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Platelet Inhibition by Insulin Is Absent in Type 2 Diabetes Mellitus

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Objective—ADP-induced $P2y_{12}$ signaling is crucial for formation and stabilization of an arterial thrombus. We demonstrated recently in platelets from healthy subjects that insulin interferes with Ca^{2+} increases induced by ADP-$P2y_1$ contact through blockade of the G-protein G_i, and thereby with $P2y_{12}$-mediated suppression of cAMP.

Methods and Results—Here we show in patients with type 2 diabetes mellitus (DM2) that platelets have lost responsiveness to insulin leading to increased adhesion, aggregation, and procoagulant activity on contact with collagen. Using Ser^{473} phosphorylation of protein kinase B as output for insulin signaling, a 2-fold increase is found in insulin-stimulated normal platelets, but in DM platelets there is no significant response. In addition, DM2 platelets show increased $P2y_{12}$-mediated suppression of cAMP and decreased $P2y_{12}$ inhibition by the receptor antagonist AR-C69931MX.

Conclusion—The loss of responsiveness to insulin together with increased signaling through $P2y_{12}$ might explain the hyperactivity of platelets in patients with DM2. (***Arterioscler Thromb Vasc Biol.* 2006;26:417-422.**)

Key Words: $P2y_{12}$ receptor ■ Ca^{2+} regulation ■ clopidogrel ■ protein kinase B/Akt ■ IRS-1

Platelet activation leads to release of components that initiate formation of a thrombus and start inflammatory responses that contribute to atherosclerosis.[1] Signaling through the $P2y_{12}$ receptor is crucial for formation and stabilization of a thrombus.[2,3] Inhibition of the $P2y_{12}$ receptor reduces collagen-induced adhesion, aggregation and thrombin generation.[3,4] Subjects with a $P2y_{12}$ deficiency have a bleeding tendency[3,5] and individuals with an increased $P2y_{12}$ receptor copy number have platelets with an increased responsiveness to agonists, and these subjects experience peripheral arterial thrombosis.[6] The CAPRIE trial shows that long-term administration of the $P2y_{12}$ antagonist clopidogrel is more effective than aspirin in reducing the combined risk of ischemic stroke, myocardial infarction, or vascular death in subjects with a prothrombotic condition such as diabetes mellitus type 2 (DM2).[7] These findings illustrate the crucial role of $P2y_{12}$ signaling in platelet activation in vitro and in vivo.

The importance of $P2y_{12}$ signaling is explained by its capacity to initiate 2 pathways that directly interfere with platelet activating or inhibiting mechanisms. First, there is the activation of the G-protein subunit $G_i\alpha$, which inhibits adenylyl cyclase and thereby formation of the platelet inhibitor cAMP.[8] This property is particularly evident after treatment with prostacyclin,[9] and also in the absence of cAMP elevating agents, $P2y_{12}$ signaling controls cAMP production through adenylyl cyclase.[10,11] cAMP inhibits platelets through cAMP-dependent protein kinase (protein kinase A [PKA]),[12] which inhibits almost all platelet functions through blockade of multiple steps in platelet activation cascades including receptor activation, signaling through the mitogen-activated protein kinases pathway, formation of thromboxane A_2 (TxA_2), and the activation of key enzymes such as phospholipase C_β and protein kinase C (PKC).[13] Second, there is the release of the $G_i\beta\gamma$ dimer leading to the activation of protein kinase B (PKB/Akt), and integrin $\alpha_{IIb}\beta_3$ via type 1B phosphatidylinositol 3-kinase (PI3-K).[14] In animal models, type 1B PI3-K is crucial for platelet activation and its absence protects against thromboembolic vascular occlusion.[15] In human platelets, the role of type 1B PI3-K is less well understood, because although being activated by ADP-$P2y_{12}$ contact, it appears under negative control by ADP-$P2y_1$ binding and activation of Src and PKC.[10,16]

A prime example of abnormal platelet responsiveness is observed in patients with DM2, who are characterized by an impaired responsiveness to insulin or even complete insulin resistance. DM2 subjects have a 2- to 4-fold increased risk for cardiovascular disease and have both microvascular (nephropathy, retinopathy, neuropathy) and macrovascular (peripheral arterial disease) complications. DM2 subjects have

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platelets that show increased adhesion, aggregation, TxA_2 production, and P-selectin expression.[17] In general, DM2 subjects have marked insulin resistance, mostly explained by their obesity.[18] There is indirect evidence that the hyperactivity of their platelets may be caused by insulin resistance. In healthy individuals, platelets are inhibited by insulin leading to reduced Ca^{2+} mobilization and aggregate formation.[9,17] Interestingly, a euglycemic hyperinsulinemic clamp fails to trigger platelet inhibition in obese insulin-resistant subjects even in the absence of DM.[19] The hyperactivity is likely to have pathological consequences, because the increased adhesion and aggregation will accelerate the formation of a thrombus and enhance the procoagulant activity that helps to stabilize the thrombus.[20]

In the present study, we investigated whether platelet hyperactivity observed in DM2[21] is associated with a defect in $P2y_{12}$ signaling. We demonstrated recently in healthy subjects that insulin interferes with ADP- and thrombin-induced platelet functions through interference with the $P2y_{12}$-mediated regulation of G_i[9]. After receptor binding, insulin activates the insulin receptor substrate-1 (IRS-1) through tyrosine phosphorylation, which initiates association with $G_i\alpha$-subunit. The result is inhibition of $G_i\alpha$ activity and impaired suppression of adenylyl cyclase through $P2y_{12}$, introducing a phenotype that resembles platelets with a congenital $P2y_{12}$ defect or platelets from normal individuals who have been treated with the $P2y_{12}$ antagonist clopidogrel.[9] Here we demonstrate that platelet hyperactivity in DM2 is likely to be caused by a defect in the mechanisms through which insulin interferes with signaling by the $P2y_{12}$ receptor.

Materials and Methods

The full methods can be found in the online data supplement at http://atvb.ahajournals.org.

Results

Disturbed Ca^{2+} Homeostasis in DM2

In healthy subjects, the increase in cytosolic Ca^{2+}, $[Ca^{2+}]_i$, by release from internal stores that accompanies platelet activation is easily disturbed by factors that interfere with $P2y_{12}$ signaling such as insulin.[9] To detect possible abnormalities in this mechanism in DM2 subjects, Ca^{2+} regulation was measured in platelets from healthy subjects and from DM2 subjects (normal and DM2 platelets, respectively; please see http://atvb.ahajournals.org for physical and biochemical characteristics of study subjects in Table I) in the absence of extracellular Ca^{2+}. The mean $[Ca^{2+}]_i$ in unstimulated DM2 platelets (64.4±21.8 nmol/L) was ≈2-fold higher than in normal controls (27.2±5.9 nmol/L; $P<0.01$). After stimulation with collagen, peak $[Ca^{2+}]_i$ levels did not differ between the 2 groups (378.2±139.0 and 305.4±208.2 nmol/L, $P>0.05$), respectively, but the time to peak was shorter in DM2 platelets than normal platelets (80.7±18.4 and 103.6±13.5 seconds, $P<0.03$). In normal platelets, addition of insulin failed to change the basal $[Ca^{2+}]_i$ but led to a dose-dependent reduction of collagen-induced Ca^{2+} mobilization (expressed as 100%) to 66±11% at 100 nmol/L ($P<0.001$). This agrees with earlier findings in platelets stimulated with ADP and thrombin.[9] In contrast, inhibition by insulin was absent in DM2 platelets (Figure I, available online at http://atvb.ahajournals.org). These results indicate that DM2 platelets have a disturbed Ca^{2+} homeostasis that is unresponsive to inhibition by insulin.

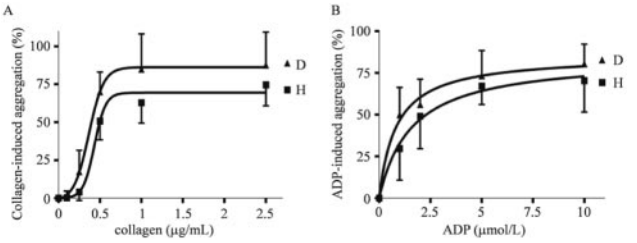

Figure 1. Increased responsiveness of DM2 platelets to aggregating agents. Aggregation of normal (H) and DM2 (D) PRP was initiated with 0.1 to 2.5 μg/mL collagen and 1 to 10 μmol/L ADP. The maximal aggregation was measured and data were fitted by nonlinear regression (H: $R^2=0.91$ and 0.79; DM2: $R^2=0.92$ and 0.86 for collagen and ADP, respectively). Means±SD; n=7 healthy and 7 DM2 subjects; (please see http://atvb.ahajournals.org for the detailed Materials and Methods).

Increased Responsiveness of DM2 Platelets to Aggregating Agents

Aggregation studies were performed to assess whether the disturbed Ca^{2+} homeostasis in DM2 platelets affected the responsiveness to collagen or ADP. Aggregation was initiated with 0.1 to 2.5 μg/mL collagen and 1 to 10 μmol/L ADP (Figure 1). Curves were fitted by nonlinear regression, which resulted in EC_{50} values for collagen-induced aggregation of 0.44 and 0.35 μg/mL for normal and DM2 platelets, respectively. For ADP-induced aggregation, these data were 1.52 and 0.79 μmol/L, respectively. The aggregation response to collagen and ADP was higher in DM2 platelets ($P<0.05$). These results indicate that the responsiveness of DM2 platelets is increased especially for ADP.

Absent Inhibition of Collagen- and ADP-Induced Platelet Aggregation by Insulin in DM2

Aggregation studies were performed to investigate whether the unresponsiveness to insulin observed in the regulation of Ca^{2+} had an effect on the role of insulin on platelet functions.[9,22] Platelets were treated with 1 nmol/L insulin and aggregation was initiated with collagen and ADP. In healthy subjects, 1 nmol/L insulin inhibited collagen- and ADP-induced aggregation to 76±11% and 75±8%, respectively ($P<0.05$). The inhibition by insulin was completely absent in platelets from DM2 subjects (Figure II, available online at http://atvb.ahajournals.org). Thus, DM2 platelets are unresponsive to insulin.

Inhibition of Collagen-Induced Platelet Deposition Under Flow by Insulin

Apart from the formation of aggregates, adhesion and generation of a pro-coagulant surface by exposure of phosphatidylserine (PS) are important steps in platelet deposition under flow. Earlier studies revealed an important role of $P2y_{12}$ signaling in these processes.[3] We determined platelet deposition and binding of annexin V-fluorescein isothiocyanate (FITC) to PS-exposing platelets after perfusion over a

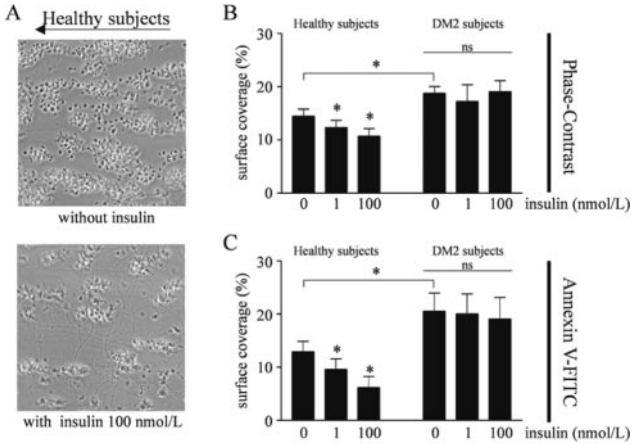

Figure 2. Inhibition of collagen-induced platelet deposition under flow by insulin. Blood was perfused at an intermediate shear rate (1000 s^{-1}) over a collagen-covered surface. A, Shown are 2 typical phase contrast images of normal blood perfused over collagen in the absence (upper panel) and presence (lower panel) of 100 nmol/L insulin. The arrow depicts the flow direction in the perfusion chamber. B, Surface coverage measured in phase contrast images. C, Surface coverage with annexin V-FITC staining platelets. Further details as in Figure 1.

collagen-coated surface at a shear rate of 1000 s^{-1}. Normal platelets rapidly adhered to collagen and formed aggregates (Figure 2A). The basal surface coverage by DM2 platelets (18.8±1.3%) was higher than by normal platelets (14.5±1.3%, $P<0.001$), which is in agreement with the hyperactivity of DM2 platelets observed in stirred suspensions (Figure 1; Figure I).[23] Insulin reduced surface coverage by normal platelets in a dose-dependent manner (10.7±1.4% at 100 nmol/L; $P<0.001$), whereas inhibition by insulin was absent in DM2 platelets (Figure 2A and 2B). Also, binding of annexin V-FITC in perfusates with DM2 platelets (20.5±3.4%) was higher than in controls (12.9±2.0%, $P<0.001$), probably as a result of the increased adhesion by DM2 platelets. In normal platelets, insulin reduced the binding of annexin V-FITC to 6.2±2.1% at 100 nmol/L ($P<0.001$), but in DM2 platelets no effect of insulin could be detected (Figure 2C). Together, these results indicate that DM2 platelets have an increased responsiveness to a collagen-coated surface under flow and that this property is insensitive to the presence of insulin.

Downstream Signaling of the Insulin Receptor/IRS-1 Complex

In adipocytes, ineffective insulin signaling or insulin resistance has been attributed to abnormalities in IRS-1 activation. To investigate whether the loss of insulin signaling to Ca^{2+} regulating mechanisms in DM platelets was accompanied by abnormal signaling initiated by the insulin receptor/IRS-1 complex, the phosphorylation of Ser^{473} on PKB was measured. Platelets were incubated with insulin for 15 minutes and samples were subjected to SDS-PAGE. In normal platelets, insulin increased the phosphorylation of PKB-Ser^{473} to 192.0±70.3% ($P<0.03$). DM2 platelets showed a complete lack of PKB phosphorylation. (Figure 3). Together with the absent interference with Ca^{2+}, probably reflecting impaired

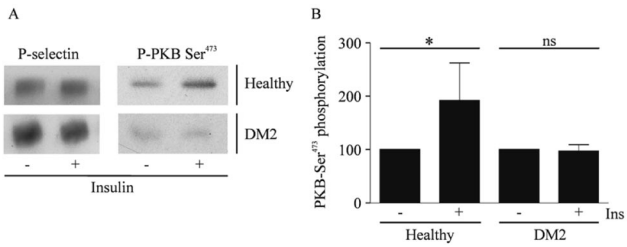

Figure 3. Downstream signaling of the insulin receptor/IRS-1 complex. Platelets were incubated with insulin for 15 minutes and subjected to SDS-PAGE. A, Blots were probed with P-PKB-Ser^{473}. B, The basal phosphorylation of individuals was expressed as 100%. Further details as in Figure 1.

signaling to $G_i\alpha_2$, the absent activation of PKB suggests that a common step in the insulin signaling machinery is affected.

Insulin Inhibits Platelet Activation by Collagen by Interfering With $P2y_{12}$ Signaling to cAMP-Dependent PKA

In platelets from healthy subjects, insulin interferes with the $P2y_{12}$-mediated suppression of cAMP formation, thereby attenuating Ca^{2+} increases and reducing aggregation induced by ADP and thrombin.[9] As shown in the present study, a similar inhibition is observed on stimulation by collagen. H89 is a specific inhibitor of PKA.[24] Pretreatment with H89 fully abolished the inhibition by insulin, confirming that insulin reduced platelet aggregation via interference with the cAMP-dependent activation of PKA. To address the question whether insulin alone changed the level of cAMP in the absence of activators or inhibitors of adenylyl cyclase, resting platelets were incubated with different concentrations of insulin followed by analysis of the phosphorylation of vasodilator-stimulated phosphoprotein (VASP), a major substrate of cAMP-dependent PKA.[13] No phosphorylated VASP could be detected in platelets treated with insulin. In contrast, addition of PGI_2 that through the IP receptor and G_s activates adenylyl cyclase induced a rapid increase in phosphorylated VASP (Figure III, available online at http://atvb.ahajournals.org). Thus, although it interferes with the regulation of cAMP and PKA, insulin is unable to change these signaling molecules in the absence of platelet agonists and antagonists.

Increased $P2y_{12}$ Signaling in DM2 Platelets

Because in normal platelets insulin interferes with platelet functions through inhibition of $P2y_{12}$ signaling,[9] the unresponsiveness to insulin in DM2 platelets might be caused by disturbances in signal transduction from $P2y_{12}$ to cAMP. To address this issue, platelets were incubated with the stable PGI_2 analog iloprost to raise cAMP and thereafter treated with increasing concentrations ADP to induce different extents of $P2y_{12}$ signaling. Basal cAMP levels did not differ between normal and DM2 platelets. In normal platelets, ADP dose-dependently reduced iloprost-induced cAMP (expressed as 100%) to 68.1±8.5% ($P<0.001$) at 10 μmol/L ADP. Addition of insulin partially reversed the effect of ADP in normal platelets leading to a reduction of cAMP to 85.1±12.7% ($P<0.02$). The decline of iloprost-induced cAMP was significantly steeper in DM2 platelets showing

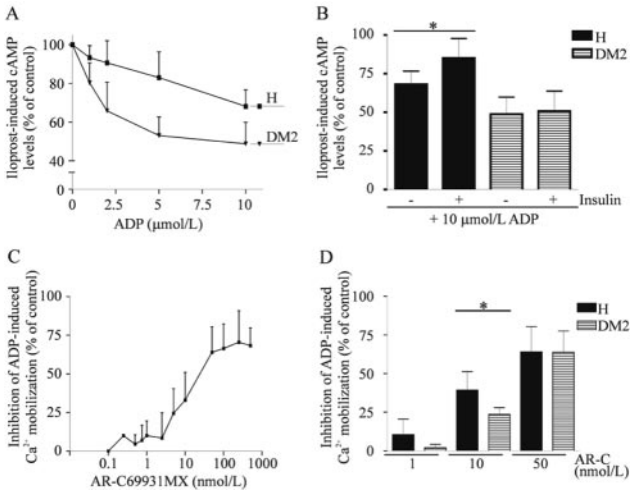

Figure 4. Increased $P2y_{12}$-signaling in DM2. Platelets were stimulated with 1 to 10 μmol/L (A) or 10 μmol/L (B) ADP in the presence or absence of iloprost. Iloprost-induced cAMP accumulation was expressed as 100%. C, Platelets were incubated with 0.1 to 500 nmol/L AR-C69931MX, and Ca^{2+} mobilization was induced by 10 μmol/L ADP. D, Platelets were incubated with 1 to 50 nmol/L AR-C69931MX, and stimulated with 10 μmol/L ADP. Further details as in Figure 1.

reduction to 48.8±11.1% ($P<0.001$) at the same ADP concentration (Figure 4A), which was unresponsive to insulin (Figure 4B). These results indicate that $P2y_{12}$ signaling is increased in DM2 platelets and not affected by the presence of insulin.

To investigate whether this abnormality affected the sensitivity to the $P2y_{12}$ receptor antagonist AR-C69931MX, dose-inhibition studies were performed in normal platelets. To this end, Ca^{2+} mobilization was induced by ADP-$P2y_1$ contact and different concentrations AR-C69931MX were added to interfere with the $P2y_{12}$-mediated support of $[Ca^{2+}]_i$ increases (Figure 4C). Optimal inhibition was observed at ≈100 nmol/L AR-C69931MX, at which point the inhibition amounted to ≈70%. Using 2 suboptimal concentrations of AR-C69931MX that induced ≈50% and 25% inhibition, studies were repeated in DM2 platelets. At 50 nmol/L AR-C69931MX normal and DM2 platelets showed a similar inhibition of ADP-$P2y_1$ Ca^{2+} mobilization, but at 10 nmol/L AR-C69931MX the inhibition in DM2 platelets was significantly lower than in normal controls (23.6±4.3% and 39.3±11.9% respectively; $P<0.05$; Figure 4D). These findings suggest that in DM2 platelets the ADP-$P2y_{12}$-mediated support of ADP-$P2y_1$ induced Ca^{2+} increases has a decreased sensitivity to the $P2y_{12}$ receptor antagonist AR-C69931MX.

Discussion

The present data show that platelets from DM2 subjects have lost their responsiveness to insulin and show increased responsiveness to ADP, which supports platelet activation by many agonists.[8] In the presence of insulin, DM2 platelets show increased collagen-induced Ca^{2+} mobilization. Collagen- and ADP-induced aggregation are slightly higher than controls and DM2 platelets show increased adhesion to surface-coated collagen under flow with a concomitant increase in PS exposure. In normal platelets, inhibition of ADP-induced aggregation by insulin is abolished by the PKA inhibitor H89. This is in line with the concept that insulin interferes with $P2y_{12}$-induced suppression of adenylyl cyclase and cAMP formation through tyrosine phosphorylation of $G_i\alpha_2$, resulting in its inhibition. In DM2 platelets, $P2y_{12}$ signaling is present and functional, but the pathway appears to be upregulated and less sensitive to $P2y_{12}$ inhibition. These findings indicate that insulin interferes with platelet activation by collagen through the same mechanism as in platelets stimulated with ADP and thrombin.[9] They also indicate that the cause for the loss of insulin sensitivity in platelets from DM2 subjects must be sought in a defect in a pathway that triggers the inhibition of $G_i\alpha_2$.

A human platelet contains ≈570 insulin receptors.[25] After receptor activation, IRS-1 is recruited and tyrosine phosphorylated leading to activation of pathways involving PKB and p38 mitogen-activated protein kinases in addition to inhibition of $G_i\alpha_2$[9,18,26]. Possibilities for abnormal insulin signaling in DM2 platelets are defects in the insulin receptor β-subunit, IRS-1, and the tyrosine phosphorylation of $G_i\alpha_2$ together with the different tyrosine kinases and phosphatases that control these processes.[9,18,27,28] Defects in the insulin receptor are associated with severe abnormalities such as growth disorders, lipodystrophy, and acanthosis nigrans, which were absent in the DM2 study group. A more likely explanation for the loss of insulin sensitivity in DM2 platelets is a defect in IRS-1. In transfected cells, the IRS-1 gene G972R variant causes impaired activation of PI3-K and PKB.[29] The same mutation is found in obese patients with DM2,[30] where it is associated with an increased risk of cardiovascular disease.[31] However, the gene variant is also found in a healthy individuals, indicating that it is not the decisive factor that makes individuals diabetic.[32] Analysis of IRS-1 and $G_i\alpha_2$ in the DM2 platelets stimulated with 100 nmol/L insulin revealed reduced and often absent tyrosine phosphorylation of these intermediates. Unfortunately, the normal controls also showed varying levels of tyrosine phosphorylation, which made it difficult to identify a precise block in insulin signaling. Instead, analysis of PKB activation consistently showed strongly impaired Ser^{473} phosphorylation in DM2 platelet but not in the controls. Thus, loss of insulin signaling to $G_i\alpha_2$ and Ca^{2+} is accompanied with loss of signaling to PKB. A likely cause is a defect in IRS-1, which is an upstream regulator of both pathways. The nature of the defect in IRS-1 regulation remains to be elucidated but apparently it is has a great effect on $G_i\alpha$. Gain- and loss-of- function defects of $G_i\alpha_2$ have been described in bipolar affective and bleeding disorders,[3,33] whereas defects in tyrosine protein phosphorylation have been described in congenital thrombocytopenia, Wiscott-Aldrich syndrome, and the Scott syndrome.[34–36]

In agreement with earlier studies in DM2 subjects,[23,37,38] we found an increased basal $[Ca^{2+}]_i$ in DM2 platelets before stimulation with collagen. Studies with thapsigargin, an inhibitor of sarco/endoplasmatic Ca^{2+}-ATPase (SERCA), made clear that the Ca^{2+} level is determined by a constant release from and re-uptake of Ca^{2+} by the endoplasmatic reticulum.[39] Apparently, in DM2 platelets these processes have reached a new steady-state with a 2-fold higher $[Ca^{2+}]_i$ than in normal platelets. This did not lead to a higher Ca^{2+}

response after collagen stimulation, but the response was faster and accompanied with increased aggregation. In addition, adhesion to collagen under flow was higher with DM2 platelets than with normal platelets suggesting that abnormalities in Ca^{2+} regulation made platelets more reactive toward a collagen-coated surface. At high shear platelet adhesion to collagen through interaction of glycoprotein (platelet glycoprotein [GP]) Ib with von Willebrand factor bound to collagen is followed by integrin $\alpha_2\beta_1$ mediated firm adhesion, which halts platelet rolling and allows collagen to interact with GPVI.[40,41] Signaling mediated via GPVI increases the binding affinity of $\alpha_2\beta_1$ to collagen and induces release of ADP.[42,43] Adhesion to collagen of platelets deficient of the $P2y_{12}$ receptor is >20% lower than with normal platelets, illustrating the potent enhancement by $P2y_{12}$ signaling through released ADP.[3] The binding of GPVI to collagen is independent of intracellular control indicating that changes in adhesion to collagen reflect modulation of $\alpha_2\beta_1$[42].

Another abnormality found in DM2 platelets is the increased signaling capacity of the $P2y_{12}$ pathway. DM2 platelets induced a steeper fall in cAMP in iloprost-treated platelets than their normal counterparts. A similar enhancement has been found in carriers of the $P2y_{12}$-H2 haplotype, which is characterized by an increased $P2y_{12}$ receptor number at the plasma membrane.[6] Carriers of the $P2y_{12}$-H2 haplotype are characterized by peripheral arterial disease due to the presence of hyper-responsive platelets that are less sensitive to pharmacological strategies that inhibit $P2y_{12}$ signaling, such as clopidogrel.[6] DM2 platelets showed a decreased responsiveness to a suboptimal concentration of the $P2y_{12}$ antagonist AR-C69931MX but at an optimal concentration the difference disappeared. Clopidogrel resistance has been described in a patient population with a general high risk for recurrent vascular events, although it was not specific for DM.[44] Our present findings might indicate that clopidogrel resistance can be overcome by applying higher doses of the drug.

Platelet hyperactivity in DM2 correlates with an increased risk of atherothrombotic complications.[45–48] In addition to hyperactive platelets, the coagulation mechanism shows abnormalities in DM2 subjects with elevated levels of coagulation factors factor VII, VIII, XI, and XII.[49] The enhanced adhesion of platelets in DM2 with a concomitant increase in PS exposure that facilitates the coagulation cascade might contribute to the hypercoagulable state observed in this disease.

Acknowledgments

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