SUPPLEMENTARY DATA

Supplementary Methods S1. Hyperinsulinemic clamp study protocol

Participants attended the Clinical Research Facility at 8am, fasted from midnight and avoided caffeine and vigorous exercise 24 hours prior to the study. Participants with DM stopped glucose lowering medication 12 hours before study and perform 4-point capillary glucose profiles to identify hypoglycemia. The study was postponed if hypoglycemia occurred within 24 hours of study.
An intravenous cannula was inserted into the antecubital fossa of the non-dominant arm for insulin and dextrose infusion. A retrograde cannula in the nondominant hand in a warming chamber was used for glucose and catecholamine sampling. A third cannula was inserted into the opposite antecubital fossa to sample prothrombotic and inflammatory markers and cortisol. In the DM group, the blood glucose was initially stabilized to a euglycemic range (6-7 mmol/l) using variable low-dose intravenous insulin infusion.

During the clamp, a primed continuous intravenous insulin infusion (Human Actrapid, Novo Nordisk, West Sussex, UK) at 120 mU/m^2/min (body surface area) along with 20% dextrose at a variable rate, was adjusted according to blood glucose concentrations every five minutes. Arterialized whole blood glucose was measured in duplicate using a glucose oxidase method (Yellow Springs Instrument 2300 STAT, Ohio, USA). During the hypoglycemic clamp, glucose was lowered to 2.5 mmol/l over 60 minutes and thereafter maintained at 2.5 mmol/l for a further 60 minutes. In the euglycemic clamp, glucose was maintained at 6 mmol/l throughout (120 minutes). Insulin was discontinued during recovery glucose returned to euglycemia over 30-minutes. The afternoon clamp procedure was identical except insulin was doubled to 240 mU/m^2/min in order to overcome acquired insulin resistance following hypoglycaemia (1). We administered intravenous insulin at similar rates in both arms and groups.
Participants continued their usual DM medications during their participation.

SUPPLEMENTARY DATA

Supplementary Methods S2. Fibrin clot properties and platelet assays

Turbidimetric and lysis assay

Venous blood was collected into tubes containing 3.2% sodium citrate (BD Vacutainer Glass Citrate Tube) on ice subjected to centrifugation at 2770 ×g for 30 minutes at 4° C within two hours of blood collection and the resultant plasma stored at -80^{o}C until analysis. Ex-vivo fibrin polymerisation characteristics of plasma samples were investigated by a validated turbidimetric clotting assay (2). Briefly, one volume of plasma was treated with 3 volumes of activation mix containing 0.03 U/ml of thrombin and 7.5 mmol/l calcium. Turbidity was monitored in a 96-well plate every 12 seconds using a microplate reader to measure optical density. A turbidimetric lysis assay was conducted as above in the presence of tPA at 83ng/ml final concentration. Plasma clot maximum absorbance (MA), as determined from the turbidity assay, reflects fibrin clot density and fibrin thickness. Plasma clot lysis time (time for initiation of clot formation to 50% fall in maximum absorbance) assesses fibrinolytic potential (3). The inter-assay CoVs for MA was 8% and lysis time 7%.

Markers of fibrin dynamics and inflammation

Fibrinogen and PAI-1 assays were performed on venous blood (4.5 mL) collected into tubes containing 3.2% buffered sodium citrate solute (BD Vacutainer Glass Citrate Tube. Fibrinogen was measured using a validated Clauss method (interassay CoV 1%). PAI-1 was assayed using an ELISA (PAI1 (SERPINE 1) Human SimpleStep ELISA Kit, Abcam, Cambridge, UK, interassay CoV 9.1%). For hsCRP, whole blood (1 mL) was collected into a serum gel tube (Vacutainer ® BD SST™ Advance) and analysed using an immunoturbidimetric assay (Cardiac C reactive protein (latex) high sensitivity, Roche Diagnostics, Indianopolis, USA) in Sheffield Teaching Hospitals Immunology laboratories within 24 hours, interassay CoV 6%). Interleukin-6 (IL6) was assayed from venous blood (5 mL) collected in EDTA tubes (BD Vacutainer™ K3E), subjected to centrifugation at 3000 ×g for 20 minutes and the resulting supernatant stored at -80^{o}C prior to analysis. IL6 was determined using cytometric bead array (Human IL6 Flex set, BD Biosciences, Oxford, UK). Complement C3 is a protein that is incorporated into the fibrin clot and modulates fibrinolysis (4). 5ml venous blood was collected in EDTA tube (BD Vacutainer™ K3E), subjected to centrifugation for 20 minute at 3000 ×g at room temperature, and samples stored at -80^{o} C prior to being assayed. Plasma C3 levels were then determined using ELISA (Biosources, San Diego, USA, interassay CoV 1.33%).

Scanning electron microscopy

Pooled samples of plasma were analysed from 10 T2DM and 10 non-DM subjects. Fibrin clots were made as previously reported (5). Samples were viewed and photographed using a field-emission scanning electron microscope (LEO1530 FEGSEM, Leo Electron Microscopy, Cambridge, United Kingdom) across four different areas of each clot captured using Leo 32 version 03.0210 software (Leo Electron Microscopy) and cropped using Paintshop Pro version 8.0 (Corel, Minneapolis, MN). Fiber diameters of all the clots were measured using the image analysis software package ImageJ 1,23y (National Institutes of Health, Bethesda, MD). In all, 160 fibrin fibres per sample (40 fibres in four clot areas) were measured with the operator blinded to the sampling time point, participant group and type of clamp. The fiber network density was estimated by counting the number of fibres crossing an arbitrary line of fixed length drawn through a single optical section. Three lines were drawn per image and this was analysed on four different micrographs from different areas of a clot for each sample (6).

SUPPLEMENTARY DATA

Platelet impedance aggregometry

Platelet aggregation was analysed using multiple electrode impedance aggregometry (Multiplate ®, Roche Diagnostics, Switzerland). 2.7 ml of venous blood was collected from subjects and transferred into a 3 ml hirudin tube (Multiplate®), with care taken to avoid any agitation of the sample. Recombinant hirudin anticoagulates the blood by direct thrombin inhibition, such that platelet function was assessed under physiological calcium conditions. The samples were analysed as soon as possible following venepuncture (within two hours) to maximise the chances of observing a catecholamine effect. Aliquots of 300 µl saline and 300 µl hirudin-anticoagulated blood were added to the cuvette and incubated at 37^{o} C for 3 minutes. 20µl of agonists (ADP 1 µmol/l and collagen 1µg/ml) were added and the assay commenced. The platelet aggregation response was measured as area under the curve.

Platelet P-selectin expression

Hirudin-anticoagulated blood was added to tubes containing PE Cy5 mouse anti-CD62P antibody (BD Biosciences, Oxford, UK) and platelet marker CD41a (BD Biosciences, Oxford UK) for measurement of platelet P-selectin expression. Platelet flow cytometric assays were performed from venous blood (2.7ml) collected in a hirudin tube (Multiplate®) . Either saline for the unstimulated sample or 5-hydroxytryptamine (5HT; 3 µmol/l) were added to assess endogenous platelet activation and platelet reactivity to 5HT respectively. 5HT was used in order to detect a synergistic effect of catecholamines on platelet activation. Aliquots of blood (5 µL) were then added to 33 µL HT buffer, 5 µL CD6 PECY5 for P-selectin, 5 µL CD41PE as a platelet marker, and either 3 µmol 5HT as agonists or 2 µL saline (unstimulated). After 20 minutes incubation in the dark at room temperature, fixing solution was added. Following fixatation, flow cytometry assay was performed within 6 hours. Flow cytometry was performed and forward light scatter, side scatter and FITC fluorescence measured (BD Accuri C6, BD Biosciences, UK). A gate was positioned around the platelet region to exclude any red cells. P-selectin expression was quantified as median CD62P fluorescence intensity of the platelet population.

SUPPLEMENTARY DATA

Supplementary Table S1. Baseline atherothrombotic markers in type 2 diabetes patients and non-diabetic participants

Data Mean±SD except age Median (range). 5HT 5-hydroxytriptamine, AUC area-under-the- curve, C3 Complement C3, hsCRP high sensitivity C reactive protein, IL6 interleukin-6, MA maximum absorbance, MFI median fluorescence intensity, PAI-1 plasminogen activator inhibitor 1. p value diabetes versus nondiabetic (non-DM) group by independent t-test or Mann-Whitney U test.

	Diabetes (n=12)	Non-DM (n=11)	p
Age (years)	54 (37-64)	52 (34-63)	0.90
Gender (no. of males)	9	5	N/A
BMI (kg/m^2)	34 ± 5	31 ± 8	0.18
Diabetes duration (years)	11 ± 7	N/A	N/A
Fibrin clot properties			
Clot MA (AU)	0.30±0.02	0.32±0.33	0.84
Clot lysis time (s)	617±168	744±219	0.13
Platelet aggregation by impedance aggregometry			
AUC ADP (U)	75±40	41±28	0.03
AUC collagen (U)	87±27	74±20	0.21
P selectin expression (CD62P MFI)			
Unstimulated	4016±1040	3236±1681	0.19
5HT	3984±1111	2866±1488	0.07
Coagulation proteins and inflammatory markers			
Fibrinogen (mg/ml)	3.01±1.12	2.66±0.72	0.39
PAI-1 (pg/ml)	3289±1386	2435±1254	0.14
C3 (mg/ml)	104±15	101±21	0.70
log hsCRP (mg/L)	0.24±0.55	0.01±0.65	0.37
log IL6 (pg/ml)	0.60±0.32	0.52±0.22	0.52

SUPPLEMENTARY DATA

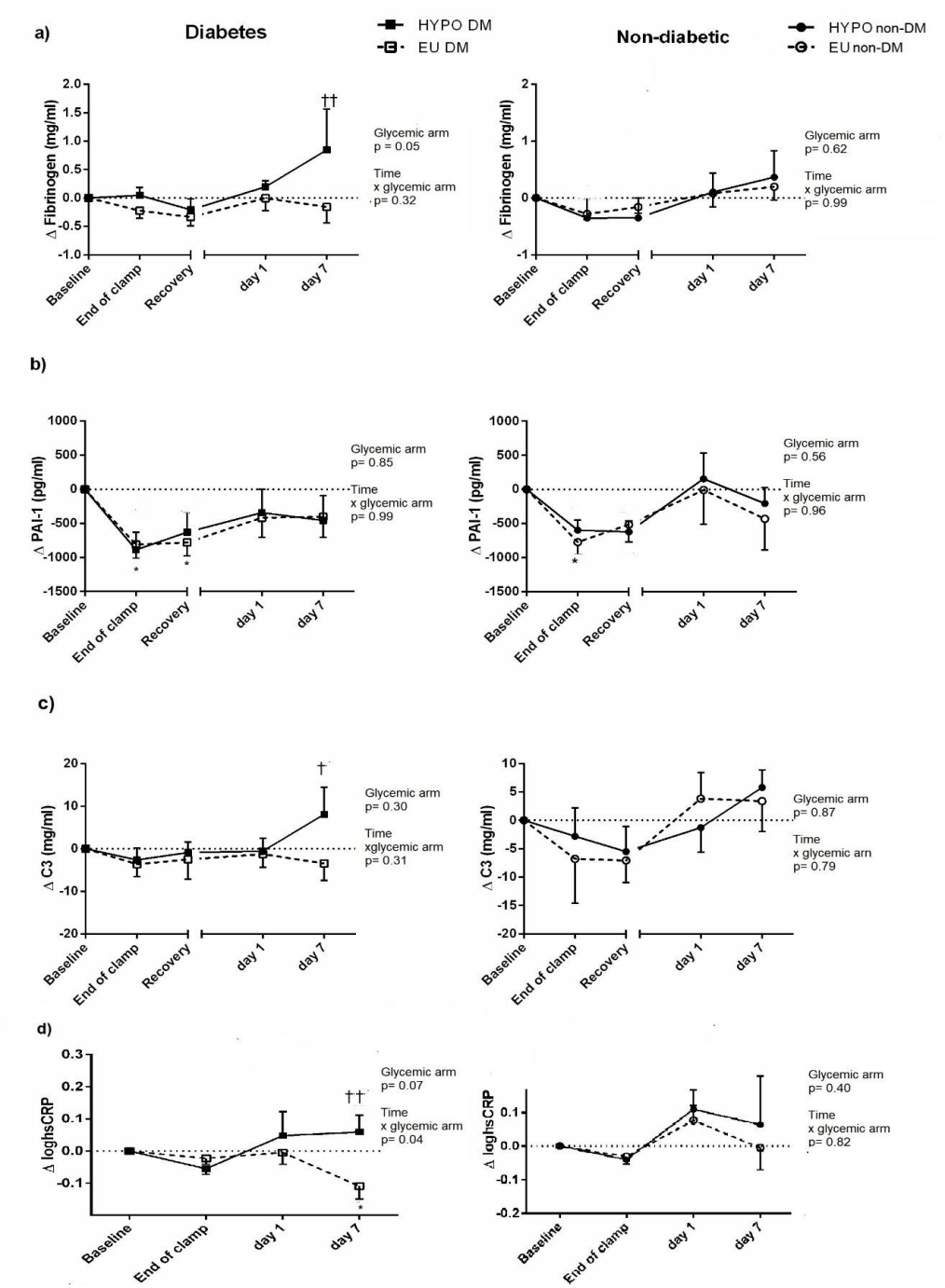
Supplementary Table S2. Blood glucose and counterregulatory hormones in euglycemic and hypoglycemic arms

Data Mean±SD *p<0.05, **p<0.01 euglycemia versus hypoglycemia within each group at the equivalent timepoint † † p<0.01 denotes significant difference between DM and non-DM groups at equivalent euglycemic or hypoglycemic timepoint by independent t-test

Blood glucose (mmol/l)	Diabetes group (n = 12)		Non-DM group (n=11)	
	EU	HYPO	EU	HYPO
Baseline	6.24±1.15	6.44±1.27	5.10±0.49†	4.87±0.31†
End of clamp	5.81±0.30	2.55±0.22**	5.96±0.18	2.56±0.10**
Recovery	6.98±1.14	6.63±1.90	6.37±0.63	8.40±1.96**
Day 1	7.88±2.52	11.4±3.76*	5.08±0.94†	5.00±0.90†
Day 7	9.30±2.70	10.3±0.94	5.09±0.37†	4.96±0.62†
Epinephrine				
(nmol/L)	EU	HYPO	EU	HYPO
Baseline	0.15±0.14	0.12±0.09	0.17±0.12	0.15±0.07
End of clamp	0.16±0.16	3.05±2.46**	0.14±0.08	3.83±2.83**
Day 1	0.23±0.43	0.17±0.21	0.17±0.11	0.20±0.25
Day 7	0.10±0.08	0.16±0.27	0.22±0.20	0.11±0.07
Norepinephrine				
(nmol/L)	EU	HYPO	EU	HYPO
Baseline	1.23±0.47	1.25±0.53	1.56±0.51	1.46±0.52
End of clamp	1.27±0.31	2.37±0.82**	1.56±0.40	2.69±1.45**
Day 1	1.97±0.52	1.93±0.35	3.08±1.48†	2.32±0.72
Day 7	2.07±0.58	2.52±1.20	2.56±0.78	2.78±1.18
Cortisol	EU	HYPO	EU	HYPO
Baseline	277±88	329±129	338±141	300±130
End of clamp	253±88	726±123**	256±107	656±148**
Day 1	243±121	178±97	312±116	230±95
Day 7	243±57	198±105	285±92	233±72

SUPPLEMENTARY DATA

Supplementary Figure S1. Effect of euglycemic and hypoglycemia on coagulation proteins and inflammatory markers in DM and non-DM subjects DM euglycemia (EU)-open square, DM hypoglycemia (HYPO)-closed square, non- DM euglycemia-open circle, non-DM hypoglycemia-closed circle. † p<0.05 †† p<0.01 euglycemia versus hypoglycemia at equivalent timepoint * p<0.05 versus baseline, ** p<0.01 versus baseline. Differences between glycemic arm and the interaction between glycemic arm and time by a mixed model with repeated measures are shown. Data mean (SE). Abbreviations: C3, complement component C3, PAI-1, plasminogen activator 1, hsCRP high sensitivity C reactive proteinsensitivity C reactive protein



SUPPLEMENTARY DATA

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