (serotonin) or CD40L expression that would connect ROS production in platelets with inflammation (Fig. 15 A, B, C).

It has been demonstrated in neutrophils that the TNF-α induced inside-out signaling leading to β2 integrin activation is regulated by ROS (Blouin, Halbwachs-Mecarelli et al. 1999; Bouaouina, Blouin et al. 2004) and in T-cells TCR (antigen receptor of T-cells) mediated ROS production results in increased integrin clustering and cell adhesion (Kwon, Qu et al. 2005). Recent evidence show that

ROS are generated after integrin engagement in outside-in manner in eosinophiles via β2 integrin (Lynch, Giembycz et al. 1999), Caco2 cells via α2β1 integrin (Honore, Kovacic et al. 2003), and in fibroblasts (Chiarugi, Pani et al. 2003) in which ROS are necessary for integrin signaling during cell adhesion. In platelets role of outside-in signaling and ROS production was observed in platelets of patients with HIV-1-related immune thrombocytopenia (Nardi, Tomlinson et al. 2001). These patients develop antibody against certain domain of β3 integrin (anti-GPIIa49-66) that causes activation of NAD(P)H oxidase, ROS production and leads to fragmentation of platelets (Nardi, Tomlinson et al. 2001). Therefore, a potential contribution of outside-in signals after thrombin induced integrin activation was investigated. A peptide RGDS that blocks fibrinogen binding was used together with control peptide RGES and chimeric human-mouse antibody ReoPro® (abciximab). ReoPro® is directed against αIIbβ3 integrin in order to block outside-in signals in platelets. RGDS and ReoPro® inhibited around 30-40% ROS production after thrombin stimulation (Fig. 30 A), while inhibition of control peptide was not significant indicating partial involvement of integrin mediated ROS production.

However, LIBS6 antibody that induces integrin activation and outside-in signals, did not induce ROS production (Fig. 30 B). Very low increase of integrin activation by LIBS6 (only 3 fold compared to 12 fold by thrombin) which is insufficient to trigger signals leading to NOX activation might be the reason for the inconsistency. Integrins can be activated by Mn^{2+}, in the presence of ligand fibrinogen to produce clear signals in platelets; however Mn^{2+} cannot be used in such experiments since it scavenges ROS.

Integrin αIIbβ3 is important for cross-linking platelets by fibrinogen binding as well as firm adhesion to collagen under high shear. However, the nature of the cytosolic stimulus that initiates integrin activation and subsequent cellular activation is not clear. Present study revealed a novel mechanism of intracellular signaling and its important role for the integrin inside-out regulation in platelets.

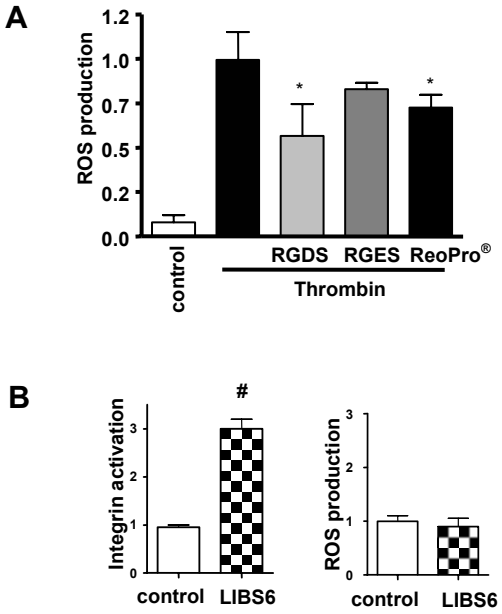

Figure 30. Outside-in signaling in ROS production. (A) Platelets were preincubated with RGDS (0.5 mM), RGES (0.5 mM), ReoPro (20 µg/ml) and stimulated with thrombin (0.005 U/ml).ROS production was measured by FACS with $DCFH_2$-DA. **(B)** Integrin αIIbβ3 activation was increased after LIBS6 (10 µg/ml), but without increase in ROS. Data are shown as arbitrary units, mean±SEM, thrombin taken as 1, * significantly different at $p<0.05$, compared to thrombin; control taken as 1 for LIBS6, # significantly different at $p<0.05$, compared to control n=3.

9.2. ROS is not Scavenging NO in Platelets

Several previous studies have shown that intracellularly produced ROS scavenges endothelial or platelet derived NO in a fast reaction forming peroxynitrite ($ONOO^-$) as an end product (Bauersachs, Bouloumie et al. 1999; Chakrabarti, Clutton et al. 2004; Clutton, Miermont et al. 2004). The NO/cGMP pathway is a well studied mechanism of platelet inhibition and can be monitored by PKG- and PKA-mediated VASP phosphorylation (Massberg, Sausbier et al. 1999). NO suppresses platelet activation by activating sGC, leading to increase of cGMP and activation of PKG Iβ (Gambaryan, Geiger et al. 2004). As a result of the activation of these signaling systems, intracellular calcium flux is inhibited, leading to suppression of P-selectin expression and αIIbβ3 activation (Keh, Gerlach et al. 1996). This led to a hypothesis that inhibition of ROS production in platelets might lead to an increase of cGMP levels and VASP phosphorylation, as more NO should be present. The

phosphorylation of VASP closely correlates with the inhibition of the integrin αIIbβ3 activation in human platelets (Horstrup, Jablonka et al. 1994). However, changes in VASP phosphorylation or GMP levels (Fig. 16 A, B) or cAMP levels were not observed despite significant inhibition of integrin αIIbβ3 activation (Fig. 12 B). This work for the first time reveals that ROS mediate its effects in platelets independently of NO/cGMP (Begonja, Gambaryan et al. 2005; Begonja, Teichmann et al. 2006). Moreover, the results suggest that ROS act through other mechanisms then scavenging NO.

9.3. Possible Mechanisms of ROS Action

Recent studies indicate that at low ROS levels oxidations occur in a specific way such as reversible modifications of amino-acid residues in the active site of enzymes. Modification of amino-acids leads to an inhibition of corresponding enzymes and thus modulation of their specific biological functions (Meng, Fukada et al. 2002). Such example is the reversible oxidation of a cystein residue in the active site of protein tyrosine phosphatase (PTP) upon reaction with ROS (Meng, Fukada et al. 2002). This oxidized cystein can be reduced again to native cystein by cellular thiols like reduced glutathione (GSH) and thioredoxin, thus reactivating the enzyme. The presence and the importance of such events in platelets were shown in the experiments with unspecific PTP inhibitor pervanadate (the complexes of vanadate Na_3VO_4 with H_2O_2). Pervanadate inhibits PTPs by irreversibly oxidizing the catalytic cysteine of PTPs and this induces overall activation of platelets (integrin activation, phosphorylation of MAP kinases, Fig. 31).

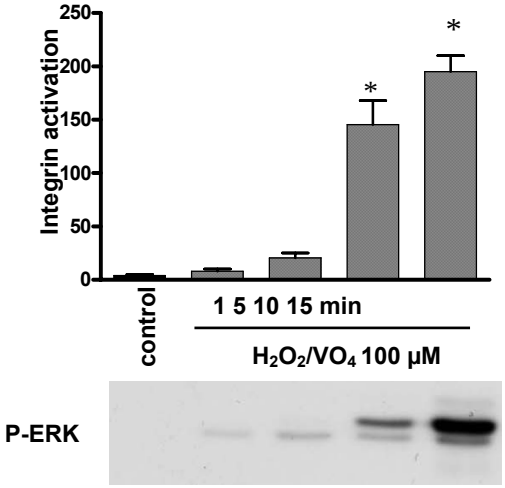

Figure 31. Overall inhibition of PTPs induces platelet activation as shown by integrin activation and ERK. Platelets were stimulated with freshly prepared pervanadate (100 µM) for different periods of times (1 to 15 min) and integrin activation was analyzed by PAC-1 binding on FACS, as well as ERK activation by Western blot. Data are expressed as arbitrary units, *significantly different as compared to control, $p<0.05$.

Chiarurgi et al. demonstrated that integrin-mediated ROS production inhibits low molecular weight PTP (LMW-PTP) thus increasing activation and downstream signaling of FAK, and finally cell adhesion and spreading in fibroblasts (Chiarugi, Pani et al. 2003). Recently, a mechanism of integrin redox regulation was demonstrated in T-cells (Kwon, Qu et al. 2005). Activation of the TCR induces ROS production which will oxidize and inhibit SHP-2 (SH2 domain-containing PTP) present in Gads-SLP-76 complex. This promotes the phosphorylation of ADAP and Vav1 leading to increased clustering of β2 integrin lymphocyte function-associated antigen 1 (LFA-1) and T-cell adhesion. SHP-1, a highly homologous phosphatase to SHP-2, has been shown to negatively regulate platelet signaling (Jones, Craik et al. 2004) and platelet agonists mediate their stimulatory signals through Gads-SLP76, ADAP, Vav1. Therefore it remains to clarify whether the mechanism shown in T-cells is also present in platelets.

Cysteine-cysteine dithiol bonds are important for the integrin conformation and may be involved in the activation-induced conformational changes. Free thiol residues are found in the integrin β_3 subunit that could represent a direct target for ROS

(Yan and Smith 2000), however these sites were shown to be regulated extracellularly by plasma GSH/GSSG system (Essex 2004).

Other possibility of ROS regulating signaling systems is by irreversibly oxidizing different types of amino-acids that targets proteins for destruction, and in this way turns off some signal transduction pathways through proteosomal degradation (Kovacic, Irani et al. 2001).

Since the presence of NOX1 in platelets was shown for the first time, it was necessary to determine its role in platelet function by analyzing platelets of NOX1 KO mice. Surprisingly, there was a tendency of lower ROS production and lower extent of integrin activation in NOX1 KO platelets, although this difference was not statistically significant as compared to WT platelets. Possible explanation for such finding might be a presence of other NOX isoform, namely NOX2 that may have more important role, or can compensate NOX1 deficiency for needed ROS production in platelets. Furthermore, different NOX isoforms might have different function in the same cells because of diverse subcellular localization. More and more studies support selective action of NOX and also of ROS substrates PTPs that are present in a specific subcellular fraction, therefore emphasizing the role of localized ROS production (Hilenski, Clempus et al. 2004; Terada 2006; Ushio-Fukai 2006).

In summary, presented data demonstrate a significant intracellular production of ROS in platelets and suggest that this production modulates platelet function as shown on Fig. 32. It was demonstrated that NAD(P)H oxidase and COX are responsible for ROS production. NAD(P)H oxidase and superoxide anions, but not COX inhibition were clearly shown to mediate integrin activation and platelet thrombus formation independently of the NO/cGMP pathway. In addition, other aspects of platelet function such as granule secretion and platelet shape change were not affected by NAD(P)H oxidase inhibition and superoxide scavenging supporting the conclusion that the observed effects are not unspecific. Thus ROS production via NAD(P)H oxidase in platelets may represent an important mechanism for the regulation of integrin activation. Whether this regulation is mediated by a direct modification of integrin subunits or by reversible inhibition of

certain PTPs in platelets or some other mechanism should be addressed by future investigations.

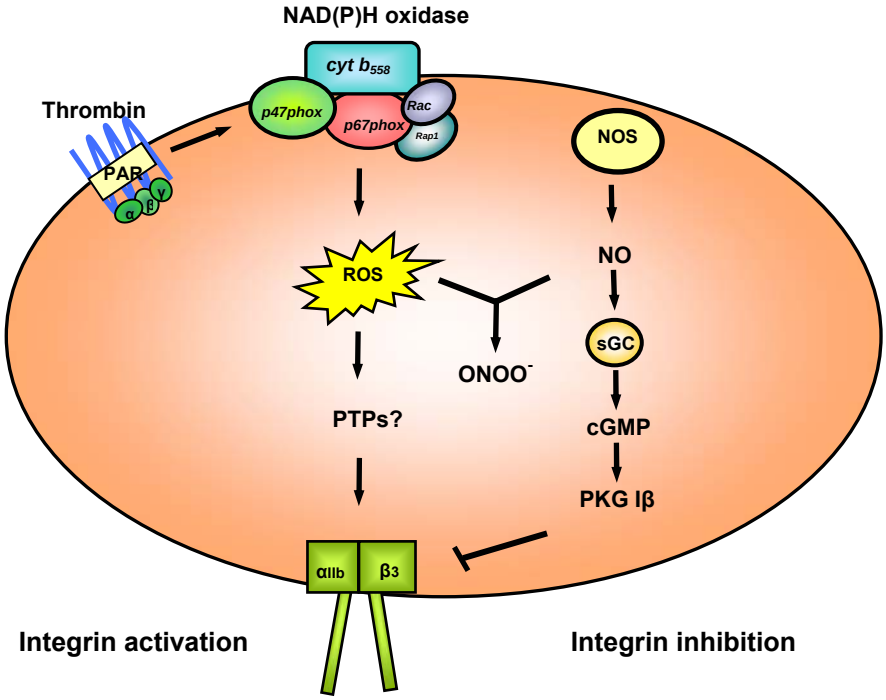

Figure 32. Proposed model for the regulatory role of platelet NO and ROS. Platelet agonists (i.e. thrombin, collagen, thromboxane) activate the NAD(P)H oxidase which results in the intracellular production of reactive oxygen species (ROS) and integrin (αIIbß3) and platelet activation. NO generated by endothelial and possibly platelet NO-synthases activates the heme-containing soluble guanylyl cyclase resulting in cGMP generation, activation of cGMP-dependent protein kinase (cGK / PKG) Iß, VASP phosphorylation and platelet inhibition. Although ROS is known to inactivate / impair (via $ONOO^-$ generation) the inhibitory NO/cGMP pathway present studies suggest that ROS activates platelet integrins by NO/cGMP-independent mechanisms.

10. Inhibitory Role of NO/cGMP in Platelets

The NO/cGMP signaling cascade regulates a variety of different physiological responses in vascular system. NO is continuously produced in the endothelial cells by eNOS and causes relaxation of vascular smooth muscle cells and inhibition of platelet aggregation. These effects are conducted by the NO receptor, sGC that produces cGMP. This leads to activation of PKG I and further transduction of NO

signals (Munzel, Feil et al. 2003). Disorders in NO/cGMP pathway are connected to endothelial dysfunction that often precedes cardiovascular diseases. Moreover, NO-releasing compounds are among the most effective drugs used for treatment of coronary heart disease.

Recently, new mechanisms for agonist-induced platelet activation and secretion were proposed to involve sequential activation of PKG that further activates MAP kinases p38 and ERK leading to integrin activation, or that Akt is upstream activator of eNOS and PKG, and this causes platelet secretion (Li, Xi et al. 2003; Li, Zhang et al. 2006; Stojanovic, Marjanovic et al. 2006). It was not possible to reproduce stimulatory role of PKG via ERK or p38 in platelets, (Gambaryan, Geiger et al. 2004; Walter and Gambaryan 2004; Begonja, Geiger et al. 2007), which was in agreement with other published data (Marshall, Senis et al. 2004; Garcia, Quinton et al. 2005). Further, the presented study shows that NO donors or cGMP analogues did not activate ERK or p38 kinase while they caused phosphorylation of VASP as marker of PKG activation (Fig. 21). Even more, in agreement with earlier published data (Schwarz, Kobsar et al. 2000) in all cases here it is rather shown that activation of PKG in platelets strongly inhibits p38 and ERK MAP kinase activity as well as secretion and aggregation (Fig. 20 and 22 A, B). This inhibitory effect was mediated by sGC since sGC inhibitor ODQ blocked SNP induced inhibition of platelet secretion, ERK and VASP phosphorylation (Fig. 20). In other cell types (lymphocytes, fibroblasts, and vascular smooth muscle cells) stimulation of PKG by NO donors or cGMP analogues can activate MAP kinase pathways including p38 MAP kinase (Komalavilas, Shah et al. 1999; Browning, McShane et al. 2000; Fischer, Palmetshofer et al. 2001). High contamination with lymphocytes in the platelet preparation of Li et al (Li, Zhang et al. 2006) study could be one of the reasons for such a discrepancy, regarding MAP kinase activation by PKG in platelets, with results presented here and with others (Schwarz, Kobsar et al. 2000; Gambaryan, Geiger et al. 2004; Marshall, Senis et al. 2004; Garcia, Quinton et al. 2005; Li, Zhang et al. 2006).

10.1. p38 is Activated by Thrombin Induced ADP and TxA_2 Secretion

Platelet p38 MAP kinase has been found to be phosphorylated and activated in response to collagen, thromboxane analogue (Saklatvala, Rawlinson et al. 1996), thrombin (Kramer, Roberts et al. 1995), vWF (Canobbio, Reineri et al. 2004) and other platelet activating agents like homocysteine (Leoncini, Bruzzese et al. 2006). Nevertheless, the functional roles, as well as, the signaling pathways upstream of p38 MAP kinase activation in agonist-stimulated platelets are largely undefined.

p38 has been shown to mediate platelet adhesion in static as well as flow conditions on low collagen density surfaces (Mazharian, Roger et al. 2005), and platelet aggregation induced by low collagen, U46619 (Saklatvala, Rawlinson et al. 1996), and thrombin (Li, Zhang et al. 2006) concentrations. However, these studies relied on the use of p38 inhibitors (SB202190, SB203580), but unfortunately did not use appropriate controls such as the inactive analogue of these inhibitors, SB202474 (Huang, Wang et al. 2002).

This study shows that p38 MAP kinase inhibitors did not significantly ($p>0.05$, ANOVA) affect platelet aggregation and P-selectin expression (Fig. 22 A, B) even at low concentration of thrombin (0.005 U/ml) when compared to control compound. These results are in agreement with data obtained with other, more selective, second generation p38 inhibitors which did not affect human platelet aggregation caused by a wide range of agonists (Kuliopulos, Mohanlal et al. 2004). Therefore, it was possible to conclude that p38 does not play a significant role in thrombin-induced activation and aggregation of human platelets.

An important characteristic of platelets is ability to amplify its own signals allowing maximal activation even by low concentration of agonists. This is of special importance in arteries where blood flow may rapidly dilute and remove soluble agonists. Contribution of amplificatory signals in p38 MAP kinase activation was not studied so far in platelets. Therefore, importance of ADP and TxA_2 in thrombin stimulated platelets in p38 MAP kinase activation was investigated, and in parallel platelets secretion was analyzed by monitoring P-Selectin expression. Observed results indicate that activation of $P2Y_{12}$, but not $P2Y_1$ or $P2X_1$ receptors by secreted

ADP and activation of thromboxane receptor by secreted TxA_2 from low dose thrombin-stimulated platelets play a significant role in p38 MAP kinase activation thus amplifying the activation of platelets at low thrombin concentrations (Fig. 23). However, stronger stimulation of platelets (high thrombin concentration), as in a case of ERK activation (Falker, Lange et al. 2004), overcomes the effect of secondary secretion and p38 MAP kinase activation could not be inhibited by ADP scavenger apyrase, $P2Y_{12}$ inhibitor AR-C69931MX or COX inhibitor indomethacin (data not shown).

In stimulated platelets numerous signaling molecules are activated and most of them are connected with different mechanisms of platelet activation including integrin activation, granule secretion, surface molecule expression etc. However, because of complexity and overlapping of different pathways, it is difficult to clearly distinguish the functional role of certain proteins. Also, it is not necessary that all expressed signaling systems in platelets are directly connected with platelet activation. Probably some of these proteins may play a role in postaggregatory platelet events such as apoptosis and elimination of stimulated and aggregated platelets. One of the most important roles of MAP kinases in nucleated cells is connected with the regulation of gene expression and apoptosis (Wada and Penninger 2004). Platelets are cells without nuclei, however in megakaryocytes p38 and ERK MAP kinase are important for their differentiation (Rojnuckarin, Drachman et al. 1999; Kirito, Fox et al. 2003), and therefore platelets might contain these proteins as left-overs needed only for megakaryocytes. Apoptosis-like events have been described in platelets (Clarke, Savill et al. 2003), however it remains to be elucidated whether p38 and other MAP kinases take part in these processes in platelets.

11. Functionally Active cGMP/cAMP Pathway in Megakaryocytes

Megakaryocytes are among least represented haematopoietic cells in bone marrow, and after platelet release they are rapidly removed by bone marrow

macrophages. This makes *in vivo* investigations of megakaryocytes very difficult and restricted to a certain model. Cutting edge approaches in the research of megakaryopoiesis and platelet fragmentation are employing *in vitro* various cells with a potential to develop megakaryocytes under certain stimuli (e.g. human CD34+ stem cells, primary bone marrow or fetal liver cells cultured with TPO will develop megakaryocytes). In this work fetal liver cells were employed as a model for *in vitro* derived megakaryocytes. Several markers of megakaryopoiesis have confirmed a successful experimental approach as shown by increased number of big cells in cultures, increased megakaryocytic marker CD61, and increased ploidity of cells as a typical feature of megakaryocytes (Fig. 24).

It has been known for a while that cyclic nucleotides cAMP and cGMP together with its targets PKA and PKG, and downstream effectors play an important inhibitory role in platelet signaling. However, it is not clear whether they are also important for megakaryocyte and platelet formation. Therefore, experiments were designed to define the role of cyclic nucleotides in two processes: development of megakaryocytes from stem cells and formation of platelets from megakaryocytes.

Presented results demonstrate increased expression of PKG I, VASP, β sGC, PKA C during megakaryocyte culturing, that was paralleled with megakaryocyte maturation. At day 4 when megakaryocytes are at high stage of ploidity (64 N) and with many proplatelet structures, expression of PKG I, PKA C, and their substrate VASP is appreciable higher then at day 2 when megakaryocytes are smaller, with lower ploidity (16N) and with very few proplatelets present. Even more interesting is a difference in VASP phosphorylation at different stages of megakaryocyte maturation implying a possible involvement of this protein and cyclic nucleotides in maturation of megakaryocytes and platelets. VASP was phosphorylated at Ser159 site at earlier stages while Ser239 was phosphorylated only at day 4 in stage of already matured megakaryocytes and platelet production. This indicated a possible role of PKA in early steps of megakaryocyte maturation, and involvement of PKG perhaps at later stages together with platelet release. It has been shown also that PKG and PKA can be stimulated further by SNP and 8-pCPT-cGMP and forskolin and PGE_1, respectively, as observed by VASP phosphorylation (Fig. 26), addressing the functionality of these pathways.

These results invoke an intriguing question: do megakaryocytes express certain proteins only because they are needed later for normal platelet function, or these proteins are also exploited, at least partially, during megakaryocyte differentiation and platelet formation, which finally would be biologically economical?

There are a number of studies and examples that can give a partial answer to this question. PI3-K plays significant role in platelet adhesion, and it is required for TPO-induced cell survival and cycle progression in megakaryocytes (Geddis, Fox et al. 2001). Small GTPase Rap1b enhances integrin-mediated adhesion of platelets and megakaryocytes, and it is needed for megakaryocyte differentiation through sustained ERK activation (Garcia, de Gunzburg et al. 2001). p38 MAP kinase was shown to be involved in TPO-mediated up-regulation of transcription factor Hoxb4 and thus involved in haematopoietic stem cell renewal (Kirito, Fox et al. 2003). ERK 1/2 is involved in megakaryocyte polyploidization (Rojnuckarin, Drachman et al. 1999) and proplatelet formation (Jiang, Jia et al. 2002). Integrin αIIbβ3 is also able to intervene in proplatelet formation when megakaryocytes are cultured on fibrinogen matrices (Larson and Watson 2006).

11.1. PKA Regulates Megakaryocyte Maturation

Although studies in mice with deletion of transcription factor GATA-1 or NF-E2 provide a strong link between megakaryocyte maturation and platelet function, at the molecular level little is known about the actual processes involved.

To seek out whether PKA and PKG play active role during maturation of megakaryocytes and generation of platelets, fetal liver cells were treated with different compounds that change cAMP and cGMP levels in the cells. However, sustained application of the compounds caused downregulation or inhibition rather then activation of certain proteins. For example, 8-pCPT-cGMP downregulated PKG I, ODQ downregulated β sGC, forskolin and H89 downregulated PKA C and VASP, and PGE_1 downregulated VASP and inhibited PKA activation as seen by decreased VASP phosphorylation (Fig. 27 B, D). Nonetheless, only forskolin, H89 and PGE_1 decreased the generation of megakaryocytes in culture indicating important role of PKA, and also VASP in this process.

Elevation of cAMP in cells finely tunes many physiological events such as metabolism, cell growth, differentiation, gene transcriptions, and synaptic release of neurotransmitters. The principal cAMP target is protein kinase A (PKA) that mediates majority of cAMP effects in the cells. However, cAMP can also activate cyclic nucleotide gated ion channels (Nakamura and Gold 1987), as well as guanine exchanging factors Epac1 and Epac2 that regulate activity of Rap1 (de Rooij, Zwartkruis et al. 1998).

Erythrocytes and megakaryocytes derive from the same progenitor cells in contrast to other haematopoietic lineages. They share the same transcriptional regulators, GATA-1, FOG-1, and NF-E2, and therefore other important molecules and pathways can be delineated on the basis of their plausible role in erythrocyte maturation. cAMP elevating agents as well as prostaglandins increase erythropoietin induced erythroid differentiation (reviewed in Boer, Drayer et al. 2003). Several regulatory mechanism have been proposed to be modulated downstream by PKA, such as Ras/ERK, signal transducer and activator of transcription 5 (STAT5) that is highly activated in erythroid cells, and transcription factor NF-E2. Transactivational activity of NF-E2 is decreased in PKA-deficient cells, and PKA is needed for its maximal activation (Casteel, Suhasini et al. 1998). Interestingly, in mice deficient in p45 subunit of NF-E2 early megakaryocyte differentiation is apparently normal although with reduced proliferation of megakaryocyte progenitors, but with defect in platelet release, enlarged cytoplasm and failure to produce proplatelets in culture (Shivdasani, Rosenblatt et al. 1995). These features are to some extend similar to the observations in here presented experiments, in conditions when cells were treated with forskolin or PGE_1. There was a decrease in megakaryocytes development; more immature megakaryocytes were present based on smaller number of cells with higher ploidity. However, endomitosis was not completely blocked, while there were still megakaryocytes with 64N. In average, megakaryocytes had enlarged cytoplasm and there was a decrease in proplatelet structures in culture. Interestingly, H89 had overall similar effect as forskolin and PGE_1, however difference can be observed by stronger ploidity inhibition (maximum is 32N), and not so large megakaryocytes. This could be a result of non selective inhibition of PKA by H89.

Earlier studies on cAMP influence in megakaryocyte development and platelet formation were mostly done in megakaryocytic cell lines producing conflicting results. For instance cAMP analogues induced megakaryocytic differentiation in human leukemic K-562 cells (Tortora, Clair et al. 1989), while in DAMI cells they caused inhibition of cell growth and GPIb expression (Vittet, Duperray et al. 1995). Proplatelet formation of guinea pig bone marrow megakaryocytes was observed to be increased by dibutyryl cAMP, and IBMX (Leven 1995), while in rat megakaryocytes H-8, PKA inhibitor, caused the same effect indicating that decrease of cAMP is required for morphological changes of megakaryocytes (Uneyama, Uneyama et al. 1995). Sauer et al. showed that TPO increases cAMP, and activates PKA in CMK cells (Sauer, Tausch et al. 2001). Increase in the expression level of PKA regulatory subunit RIIβ were observed during maturation of human CD34+ stem cells into megakaryocytes, however the meaning of this increase for megakaryocyte development was not further studied (den Dekker, Gorter et al. 2002). Recent investigations depicted inhibitory effect of cAMP on apoptosis of CD34+ stem cells, and CD34+ -derived megakaryocytes mediating TPO or SCF induced cell survival (Pozner, Negrotto et al. 2005; Negrotto, Pacienza et al. 2006). cAMP increases expression of chemokine receptor CXCR4, which augments the ability of CD34+ cells to transmigrate the bone marrow endothelial cells and adhere to stroma (Goichberg, Kalinkovich et al. 2006).

11.2. PKG and PKA Regulate Platelet Release

Localization of megakaryocytes to bone marrow vascular niche was shown to be necessary for their final maturation and platelet release (Avecilla, Hattori et al. 2004). Recently, several microRNAs (newly recognized small regulatory RNAs) were showed to be down-regulated during *in vitro* differentiation of CD34+ -derived megakaryocytes that hypothetically unblock target genes involved in differentiation (Garzon, Pichiorri et al. 2006). Curiously, inhibition of PKA attenuated one of the microRNAs studied, pre-miR-181a and this was connected with inhibited differentiation of Meg-01 cells (Guimaraes-Sternberg, Meerson et al. 2006).

Although cGMP regulates gene expression, and dedifferentiation of vascular smooth muscle cells is associated with loss of PKG expression (Boerth, Dey et al. 1997), NO/cGMP pathway did not take part in process of megakaryocyte maturation from stem cells. Results presented here rather support a role of NO/cGMP in final step of platelet release. This was evidenced by increased PKG activity and increased VASP phosphorylation only when megakaryocytes formed proplatelet structures, in their final mature stage. Also 8-pCPT-cGMP stimulation increased platelet production around 30% at day 3 cultured megakaryocytes. DEA/NO or sildenafil effect was weaker in induction of platelet production most probably because of the short nature of the signals they produce. DEA/NO releases short living NO, which stimulates sGC and PKG; however these signals go back to basal levels if there is no constant stimulation. Similar is with sildenafil, that blocks PDE5, however if there is a low constitutive activity of sGC, there is no enough accumulation of cGMP that would conduct the signal. On the other hand, cGMP analog provides constant stimulation of PKG and therefore has a biological effect on megakaryocytes producing platelets. These results suggest that megakaryocytes need a signal from outside to stimulate sGC/PKG, but not from within megakaryocytes. This is also supported by the observation that stem cells from fetal livers expressed eNOS comparable to control cells (endothelial cells) while at higher maturation stages of megakaryocytes eNOS was not detectable (Fig. 25 A).

In vivo polyploid megakaryocytes localize to sinusoidal bone marrow endothelial cells, forming transendothelilal pseudopods, or migrate through endothelial cells and release platelets into intravascular sinusoidal space (Tavassoli and Aoki 1989). This translocation of megakaryocytic progenitors to the vascular sinusoids is mediated by chemokines FGF-4 and SDF-1 and is sufficient to induce megakaryocytes maturation and platelet release even in the absence of TPO (Avecilla, Hattori et al. 2004). However, despite the evidence for the requirement of megakaryocyte-vascular sinus interactions and proplatelet release, nothing is known about the molecules that regulate these events. NO was shown to induce apoptosis in Meg-01 cells that was associated with increased platelet production and iNOS knockout mice have around 50% decreased platelet count (Battinelli,

Willoughby et al. 2001). Therefore, localization of megakaryocytes to vascular microenvironment brings them closer to endothelium, that produces NO and prostaglandins that then, at least partially, regulate megakaryocyte development and platelet release in the sinusoids.

Double and triple NOS knockout mice have decreased viability indicating its role in development. The expression of sGC and PKG increases after differentiation of embryonic stem cells into cardiomyocytes (Krumenacker, Katsuki et al. 2006), results that are similar to here presented ones in respect to differentiation of haematopoietic stem cells. It was shown that mice deficient in eNOS have a defect in mobilization of progenitor cells from bone marrow, and reduced colony-forming activity and proliferation of bone marrow haematopoietic stem cells (Aicher, Heeschen et al. 2003). Exposure to NOS inhibitors *in vivo* increased the number of stem and progenitor cells in mice bone marrow (Michurina, Krasnov et al. 2004). Moreover, previous investigations observed decreased number of platelets in rats treated with L-nitroarginin and L-NAME (Molnar, Suto et al. 1994; Nagase, Isobe et al. 1995).

In summary, here presented results show increased expression during megakaryocyte maturation of proteins involved in inhibitory pathways of platelets, including cAMP and cGMP pathway. *In vitro* differentiated megakaryocytes had developed functional cAMP and cGMP pathways since their stimulation resulted in increased VASP phosphorylation. Even more, results demonstrate for the first time involvement of cAMP/PKA in development of megakaryocytes from stem cells, regulating megakaryocyte endomitosis and cytoplasm maturation. cGMP/PKG pathway seems to be partially enrolled in final step of platelet release. These data assure future investigations to find out mechanisms by which cAMP regulates maturation processes in megakaryocytes and by which cGMP is involved in thrombopoiesis. PKG I and sGC knockout mice would be appreciated models to study proposed NO/cGMP effects *in vivo*.

CONCLUDING REMARKS

The presented series of studies were carried out to define the physiological role of ROS in platelet signaling, point out the relation of NO/cGMP/PKG I pathway to ROS and to MAP kinases, and to investigate the role of cyclic nucleotides (cGMP and cAMP) in development of platelet-parent cells, megakaryocytes.

The first part of presented work demonstrates significant intracellular production of ROS in platelets and suggests that this production modulates platelet function. NAD(P)H oxidase and COX were responsible for ROS production. However, only inhibition of NAD(P)H oxidase but not COX was clearly shown to inhibit integrin activation and platelet thrombus formation. This inhibition was independent of the NO/cGMP pathway as there was no increase in VASP phosphorylation and cGMP levels. ROS production via NAD(P)H oxidase in platelets may represent an important mechanism for the regulation of integrin activation. Clearly, further investigations are required to determine the exact mechanism involved in ROS regulation of integrin activation. As mentioned earlier, PTPs such as SHP-1, SHP-2 are potential interesting targets of ROS to be studied in platelets. Also a more global approach including mass spectrometry may help in identifying new ROS targets by determining changes in disulfide bonds of proteins during platelet activation.

MAP kinases play important role in gene expression and apoptosis. Recently, NO/cGMP/PKG I dependent activation of MAP kinases was suggested. The study presented here clearly shows rather an inhibitory role of NO/cGMP/PKG I in p38 and ERK activation in platelets. Secondary secretion of ADP and TxA_2 mediates p38 activation upon thrombin stimulation, thus strengthening the platelet reaction to stimulus. However, activation of p38 MAP kinase independently of the thrombin concentration has no significant effect on platelet stimulation, calcium mobilization and platelet aggregation, suggesting a possible role in later events, such as procoagulant activity, clot retraction or clearance of stimulated platelets.

Bone marrow vascular niche allows maturation of haematopoietic stem cells and mobilization of developed blood cells to the circulation. Endothelial cells produce NO constitutively and prostacycline in response to different stimuli, that give rise to

cGMP and cAMP, respectively. This work demonstrates that cGMP and cAMP are differentially involved in megakaryocyte and platelet development. cAMP is engaged in haematopoietic stem cell differentiation to megakaryocytes, in contrast to cGMP that has no impact on this process. While PKA is already present in stem cells, expression of proteins involved in cGMP signaling (sGC, PKG) increases with maturation of megakaryocytes. In final step of megakaryocyte maturation that includes release of platelets, cGMP and cAMP have mild but opposing effects: cGMP increases platelet production while cAMP decreases it indicating finely regulated process that could depend on stimulus coming from adjacent endothelial cells of sinusoids. These results contribute to a better understanding of possible molecular mechanisms involved in megakaryocyte maturation in bone marrow vascular microenvironment. However, it remains to clarify the functional role of cyclic nucleotides and related proteins in megakaryocytes and platelets production *in vivo.* Lineage-restricted gene knockouts for studying megakaryocytes and platelets function have been recently developed and could be useful in answering this question. Megakaryocytes analyzed here show enhanced VASP phosphorylation during their maturation process. Further experiments with VASP knockouts are under way to investigate if VASP is involved in *in vitro* differentiation of megakaryocytes. Finally, phosphorylation of VASP at different sites may be responsible for distinct functions of VASP at various megakaryocyte maturation stages and needs to be investigated in more detail.

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Abbreviations

ADP	Adenosine diphosphate
APS	Ammoniumpersulfate
ASA	Acetysalicylic acid
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
COX	Cyclooxigenase
DAG	Diacylglycerol
DMEM	Dulbecco´s modified Eagle´s medium
DMSO	Dimethylsulfoxide
EDTA	Ethylendiaminetetraacetic acid
Epac	Exchange protein directly activated by cAMP
ERK	extracellular signal-regulated kinases
EtOH	Ethanol
FcR	Fc receptor
FCS	Fetal calf serum
GPDH	Glycerol 3-phosphate dehydrogenase
H2DCF-DA	2´-7´- dichlorodihydrofluorescein diacetate
Hepes	4-(2-hydroxylethyl)-1-piperazineethanesulfonic acid
HSC	Hematopoietic stem cell
Hsp27	Heat shock protein 27
IP_3	Inositotriphosphate
MAPK	Mitogen-activated protein kinase
MK	Megakaryocyte
NAD(P)H	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
NOS	Nitric oxide synthase
NOX	NAD(P)H oxidase
PAGE	Polyacrylamide gel electrophoresis
PAR	Protease activated receptor
PBS	Phosphate buffered saline
PDE	Phosphodiesterase
PGE_1	Prostaglandin E_1
PGI_2	Prostacyclin
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
ROS	Reactive oxygen species
RT	Room temperature
sGC	Soluble guanylyl cyclase
SNP	Sodium nitropruside
TAE	Tris-acetate-EDTA
TPO	Thrombopoietin
Tris	Tris-(hydroxymethyl)-aminomethan
TxA_2	Thromboxane A_2
VASP	Vasodilator-stimulated phosphoprotein
vWF	von Willebrand factor

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A) Originalartikel

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