cGMP Signaling in Platelets

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Abstract

The cyclic nucleotide cGMP is a key intracellular signaling molecule in mammals. It mediates many effects of nitric oxide (NO) including the regulation of vascular tone and platelet activity. Pharmacological and genetic studies have indicated that the NO-cGMP pathway could be an attractive target for antithrombotic drugs. Here, we summarize the biochemistry and (patho-) physiology of cGMP signaling in platelets. These cells generate and degrade cGMP by the NO-activated soluble guanylate cyclase and several phosphodiesterases (PDE2, PDE3, and PDE5), respectively. An increase of the cGMP concentration activates cGMP-dependent protein kinase type I (cGKI), which phosphorylates several platelet proteins. Among the cGKI substrates are small G-proteins (e.g., Rap1B), regulators of G-protein signaling (e.g., RGS18) and intracellular Ca^{2+} release (e.g., IRAG), and actin-binding proteins (e.g., VASP). According to the prevalent view, cGKI-dependent substrate phosphorylation limits platelet activation and thrombus formation through the inhibition of intracellular Ca^{2+} release, integrin activation, cytoskeletal remodeling, and granule secretion. Interestingly, several studies suggest that cGMP also promotes specific aspects of platelet activation. We discuss these seemingly contradictory findings and propose a new model of cGMP-regulated hemostasis that leads to optimal platelet activation in response to vascular injury. This model integrates both platelet stimulation and inhibition by dynamic shear stress-regulated cGMP signals that are generated during different phases of thrombus formation under flow in vivo.

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Contents

15.1 Introduction

Platelets are key players in hemostasis and thrombosis [1–4]. After vascular injury, platelets rapidly adhere to the subendothelial matrix exposed at the site of injury and become activated. Initially, platelets adhere to von Willebrand factor (vWF) via the glycoprotein Ib/IX/V (GPIb/IX/V) complex and to collagen via GPVI. This results in platelet activation and transformation of integrin $\alpha_{IIb}\beta_3$ (fibrinogen receptor, also called GPIIb/IIIa) and integrin $\alpha_2\beta_1$ (collagen receptor), which firmly bind to their respective extracellular matrix ligands. Then, platelets spread and form a surface for the recruitment of additional platelets via fibrinogen bridges between $\alpha_{IIb}\beta_3$ receptors leading to platelet aggregation and formation of a hemostatic plug that stops bleeding. Arterial thrombosis is primarily an exaggerated hemostatic response at the site of vascular injury. Thrombosis plays a causative role in myocardial infarction, and antithrombotic therapy takes center stage in the management of acute coronary syndromes. To avoid vessel occlusion, thrombus growth is limited by two endogenous inhibitors supplied by the endothelium, prostacyclin, and nitric oxide (NO). Prostacycylin and NO increase the levels of cAMP and cGMP in platelets, respectively. Here, we focus on the biochemistry, pharmacology, and (patho-)physiological role of the NO-cGMP signaling cascade in platelets. We will also discuss recent developments and controversies in this field, in particular whether an increase in platelet cGMP inhibits and/or promotes hemostasis and thrombosis.

NO is generated from L-arginine by NO synthases (NOS). There are three known NOS enzymes encoded by distinct genes: the neuronal NOS (nNOS or NOS1), the inducible NOS (iNOS or NOS2), and the endothelial NOS (eNOS or NOS3) [5]. nNOS and eNOS are constitutively expressed in various tissues and both enzymes are activated by Ca^{2+}/calmodulin. In contrast, expression of iNOS is inducible and the enzyme is constitutively active. Its expression is induced in several cell types including macrophages, vascular smooth muscle cells (VSMCs), and endothelial cells after exposure to lipopolysaccharide (LPS) or cytokines. eNOS is the major enzyme responsible for NO production in the vascular endothelium. NO generated in endothelial cells rapidly diffuses across cell membranes into

VSMCs, where it activates the NO receptor, soluble guanylate cyclase (sGC). sGC produces cyclic guanosine monophosphate (cGMP) in VSMCs resulting in vasodilation. Endothelium-derived NO also diffuses into the vessel lumen, where it interacts with several blood cell types including platelets.

cGMP was isolated from rat urine in the 1960s [6]. Later, it was found that cGMP mediates many effects of NO in platelets and other cell types. cGMP is formed by guanylate cyclases via cyclization of guanosine triphosphate (GTP) (Fig. 15.1). The NO-activated sGC is mainly located in the cytosol [7, 8], while

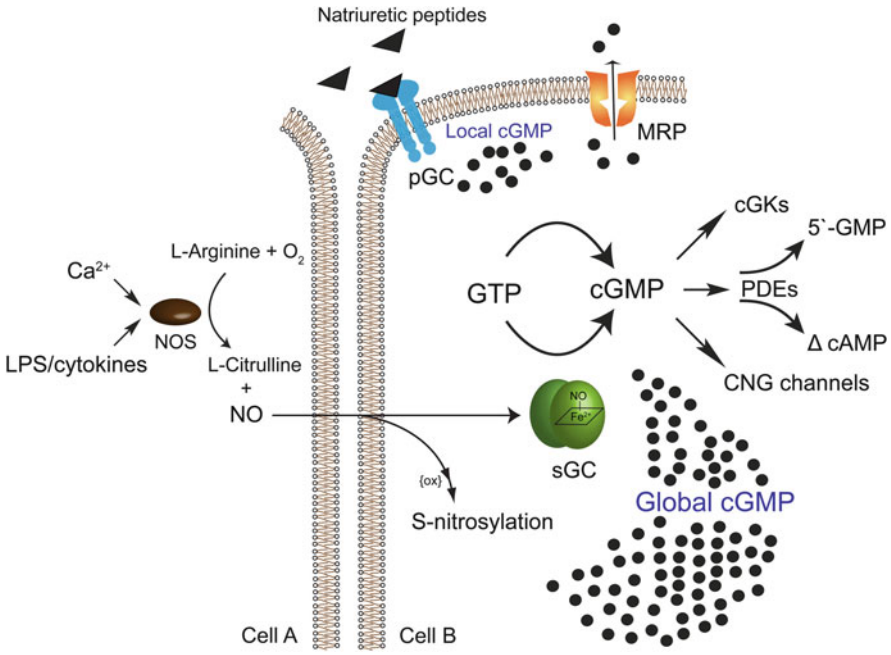

Fig. 15.1 Basic principles of cGMP signaling. NO is synthesized by a NOS in cell A (e.g., Ca^{2+}-activated eNOS in an endothelial cell, or iNOS, whose expression is induced by LPS/cytokines, in a macrophage) and diffuses across cell membranes to a nearby target cell B (e.g., a VSMC or platelet). NO binds to the Fe^{2+} of the heme group of sGC and induces the generation of a global cGMP pool (*black balls*) in the cytosol. Binding of natriuretic peptides to pGCs triggers the formation of local cGMP microdomains at the plasma membrane. cGMP exerts its functions via several effector proteins, mainly cGKs, PDEs, and CNG channels. Removal of cGMP is accomplished via hydrolysis to 5′-GMP by PDEs (e.g., PDE5) and via excretion by transporters in the plasma membrane including members of the MRP family (e.g., MRP4). cGMP can also modulate cAMP levels through stimulation or inhibition of cAMP-degrading PDEs (e.g., PDE2 or PDE3, respectively). NO can also undergo oxidation and react with thiols of cysteine side chains leading to S-nitrosylation of proteins. The major elements of cGMP signaling in platelets are NO, sGC (predominantly the $\alpha_1\beta_1$ isoform), cGKI (predominantly the cGKIβ isoform), and PDEs (predominantly PDE2, PDE3, and PDE5). Note that pGCs have not been detected in platelets. *cGKs*, cGMP-dependent protein kinases; *CNG* channels, cyclic nucleotide-gated cation channels; *LPS*, lipopolysaccharide; *MRP*, multidrug resistance protein; *NOS*, nitric oxide synthase; PDEs, phosphodiesterases; *pGC*, particulate guanylate cyclase; *sGC*, soluble guanylate cyclase

particulate guanylate cyclases (pGCs) are transmembrane receptors activated by peptides such as atrial, brain, and C-type natriuretic peptide (ANP, BNP, and CNP, respectively) [9, 10]. Accumulating evidence suggests that cGMP produced by different guanylate cyclases has different functional outcomes, even in the same cell, thus indicating the existence of subcellular cGMP signaling compartments [11, 12]. One of the simplest models predicts that the NO-sGC system generates global cGMP signals in the cytosol, whereas the natriuretic peptide-pGC system produces localized cGMP microdomains at the plasma membrane (Fig. 15.1). In order to terminate cGMP signaling, cGMP is efficiently removed, either by degradation into 5′-GMP by phosphodiesterases (PDEs) or by excretion via nucleotide transporters present in the plasma membrane [13, 14]. The mechanisms for cGMP removal are also important to shape and maintain cGMP compartments. According to the current view, distinct PDEs selectively regulate either plasma membrane or cytosolic cGMP pools. cGMP elicits its functional effects via binding to cGMP receptor proteins. It activates cyclic nucleotide-gated (CNG) cation channels and cGMP-dependent protein kinases (cGKs) [12, 15, 16]. The cGK type I (cGKI) is the principal cGMP effector in platelets. cGMP can also activate or inhibit PDEs that hydrolyze cAMP. For instance, cGMP inhibition of the cAMP-hydrolyzing PDE3 leads to an increase of the intracellular cAMP concentration, thereby providing a mechanism for cross talk between cGMP and cAMP signaling (Fig. 15.1).

As a widely distributed second messenger, cGMP controls many physiological functions ranging from smooth muscle relaxation and platelet activation to neuronal plasticity and sensory axon bifurcation [12, 17]. The importance of cGMP signaling for health and disease has also driven the development of cGMP-elevating drugs used in the clinic, particularly for the treatment of cardiovascular diseases. Organic nitrates release NO resulting in increased cGMP levels in VSMCs and vasodilation, thereby alleviating chest pain associated with coronary heart disease. Sildenafil (Viagra®), an inhibitor of the cGMP-specific PDE5, is used for the treatment of erectile dysfunction and pulmonary hypertension. Recently, the sGC stimulator riociguat has been approved for the treatment of two forms of life-threatening pulmonary hypertension. Interestingly, the physiological relevance and therapeutic potential of platelet NO-cGMP signaling for the regulation of platelet activity, hemostasis, and thrombosis is still elusive.

15.2 Shaping cGMP Signals in Platelets

15.2.1 Generation of cGMP

The intracellular cGMP concentration is determined by the balance of its synthesis and removal. To date, pGCs have not been detected in platelets. However, platelets do express sGC, the only definitive receptor for NO. Thus, cGMP synthesis in platelets is exclusively triggered by NO-activated sGC. sGC is a heterodimer consisting of two homologous subunits: an α-subunit of 73–82 kDa and a heme-binding β-subunit of ≈70 kDa [8, 18, 19]. For each subunit, two isoforms have

been identified (α_1 and α_2, β_1 and β_2), but the β_2 isoform, which is preferentially expressed in kidney, does not form functional heterodimers [20]. The $\alpha_1\beta_1$ heterodimer is expressed in platelets, VSMCs, and many other cell types, while expression of the $\alpha_2\beta_1$ isoform is more restricted and most abundant in brain, lung, and placenta [21–24]. The catalytic activity of sGC requires both an α- and a β-subunit. Each subunit consists of four distinct regions that are conserved among eukaryotes. The β_1-subunit contains an N-terminal heme-binding domain, a Per/Arnt/Sim (PAS) domain, a putative amphipathic helix/coiled-coil, and a C-terminal catalytic domain. The α_1-subunit shares 30% sequence identity with the β_1-subunit and has a similar structural organization, except that its N-terminus does not bind heme and is of unknown function. The heme-binding domain of the β_1-subunit is also termed H-NOX (heme nitric oxide/oxygen) domain based on its ligand binding properties, which are conserved among similar proteins found in prokaryotes and eukaryotes [8, 25]. NO binds to the Fe^{2+} of the heme moiety forming a NO-Fe^{2+}-His complex and converting sGC into an active enzyme with over 200-fold increase in activity compared to the basal state [26]. A reduced Fe^{2+}-bound heme is essential for the activation of sGC by NO. Oxidation of the heme Fe^{2+} to Fe^{3+} strongly attenuates the enzyme's sensitivity to NO [27, 28]. The commonly used sGC inhibitor ODQ (1H-[1, 2, 4]oxadiazolo[4,3-a]quinoxalin-1-one) oxidizes the sGC heme Fe^{2+} to Fe^{3+} resulting in irreversible desensitization of sGC to NO [27].

Platelets predominantly express the $\alpha_1\beta_1$ isoform of sGC [29]. In line with this expression profile, platelets of sGC α_2-subunit knockout mice showed no functional difference compared to wild-type platelets [29]. However, genetic inactivation of the β_1-subunit resulted in impaired inhibition of platelet aggregation by NO and decreased tail bleeding times in whole body knockout animals [30, 31]. Consistent with the results from sGC β_1-deficient mice, mouse mutants expressing heme-deficient NO-unresponsive sGC also exhibited loss of NO-mediated platelet inhibition and shorter tail bleeding times than wild-type littermates [32]. These results indicated an inhibitory role of the NO-cGMP pathway in hemostasis and thrombosis. How sGC activity is regulated in intact platelets under native conditions during platelet activation and thrombus formation is not well understood. Besides the dramatic stimulation of sGC by NO, it is likely that sGC activity is also affected by alternative mechanisms. Interestingly, elevation of platelet cGMP has been detected after exposure of platelets to platelet-activating agonists including vWF, collagen, and thrombin [33–35]. Binding of vWF to platelet GPIb leads to phosphorylation of the sGC β_1-subunit and cGMP generation in an NO-independent manner [34]. It has also been suggested that several proteins including CCTη [36], Hsp70 [37], PSD95 [38], LGN [39], and PDI [40] are able to interact with sGC and, thereby, modulate cGMP synthesis in intact cells.

15.2.2 Removal of cGMP

Cyclic nucleotide PDEs catalyze the hydrolysis of one of the phosphoester bonds of cAMP or cGMP, producing inactive 5′-AMP or 5′-GMP, respectively. There are

21 genes known to encode PDEs, and they are grouped into 11 gene families based on their amino acid sequence, regulatory properties, and catalytic characteristics. PDE 4, 7, and 8 are highly specific for cAMP, while PDE 5, 6, and 9 are highly specific for cGMP, and the remaining five families (PDE 1, 2, 3, 10, and 11) hydrolyze both cGMP and cAMP, although with different affinities and efficiencies [13].

Platelets express three cGMP-hydrolyzing PDEs: PDE2, PDE3, and PDE5 [41, 42]. PDE2 is a cGMP-activated cGMP/cAMP PDE. Binding of cGMP to an allosteric site of PDE2 promotes the hydrolysis of both cGMP and cAMP. In contrast, PDE3 is a "cGMP-inhibited" cGMP/cAMP PDE. In fact, PDE3 has similar affinities for cGMP and cAMP, but the reaction rate for cGMP is only ≈10% of that for cAMP. Thus, cGMP exerts competitive inhibition of cAMP hydrolysis and PDE3 is usually referred to as "cGMP-inhibited" PDE. The cGMP-specific PDE5 is abundantly expressed in platelets [43]. By binding to an allosteric site, cGMP increases the catalytic activity of the enzyme and, thus, promotes its own degradation [44]. Activation of PDE5 by cGMP is further augmented via phosphorylation of a specific serine residue (Ser92 or Ser102 of bovine or human PDE5, respectively) by cGKI [45].

Intracellular cGMP can also be removed through cGMP efflux. Several cGMP transporters located in the plasma membrane have been identified including members of the multidrug resistance protein (MRP) family such as MRP4/5/8 [46]. MRP4-mediated removal of cGMP in VSMCs contributes to the control of muscle tonus to an extent similar to PDE5-mediated cGMP degradation [47]. Efflux of cGMP from activated human platelets was reported more than 20 years ago [48]. While MRP5 and MRP8 were not detected in platelets [49], MRP4 is highly abundant in dense granules and also at lower levels in the plasma membrane of human platelets [50]. MRP4-mediated cGMP efflux reduces the inhibitory effects of cGMP in human platelets [50, 51], but the relative importance of this process in comparison to cGMP removal via PDEs remains to be established.

15.3 cGMP Effector Mechanisms in Platelets

cGKs are central mediators of cGMP signaling [12]. They belong to the AGC subfamily of serine/threonine protein kinases and are activated by cGMP concentrations in the range of ≈0.1–1 μM [52, 53]. Mammals have two cGK genes, *prkg1* and *prkg2*, encoding cGKI and cGK type II, respectively. In the cardiovascular system, cGKI is more commonly expressed than cGK type II, in particular in VSMCs, cardiomyocytes, and platelets. The *prkg1* gene encodes two cGKI isoforms, termed cGKIα and cGKIβ, which differ in their N-terminal domains (≈100 amino acids). Human platelets only express cGKIβ, whereas both cGKIβ and a small amount of cGKIα were detected in mouse platelets [54, 55]. In an ischemia-induced thrombosis model, cGKI-deficient mouse platelets showed increased adhesion and aggregation compared to wild-type platelets indicating an inhibitory role of cGKI-mediated signaling in platelets [56]. cGKI substrates such

as vasodilator-stimulated phosphoprotein (VASP) and inositol-1,4,5-trisphosphate (IP_3) receptor-associated cGMP kinase substrate (IRAG) are also abundantly expressed in platelets. Consistent with the analysis of cGKI mouse mutants, mice deficient in VASP or IRAG showed impaired NO/cGMP-dependent inhibition of platelet aggregation in vivo [55, 57]. Taken together, the data from genetic manipulation of sGC, cGKI, and cGKI substrates have clearly established the importance of the NO-sGC-cGMP-cGKI pathway for platelet inhibition in vivo. What are the molecular mechanisms of cGMP inhibition of platelet activity? Many studies have shown that activation of NO-cGMP signaling interferes with several key events during platelet aggregation under in vitro conditions including Ca^{2+} release from intracellular stores, granule secretion, and activation of small G-proteins and integrins.

Ca^{2+} plays a central role in platelet aggregation by promoting platelet adhesion, granule release, soluble agonist-induced platelet activation, cytoskeleton reorganization, and integrin activation [2]. Soluble platelet agonists that activate $G\alpha_q$-coupled receptors (ADP, thrombin, thromboxane A_2) as well as collagen induce activation of phospholipase C, which generates IP_3. IP_3 binds to and activates the IP_3 receptor, a Ca^{2+} channel in the endoplasmic reticulum membrane mediating Ca^{2+} release from intracellular Ca^{2+} stores followed by store-operated Ca^{2+} entry from the extracellular space [58, 59]. Sustained cytosolic Ca^{2+} elevation is required for both platelet aggregation and blood coagulation. Platelets provide a suitable surface for the assembly of coagulation protein complexes by expressing phosphatidylserine. The exteriorization of phosphatidylserine is mediated by the Ca^{2+}-dependent regulation of a phospholipid scramblase called TMEM16F. Phosphatidylserine exposure accelerates thrombin formation via the coagulation cascade, through which fibrinogen is converted into fibrin, thereby stabilizing the thrombus [4]. It is well known that activation of cGKI suppresses agonist-induced intracellular Ca^{2+} release in VSMCs [60–62], at least in part, by forming a complex containing cGKIβ, its substrate IRAG, and the IP_3 receptor type I (IP_3RI) [63, 64]. A similar mechanism is also active in platelets. It has been shown that cGKI phosphorylates IRAG in intact platelets and that thrombin-induced Ca^{2+} release was attenuated by NO or cGMP in wild-type platelets but not in IRAG-deficient platelets [55]. Thus, the interaction of cGKI-phosphorylated IRAG and the IP_3RI appears to suppress IP_3-induced Ca^{2+} release from the endoplasmic reticulum of platelets.

Following platelet activation, platelets undergo a dramatic shape change mediated by cytoskeletal remodeling and accompanied by the release of platelet granule contents. The release of soluble agonists such as thromboxane A_2 and ADP is crucial for irreversible platelet activation at the site of vascular injury. These agonists activate specific G protein-coupled receptors and act in an autocrine or paracrine manner to enhance platelet activation and thrombus formation [4]. The platelet cytoskeleton binds and positions specific signaling molecules. In particular, reorganization of the actin cytoskeleton promotes the formation of active integrin signaling complexes. An increase in cGMP also affects the cytoskeleton through cGKI-mediated phosphorylation of cytoskeleton-associated proteins. The actin-binding protein VASP is a major cGKI substrate in platelets [65]. cGKI

phosphorylates VASP at Ser157 and Ser239. VASP participates in actin fiber formation and VASP phosphorylation affects its own intracellular localization [66]. Phosphorylation of VASP at Ser157 correlates with inhibition of the fibrinogen receptor integrin $\alpha_{IIb}\beta_3$ [67]. VASP-deficient platelets show enhanced binding of integrin $\alpha_{IIb}\beta_3$ to fibrinogen revealing an inhibitory function of VASP in integrin activation [68, 69]. Deletion of VASP in mice also leads to enhanced adhesion of platelets to the vascular wall in an ischemia-induced thrombosis model [57]. These findings suggest that cGMP inhibits platelet aggregation, at least in part, via cGKI-mediated VASP phosphorylation and interference with cytoskeletal remodeling, which is normally associated with platelet activation. However, the exact functional role of VASP phosphorylation at Ser157 and/or Ser239 in hemostasis and thrombosis in vivo is unknown.

In addition to IRAG and VASP, several other cGKI substrates have been identified that might contribute to the inhibitory actions of cGMP in platelets [70, 71]. Many of these proteins represent G-proteins or modulators of G-protein activity known to control platelet activation via their effects on integrins, intracellular Ca^{2+} release, and the cytoskeleton. For instance, the small G-protein Rap1B is involved in the activation of integrin $\alpha_{IIb}\beta_3$ [72]. Inhibition of integrin $\alpha_{IIb}\beta_3$ by NO/cGMP in human platelets is, in part, attributed to cGKI-mediated phosphorylation of Rap1B [73] or Rap1GAP2 [74], a GTPase-activating protein for Rap1B. Moreover, cGKI has been shown to phosphorylate regulator of G-protein signaling 18 (RGS18) [75]. RGS18 is a GTPase-activating protein for the $G\alpha_q$-subunits of heterotrimeric G-proteins that interact with G protein-coupled receptors at the platelet plasma membrane. Phosphorylation of RGS18 by cGKI potentiates RGS18 function and, consequently, attenuates $G\alpha_q$ signaling leading to reduced receptor-mediated Ca^{2+} release from intracellular stores triggered by platelet agonists like thrombin [75]. Recently, two novel cGKI substrates in platelets have been identified, ARHGAP17, a GTPase-activating protein, and ARHGEF6, a guanine nucleotide exchange factor, which regulate the activity of the small G-protein Rac1 [76]. Phosphorylation of ARHGAP17 and ARHGEF6 by cGKI induces a rearrangement of their associated protein complexes, resulting in a reduced level of active Rac1, a key player in cytoskeletal remodeling and platelet activation [77].

In addition to cGMP, cAMP is another important cyclic nucleotide messenger playing an inhibitory role in platelet activation [71]. cGMP and cAMP activate cGMP- and cAMP-dependent protein kinases, respectively, and most of the abovementioned cGKI substrate proteins can also be phosphorylated by cAMP-dependent protein kinase. Thus, it appears that in many cases cGMP and cAMP signals converge at the level of downstream mechanisms. There are also other types of interplay between cGMP and cAMP signaling in platelets. Perhaps the most significant cGMP-cAMP cross talk is mediated through the stimulatory or inhibitory effect of cGMP on cAMP hydrolysis by PDE2 or PDE3, respectively. A rise in the intracellular cGMP concentration can decrease the cAMP level via cGMP-activated PDE2, and it can increase the cAMP level via cGMP-inhibited PDE3 [41, 78]. Thus, cGMP might affect platelet activity not only via activation of cGKI

but also via modulation of cAMP signaling (Fig. 15.1). This mode of cGMP-cAMP cross talk has been best studied in cardiomyocytes, where it might contribute to the spatiotemporal control of cyclic nucleotide signaling compartments and cardiac (patho-)physiology [79]. Indeed, cGMP-mediated inhibition of PDE3 has also been implicated in elevation of cAMP levels and activation of cAMP-dependent protein kinase in human platelets, which could mediate at least some aspects of NO-induced platelet inhibition [80, 81]. Another study has described a compartment-specific signaling complex in human platelets containing PDE5, cGKIβ, and the IP_3RI and proposed a model in which cGKI selectively activates PDE5 within a defined microdomain allowing spatial and temporal control of cGMP signaling in platelets [82]. However, the in vivo relevance of cGMP-cAMP cross talk and compartmentalized cyclic nucleotide signaling in platelets remains to be established.

15.4 Relevance of cGMP in Hemostasis, Thrombosis, and Antithrombotic Therapy

The biochemical, genetic, and pharmacological studies described above demonstrate that platelets express a robust NO-sGC-cGMP-cGKI pathway and that activation of this signaling cascade results in platelet inhibition. It is widely accepted that a cGMP increase works as a brake to limit thrombosis. Several clinically used antiplatelet drugs elevate cGMP and/or cAMP levels. For instance, dipyridamole and cilostazol inhibit cyclic nucleotide PDEs. Moreover, recent evidence indicates that activation of the NO-cGMP axis contributes to the antithrombotic effects of certain β-blockers like nebivolol [83] as well as to the action of $P2Y_{12}$ receptor blockers that are commonly used in antiplatelet therapy (e.g., clopidogrel, prasugrel, ticagrelor) [84]. On the other hand, disabling the cGMP brake is predicted to promote platelet activation and aggregation. Indeed, a recent report suggests that hyperlipidemia and oxidized low-density lipoprotein promote platelet hyperactivity by inducing reactive oxygen species that desensitize cGKI-mediated platelet inhibition [85].

It is important to note that most of our knowledge about cGMP signaling in platelets has been derived from in vitro analysis of wild-type and mutant platelets isolated from mice and humans, for instance, by biochemical or aggregometry assays. In vitro experiments with isolated platelets do probably not completely mimic the in vivo situation, in particular with regard to platelet interactions with immobilized substrates, other cell-types, blood flow, and shear forces that influence platelet activity under in vivo conditions [3, 4, 86]. Moreover, the interpretation of experimental results is often complicated by technical and biological issues. (1) The selectivity and efficacy of several cGMP analogs commonly used to study cGMP signaling in platelets is questionable [87–89]. (2) NO, especially if used at high concentrations, can also exert cGMP-independent effects [90], for instance, via protein S-nitrosylation [91]. (3) Conventional methods for detection of cGMP (e.g., RIA or ELISA) are performed with platelet extracts. These cGMP assays have low

temporal resolution and lack spatial resolution. They are not able to monitor dynamic cGMP signals in real time in living platelets during activation and aggregation. The transferability of cGMP levels determined in platelet extracts to cGMP signaling in living platelets appears limited if one considers that platelet cGMP is very rapidly degraded by PDEs [43] and that changes of the cGMP concentration in subcellular compartments might remain undetected when cGMP levels are determined in cell extracts [82]. Moreover, it has been noted that some commercially available ELISA kits for cGMP can give false-positive results [92]. (4) Last but not least, the interpretation of experiments with gene-targeted mice or with platelets isolated from these mice might be complicated due to additional, probably unknown, phenotypes of the mutant mice. For instance, cGKI-deficient mice show significantly elevated IL-6 serum levels [93]. The high IL-6 concentration is not due to dysfunctional cGMP signaling in platelets and leads to thrombocytosis [94]. The high platelet count and potential alterations of platelet functions secondary to the high IL-6 levels might lead to thrombosis and bleeding phenotypes in global cGKI knockout mice that are not caused by a lack of cGMP-cGKI signaling in platelets. Furthermore, it should be considered that vascular endothelial-generated NO and the smooth muscle sGC-cGMP-cGKI pathway is important in vasodilation. Thus, the whole body deficiency of sGC or cGKI may cause vasoconstriction and consequently reduce bleeding time. To specifically address the role of cGMP signaling proteins in platelets, it is recommended to analyze platelet-specific knockout mice.

To date, several studies have investigated platelet cGMP signaling by using in vivo models of hemostasis and thrombosis. One study applied intravital microscopy in mice after intestinal ischemia/reperfusion injury and found that platelet cGKI but not endothelial or smooth muscle cGKI is required to prevent intravascular adhesion and aggregation of platelets after ischemia in vivo [56]. Surprisingly, another study reported that cGKI-deficient platelets showed impaired activation in response to vWF and low-dose thrombin and that global cGKI knockout mice had prolonged tail bleeding times [33]. A follow-up study of the same group reported that aggregation of sGC-deficient platelets was reduced at low concentrations of collagen and thrombin, and tail bleeding times and thrombus formation were increased in platelet-specific sGC knockout mice [35]. These findings indicated a stimulatory role for the sGC-cGMP-cGKI pathway in platelet activation and provoked an ongoing controversy about cGMP's function(s) in platelets [95–98]. It is well known that platelet cGMP levels increase in response to various platelet agonists (e.g., vWF, collagen, thrombin, ADP) [33–35, 99, 100]. Unfortunately, the functional effect of agonist-induced cGMP elevation was not always investigated in these studies, thus leaving the question open whether the cGMP increase associated with a specific agonist leads to platelet activation or inhibition. In general, the cGMP level reached during the initial phase of agonist-induced platelet activation appears to be much (≈tenfold) smaller than the cGMP concentration reached during NO-induced platelet inhibition. Mechanistic details as to how platelet agonists increase cGMP and how cGMP might promote the platelet activation process are not well understood. It is a matter of debate whether platelet agonists activate a platelet NOS to produce NO or

whether platelet sGC is activated in an NOS/NO-independent manner [92]. A recent study indicated that platelets produce NO during adhesion to immobilized collagen in flowing blood under high shear rates, but under these conditions NO production limited rather than promoted further platelet deposition [101]. Other work indicated that vWF stimulates cGMP production in platelets independent of NOS and NO via Src kinase-mediated Tyr192 phosphorylation and activation of sGC [34]. It was suggested that the increase in cGMP upon stimulation of GPIb by vWF activates cGKI, which in turn leads to the stimulation of mitogen-activated protein kinases (MAPKs) and activation of the platelet integrin $\alpha_{IIb}\beta_3$ [102]. However, other studies could not confirm a stimulatory role of the cGMP-cGKI-MAPK axis in platelet activation by thrombin or low-dose collagen [103, 104].

Interestingly, an increasing number of publications describe a stimulatory role of cGMP in platelet activation elicited by ligands of pattern recognition receptors such as TLR4 and NOD2. These receptors play a key role in innate immunity [105] and might provide a bridge between infection/inflammation and thrombotic events. Bacteria-derived LPS stimulates platelet aggregation and thrombus formation via binding to TLR4 on the platelet surface. TLR4-induced intracellular signaling in platelets required the adaptor protein MyD88 and was associated with elevated cGMP and cGKI activity, which may initiate platelet activation [106]. Very recently, it was shown that the damage-associated molecular pattern molecule HMGB1 is a critical mediator of thrombosis, whose effects are transmitted via TLR4/MyD88-dependent recruitment of sGC toward the platelet plasma membrane, followed by cGMP synthesis and activation of cGKI [107]. Another study found that platelets also express the pattern recognition receptor NOD2 and that activation of platelet NOD2 by bacteria-derived muramyl dipeptide elicits an increase of cGMP-cGKI signaling associated with enhanced platelet activation and thrombosis [108]. In sum, these studies support the notion that activation of the cGMP-cGKI pathway via certain upstream triggers can have a stimulatory effect on platelets, which may be critical for abnormal platelet activation and aggregation in response to bacterial infection and inflammation.

The pathophysiological relevance of platelet cGMP signaling in humans is supported by recent human genetic analysis. One study identified the segregation of heterozygous mutations in two genes functionally related to NO-cGMP signaling in an extended myocardial infarction family [109]. The mutated genes encode the sGC α_1-subunit and the CCTη chaperone, which stabilizes sGC. Platelets from digenic mutation carriers contained less sGC protein and displayed reduced NO-induced cGMP formation, and sGC α_1-deficient mice showed accelerated thrombosis in the microcirculation after local trauma [109]. These findings indicate that dysfunctional NO-cGMP signaling increases the risk of myocardial infarction, perhaps through increased thrombus formation. Unfortunately, the platelet function of mutated carriers was not analyzed in this study. Therefore, it is not clear whether variants in sGC α_1 and CCTη promote myocardial infarction through platelet activation or other mechanisms. Another report described an autosomal-recessive syndrome resulting in severe moyamoya (a cerebrovascular condition leading to stroke) and early-onset achalasia (a rare disease characterized by aperistalsis of the

esophagus) that is associated with homozygous mutations in the sGC α_1 gene [110]. Mutated carriers had a complete loss of sGC protein in their platelets. Interestingly, loss of platelet sGC led to a defect in platelet activation, strongly suggesting that sGC-cGMP signaling has a stimulatory role in human platelets [110]. Two recent large-scale genome-wide association studies identified a common variant on chromosome four overlapping with the sGC α_1 gene, and this variant showed a significant association with coronary artery disease and myocardial infarction [111, 112]. Thus, human genetic studies provide substantial evidence for a causal involvement of sGC and cGMP signaling in the pathogenesis of atherothrombotic diseases. cGMP-elevating drugs, such as the PDE5 inhibitor sildenafil or the sGC stimulator riociguat, are increasingly recognized as a treatment option for cardiovascular and cardiopulmonary disorders. However, it is not clear whether an increase of cGMP in platelets specifically would have beneficial and/or detrimental effects on these diseases.

Taken together, the in vitro and in vivo data discussed above strongly suggest a biphasic role of cGMP signaling in both platelet activation and inhibition. We speculate that biphasic cGMP signaling in platelets is indeed highly relevant for an optimal hemostatic platelet response in vivo and can be best explained when we consider the dynamics of platelet cGMP signals and thrombus formation under flow. It is now widely recognized that blood flow and the resulting shear forces are key factors affecting platelet aggregation [113, 114]. Shear rates in arteries and arterioles are in the range of 300–800 s^{-1} and 500–1600 s^{-1}, respectively, whereas those in veins are about ten times lower at 20–200 s^{-1} [115]. During thrombus formation, there is a dramatic increase of shear rates, which can surge above 10,000 s^{-1} in stenotic arteries [116]. It has long been known that shear stress activates eNOS in endothelial cells increasing NO production [117] and that shear stress-induced NO can diffuse into the vessel lumen and stimulate cGMP synthesis in platelets [118]. Thus, it is likely that endothelial NO release and platelet cGMP signals are modulated by the changing shear forces during thrombus formation.

Based on these assumptions and the myriad of data published in the field, we propose a new model that integrates seemingly contradictory findings as well as cGMP compartmentation and blood flow to describe dynamic platelet cGMP signaling during hemostasis and thrombosis in vivo (Fig. 15.2). In this model, a small/compartmentalized increase of platelet cGMP during the initial phase of platelet activation promotes platelet aggregation at the site of injury and is then followed by a stronger cGMP signal that inhibits further recruitment of platelets and limits thrombus growth. How is the spatiotemporal dynamics of platelet cGMP signals regulated, and how can an increase of cGMP both stimulate and block platelet aggregation? To provide answers to these questions, our model incorporates two important features: (1) adjustment of the intraplatelet cGMP concentration by shear-dependent NO release from the endothelium and (2) generation of different cGMP pools during early and late stages of platelet aggregation. In the initial phase of platelet adhesion and activation, shear stress and endothelial NO production at the site of injury are relatively low (Fig. 15.2, *left*). In response to adhesive ligands

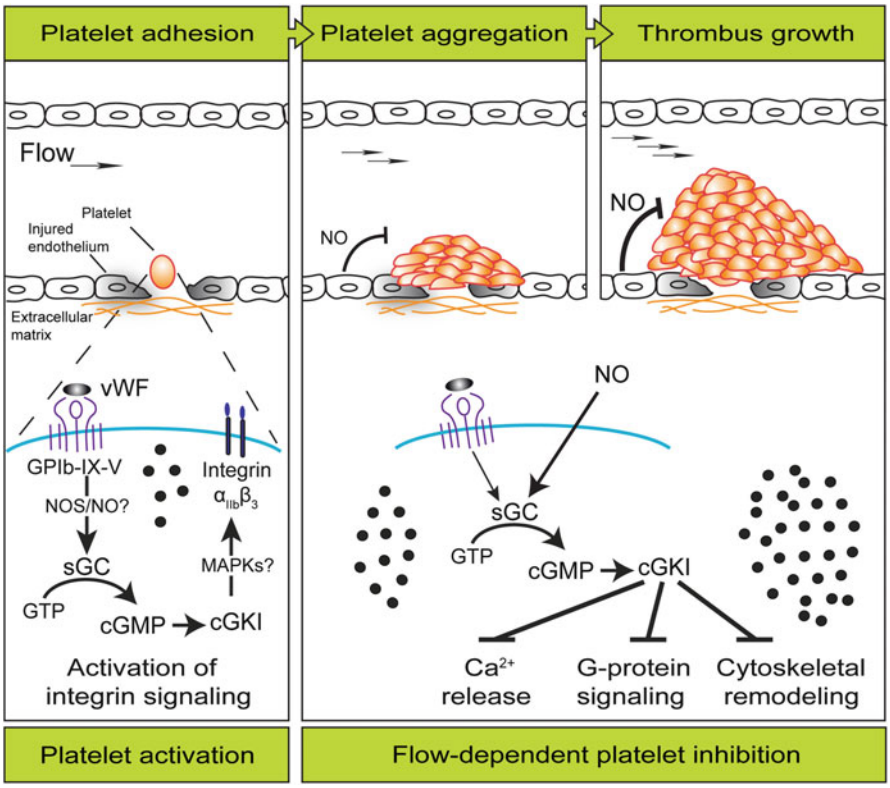

Fig. 15.2 A model for autoregulated hemostasis and thrombosis by dynamic flow-dependent cGMP signaling. The regulation of platelet activity by cGMP (*black balls*) is flow dependent. An important factor that adjusts the intraplatelet cGMP concentration is endothelial NO release, which is augmented with increasing shear stress during thrombus growth. The increasing shear stress exerted by blood flow during thrombosis is indicated by *horizontal arrows* in the vessel lumen. In the early phase of platelet activation, a mild cGMP increase is stimulatory (*left*). At this stage, shear stress and NO production at the site of injury are low. In response to adhesive ligands such as vWF and collagen, sGC is weakly activated via a not well-defined mechanism, perhaps via intraplatelet NOS/NO, resulting in a relatively small and/or compartmentalized cGMP increase at the platelet plasma membrane. This early cGMP signal promotes platelet adhesion via activation of integrin $\alpha_{IIb}\beta_3$ signaling, perhaps via cGKI and MAPKs. However, in later stages of platelet aggregation and thrombus growth, strong inhibitory cGMP signals are generated (*mid* and *right*). Flow-induced shear rates and endothelial NO production increase with thrombus growth. The high local NO concentration results in strong stimulation of sGC activity in aggregated and freshly recruited platelets. The high cGMP concentration in these platelets strongly activates cGKI, which phosphorylates multiple substrates resulting in inhibition of intracellular Ca^{2+} release, G-protein signaling, and cytoskeletal remodeling, so that further recruitment of platelets and overgrowth of hemostatic thrombi is prevented. With decreasing thrombus size, the shear forces acting on the endothelium and the production of cGMP decline, and the intraplatelet cGMP concentration drops due to hydrolysis mainly by PDE5 (not shown). Thus, the shear stress dependency of the sGC-cGMP-cGKI pathway could provide a mechanism for a biphasic role of cGMP, whereby weak/compartmentalized cGMP signals stimulate initial platelet

such as vWF and collagen, sGC is weakly activated via a not well-defined mechanism resulting in the formation of a membrane-associated cGMP microdomain. This local cGMP pool stimulates integrin activation and, thereby, promotes platelet activation and aggregation. A localized cGMP compartment is also consistent with the fact that the cGMP elevation measured in cell extracts after agonist stimulation of platelets is relatively mild. With thrombus growth, the adjacent endothelium is exposed to increasing shear stress (Fig. 15.2, *mid and right*). Thus, high amounts of NO are released from the endothelium resulting in strong activation of sGC and cGMP production in aggregated and newly recruited platelets. This generates a second, global cGMP pool in the platelet cytoplasm that overrides the local stimulatory cGMP pool and prevents further platelet aggregation via the classical inhibitory cGMP pathway. Mechanistically, both cGMP pools could act via activation of cGKI and modulation of the intracellular Ca^{2+} concentration, the local stimulatory cGMP pool by augmenting Ca^{2+} influx, and the global inhibitory cGMP pool by suppressing Ca^{2+} release from the endoplasmic reticulum [119]. Taken together, our model proposes that the cGMP signaling system acts as both a gas pedal and a brake that are autoregulated via blood flow/shear stress to achieve optimal platelet activation during the hemostatic response after vascular injury.

15.5 Concluding Remarks

During the last decades, tremendous progress has been made in elucidating the functions of cGMP signaling in platelets. Biochemical, pharmacological, and genetic studies have clearly established an inhibitory role of the canonical NO-sGC-cGMP-cGKI pathway in platelet aggregation, mainly by inhibiting intracellular Ca^{2+} release and cytoskeletal remodeling. However, accumulating evidence indicates that cGMP can also promote initial platelet activation. The mechanism of this stimulatory action of cGMP is less well understood than inhibitory cGMP signaling. These contradictory findings combined with the fact that many of the previous studies were performed under in vitro conditions, which probably do not completely mimic the in vivo situation, make it difficult to conclude whether an increase in platelet cGMP has beneficial and/or detrimental effects on hemostasis and thrombosis in vivo. Major questions in platelet cGMP signaling remain to be answered, for instance:

- Can platelets produce NO?
- Does NO also exhibit cGMP-independent effects on platelet activity?
- Does cGMP also exhibit cGKI-independent effects on platelet activity?

Fig. 15.2 (continued) activation at the site of vascular injury followed by stronger cGMP signals that limit an exaggerated hemostatic response and occlusive thrombosis. As such, the cGMP signaling system could serve as both a gas pedal and a brake that are autoregulated via blood flow/shear stress to achieve optimal platelet activation during the hemostatic response after vascular injury

- How exactly does cGMP signaling interact with other signaling pathways to control platelet functions? For instance, what is the role of cGMP-cAMP cross talk? What are the in vivo substrates of cGKI?
- In addition to cGMP hydrolysis via PDEs, how important is cGMP efflux via cyclic nucleotide transporters in shaping cGMP signals in platelets?
- Do platelets have subcellular cGMP signaling compartments? If so, are they functionally relevant?
- How does cGMP promote initial platelet activation? How is sGC activated in response to platelet agonists and is the resulting cGMP production compartmentalized, for instance, at the plasma membrane? How does the cGMP increase promote integrin activation and platelet aggregation?
- Are cardiovascular diseases associated with dysfunctional cGMP signaling in platelets? Can we treat these disorders by targeting platelets with cGMP-elevating drugs?

To improve our understanding of cGMP's function in hemostasis and thrombosis, it is important to study cGMP signaling in platelets under in vivo conditions, in the context of platelet interactions with the vessel wall and blood flow. We propose a new model of cGMP-regulated hemostasis that integrates both platelet stimulation and inhibition by dynamic shear stress-regulated cGMP signals during different phases of thrombus formation under flow in vivo (Fig. 15.2). In this model, rapidly changing and compartmentalized cGMP signals are crucial for the appropriate functioning of platelets under native conditions. This hypothesis has to be tested in the future. However, the spatiotemporal dynamics of cGMP signals in living platelets cannot be monitored with conventional cell-destructive cGMP assays such as RIA and ELISA. In recent years, cGMP sensor proteins have been developed for the visualization of cGMP signals in real time in living cells [120, 121]. Transgenic mice expressing such a cGMP biosensor are available [122, 123] and should allow for the imaging of dynamic cGMP signals in platelets under flow conditions. The spatiotemporal cGMP profile within platelets can then be correlated with changes in platelet behavior during platelet aggregation in vitro or even during thrombus formation in vivo. The study of cGMP in hemostasis and thrombosis should benefit from these technical advances.

What is the therapeutic potential of cGMP-elevating drugs for the treatment of thrombotic conditions? An inherent weakness with all currently used antiplatelet agents is their deleterious impact on hemostasis, with the most potent antithrombotic drugs typically conferring the greatest bleeding risk [4]. Optimal platelet activation in response to vascular injury in the context of hemostasis means preventing circulating platelets from activating needlessly, allowing them to respond quickly when necessary and limiting platelet activation to avoid excessive platelet accumulation and thrombus growth [124]. Dynamic flow-regulated NO-cGMP signaling might indeed provide a self-regulating gas and brake for optimal platelet activation. As such, pharmacological stimulation of platelet cGMP signaling, perhaps with innovative compounds that selectively trigger inhibitory cGMP signals in a growing thrombus without affecting stimulatory cGMP

signals during initial formation of the hemostatic plug, is an interesting strategy for antithrombotic therapy with a lower risk of bleeding. Indeed, novel sGC-stimulating drugs have been shown to reduce thrombus formation in animal models [125].

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Compliance with Ethical Standards

Conflict of Interest: Lai Wen, Susanne Feil, and Robert Feil declare that they have no conflict of interest.

Ethical Approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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