Chapter 9

Mechanisms Involved in Diabetes-Associated Platelet Hyperactivation

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1. Introduction

Diabetes mellitus is a multi-factorial disease caused by a combination of genetic and environmental factors. Although insulin resistance and dysregulation of glucose and lipid homeostasis are the primary hallmarks of the disease, it is now well accepted that the morbidity and mortality associated with diabetes mostly result from micro- and macro-vascular complications [1]. An early step in the pathogenesis of the vascular complications of diabetes is the development of endothelial dysfunction which is characterised by a decrease in nitric oxide (NO) bioavailability, prostacyclin production and a general reduction in the anti-thrombogenic properties of vascular wall [2]. Diabetes is also characterised by an alteration of platelet function. Indeed, platelets from patients with type 1 or type 2 diabetes are hyperreactive and demonstrate increased adhesiveness as well as exaggerated aggregation and thrombus formation. Several mechanisms have been reported to mediate the hyperreactivity of platelets from diabetic patients including morphological changes such as increased mean platelet volume and accelerated platelet turnover, biochemical changes such as increased reactive oxygen species (ROS) production, increased synthesis of thromboxane A2 (TXA2) and thrombin and a dysregulated Ca^{2+} homeostasis. Platelets from diabetic patients also demonstrate increased surface expression of adhesion proteins such as P-selectin and the αIIbβ3 integrin and reduced membrane fluidity. These changes characteristic of the "diabetic platelet" have been mostly attributed to the metabolic dysregulation associated to the insulin resistance and dyslipidemia. However, given that platelet hyperreactivity has also been found in patients with type 1 diabetes mellitus it is suggested that hyperglycemia alone can account for at least part of the altered platelet response in patients with diabetes mellitus. Oxidative stress which characterizes both types of diabetes has also been shown to be an important factor mediating the phenotypic changes of diabetic platelets. In this chapter I will first give an overview of the

physiological platelet activation, and then discuss the role of factors such as insulin resistance, hyperglycemia, dyslipidemia on platelet function. Next, I will describe the intracellular mechanisms underlying platelet hyperreactivity in diabetes. Finally, the impact of diabetes on the responsiveness to anti-platelet therapy will be discussed.

2. Physiological platelet activation

Platelets are anucleated cells generated from megakaryocytes, and after their release into the blood, circulate for approximately 10 days. The main role of platelets is to maintain hemostasis. Under normal conditions, platelet adhesion to the vascular wall is inhibited due to the anti-thrombotic nature of the endothelial cell surface and the permanent release of anti-platelet factors by the endothelium [3]. Following vascular injury, especially under the influence of high shear stress, platelets tether and adhere to the exposed subendothelial collagen via the von Willebrand factor (vWF)-mediated binding to platelet glycoprotein Ib/V/IX complex. The initial interaction is subsequently strengthened by the interaction of collagen to its receptor glycoprotein VI (GPVI) and the integrin $\alpha 2\beta 1$. The ligation of these receptors activates Src family tyrosine kinases (SFKs) which lead to the phosphorylation and activation of the phospholipase Cγ2 (PLC γ2). The latter hydrolyses membrane Phosphatidylinositol-4, 5-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol 1, 4, 5-triphosphate (IP3) which respectively activates the protein kinase C (PKC) and stimulates the release of Ca^{2+} from the intracellular stores. PKC binds to and phosphorylates the cytoplasmic tail of the $\beta 3$ subunit of the αIIb$\beta 3$ integrin complex, which then recruits talin and kindlin-3. This in turns causes a conformational change in the extracellular domain of the integrin and enables the binding of circulating fibrinogen (inside-out signalling). Fibrinogen links activated αIIb$\beta 3$ integrins of neighbouring platelets and initiates platelet aggregation. After its binding, fibrinogen initiates the so-called "outside-in" signalling of the integrin. This step includes the activation of SFKs which promotes the tyrosine phosphorylation of $\beta 3$ integrin and the binding of cytoskeletal proteins leading to the activation of the phosphatidylinositide 3-kinases (PI3K) and platelet adhesion. Ca^{2+} and DAG also act together to activate the calcium- and diacylglycerol-regulated guanosine exchange factor (CalDAGGEF), a guanosine exchange factor important for the activation of the small GTPase Rap1. The latter activates mitogen-activated protein kinases (MAPKs) which are known to be upstream of the phospholipase A2 (PLA2)/cyclooxygenase (COX) signalling cascade resulting into the production of thromboxane A2. PKC and MAPK act in concert to stimulate the release of different second mediators from platelet granules. These agonists; including adenosine diphosphate (ADP), serotonin and the formed thromboxane A2 bind to their respective G protein–coupled receptors (GPCRs). Through the activation of G protein-mediated signalling pathways, they can further increase their own formation and/or release, thus acting as positive-feedback amplifying platelet responses by recruiting additional platelets and promoting aggregation. (for review see references [4, 5]) (figure 1). In addition to stimulating degranulation, the increase in platelet $[Ca^{2+})_i$ also leads to the activation of calpains, a family of Ca^{2+}-dependent neutral cysteine proteases. While some calpains are expressed only in specific tissues, platelets are known to express at least two

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isoforms of this enzyme i.e μ-calpain (calpain 1) and m-calpain (calpain 2). The μ- and m-calpain were initially named for the Ca^{2+} concentration (micromolar versus millimolar) required for their activation in vitro [6]. However it is now clear that additional mechanisms such as phosphorylation also regulate their proteolytic activity [7]. Several reports have highlighted the importance of μ- and m-calpain in platelet activation. Indeed, once activated, calpains induce the limited proteolysis of a number of proteins implicated in cytoskeletal rearrangement, degranulation and aggregation. Proteins identified to-date that are targeted by calpain include spectrin, adducin and talin as well as platelet-endothelial cell adhesion molecule-1 (PECAM-1), the myosin light chain kinase and N-ethylmaleimide-sensitive-factor attachment receptor proteins such as N-ethylmaleimide sensitive factor attachment protein-23 and vesicle-associated membrane protein-3. Furthermore, μ-calpain modulates αIIbβ3 integrin-mediated outside-in signaling and platelet spreading by cleaving the β3 subunit of the αIIbβ3 integrin [8]. In the final stages of platelet activation, phosphatidylserine, which is normally sequestered in the inner leaflet of the plasma membrane, is relocated to the outer leaflet leading to the shedding of microparticles and giving platelets a procoagulant surface. After stimulation, Ca^{2+} is removed from the cytosol and sequestred into the intracellular stores and/or extruded into the extracellular space by the action of the sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) and the plasma membrane Ca^{2+}-ATPase (PMCA), respectively. Formation of platelet plug or primary hemostasis is associated with the activation of the coagulation cascade which results into fibrin deposition and linking (secondary hemostasis) and the formation of a red clot. After the clot has been formed, platelets rearrange and contract their intracellular actin/myosin cytoskeleton. Given that the intracellular actin network is connected to the internal part of the integrin αIIbβ3, platelet contractile force on the fibrin network will lead to "clot retraction". Finally, the fibrin is slowly dissolved by the fibrinolytic enzyme, plasmin, and the platelets are cleared by phagocytosis and wound healing will take place.

Platelets store quite high concentrations of chemokines, cytokines, growth factors and vasoactive substances. The latter are sequestered into 2 major types of granules; α-granules and dense granules. On the one hand, alpha-granules are known to contain growth factors including vascular endothelial growth factor, platelet-derived growth factor, endostatin, transforming growth factor-β; chemokines such as platelet factor 4 (CXCL4) and CCL5 and adhesion molecules such as P-selectin. Dense granules, on the other hand, store mainly small molecules (e.g ADP and serotonin) and ions (such as Ca^{2+} and Mg^{2+}). Upon activation, platelets release soluble proteins contained in their granules and redistribute some α-granules contents to the membrane (e.g P-selectin, CD40 ligand). Given the variety of proteins released upon platelet activation, it is clear that platelets may affect the vascular wall in different ways. Indeed, beyond their role in hemostasis, platelets are involved in angiogenesis as well as in vascular inflammation.

3. Effect of insulin resistance on platelet function

Insulin exerts an inhibitory effect on platelets but the intracellular mechanisms remain not fully characterized and whether the effects are mediated by the insulin receptor is unclear.

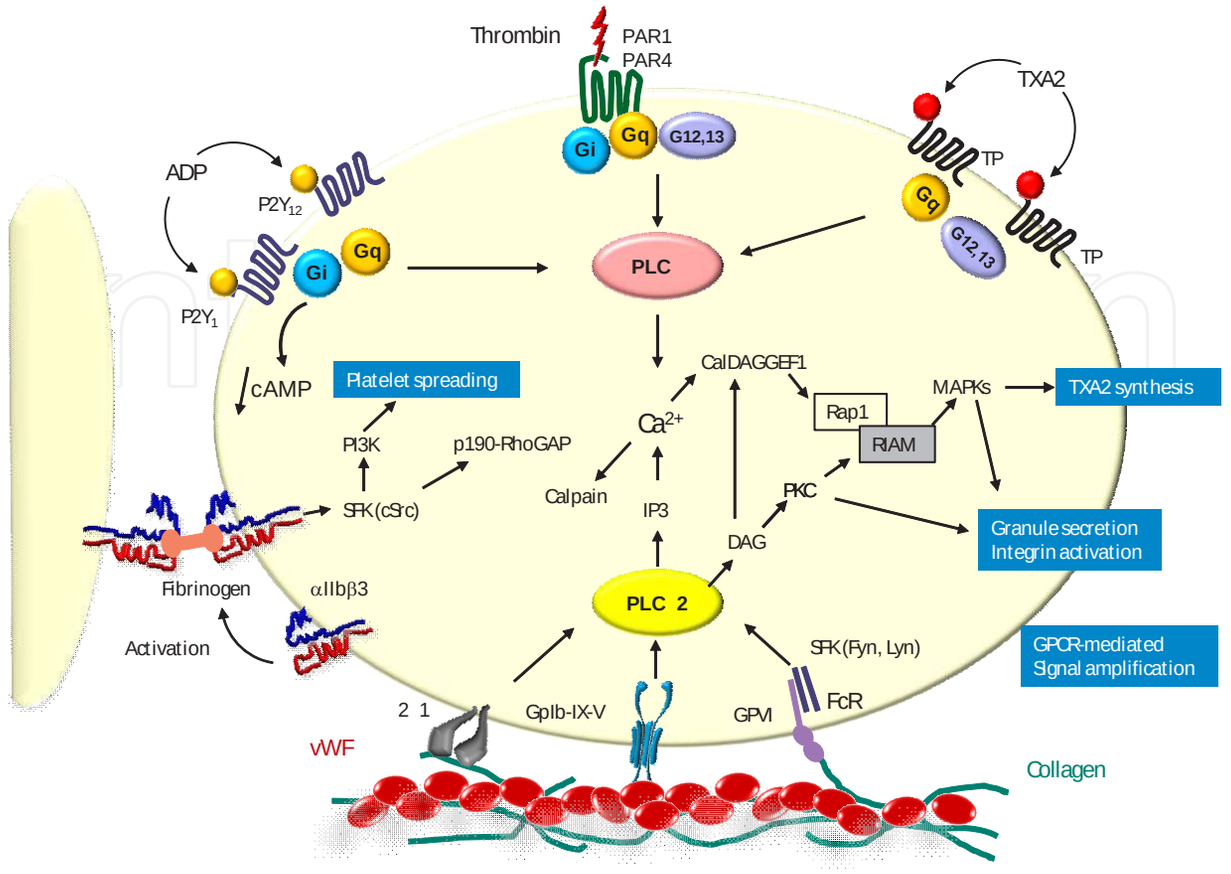

Figure 1. Platelet major signalling pathways. Collagen binding either to GPVI or $\alpha 2\beta 1$ integrin or vWF binding to Gp1b-IX-V leads to the activation of Src family kinases and tyrosine phosphorylation of the phospholipase C$\gamma 2$. PLC$\gamma 2$ cleaves phosphatidylinositol (4, 5)-bisphosphate PIP2 to generate inositol (1, 4, 5)-trisphosphate (IP3) and diacylglycerol (DAG). IP3 and DAG are responsible for the mobilization of calcium from intracellular stores leading to calpain activation and the activation of isoforms of protein kinase C (PKC), respectively. Ca^{2+} and DAG activate the Ca^{2+} and DAG-regulated guanosine exchange factor (CalDAGGEF) which in turn activate the small GTPase Rap1. The latter activates the mitogen-activated kinase (MAPK) which leads to thromboxane A2 generation, granule secretion and integrin activation. The released factors potentiate platelet signaling via the activation of G protein-coupled receptors/phospholipase Cβ pathway. The binding of fibrinogen to activated integrin initiates platelet spreading by activating the Phosphoinositide 3-kinase (PI3K) pathway.

Indeed, there is no evidence of the expression of insulin receptor on platelets [9] and it is speculated that the effects are rather mediated by the activation of insulin-like growth factor (IGF) receptor [10, 11]. Insulin decreases thrombin-induced increase in Ca^{2+} and attenuates agonist-induced platelet aggregation [12]. One of the mechanisms described to mediate the anti-platelet effect of insulin is the activation of the AMP-activated protein kinase (AMPK) and Akt by the PI3K [13]. Insulin can also inhibit Ca^{2+} mobilization by activating the inhibitory G-protein Gi [14]. However, given that insulin can also stimulate the release of ADP [15], it is assumed that whether insulin activates or inhibits platelets may depend on its concentration. Although the insulin effect in platelets has been initially believed to involve the activation of nitric oxide synthase (NOS), there are contradictory reports on the expression and function of NOS in human platelets [13, 16]. Insulin resistance refers to the loss of response to insulin stimulation. The molecular mechanisms of insulin resistance are complex but it has been

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shown that the faculty of insulin to inhibit platelet activation is lost in diabetic patients [17] and the inhibitory effect of insulin on the interaction of platelet with collagen and other agonists is blunted by insulin resistance in obese subjects [18]. Moreover, in non diabetic, obese women, there is a direct correlation between platelet reactivity assessed by thromboxane A2 generation and insulin resistance [19].

4. Effect of hyperglycemia and AGEs

Despite the fact that diabetes is characterized by chronic hyperglycemia, there is evidence that acute hyperglycemia can directly affect platelet reactivity. Indeed, a prospective randomised double blind controlled study has shown that 24h euglycaemic treatment significantly increased the plasma levels of the platelet and endothelial activation markers soluble P-selectin and vWF [20]. Moreover, it has been shown that challenging healthy non-diabetic subjects with 24h hyperglycemia-hyperinsulinemia altered the insulin signaling pathway [21]. Indeed, the glycogen synthase kinase 3β (GSKβ3) and the tyrosine phosphatase SHP2 as well as tissue factor were upregulated on mRNA and protein levels while mRNA for the syntaxin 4-binding protein was downregulated. High glucose can also increase calcium influx in platelets by enhancing a PI3K-dependent transient receptor potential channel canonical type 6 [22]. The latter is known to be significantly highly expressed in diabetic platelets. Hyperglycemia is able to increase the expression and/or activity of PKC [23, 24], a central kinase in the regulation of platelet activity. Another consequence of hyperglycemia in platelets is the induction of mitochondrial dysfunction. One of the recent mechanisms linking hyperglycemia and mitochondrial dysfunction is the activation of aldose reductase and subsequent ROS production which result to p53 phosphorylation [25] or the stimulation of the PLCγ2/PKC/p38α MAPK pathway and the increase in TXA2 production [26]. More recent findings showed that the hyperglycemia-induced platelet activation could be attributed to the downregulation of different micro-RNA (miR) such as miR-223 and miR-146a. Indeed, low platelet and plasma miR-223 and miR-146a expression has been associated with an increased risk for ischemic stroke in patients with diabetes mellitus [27]. Many of the deleterious effects of glucose have been attributed to its metabolite methylgyloxal (MG), a highly reactive dicarbonyl metabolite that is generated endogenously by the nonenzymatic degradation of the glycolytic intermediates, dihydroxyacetone phosphate and glyceraldehyde-3-phosphate [28]. It is known that plasma levels of MG in diabetic individuals are enhanced. Study investigating the effect of MG on platelets showed that acute application of MG to platelets increases intracellular Ca^{2+} levels and activates classical PKCs at the same time as inhibiting PI3K/Akt and the β3-integrin outside-in signalling. Moreover, *in vivo*, MG increases thrombus size but reduces its stability in mice [29]. Although most of the effects of MG have been attributed to the formation of advanced glycation end-products (AGEs) and the subsequent activation of the AGE receptor; RAGE, this study has highlighted a direct effect of MG on platelets which may contribute to the diabetes-associated platelet hyperaggregability. Several studies have shown that AGEs can activate platelets and that platelets express RAGE. The expression of surface markers such as P-selectin (CD62) and CD63 (a lysosomal glycoprotein) has been shown to be significantly

increased by AGE stimulation [30] suggesting AGE-induced platelet degranulation. However, this study failed to report the detailed intracellular mechanism involved. Studies performed in mice could show that AGEs induce a prothrombotic phenotype via interaction with platelet glycoprotein IV (CD36) [31]. The serum- and glucocorticoid-inducible kinase 1 (SGK1) has been also suggested to mediate AGE-induced platelet hyperactivation [32]. Indeed, SGK1 expression is known to be regulated by hyperglycaemia and AGEs. In platelets, SGK1 increases store-operated calcium entry (SOCE) and thereby regulates several Ca^{2+}-dependent platelet functions such as degranulation, integrin $\alpha_{IIb}\beta_3$ activation, phosphatidylserine exposure, aggregation and thrombus formation.

5. Effect of dyslipidemia

The fact that glycemic control alone has proven insufficient to reduce thrombotic complications in diabetic patients suggests that other factors may contribute to platelet hyperreactivity in diabetes [33]. One feature of diabetes is the presence of dyslipidemia which is characterized by high plasma triglyceride concentration, reduced high density lipoprotein (HDL) concentration, and increased concentration of low density lipoprotein (LDL)[34]. There is evidence that dyslipidemia contributes to the diabetes-associated platelet hyperactivation. By binding to a pertussis sensitive G-protein coupled receptor on platelets, LDL induces an increase in cytosolic $[Ca^{2+}]_i$, IP3 formation and activation of PKC [35]. However, the pro-thrombotic properties of LDL seem to be rather associated to its oxidation. Indeed, oxidized-LDL can directly interact with platelets specific receptors such as the lectin-like oxidized LDL receptor-1 [36] or the CD36 [37, 38]. The latter involves the activation of the MAPK c-Jun N-terminal kinase (JNK)2 and its upstream activator MKK4. Not only are platelets activated by ox-LDL but activated platelets are also known to be able to form ox-LDL via platelet gp91phox (NOX2)-dependent ROS generation [39] suggesting the contribution of platelets to circulating ox-LDL. The formed ox-LDL has been demonstrated to be either uptaken by monocytes [39] or amplify platelet activation [40]. On the molecular levels, LDL activates the platelet arachidonic acid signalling cascade, i.e phosphorylation of p38 MAPK and cytosolic phospholipase A2, leading to increased TXA2 formation [41]. Interestingly, there is less information on the direct effects of triglycerides on platelets. However, the link between hyperlipidemia and platelet hyperactivation is supported by the fact that lipid-lowering agents possess anti-thrombotic properties [42, 43].

6. Dysregulation of calcium signalling

One of the characteristics of platelets from patients with type 2 diabetes is the alteration of the intracellular Ca^{2+} homeostasis. Different mechanisms have been reported to be responsible for this abnormality. One of the mechanisms leading to the latter phenomenon is the reduction in platelet Ca^{2+}-ATPase activity in diabetic subjects [44, 45]. Moreover, the function of the Na^+/Ca^{2+} exchanger is known to be significantly altered in platelets from diabetic

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patients [46]. Another mechanism contributing to the enhanced resting cytosolic calcium in platelets from diabetic patients is the increased passive Ca^{2+} leakage rate from the intracellular stores [47]. Given that most of the intracellular signalling in platelets is regulated by calcium, it is more than expected that a dysregulation in calcium homeostasis would affect platelet function in many ways. One of the consequences of the increased $[Ca^{2+}]_i$ in platelet is the activation of calpains. Although calpain-mediated proteolysis is involved in physiological platelet activation, type 2 diabetes has been shown to be associated with the overactivation of calpain in platelets [45] leading to marked changes in the platelet proteome [48]. In platelets from diabetic patients, the integrin-linked kinase and septin-5 were found to be new calpain substrates and their cleavage was shown to be involved in the enhanced platelet adhesion and spreading as well as enhanced α-granule secretion, respectively. Moreover, calpain was able to cleave the chemokine RANTES into a variant with an enhanced chemotactic activity. The *in vivo* relevance of calpain in inducing the hyperreactivity of platelets from diabetic patients was supported by the finding that treatment of diabetic mice with calpain inhibitor preserved the platelet proteome, and reversed the diabetes-associated platelet hyperactivation [48].

7. Increased apoptosis

Although being anucleated, there is evidence demonstrating that platelets possess the necessary machinery to undergo apoptosis [49]. Among other mechanisms, calpain seems to play an important role in platelet apoptosis [50, 51]. Indeed, although caspase is activated during platelet apoptosis, this seems to be downstream of calpain activation. The increased calpain activation in platelets from diabetic patients described above suggests that diabetic platelets may be more prone to apoptosis. Several factors have been reported to induce platelet apoptosis including the diabetes-associated oxidative stress which is an important stimulus for inducing mitochondrial damage [52]. Mitochondria not only are the target of oxidative stress but are also able to generate ROS therefore amplifying the reaction to oxidative stress [52, 53]. Platelets from patients with type 2 diabetes demonstrate an increased ATP content but decreased mitochondrial membrane potential [54] supporting the alteration of mitochondrial function. Another mechanism involved in platelet apoptosis is the development of endoplasmic reticulum (ER) stress. It has been shown that diabetes mellitus was associated with the production of hyperreactive platelets expressing an altered protein disulfide isomerase and 78-kDa glucose-regulated protein [55]. Moreover, homocysteine, which levels are known to be significantly increased in diabetic patients, has been shown to stimulate ER stress-mediated platelet apoptosis by activating the caspase pathway [56]. One of the consequences of platelet activation and apoptosis is the generation of intact membrane vesicles known as microparticles. The formation of platelet-derived microparticles (PMPs) is known to be Ca^{2+}- and calpain-dependent. Although PMPs are involved in hemostasis due to their procoagulant properties, elevated levels of PMPs in blood from diabetic patients has been suggested to participate in the increased vascular complications in diabetes [57, 58].

8. Increased mean platelet volume

Platelet reactivity and size have been shown to directly correlate. Indeed, young and large platelets exhibit higher activity than old and small ones. The mean platelet volume (MPV) is an indicator of the average size of platelets which has been largely used to investigate the relationship between platelet size and activity. There is evidence that MPV is significantly increased in diabetic patients and that it directly correlates with glycemic control [59-61].

9. Hyporesponsiveness to anti-platelet therapy

One feature of platelets from diabetic patients is their hyporesponsiveness to anti-platelet therapy. Indeed, there is evidence that anti-platelet therapy is less effective in diabetic patients when compared with patients without diabetes [62]. One example is the so-called "aspirin resistance" in which diabetic patients are refractory to the anti-platelet effect of aspirin [63]. Aspirin or salicylic acid acetylates and irreversibly inhibits cyclooxygenase thereby inhibiting the TXA2 formation. Aspirin has been also shown to activate the NO/cGMP pathway. Although aspirin resistance is seen in the majority of diabetic patients, the exact molecular mechanism is still unclear. One of the mechanisms proposed to mediate aspirin-resistance is the increased glycation of platelet proteins which may alter the acetylation process [64]. Some *in vitro* studies have also shown a direct link between hyperglycemia and aspirin resistance. Certainly, high glucose can acutely reduce the antiaggregating effect of aspirin by inhibiting the aspirin-induced activation of the NO/cGMP/PKG pathway without affecting the aspirin-induced inhibition of TXA2 synthesis [65]. Given that acute stimulation of platelets with other monosaccharides such as fructose and galactose was shown to lead to a similar alteration of the aspirin effect on platelets and that lactic acid also impaired the inhibition of platelet aggregation with aspirin, it has been suggested that lactic acid might be the mediator of the glucose-induced inhibition of the aspirin effect in platelets [66]. Interestingly, the platelet resistance seems to be specific to aspirin since hyperglycemia-induced platelet hyperactivation in type 2 diabetes could be reversed by a nitric oxide-donating agent [67]. More recently, non-HDL cholesterol has been reported to be an independent risk factor for aspirin resistance in patients with type 2 diabetes [68].

Diabetes is also known to be associated to a reduced responsiveness of platelets to the P2Y12 ADP receptor antagonist clopidogrel [69, 70]. Although not directly investigated in diabetic patients, upregulation of ADP receptor levels, increase in ADP exposure or accelerated platelet turnover may contribute to clopidogrel resistance.

10. Conclusions

The fact that the diabetic milieu can affect platelet function in several ways explains the failure of glycemic control alone to reduce the risk of atherothrombotic events in diabetic patients.

Indeed, the increased platelet hyperreactivity is the result of complicated inter-regulated mechanisms. Moreover, given that diabetic platelets are resistant to most anti-platelet therapy, there is a need of new therapeutical strategies to improve platelet function in diabetes. Certainly, management of both glycemia and dyslipidemia would improve the effects of anti-platelet therapy. Moreover, the facts that calpain plays a key role in platelet activation and that calpain activity is elevated in diabetic platelets, makes it tempting to suggest the Ca^{2+}-activated proteases as a promising therapeutic target to prevent thrombotic complications in diabetic patients.

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