Online Supplement of:

Platelet Inhibition by Insulin is Absent in Type 2 Diabetes Mellitus

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Running title: Impaired insulin signaling in DM2 platelets

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**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 \( n=7 \), University of Maastricht) and a group for the other measurements \( n=7 \), 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 \( \mu \)mol/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) Ser473 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 P2Y12 antagonist, the ATP analogue N6-(2-methylthioethyl)-2-(3,3,3-trifluoropropylthio)-β,γ-dichloromethylene ATP (AR-C69931MX) was a kind gift from Astra Zeneca (Loughborough, UK) and the PGI2 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 Na2HPO4, 1 mmol/L MgSO4, 10 mmol/L HEPES, pH6.5) containing 5 mmol/L D-glucose. Prior to centrifugation (330xg, 15 minutes, 20°C), 0.001 vol of PGI2 (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 Ca2+ 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 previously.³ 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


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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\(^{2+}\)]\(_i\). (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>
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<tr>
<td>Systolic Blood Pressure (mm Hg)</td>
<td>126±15</td>
<td>145 ± 9</td>
<td>&lt; 0.0001</td>
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<tr>
<td>Diastolic Blood Pressure (mm Hg)</td>
<td>81±5</td>
<td>85 ± 8</td>
<td>0.11</td>
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<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>
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<tr>
<td>Serum Triglycerides (mmol/L)</td>
<td>1.5 ± 0.5</td>
<td>1.9 ± 0.7</td>
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<tr>
<td>Serum Creatinine (mmol/L)</td>
<td>93.2 ± 10.7</td>
<td>72.8 ± 7.1</td>
<td>&lt;0.001</td>
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</tbody>
</table>
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Online Supplement Figure 1: Andrade Ferreira et al.
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