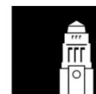
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Platelet function following induced hypoglycaemia in type 2 diabetes.

Short title: Hypoglycaemia induced platelet function in T2DM

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Abstract:

Aim. - Strict glycaemic control has been associated with an increased mortality rate in subjects with type 2 diabetes (T2DM). Here we examined platelet function immediately and 24 hours following induced hypoglycaemia in people with type 2 diabetes compared to healthy age-matched controls.

Methods. - Hyperinsulinaemic clamps reduced blood glucose to 2.8 mmol/L (50mg/dl) for 1 hour. Sampling at baseline; euglycaemia 5mmol/L (90mg/dl); hypoglycaemia; and at 24 post clamp were undertaken. Platelet function was measured by whole blood flow cytometry.

Results. - 10 subjects with T2DM and 8 controls were recruited. Platelets from people with T2DM showed reduced sensitivity to prostacyclin (PGI_2, 1nM) following hypoglycaemia. The ability of PGI_2 to inhibit platelet activation was significantly impaired at 24 hours compared to baseline in the T2DM group. Here, inhibition of fibrinogen binding was 29.5% (10.3 – 43.8) compared to 50.8% (36.8 – 61.1), ($P < 0.05$), while inhibition of P-selectin expression was 32% (16.1 – 47.6) vs. 54.4% (42.5 – 67.5) ($P < 0.05$). No significant changes in platelet function were noted in controls.

Conclusion. - Induced hypoglycaemia in T2DM enhances platelet hyperactivity through impaired sensitivity to prostacyclin at 24 hours.

Key words: Hypoglycaemia; Platelets; Type 2 diabetes.

Abbreviations: PGI_2 = prostacyclin,

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Introduction:

Strict glycaemic control is associated with increased risk of hypoglycaemia. Although, hypoglycaemia has traditionally been considered a complication of the treatment for type 1 diabetes, it has recently been recognised as a problem in people with type 2 diabetes (T2DM) particularly those on insulin therapy [1]. A lack of cardiovascular benefit with strict glycaemic control in recent studies in people with T2DM has raised questions about the role of hypoglycaemia in thrombogenesis [2-4].

Platelets circulate the blood in a quiescent state, maintained by fine balance between antithrombotic and prothrombotic molecules synthesised by endothelial cells [5], and play a key role in both acute thrombus formation at the site of vascular injury and atherogenesis [5]. Endothelial dysfunction, inflammation, oxidative stress, and metabolic alterations may cause platelets to be more activated [5]. Studies in people with type 1 diabetes and healthy controls report an increase in platelet activation with hypoglycaemia [6, 7]; through mechanisms including an elevation in the levels of the counter-regulatory hormone adrenaline [8, 9]. Activated platelets express P-selectin, released from α-granules, and bind to plasma molecules such as fibrinogen. These interactions promote the adhesion of leukocytes to the vessel wall and atherosclerosis [5]. Platelet surface expression of P-selectin and fibrinogen binding correlates with subsequent cardiac risk [10], and increases in those with cardiovascular disease [11]. Key to the inhibition of platelet activation is prostacyclin (PGI_2) and nitric oxide [5]. As most studies assessing the effects of hypoglycaemia on platelet function and thrombotic risk are conducted in people with type 1 diabetes and healthy controls [6-8], their findings may not necessarily be applicable to people with T2DM. In this study, we hypothesised that acute hypoglycaemia would result in increased platelet activation in T2DM compared to controls, with residual effects at 24 hours.

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Material and methods:

Study participants:

A case-control study was undertaken in people with T2DM and age-matched controls, recruited by advert. Study inclusion criteria included non-smoking men and women aged between 40 – 60 years. Subjects in the normal control group had no medical conditions and were not taking any medications. Subjects in the T2DM group had T2DM for less than 10 years and no history of microvascular disease (retinopathy, nephropathy, and neuropathy), HbA1C ≤ 9.5% (80.3 mmol/mol), and were treated with diet or metformin (at least 6-month duration). Exclusion criteria included pregnancy, lack of contraception in women of child bearing age, chronic medical conditions, use of anti-platelet medications, smoking, drop attacks, alcohol or drug abuse, psychiatric illness, or previous history of seizure. All subjects had an ECG to exclude overt ischaemia, and venesection to exclude anaemia, hyperlipidaemia, renal or hepatic impairment. Subjects in the control group were non-diabetic and had an oral glucose tolerance test to excluded diabetes or impaired glucose tolerance. Subjects with diabetes were examined to exclude diabetic neuropathy; urine testing to exclude microalbuminuria and retinal screening to exclude diabetic retinopathy were undertaken.

Glucose clamping was performed after a 10 hour fast and all participants were asked to avoid exercise, caffeine and alcohol for 24 hours prior to the clamp. Individuals with T2DM stopped their metformin two days prior to the visit. Following the clamp subjects were given lunch. Twenty-four hours after the clamp, and after a 10 hour fast, subjects attended for venesection and an early morning urine sample.

The study was approved by the Yorkshire and the Humber Research Ethics Committee and all study participants gave their signed informed consent.

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Hyperinsulinaemic euglycaemic-hypoglycaemic clamp studies:

Three polyethylene catheters were inserted in each antecubital fossa and back of the hand veins for blood sampling, insulin/dextrose infusions, and blood glucose measurements, respectively. The hand which had the catheter was constantly warmed at 60°C, using a heat box to arterialize the veins, and small blood volume withdrawn every 5 minutes from this catheter. The blood was analysed instantly for glucose measurement using HemoCue® Glucose 201+ (HemoCue AB, Angelholm, Sweden) to guide the rate of the dextrose infusion. The HemoCue's microcuvettes were stored and handled according to the manufacturer's protocol and the machine was calibrated before each session using manufacturer's control solutions. The HemoCue system correlates well with the Yellow Springs Instrument (YSI 2300 STAT), particularly in hypoglycaemia [12].

The insulin infusion rate was constant throughout the clamp at [60mU/body surface area (m^2)/min], while the rate of the 20% dextrose infusion was adjusted every 5 minutes to achieve the target blood glucose level. Body surface area (m^2) was calculated as [0.007184 x (height(cm)^0.725) x (weight(kg)^0.425)] [13]. The duration of the hyperinsulinaemic clamp was 4 hours and included 4 stages, each lasting 1 hour. In stage 1, the rate of glucose infusion was adjusted to achieve a stable blood glucose level of 5 mmol/L (90mg/dl). In stage 2 blood glucose was maintained at 5 mmol/L (90mg/dl) for 1 hour (euglycaemic clamp). In stage 3 blood glucose level was dropped gradually, over 1 hour, to 2.8 mmol/L (50mg/dl). In stage 4 blood glucose was maintained at 2.8 mmol/L (50mg/dl) for 1 hour (hypoglycaemic clamp).

Biochemical markers:

Urine samples were collected and aliquots stored at -20°C until batch analysis. Blood samples were separated immediately by centrifugation at 2000g for 15 minutes at 4°C, and the aliquots were stored at –80°C within 30 minutes of blood taking until batch analysis. Fasting plasma

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glucose (FPG) was measured using a Synchron LX 20 analyzer (Beckman-Coulter) using the manufacturer's recommended protocol. Total cholesterol, triglycerides, and high-density lipoprotein (HDL) cholesterol levels were measured enzymatically using a Synchron LX20 analyzer (Beckman-Coulter, High Wycombe, UK). Plasma metanephrine and normetanephrine were measured by tandem mass spectrometry. The between-run coefficients of variation (CV) for the metanephrine and normetanephrine measurements were 6.5–12.2% and 4.7–11.5%, respectively.

Platelet function:

Fluorescein isothiocyanate (FITC)-conjugated anti human CD42b, phycoerythrin (PE)-conjugated anti human CD62P, PE anti-human CD45, FITC-anti IgG_{1k}, and PE-anti IgG_{1k} isotope controls were obtained from BD bioscience (Oxford, UK). FITC-anti human fibrinogen was obtained from Dako (Stockport, UK). PGI_2 was obtained from Cayman (USA) and Adenosine 5′-diphosphate (ADP) from Sigma (Poole, UK).

Platelet function was analysed in whole blood by flow cytometry as described previously [14, 15]. Venous blood was collected without stasis from the antecubital vein into 3.8% sodium citrate using a 20-gauge intravenous cannula. The first 2ml of blood was discarded to avoid artifactual platelet activation. Samples were prepared within 5 minutes of blood collection.

For P-selectin expression and fibrinogen binding, 5μL of citrated blood was diluted in 50μL of modified Tyrode's buffer (150mM NaCl, 5mM HEPES [N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid], 0.55mM NaH2PO4, 7mM NaHCO3, 2.7mM KCl, 0.5mM MgCl2, 5.6mM glucose, pH 7.4) and mixed with 2μL of FITC anti-CD42b monoclonal antibody, FITC anti-fibrinogen polyclonal antibody, PE anti-P-selectin monoclonal antibody or IgG isotype control. The platelet population was identified by forward and side scatter characteristics and confirmed by expression of the platelet specific surface marker CD42b. Fibrinogen binding and P-selectin

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expression were measured in 10,000 platelet events. To study the sensitivity of platelets to activation, diluted blood was stimulated with ADP 0.1 – 10µM and samples were then fixed with 500µl of 0.2% paraformaldehyde after 10 minutes. In some experiments samples were incubated with PGI_2 0.1 – 10nM for 2 minutes prior to adding ADP 1µM followed by fixation with 0.2% formaldehyde after 10 minutes. Samples were analysed within 3 hours of fixation by flow cytometry. P-selectin expression and fibrinogen binding were expressed as the percentage of positive platelets above a predefined threshold, which was set at 2% on the appropriate negative controls as previously described [15].

Statistical analysis:

Baseline demographic data were summarised by the median and interquartile range for continuous data; percentages otherwise. Data were checked for normality using Kolmogorov-Smirnov test. Missing values were excluded on a case wise basis. An area under the curve was calculated [16]. Two broad statistical approaches were made (within cases; between T2DM cases and controls). Within group comparisons are as follows: changes from baseline at each stage (euglycaemia, hypoglycaemia and 24 hours) were compared using the paired t-test (or Wilcoxon signed-rank test for non-normally distributed data). Repeated measures were compared by analysis of variance (ANOVA) (or Friedman test for non-normally distributed data). Between groups' comparisons are as follows: for each group (T2DM and controls) a difference between baseline and nadir/peak was calculated. The between group differences were compared using the independent t-test (or Mann-Whitney U test for non-normally distributed data). A similar approach was made for the area under the curve. Correlations were evaluated using Pearson's coefficient (or Spearman's coefficient for non-normally distributed data). The sample size was too small to adjust for baseline covariates. No subgroup comparisons were planned. A two tailed P value of < 0.05 was considered statistically significant. ANOVA post-

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hoc comparisons were presented in two different ways: unadjusted [17] and Sidak test corrected for multiple comparisons. Statistical analysis was performed using the PASW statistics 19 package (SPSS Inc., Chicago, USA).

Results:

20 participants were screened, 18 recruited (10 people with T2DM, 8 controls). Concomitant use of antidepressant medication and an inability to understand the study protocol resulted in 2 screen failures. In the T2DM group, the median duration of diabetes was 10 months (5 – 24); seven participants (70%) were on metformin therapy, while three were diet controlled. Participants' demographics and baseline characteristics are summarised in Table I.

Blood glucose:

For the control group, target blood glucose achieved was 4.9 ± 0.2 mmol/L (87 ± 3mg/dl) during euglycaemia and 2.9 ± 0.1 (52 ± 2mg/dl) mmol/L during hypoglycaemia. For the T2DM group, target blood glucose achieved was 4.9 ± 0.2 mmol/L (87 ± 3mg/dl) and 3.0 ± 0.1 mmol/L (54 ± 2mg/dl) during euglycaemia and hypoglycaemia, respectively.

Platelet function:

Within each group, there was no significant change in unstimulated, or ADP (0.1 – 10μM) stimulated, platelet surface expression of P-selectin or fibrinogen binding during euglycaemia, hypoglycaemia or at 24 hours after the clamp (Table S1; see supplementary materials associated with this article on line).

In subjects with T2DM, platelet sensitivity to PGI_2 (1nM), measured as percentage inhibition in fibrinogen binding and P-selectin expression compared to ADP (1μM), was significantly lower

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at 24 hours 29.5% (10.3 – 43.8) compared to baseline 50.8% (36.8 – 61.1) for fibrinogen binding; and 32.0% (16.1 – 47.6) at 24 hours compared to 54.4% (42.5 – 67.5) at baseline for P-selectin expression (Table II). Although there was a trend in the T2DM group for a reduction in platelet sensitivity to PGI_2 0.1nM and 1nM at euglycaemia and hypoglycaemia compared to baseline, these changes were not statistically significant when multiple comparisons were adjusted for (Table II). In contrast, in the control group, platelet sensitivity to PGI_2 did not change acutely during the euglycaemic clamp, hypoglycaemic clamp or at 24 hours afterwards (Table II).

Platelets sensitivity to PGI_2 1nM, for both groups combined at baseline and 24 hours, negatively correlated with hsCRP levels: rho = -0.318, $P = 0.059$ for P-selectin expression, rho = -0.396, $P = 0.017$ for fibrinogen binding; and with isoprostane levels: r = -0.51, $P < 0.01$ for fibrinogen binding and r = -0.60, $P < 0.01$ for P-selectin expression.

Hormonal markers:

In both groups, plasma metanephrine significantly increased at hypoglycaemic and returned to baseline after 24 hours (Table III). There was no significant change in plasma normetanephrine during the study in either group.

Discussion:

In this study platelets sensitivity to PGI_2 was significantly impaired at 24 hours after induced hypoglycaemia in the T2DM group only. Impaired platelet function was due a dysfunction in the inhibitory pathway (sensitivity to PGI_2), while the stimulatory pathway (stimulation with ADP) remained unaffected, resulting in increased blood coagulability. These results are important as platelets play a key role in atherothrombosis [5] and hypoglycaemia is a common complication

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of strict glycaemic control and insulin therapy in people with T2DM [1]. In a large observational study in people with T2DM, strict glycaemic control, with a median HbA1C 6.4% (interquartile range 6.1 – 6.6), was associated with increased all-cause and cardiovascular mortality [18]. In the ACCORD study, strict glycaemic control in people with T2DM with significantly more episodes of severe hypoglycaemia was associated with increased mortality [19]; and although it was suggested that hypoglycaemia did not explain the increased mortality rate [20]; no cause for the increased deaths has been identified despite further analysis [20-23]. A recent joint position statement by the American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD) stated that “a glucose concentration < 54 mg/dL (3.0 mmol/L) is sufficiently low to indicate serious, clinically important hypoglycaemia” [24]. Hypoglycaemia may lead to vascular disease through mechanisms including sympatho-adrenal activation and catecholamine release, endothelial dysfunction, inflammation, oxidative stress, increased coagulation and platelet activation [25]. Our data suggest that an adverse cardiovascular effect of hypoglycaemia may persist well beyond the normalisation of blood glucose levels. The fact that the observed changes in platelet function in our experiment were only significant with 1nM of PGI_2 is expected, as the higher dose of PGI_2 (10nM) cause maximum inhibition masking small changes in platelet function, whilst lower dose of PGI_2 (0.1nM) caused minimal inhibition.

An increase in adrenaline levels with hypoglycaemia has been found to impair platelet function in previous studies [8, 26]; however, it is unlikely to fully explain the changes in platelet function observed in our study. While metanephrine levels significantly increased in both groups at hypoglycaemia in our study, platelets sensitivity to PGI_2 at 24 hours was only impaired in the T2DM group. Furthermore, any effects of metanephrine are likely to be also observed in the absence of PGI_2, as adrenaline is known to potentiate the activatory capacity of ADP [9]. However, we found that ADP reactivity was similar in all groups suggesting that the observed increase in metanephrine does not have a major influence on platelet function under our

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experimental conditions. Further studies are needed to examine the underlying mechanisms for impaired platelet function with hypoglycaemia in people with T2DM.

A few studies have looked into the effects of acute hypoglycaemia on platelet function using either insulin stress test [7] or hyperinsulinaemic hypoglycaemic clamps [6]. These studies were mainly performed in people with type 1 diabetes or healthy controls and showed increased platelet aggregation [7]; or increased plasma soluble P-selectin levels with acute hypoglycaemia [27, 28]. It is worth noting the plasma soluble P-selectin is an indirect measure of platelet function and it is also produced by the endothelial cells [29]. Our data are in accord with a study of platelet function in people with type 1 diabetes that showed an increase in platelet activation after acute hypoglycaemia with a peak at 24 hours, though their data did not reach significance [6]. A delayed effect of hypoglycaemia was suggested in a report of impaired autonomic function 16 hours after induced hypoglycaemia in healthy men and women [30]. The changes in platelet function observed at 24 hours in the T2DM group in our study could be related to an increase in inflammation in response to hypoglycaemia [31], or insulin resistance as insulin is thought to have an inhibitory effect on platelet activation [32]. The T2DM group in our study had a higher BMI and waist circumference compared to controls that may have contributed to their impaired platelet function, as obesity is associated with platelet dysfunction probably because of increased levels of insulin resistance, inflammation and oxidative stress [33].

The reduction in platelet sensitivity to PGI_2 at 24 hours in the T2DM group in our study could also, in theory, be related to insulin. Our data suggest that euglycaemia is associated with reduced platelet sensitivity to PGI_2 in T2DM but not controls. This is also evident in the P-selectin data, although since this is a less sensitive marker the data are not significant. To the best of our knowledge there is no documented evidence that hyperinsulinaemia alters platelet cyclic adenosine monophosphate (cAMP) response, and while platelets express the insulin receptor [34], little is known of the signalling events it is linked to. However, studies in people

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with type 1 diabetes and healthy controls have suggested that insulin infusion has either a neutral or even a favourable effect on platelet function [6, 35].

The strengths of this study include the inclusion of a group of people with T2DM, who were relatively treatment naïve and not on poly-pharmacy, and an age-matched healthy control group. We also examined different markers of platelet function using well-established methods. The main study limitation was the small study number. Another limitation is measuring platelet function at euglycaemia and hypoglycaemia in the same clamp study, rather than in two separate studies, making it difficult for us to exclude an effect for insulin clamp on platelet function. However, our study design mimics what happens in a 'real life' situation where euglycaemia is followed by hypoglycaemia. By comparing platelet function at each time point to the same baseline, it reduces the risk of data variability which could be a problem for studies with a small sample size [6].

In conclusion, induced hypoglycaemia in T2DM enhances blood coagulability through impaired platelet sensitivity to prostacyclin at 24 hours. This may lead to increased blood coagulability and so enhance the risk for cardiovascular events in this patient group.

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Legends

Table I. Study participants' demographics and baseline characteristics. Data presented as median (25th/75th centiles). Waist/hip, waist to hip ration; BP, blood pressure; HDL, high density lipoprotein; LDL, low density lipoprotein; Chol/HDL, cholesterol to HDL ratio; HbA1C, haemoglobin A1C. * $P < 0.05$.

Table II. Platelet sensitivity to prostacyclin. Whole blood samples were incubated with PGI_2 (0.1 to 10nM) before stimulation with ADP 1µM. Data presented as percentage inhibition of fibrinogen and P-selectin expression compared to ADP 1µM only samples (higher numbers indicate higher sensitivity to PGI_2). Data presented as median (25th/75th centiles). T2DM, type 2 diabetes mellitus; Fib, fibrinogen binding; PGI_2, PGI_2; %inhib, percent inhibition. Statistically significant changes are highlighted. *$P < 0.05$ compared to baseline (non-adjusted). ^ $P < 0.05$ compared to baseline (post-hoc comparisons Sidak test adjusted).

Table III. A comparison of hormonal markers during insulin clamp. Data presented as median (25th/75th centiles). T2DM, type 2 diabetes mellitus; All significant *P* values are highlighted. * $P < 0.05$ compared to any other time point (non-adjusted). ^ $P < 0.05$ compared to any other time point (post-hoc comparisons Sidak test adjusted). ^^ $P < 0.05$ compared to euglycaemia and 24 hours (post-hoc comparisons Sidak test adjusted).

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Author contributions: HK– contributed to study design, performed experiments, collected, analysed, and interpreted data and wrote manuscript; AA, BS, and KSW– performed experiments and contributed to the writing of manuscript; ASR– contributed to statistical analysis and the writing of manuscript; TS– contributed to the writing of manuscript; ESK, KMN and SLA– contributed to study design, data interpretation and the writing of the manuscript. All authors approved the final version of the manuscript.

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Appendix supplementary material

Supplementary materials (Table S1) associated with this article can be found at

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http://www.scincedirect.com at doi . . .

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Table I. Study participants' demographics and baseline characteristics.

	T2DM (n=10)	**Controls (n=8)**
Age (year)	47.0 (42.0 – 51.5)	47.5 (40.8 – 52.8)
Males (%)	8 (80%)	5 (62.5%)
Weight (kg)	103.1 (87.0 – 109.1)	85.5 (71.7 – 99.2)
BMI (kg/m^2)	35.8 (27.3 – 40.9)	28.2 (24.2 – 32.8)*
Waist circumference (cm)	117.0 (99.5 – 124.7)	91.0 (82.9 – 111.3)*

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(cm)		
Hips circumference (cm)	113.9 (105.8 – 128.2)	103.0 (99.5 – 113.7)
Waist/hip	0.99 (0.91 – 1.1)	0.90 (0.83 – 0.98)
Systolic BP (mmHg)	132 (111 – 142)	123 (116 – 132)
Diastolic BP (mmHg)	74 (68 – 85)	76 (69 – 82)
Cholesterol (mmol/L)	5.3 (4.4 – 5.7)	5.6 (4.2 – 5.7)
Triglycerides (mmol/L)	1.3 (0.98 – 2.2)	1.3 (0.78 – 1.5)
HDL (mmol/L)	1.2 (0.98 – 1.4)	1.3 (1.2 – 1.3)
LDL (mmol/L)	3.2 (2.8 – 3.7)	3.5 (2.7 – 3.7)
Chol/HDL	3.9 (3.6 – 5.8)	4.0 (3.4 – 4.3)
HbA1C (mmol/mol)	45.5 (39 – 56.3)	34 (31 – 36)*
HbA1C (%)	6.3 (5.7 – 7.3)	5.3 (5.0 – 5.4)*

Data presented as median (25th/75th centiles). Waist/hip, waist to hip ration; BP, blood pressure; HDL, high density lipoprotein; LDL, low density lipoprotein; Chol/HDL, cholesterol to HDL ratio; HbA1C, haemoglobin A1C. * $P < 0.05$.

Table II. Platelet sensitivity to prostacyclin.

	Baseline	**Euglycaemia**	**Hypoglycaemia**	**24 hours**
T2DM				
Fib PGI_2 10nM	93.9 (70.2 – 95.5)	89.0 (74.1 – 94.8)	89.1 (76.9 – 93.8)	88.7 (84.6 – 93.7)
Fib PGI_2 1nM	50.8 (36.8 – 61.1)	36.5 (12.1 – 51.8)*	29.1 (10.5 – 64.2)	29.5 (10.3 – 43.8)^
Fib PGI_2 0.1nM	12.5 (3.9 – 15.7)	3.2 (0 – 13.8)*	0.0 (0.0 – 12.9)	7.9 (1.6 – 13.3)
P-selectin PGI_2 10nM	95.5 (73.5 – 96.4)	90.2 (80.4 – 94.1)	90.7 (76.4 – 93.5)	91.3 (89.8 – 93.4)
P-selectin PGI_2 1nM	54.4 (42.5 – 67.5)	44.2 (16.5 – 60)	36.1 (13.9 – 64.5)	32.0 (16.1 – 47.6)^
P-selectin PGI_2 0.1nM	18.1 (10.1 – 22.4)	15.3 (0 – 31.7)	4.3 (0 – 19.1)	13.4 (0.9 – 17.4)

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Controls				
Fib PGI_2 10nM	94 (91.1 – 96)	95.7 (91.6 – 96.6)	92 (76 – 95.3)	95.3 (87.6 – 96.4)
Fib PGI_2 1nM	41.8 (19.6 – 80)	43.4 (33.1 – 78.8)	49.9 (31.8 – 56.4)	35.3 (6.1 – 54.5)
Fib PGI_2 0.1nM	8.2 (0.8 – 45.5)	11.6 (1.7 – 30.1)	15.9 (6.5 – 22.3)	5.8 (0.7 – 13.5)
P-selectin PGI_2 10nM	90.3 (74.8 – 97.2)	92.4 (83.4 – 93.8)	87.3 (72.7 – 96.3)	95.1 (71.9 – 97)
P-selectin PGI_2 1nM	51.7 (22.1 – 75.7)	46.7 (44.6 – 67.7)	48.4 (31.1 – 65.9)	47.8 (13.3 – 65.5)
***P*-selectin PGI_2 0.1nM**	13.9 (1.7 – 44.2)	15.4 (3.8 – 30.8)	21.3 (7.2 – 22.5)	14.6 (7.1 – 16.0)

Whole blood samples were incubated with PGI_2 (0.1 to 10nM) before stimulation with ADP 1µM. Data presented as percentage inhibition of fibrinogen and P-selectin expression compared to ADP 1µM only samples (higher numbers indicate higher sensitivity to PGI_2). Data presented as median (25th/75th centiles). T2DM, type 2 diabetes mellitus; Fib, fibrinogen binding; PGI_2, PGI_2; %inhib, percent inhibition. * $P < 0.05$ compared to baseline (non-adjusted). ^ $P < 0.05$ compared to baseline (post-hoc comparisons Sidak test adjusted).

Table III. A comparison of hormonal markers during insulin clamp.

	Baseline	Euglycaemia	Hypoglycaemia	24 hours	Comments
T2DM					
Metanephrine (80 – 510) pmol/L	86 (42 – 122)	121 (71 – 147)	284 (199 – 308)*^	101 (58 – 128)	ANOVA (*P*= 0.001)
Normetanephrine (120 – 1180) pmol/L	153 (127 – 223)	143.5 (81 – 262)	157 (128 – 282)	202 (133 – 232)	
Controls					
Metanephrine (80 – 510) pmol/L	153 (88 – 225)	150 (68 – 222)	415 (250 – 607)*^^	107 (66 – 183)	ANOVA (*P* = 0.048)
Normetanephrine (120 – 1180) pmol/L	234 (157 – 324)	225 (183 – 241)	225 (154 – 358)	231 (177 – 301)	

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Data presented as median (25th/75th centiles). T2DM, type 2 diabetes mellitus; All significant *P* values are highlighted. * $P < 0.05$ compared to any other time point (non-adjusted). ^ $P < 0.05$ compared to any other time point (post-hoc comparisons Sidak test adjusted). ^^ $P < 0.05$ compared to euglycaemia and 24 hours (post-hoc comparisons Sidak test adjusted).

Table S1. Platelet activation and response to stimulation with ADP.

Group	Sample	Baseline	Euglycaemia	Hypoglycaemia	24 hours
T2DM	**Fib unstimulated**	2.6 (1.8 – 3.2)	3.0 (2.3 – 3.6)	2.8 (1.9 – 5.0)	1.9 (1.5 – 2.6)
	Fib ADP 0.1µM	6.1 (3.6 – 9.8)	4.2 (2.9 – 9.1)	3.1 (2.7 – 10.8)	4.9 (3.4 – 14.0)
	Fib ADP 1µM	52.6 (41.2 – 78)	40.6 (31.3 – 66.2)	46.0 (23.9 – 74.2)	51.3 (43.5 – 71.6)
	Fib ADP 10µM	75.3 (66.9 – 88.1)	72.5 (60.9 – 80.8)	70.0 (59.7 – 93.2)	79.1 (70.4 – 83.8)
	P-selectin unstimulated	1.5 (1.2 – 3.0)	2.0 (1.8 – 2.5)	2.6 (1.6 – 3.4)	1.5 (1.0 – 3.3)
	P-selectin ADP 0.1µM	3.9 (2.8 – 7.1)	2.7 (2.2 – 4.6)	3.9 (2.9 – 5.6)	4.9 (3.7 – 7.7)
	P-selectin ADP 1µM	47.4 (33.5 – 67.3)	41.9 (27.8 – 54.8)	47.2 (22.4 – 60.2)	49.2 (36.8 – 61.4)
	P-selectin ADP 10µM	74.6 (59.2 – 81.0)	66.1 (57.9 – 77.5)	74.6 (61.5 – 85.3)	74.1 (64.5 – 79.6)
	PMA unstimulated (%)	7 (4.1 – 15.6)	13.8 (11.2 – 18.3)	11.5 (8.9 – 18.9)	10.8 (7.0 – 14.5)
	PMA ADP 1µM (%)	17.5 (11.7 – 34.9)	27.1 (24.7 – 29.5)	26.9 (19.1 – 32.9)	26.8 (15.3 – 43.7)
Controls	**Fib unstimulated**	2.1 (1.8 – 2.7)	3.3 (1.7 – 7.4)	2.4 (1.7 – 6.5)	2.1 (1.7 – 4.0)
	Fib ADP 0.1µM	5.1 (3.3 – 8.2)	5.8 (2.7 – 10.3)	5.9 (2.9 – 22.4)	8.5 (3.6 – 18.3)

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	8.2)			18.3)
Fib ADP 1μM	59.4 (34.1 – 70.1)	54.8 (38.3 – 76.4)	62.8 (33.9 – 78.2)	60.0 (46.6 – 80.2)
Fib ADP 10μM	80.8 (70.1 – 87.2)	82.0 (63.0 – 92.0)	81.9 (61.3 – 91.5)	81.0 (68.9 – 91.6)
P-selectin unstimulated	3.2 (1.8 – 2.7)	3.3 (2.5 – 4.4)	3.1 (1.5 – 5.0)	1.9 (1.4 – 4.5)
P-selectin ADP 0.1μM	6.5 (3.3 – 8.3)	5.4 (3.1 – 6.5)	6.2 (4.1 – 13.2)	6.6 (4.6 – 10.0)
P-selectin ADP 1μM	50.0 (39.3 – 58.8)	44.6 (33.0 – 52.9)	52.4 (32.4 – 64.5)	46.4 (40.0 – 60.5)
P-selectin ADP 10μM	76.2 (64.8 – 80.0)	71.2 (63.8 – 82.5)	71.9 (61.8 – 84.5)	71.7 (62.8 – 78.1)

Fibrinogen binding and P-selectin expression on platelets' surface (% of positive cells) were measured in unstimulated samples (basal activation) and in response to stimulation with ADP 1-10μM. Data were presented as median (25th/75th centiles). All changes were not significant, $P > 0.05$. T2DM, type 2 diabetes mellitus; Fib, fibrinogen binding; ADP, Adenosine 5′-diphosphate; PGI_2, prostacyclin.

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