Platelet Inhibition by Insulin Is Absent in Type 2 Diabetes Mellitus

Irlando Andrade Ferreira, Astrid I.M. Mocking, Marion A.H. Feijge, Gertie Gorter, Timon W. van Haeften, Johan W.M. Heemskerk, Jan-Willem N. Akkerman

Objective—ADP-induced P2Y12 signaling is crucial for formation and stabilization of an arterial thrombus. We demonstrated recently in platelets from healthy subjects that insulin interferes with Ca2+ increases induced by ADP-P2Y1, contact through blockade of the G-protein Gβγ, and thereby with P2Y12-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 Ser473 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 P2Y12-mediated suppression of cAMP and decreased P2Y12 inhibition by the receptor antagonist AR-C69931MX.

Conclusion—The loss of responsiveness to insulin together with increased signaling through P2Y12 might explain the hyperactivity of platelets in patients with DM2. (Arterioscler Thromb Vasc Biol. 2006;26:000-000.)

Key Words:

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 P2Y12 receptor is crucial for formation and stabilization of a thrombus.2,3 Inhibition of the P2Y12 receptor reduces collagen-induced adhesion, aggregation and thrombin generation.4,5 Subjects with a P2Y12 deficiency have a bleeding tendency5,5 and individuals with an increased P2Y12 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 P2Y12 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 P2Y12 signaling in platelet activation in vitro and in vivo.

The importance of P2Y12 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 Gαi, 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, P2Y12 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 A2 (TxA2),13 and the activation of key enzymes such as phospholipase Cβ and protein kinase C (PKC).13 Second, there is the release of the Gβγ dimer leading to the activation of protein kinase B (PKB/Akt) and integrin αIIbβ3 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.14 In human platelets, the role of type 1B PI3-K is less well understood, because although being activated by ADP-P2Y12 contact, it appears under negative control by ADP-P2Y1 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 (neuropathy, retinopathy, nephropathy) and macrovascular (peripheral arterial disease) complications. DM2 subjects have

Original received August 4, 2005; final version accepted November 17, 2005.

From the Laboratory for Thrombosis and Haemostasis (I.A.F., A.I.M.M., G.G., J.-W.N.A.), Department of Hematology, University Medical Center Utrecht and the Institute for Biomembranes (I.A.F., A.I.M.M., G.G., J.-W.N.A.), Utrecht University, the Netherlands; Department of Biochemistry and Human Biology (M.A.H.F., J.W.M.H.), University of Maastricht, the Netherlands; Department of Internal Medicine (T.W.v.H.), University Medical Center Utrecht, the Netherlands. Correspondence to Dr J.W.N. Akkerman, Thrombosis and Haemostasis, Department of Hematology, University Medical Center Utrecht, Heidelberglaan 100, 3584 CX Utrecht, the Netherlands. E-mail j.w.nakkerman@azu.nl

© 2005 American Heart Association, Inc.

Arterioscler Thromb Vasc Biol. is available at http://www.atvbaha.org

DOI: 10.1161/01.ATV.0000199519.37089.a0
platelets that show increased adhesion, aggregation, TxA₂ production, and P-selectin expression. In general, DM2 subjects have marked insulin resistance, mostly explained by their obesity. 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 Ca²⁺ mobilization and aggregate formation. Interestingly, a euglycemic hyperinsulinemic clamp fails to trigger platelet inhibition in obese insulin-resistant subjects even in the absence of DM. 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.

In the present study, we investigated whether platelet hyperactivity observed in DM2 is associated with a defect in P₂Y₁₂ signaling. We demonstrated recently in healthy subjects that insulin interferes with ADP- and thrombin-induced platelet functions through interference with the P₂Y₁₂-mediated regulation of Gᵢ₉. After receptor binding, insulin activates the insulin receptor substrate-1 (IRS-1) through tyrosine phosphorylation, which initiates association with Gα- subunit. The result is inhibition of Gᵢ₉ activity and impaired suppression of adenylyl cyclase through P₂Y₁₂, introducing a phenotype that resembles platelets with a congenital P₂Y₁₂ defect or platelets from normal individuals who have been treated with the P₂Y₁₂ antagonist clopidogrel.

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 P₂Y₁₂ receptor.

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

Results
Disturbed Ca²⁺ Homeostasis in DM2
In healthy subjects, the increase in cytosolic Ca²⁺, [Ca²⁺]ᵢ, by release from internal stores that accompanies platelet activation is easily disturbed by factors that interfere with P₂Y₁₂ signaling such as insulin. To detect possible abnormalities in this mechanism in DM2 subjects, Ca²⁺ regulation was measured in platelets from healthy subjects and from DM2 subjects (normal and DM2 platelets, respectively; please see http://atvb.ahajournals.org for the detailed materials and methods). Ca²⁺ levels were elevated in unstimulated DM2 platelets (14.4±2.18 μmol/L) compared to normal controls (27.2±5.9 μmol/L; P<0.01). After stimulation with collagen, peak [Ca²⁺]ᵢ was higher in normal platelets (27.2±5.9 μmol/L; P<0.01) than in DM2 platelets (19.3±3.4 μmol/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 [Ca²⁺]ᵢ, but led to a dose-dependent reduction of collagen-induced Ca²⁺ mobilization (expressed as 100%) to 66±11% at 100 μmol/L (P<0.001). This agrees with earlier findings in platelets stimulated with ADP and thrombin. 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 Ca²⁺ homeostasis that is unresponsive to inhibition by insulin.

Increased Responsiveness of DM2 Platelets to Aggregating Agents
Aggregation studies were performed to assess whether the disturbed Ca²⁺ 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. The maximal aggregation was measured and data were fitted by nonlinear regression (H; R²=0.91 and 0.79; DM2: R²=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).

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 Ca²⁺ had an effect on the role of insulin on platelet functions. 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 P₂Y₁₂ signaling in these processes. We determined platelet deposition and binding of annexin V-fluorescein isothiocyanate (FITC) to PS-exposing platelets after perfusion over a
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). 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 Ca²⁺ regulating mechanisms in DM platelets was accompanied by abnormal signaling initiated by the insulin receptor/IRS-1 complex, the phosphorylation of Ser473 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-Ser473 to 192.5±70.3% (\(P<0.03\)). DM2 platelets showed a complete lack of PKB phosphorylation. (Figure 3). Together with the absent interference with Ca²⁺, probably reflecting impaired signaling to G_\(_{i/o}\), 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 P2y₁₂ Signaling to cAMP-Dependent PKA**

In platelets from healthy subjects, insulin interferes with the P2Y₁₂-mediated suppression of cAMP formation, thereby attenuating Ca²⁺ increases and reducing aggregation induced by ADP and thrombin. As shown in the present study, a similar inhibition is observed on stimulation by collagen. H89 is a specific inhibitor of PKA. 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 adenylly 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. No phosphorylated VASP could be detected in platelets treated with insulin. In contrast, addition of PGI₂ that through the IP receptor and G stimulates cAMP formation, thereby disturbing signal transduction from P2y₁₂ to cAMP. To address this issue, platelets were incubated with the stable PGI₂ analog iloprost to raise cAMP and thereafter treated with ADP and thrombin. Because in normal platelets insulin interferes with platelet functions through inhibition of P2Y₁₂ signaling, the unresponsiveness to insulin in DM2 platelets might be caused by disturbances in signal transduction from P2Y₁₂ to cAMP. To address this issue, platelets were incubated with the stable PGI₂ analog iloprost to raise cAMP and thereafter treated with increasing concentrations ADP to induce different extents of P2Y₁₂ 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...
ADP-induced aggregation by insulin is abolished by the PKA inhibitor H89. This is in line with the concept that insulin interferes with P2Y₁₂-induced suppression of adenylyl cyclase and cAMP formation through tyrosine phosphorylation of G₁₃₂, resulting in its inhibition. In DM2 platelets, P2Y₁₂ signaling is present and functional, but the pathway appears to be upregulated and less sensitive to P2Y₁₂ inhibition. These findings indicate that insulin interferes with platelet activation by collagen through the same mechanism as in platelets stimulated with ADP and thrombin.³⁷ 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 G₁₃₂.

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 G₁₃₂.²⁹ Possibilities for abnormal insulin signaling in DM2 platelets are defects in the insulin receptor β-subunit, IRS-1, and the tyrosine phosphorylation of G₁₃₂ together with the different tyrosine kinases and phosphatases that control these processes.²⁹,³⁸,⁷⁷,⁷⁸ Defects in the insulin receptor are associated with severe abnormalities such as growth disorders, lipodystrophy, and acanthosis nigricans, 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 G₁₃₂ 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 Ser⁴⁷³ phosphorylation in DM2 platelet but not in the controls. Thus, loss of insulin signaling to G₁₃₂ and Ca²⁺ 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 G₁₃₂. Gain- and loss-of- function defects of G₁₃₂ have been described in bipolar affective and bleeding disorders,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 [Ca²⁺] in DM2 platelets before stimulation with collagen. Studies with thapsigargin, an inhibitor of sarco/endoplasmatic Ca²⁺-ATPase (SERCA), made clear that the Ca²⁺ level is determined by a constant release from and re-uptake of Ca²⁺ by the endoplasmatic reticulum.³⁹ Apparently, in DM2 platelets these processes have reached a new steady-state with a 2-fold higher [Ca²⁺], than in normal platelets. This did not lead to a higher Ca²⁺

**Figure 4.** Increased P2Y₁₂-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 Ca²⁺ 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.

**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 Ca²⁺ 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
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 \(\alpha_{IIb}\beta_3\) 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 \(\alpha_{IIb}\beta_3\) 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 \(\alpha_{IIb}\beta_3\).\(^{42}\)

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 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

The support of the Dutch Diabetes Research Foundation (1999.046; Amersfoort, the Netherlands) and the “Fonds voor het Hart” (The Hague, the Netherlands) is gratefully acknowledged. J.W.N.A. is supported by the Netherlands Thrombosis Foundation. We thank Dr S.J. Korporaal, M. van Lier, Dr E.J.P. de Koning, and J.P. van Wijk (UMC Utrecht, the Netherlands) for their assistance, and the volunteers for their help.

References


Platelet Inhibition by Insulin Is Absent in Type 2 Diabetes Mellitus
Irlando Andrade Ferreira, Astrid I.M. Mocking, Marion A.H. Feijge, Gertie Gorter, Timon W. van Haeften, Johan W.M. Heemskerk and Jan-Willem N. Akkerman

Arterioscler Thromb Vasc Biol. published online December 8, 2005;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/early/2005/12/08/01.ATV.0000199519.37089.a0.citation

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2005/12/12/01.ATV.0000199519.37089.a0.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org/subscriptions/
Online Supplement of:

Platelet Inhibition by Insulin is Absent in Type 2 Diabetes Mellitus

Irlando Andrade Ferreira¹, Astrid I. M. Mocking¹, Marion A. H. Feijge², Gertie Gorter¹, Timon W. van Haeften¹, Johan W. M. Heemskerk², and Jan-Willem N. Akkerman¹

From the ¹Laboratory for Thrombosis and Haemostasis, Department of Hematology, University Medical Center Utrecht, and the ¹Institute for Biomembranes, Utrecht University, the Netherlands; ²Department of Biochemistry and Human Biology, University of Maastricht, the Netherlands; ³Department of Internal Medicine, University Medical Center Utrecht, the Netherlands

Running title: Impaired insulin signaling in DM2 platelets

Corresponding author:
Prof. Dr. J. W. N. Akkerman Thrombosis and Haemostasis, Department of Hematology, University Medical Center Utrecht, Heidelberglaan 100, 3584 CX Utrecht, the Netherlands, phone: +31 30 250 6512, fax: +31 30 251 1893, e-mail: j.w.n.akkerman@azu.nl

Word Count:
Abstract 150
Condensed abstract 48
Total word count MS 4949
Online text word count 2050
**Materials and Methods**

**Subjects**

The healthy \((n=14)\) and DM2 subjects, diagnosed according to the guidelines of the Expert Committee on the diagnosis and classification of DM \(^{21}\), without clinical signs of peripheral arterial disease \((n=14)\) recruited for the present study did not take any platelet-inhibiting medication 10 days prior to blood-collection. All DM2 subjects were treated with oral glucose lowering agents (biguanides or sulfonylurea derivatives). The physical and biochemical characteristics are available in the online supplement Table 1. The DM2 subjects had a higher BMI and systolic blood pressure than healthy subjects. The biochemical characteristics did not differ between the two study groups with the exception of fasting serum insulin, glucose and serum creatinine. The degree of insulin resistance was estimated by homeostasis model assessment (HOMA), which is an independent marker of CVD in DM2, by the following formula: fasting plasma glucose (mmol/L) times fasting plasma insulin (mU/L) divided by 22.5\(^{1}\). Low HOMA values represent a high insulin efficiency, whereas high values represent insulin inefficiency or insulin resistance. HOMA was increased 3-fold in DM2 subjects, thus reflecting the insulin resistant state in these subjects.

Exclusion criteria included a previous cardiovascular event, pregnancy, and the use of insulin, anti-epileptic drugs, acetyl salicylic acid or other non-steroidal anti-inflammatory drugs. Written informed consent was obtained after the purpose, characteristics, and potential risks of the experiments had been explained to the subjects. No sex-based or racial/ethnic-based differences were present. The study protocol was approved by the Medical Ethics Committee of the University Medical Center Utrecht (UMCU, the Netherlands) and the Academic Hospital Maastricht (the Netherlands). For logistic reasons the patients group was divided in a group for adhesion analysis under flow \((n=7, \text{University of Maastricht})\) and a group for the other measurements \((n=7, \text{University Medical Center Utrecht})\). The characteristics of the patient groups did not differ significantly.

**Materials**

We obtained collagen reagent Horm (collagen in short), as native collagen type I/III fibrils from equine tendons, from Nycomed Pharma (Munich, Germany); D-Phe-Pro-Arg-chloromethylketone (PPACK), heparin and the monoclonal phosphospecific vasodilator phosphoprotein (VASP P-Ser\(^{157}\)) antibody from Calbiochem (San Diego, CA, USA); prostacyclin (PGI\(_2\)) and iloprost (PGE\(_2\)) from Cayman Chemical (Ann Arbor, MI, USA); human recombinant insulin (solubilized according to the recommendations of the manufacturer in 10 mmol/L acetic acid, 100 mmol/L NaCl, and 0.01% BSA to reach the stock concentration of 100 µmol/L), adenosin-5'-
diphosphate (ADP) and Fura 2-AM from Sigma (St. Louis, MO, USA); FITC labeled annexin V from Nexus Research (Hoeven, the Netherlands); the isoquinolinesulfonamide compound H89 (N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide) from Alexis Biochemicals (Lausen, Switzerland); phosphospecific protein kinase B (P-PKB) Ser\(^{473}\) and P-selectin (C-20) antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, USA); goat anti-mouse horseradish peroxidase (HRP) (GAMPO), rabbit anti-goat HRP (RAGPO) from DAKO A/S (Glostrup, Denmark) and goat anti-rabbit HRP from Cell Signaling Technology Inc. (Beverly, MA, USA). The ADP receptor P2Y\(_{12}\) antagonist, the ATP analogue N\(^{6}\)-2-methylthioethyl)-2-(3,3,3-trifluoropropylthio)-β,γ-dichloromethylene ATP (AR-C69931MX) was a kind gift from Astra Zeneca (Loughborough, UK) and the PGI\(_2\) analog iloprost was kindly provided by Schering AG (Berlin, Germany). All other chemical reagents were of analytical grade.

### Preparation of Washed Platelets

Freshly drawn venous blood was collected into 0.1 volume of 130 mmol/L trisodium citrate. Citrated blood was centrifuged (150xg, 15 minutes, 20°C), and the platelet-rich plasma (PRP) was collected. The remaining blood was centrifuged (800xg, 10 minutes, 20°C) to obtain platelet-poor plasma (PPP; see below). PRP was supplemented with 0.1 vol of ACD (2.5% trisodium citrate, 1.5% citric acid, 2% D-glucose) and centrifuged (330xg, 15 minutes, 20°C). The supernatant was removed and the platelet pellet was resuspended in HEPES/Tyrode buffer (145 mmol/L NaCl, 5 mmol/L KCl, 0.5 mmol/L Na\(_2\)HPO\(_4\), 1 mmol/L MgSO\(_4\), 10 mmol/L HEPES, pH6.5) containing 5 mmol/L D-glucose. Prior to centrifugation (330xg, 15 minutes, 20°C), 0.001 vol of PGI\(_2\) (10 ng/mL, final concentration) was added. The platelet pellet was resuspended in HEPES/Tyrode buffer (pH7.25) containing 5 mmol/L D-glucose. The final platelet concentration was adjusted to 2.0x10\(^{11}\) cells/L. Prior to the experiments, platelets were kept at 20°C for 45 minutes to ensure a resting state. Where indicated, cells were incubated with 0.1-1000 nmol/L AR-C69931MX for 30 seconds, 10 µmol/L H89 for 10 minutes, 1-100 nmol/L insulin for 5 minutes, or 10 µg/L iloprost for 1 minute. These treatments were followed by stimulation with 2.5 µg/mL collagen or 10 µmol/L ADP unless stated otherwise.

### Measurement of Ca\(^{2+}\) Mobilization

PRP was prepared as described above and incubated with 3 µmol/L Fura 2-AM (45 minutes, 37°C, light-protected). Then PRP was supplemented with ACD, centrifuged again (330xg, 15 minutes, 20°C) and resuspended in HEPES/Tyrode buffer (pH7.25) containing 5 mmol/L D-glucose. The final platelet concentration
was adjusted to 2.0x10^{11} cells/L. Fura-2-fluorescence was recorded in 1.0 mL aliquots of stirred platelet suspension in the absence of extracellular Ca^{2+} at 20°C in a F-4500 fluorescence spectrophotometer (Hitachi Ltd., Tokyo, Japan) with excitation wavelengths of 340 and 380 nm and emission at 510 nm. Changes in Ca^{2+} levels were monitored using the Fura-2 fluorescence ratio and calibrated according to the method of Grynkiewicz et al.².

**Measurement of Platelet Aggregation**

PRP was prepared as described above and the platelet concentration was adjusted to 2.0x10^{11} cells/L with PPP. Aliquots of 0.5 mL were warmed to 37°C for 5 minutes, followed by stimulation with collagen of ADP at the indicated concentrations. Pretreatment of platelets with insulin was shortened by 2 minutes to account for the higher temperature of these experiments. Platelet aggregation was monitored continuously for 7 minutes at 900 rpm in an optical aggregometer (model 570 VS, Chrono-Log Corporation, Havertown, PA, USA).

**Platelet Adhesion under Flow Conditions**

Whole blood perfusion experiments were performed at 20°C as described previously³. In short, glass coverslips were coated with collagen (12.5 µg/cm²) and blocked with Hepes buffer pH7.45 containing 1% BSA. Venous blood was freshly drawn in 0.1 vol of PPACK (40 µmol/L). Where indicated, blood was preincubated for 1 minute with insulin and perfused for 4 minutes over the coverslip through a 50 µm deep chamber using a pulse-free pump at a shear rate of 1000 s⁻¹ after which flow chambers were rinsed at the same shear rate for 4 minutes with Hepes buffer pH 7.45 supplemented with 1U/mL heparin and 2 mmol/L CaCl₂. High resolution microscopic bright-field images were subsequently recorded in real-time with a Visitech digital imaging system (Sunderland, United Kingdom). Exposure of phosphatidylserine (PS) was detected postperfusion with a heparinized rinsing buffer containing Annexin V-FITC (0.5 µg/mL). Phase contrast and fluorescence microscopic images were captured for concurrent monitoring of two independent parameters of platelet deposition under flow, i.e. surface coverage of deposited platelets and procoagulant PS-expressing platelets stained with annexin V-FITC. Changes in surface coverage of platelets in the phase contrast and fluorescent images were obtained from at least 10 different collagen-containing microscopic fields chosen at random and analyzed off-line using ImagePro software (Media Cybernetics, Silver Spring, MD, USA) and Quanticell software (Visitech International, Sunderland, UK) respectively.
Immunoblotting

Washed platelets were incubated with insulin or PGI₂ and samples were collected in 3x Laemmli-based sample buffer. Aliquots were subjected to SDS PAGE on 10% gels. Proteins were transferred from the gel to nitrocellulose sheets and blocked in 5% PY-BSA/TBST 0.1%. Blots were divided into two parts at about 75 kD and probed separately with a primary antibody for phosphorylated VASP (50 kD) and P-Selectin (140 kD) as a control for lane loading. The proteins were detected by enhanced chemiluminescence with horseradish peroxidase labeled secondary antibodies (respectively GAMPO and RAGPO). The intensity of the bands was quantitated with ImageQuant-TL software (Amersham Biosciences, Uppsala, Sweden). For the measurement of PKB phosphorylation, washed platelets were incubated with 100 nmol/L insulin for 15 minutes at 22°C. Then, samples were fixed in 1% formaldehyde (15 minutes, 4°C), centrifuged (8000xg, 30 seconds) and resuspended in 1x Laemmli-based sample buffer. After SDS PAGE on 10% gels, proteins were transferred to nitrocellulose sheets, blocked in 5% PY-BSA/TBST 0.1% and probed separately with a primary antibody for P-PKB-Ser⁴⁷³ and P-selectin as a control for lane loading.

Measurement of cAMP levels

PRP was incubated at 22°C with 100 nmol/L insulin for 4 minutes, iloprost for 1 minute followed by ADP for 3 minutes as described earlier with modifications⁴. Samples were lysed in 0.33 vol of 7% perchloric acid followed by centrifugation (11000xg, 10 minutes, 4°C). cAMP levels, [cAMP], were determined in a cyclic AMP [³H] assay system (Amersham Pharmacia Biotech, Uppsala, Sweden).

Statistical Analysis

Statistical analysis was performed using one-way ANOVA with Tukey’s multiple comparisons test as post-test for repeated measurements. Results are expressed as means±SD of observations in 7 healthy and 7 DM2 subjects unless indicated otherwise. Differences were considered significant at P<0.05.
References Online Supplement


Online Supplement
Impaired insulin signaling in DM2 platelets

Figure Legends Online Supplements

Online supplement Figure I. Disturbed Ca$^{2+}$ Homeostasis in Diabetes Mellitus Type 2
Platelets were stimulated with collagen with or without insulin. (A) The curves represent typical collagen-induced Ca$^{2+}$ traces in platelets in the absence of insulin. (B) Basal [Ca$^{2+}$]$_i$ in resting platelets. (C) Time following collagen addition to reach a peak [Ca$^{2+}$]. (D) Inhibition of collagen-induced Ca$^{2+}$ mobilization by insulin. An asterisk indicates a significant difference between groups (Means±SD; n=7 healthy and 7 DM2 subjects; please see www.ahajournals.org for the detailed Materials and Methods in the Online Supplement).

Online Supplement Figure II. Absent Inhibition of Collagen- and ADP-induced Platelet Aggregation by Insulin in DM2
PRP was incubated with insulin and aggregation was initiated with collagen (A-C) or ADP (D-F). The curves are typical for the effect of insulin on aggregation observed in normal (A/D) and DM2 platelets (B/E). Further details as in Figure 1.

Online supplement Figure III. Inhibition of Collagen-induced Platelet Activation by Insulin and the interference by H89, an inhibitor of PKA.
(A) PRP was incubated with the PKA-inhibitor H89 and aggregation was initiated with collagen with or without insulin at 37°C. Further details as in Figure 2. (B) Platelets were incubated with insulin or PGI$_2$ and samples were subjected to SDS-PAGE and probed for the phosphorylation of VASP P-Ser$^{157}$ and P-selectin as a control for lane loading (n=3).
**Online supplement Table I. Baseline Characteristics of the Study Subjects**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Healthy subjects (n=14)</th>
<th>DM2 Subjects (n=14)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>39 ± 16</td>
<td>43 ± 13</td>
<td>0.43</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>22.8 ± 1.7</td>
<td>30.1 ± 4.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Systolic Blood Pressure (mm Hg)</td>
<td>126±15</td>
<td>145 ± 9</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Diastolic Blood Pressure (mm Hg)</td>
<td>81±5</td>
<td>85 ± 8</td>
<td>0.11</td>
</tr>
<tr>
<td>Fasting Serum Insulin (mU/L)</td>
<td>5.7 ± 1.8</td>
<td>9.8 ± 5.0</td>
<td>&lt;0.007</td>
</tr>
<tr>
<td>Fasting Serum Glucose (mmol/L)</td>
<td>4.6 ± 0.6</td>
<td>8.2 ± 2.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HOMA – Insulin Resistance Index</td>
<td>1.1 ± 0.2</td>
<td>3.2 ± 1.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Serum Cholesterol (mmol/L)</td>
<td>4.5 ± 0.5</td>
<td>4.8 ± 0.8</td>
<td>0.23</td>
</tr>
<tr>
<td>Serum Triglycerides (mmol/L)</td>
<td>1.5 ± 0.5</td>
<td>1.9 ± 0.7</td>
<td>0.06</td>
</tr>
<tr>
<td>Serum Creatinine (mmol/L)</td>
<td>93.2 ± 10.7</td>
<td>72.8 ± 7.1</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
Online Supplement
Impaired insulin signaling in DM2 platelets

Online Supplement Figure 1: Andrade Ferreira et al.
Online Supplement
Impaired insulin signaling in DM2 platelets

Online Supplement Figure II: I. Andrade Ferreira et al.
Online Supplement
Impaired insulin signaling in DM2 platelets

Online Supplement Figure III: I. Andrade Ferreira et al.