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Prolonged prothrombotic effects of antecedent hypoglycemia in individuals with type 2 diabetes mellitus

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Abstract

Objective: Hypoglycemia has been linked to persistent increases in cardiovascular (CV) mortality in type 2 diabetes after the event. Our aim was to examine acute and downstream effects of hypoglycemia on markers of thrombosis risk and inflammation in type 2 diabetes.

Research Design and Methods: Twelve type 2 diabetes individuals with no history of CV disease and 11 age- and BMI-matched nondiabetic volunteers underwent paired hyperinsulinemic euglycemic (glucose 6mmol/l for two 60 minute periods) and hypoglycemic clamps (glucose 2.5mmol/l for two 60 minute periods) on separate occasions on Day 0. Fibrin clot properties, platelet reactivity and inflammatory markers were measured at baseline, end of and following recovery from initial clamp, day 1 and day 7 using validated assays and electron microscopy.

Results: Euglycemic hyperinsulinemia reduced platelet reactivity, decreased fibrin clot density and improved fibrinolytic efficiency in both groups. Platelet reactivity and aggregation increased during acute hypoglycemia in both groups, resolving at recovery. In type 2 diabetes, clot lysis times and clot maximum absorbance increased up to day 7 ($p=0.002$ and $p=0.001$ versus euglycemia respectively), but clots from nondiabetic controls showed limited changes. Fibrin network density increased Δ 1.15±0.28 fibers/μm^2 at day 7 following the hypoglycemic clamp ($p<0.01$ for glycemic arm) while fibrinogen and complement C3 increased following hypoglycemia up to day 7 in type 2 diabetes only.

Conclusions: Antecedent hypoglycemia has acute and persistent prothrombotic effects, lasting at least 7 days, that were enhanced in individuals with type 2 diabetes. These findings

identify mechanisms by which hypoglycemia might increase short and medium-term risk of CV mortality.

Abbreviations

5HT – 5 hydroxytryptamine

AUC- Area under the curve

ADP adenosine diphosphate

CoV – coefficient of variation

CV - cardiovascular

CRP - C reactive protein

IL6 - interleukin 6

MA - Maximum absorbance

PAI-1 - Plasminogen activator inhibitor 1

T1DM – Type 1 diabetes mellitus

type 2 diabetes – Type 2 diabetes mellitus

vWF von Willebrand factor

Cardiovascular (CV) disease is the leading cause of death in type 2 diabetes mellitus (type 2 diabetes). A number of large clinical trials have attempted to address the role of intensive glucose control on vascular events and have shown either no reduction (1; 2) or an increase in mortality (3). Hypoglycemia is associated with increased cardiovascular mortality (2; 4) but evidence establishing cause and effect is lacking. In interventional trials, the increased mortality associated with hypoglycemia extended well beyond the acute event, with elevated risk months later (2). Mechanisms by which hypoglycemia might lead to increased cardiovascular mortality in the short-to-medium term are unclear.

Platelet hyperreactivity, altered fibrin clot characteristics and hypofibrinolysis have been linked to excess cardiovascular events, especially in type 2 diabetes (5). Experimental hypoglycemia in people with type 1 diabetes can increase platelet aggregation (6) and inflammatory markers, including CD40 expression and soluble CD40 ligand, platelet-monocyte aggregates, plasma levels of vascular adhesion molecules and P-selectin (7; 8). Repeated episodes of hypoglycemia impair nitric oxide-mediated endothelial function and increase thrombin/antithrombin complex(9). In clinical studies, hypoglycemia increased factor VIII and von Willebrand factor (vWF), which are procoagulant (10).

However, studies examining the longer-term effects of hypoglycemia on thrombotic and inflammatory markers remain scarce. In particular, no study to date has studied prothrombotic changes in individuals with type 2 diabetes in response to hypoglycemia, where insulin resistance and clustering of metabolic risk factors may differentiate them from those with T1DM, predisposing them to a state of depressed fibrinolysis. Antecedent hypoglycemia has been shown to alter autonomic responses and decrease vagal activity downstream of the episode

(11; 12). Suppression of cholinergic anti-inflammatory pathways (13) may lead to prothrombotic and proinflammatory responses following hypoglycemia.

We hypothesised that hypoglycemia might oppose the CV benefits of intensive glycemic control by inducing prothrombotic responses both during the episode and downstream. Thus the aim of this study was to investigate the effects of hypoglycemia in type 2 diabetes patients on key thrombotic mechanisms including i) fibrin clot structure and fibrinolysis, ii) platelet reactivity and activation, and iii) plasma levels of inflammatory markers. We studied prothrombotic changes during acute hypoglycemia and up to seven days after experimentally-induced moderate hypoglycemia in both individuals with type 2 diabetes and matched healthy controls.

Research Design and Methods

Participants

12 participants with type 2 diabetes, aged between 18 and 65 years and with HbA1c 6.5-10.5% (48mmol/mol-91mmol/mol), were recruited from Sheffield Teaching Hospitals (Sheffield, United Kingdom) outpatient clinics between 2011 and 2014. Eleven age- and BMI-matched non-diabetic individuals were recruited as controls from staff at Sheffield Teaching Hospitals and The University of Sheffield (Sheffield, United Kingdom). The same participants also had cardiac electrophysiological measurements recorded during the morning hypersinsulinemic clamps in a study of the effect of hypoglycemia on autonomic function and repolarisation (14). Participants with diabetes were taking oral hypoglycemic agents and/or glucagon-like peptide -1 (GLP-1) analogues and/or insulin for ≤ two years. Exclusion criteria included: previous myocardial infarction, ischemic heart disease, cardiac

arrhythmias, stroke or peripheral vascular disease, or other known cardiovascular disease; epilepsy; untreated hyperthyroidism; pregnancy; serious intercurrent illness. None were taking antiplatelet agents or anticoagulants apart from two patients with diabetes taking aspirin. All subjects had normal full blood count, renal function (eGFR >60 mL/min/1.73 m^2), electrocardiogram at baseline, and cardiac autonomic function using standard autonomic function tests (15). All participants gave written informed consent. The study was approved by the local Research Ethics committee.

Study design

Individuals participated in paired hyperinsulinemic euglycemic and hypoglycemic clamp studies separated by at least 4 but no more than 8 weeks to minimize carry-over effects. We used a cross-over design such that each participant served as his or her own control to reduce variability. Euglycemic studies preceded hypoglycemic studies as hypoglycemia may have persistent effects of the cardiovascular system. In previous studies, two periods of hypoglycemia resulted in altered autonomic and vagal function that could lead to proinflammatory and prothrombotic effects in the days following hypoglycemia (11; 12). In one study, these changes were observed up to 6 days later (11). Thus, in the hypoglycemic arm, arterialized blood glucose was maintained at 2.5 mmol/l for two 60-minute periods in the morning and afternoon with rapid-acting intravenous insulin at 120 and 240 mU/m^2/min respectively, alongside a variable infusion of 20% dextrose. Participants were blinded to blood glucose levels. Blood glucose was raised to euglycemic levels between morning and afternoon hypoglycemic clamps. In the euglycemic arm, arterialized blood glucose was maintained at 6 mmol/l for 60 minutes during both morning and afternoon, using similar rates of insulin infusion used during hypoglycemia. Insulin was administered at the same rates in both subject

groups. Prothrombotic and inflammatory markers were measured at the end of the morning clamp and at recovery (30 minutes after end of morning clamp) but not during the afternoon clamp. To investigate downstream effects, prothrombotic and inflammatory markers were also measured in the morning at 1 and 7 days following both euglycemic and hypoglycemic studies. The study design is outlined in Fig. 1 and details of the hyperinsulinemic clamp protocol are further described in supplementary methods S1.

Biochemical measurements

For epinephrine and norepinephrine, whole blood (6 mL) was collected into chilled lithium-heparin tubes containing 50 μL EGTA/glutathione as a preservative. These were assayed by high-performance liquid chromatography (inter-assay coefficient of variation (CoV) norepinephrine 6.03% and epinephrine 15.9%). For free insulin, whole blood (3 mL) was collected into a 6-mL lithium-heparin tube and immediately subjected to centrifugation. The resulting plasma (0.5 mL) was added to a chilled plastic tube containing 0.5 mL polyethylene glycol (PEG) for precipitation of immune complexes and mixed. The product was analysed by an immunometric assay (Invitron Insulin ELISA, Invitron Ltd, Monmouth, UK, interassay CoV 7.1%). Serum cortisol analysed from 4ml venous blood collected in serum separator tube was measured using a commercial immunoassay (Roche e602 serum cortisol assay, Roche Diagnostics, West Sussex, UK, interassay CoV 3.2%). Biochemical parameters were measured at baseline and at end of euglycemic and hypoglycemic arms in both groups.

Turbidimetric and lysis assay

Venous blood was collected into tubes containing 3.2% sodium citrate (BD Vacutainer Glass Citrate Tube) on ice. *Ex-vivo* fibrin polymerisation characteristics of plasma samples were

investigated by a validated turbidimetric clotting assay (16), described in detail under supplementary methods S2.

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). High-sensitivity C-reactive protein (hsCRP) was analysed using an immunoturbidmetric assay (Cardiac C reactive protein (Latex) high sensitivity, Roche Diagnostics, Indianopolis, USA). Interleukin-6 (IL6) was determined using cytometric bead array (Human IL6 Flex set, BD Biosciences, Oxford, UK). Complement C3 plasma levels, a protein that is incorporated into the fibrin clot and modulates fibrinolysis (17), were determined using ELISA (Biosources, San Diego, USA). Further details are described in supplementary methods S2.

Scanning electron microscopy

Pooled samples of plasma were analysed from 10 type 2 diabetes and 10 non-DM subjects. Fibrin clots were made as previously reported (18) and described in detail in the supplementary material (S 2).

Platelet impedance aggregometry

Platelet aggregation was analysed using multiple electrode impedance aggregometry (Multiplate ®, Roche Diagnostics, Switzerland) and described in detail in supplementary methods S2.

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, with detailed description of the assay provided in supplementary methods S2.

Statistical analysis

Baseline demographic data and prothrombotic and inflammatory markers are summarised as mean (SD) for parametric data, unless otherwise stated, or median (interquartile range) for nonparametric data. hsCRP and IL6 were logarithmically transformed due to a skewed distribution. End-of-clamp blood glucose and hormone concentrations were compared between euglycemic and hypoglycemic timepoints using two-way ANOVA.

We used a linear mixed model with repeated measures to compare i) whether there was significant change in prothrombotic markers over time within glycemic arms in each subject group and ii) effect of glycemic arm on changes in prothrombotic markers in each subject group. For analysis i), timepoint was specified as a fixed effect with multiple comparisons against baseline adjusted by the Sidak correction. In mixed-model analysis ii), glycemic arm, time and interaction between glycemic arm and time were specified as fixed effects and the subject as random effect. Glycemic arm and timepoint were specified as repeated measures within each subject. Repeated measures were fitted with an unstructured, compound symmetry or autoregressive 1 covariance structure and the model with the best fit (lowest Akaike's information criterion) was selected. p values were obtained by restricted maximum likelihood estimation. The effect of glycemic arm and interaction between time and glycemic arm on fiber network density and fibrin thickness were analysed using two-way repeated-measures ANOVA. We further calculated the within-individual correlations between clot MA or clot lysis time with fibrinogen, PAI-1 and C3, taking into account repeated measures,

based on the ANCOVA method (19). A p value of < 0.05 was considered significant. Results were analysed using SPSS (IBM, SPSS Statistics v20).

Based on our initial pilot data, a sample size of 10 subjects per group had a power of 81% to detect a clinically-relevant 15% difference in clot lysis time, assuming a SD of 100s. We aimed to recruit 12-13 subjects in each group, allowing for a 20-30% drop out rate.

Results

Baseline characteristics

Twelve patients with type 2 diabetes (9 male) and 11 individuals without diabetes (5 male) participated in the study. Participants with diabetes were similar in age, 54 (50-58) years, compared with 52(47-59) years in the control group ($p = 0.90$). The mean BMI was comparable: 34±5 kg/m^2 in the diabetes group versus 31±8 kg/m^2 in the non-DM group ($p=0.18$). The median duration of diabetes was 10 (8-12) years and mean HbA1c was 7.8±1.3% (62±14 mmol/mol). Among patients with diabetes, five were taking oral hypoglycemic agents only, 5 were taking oral hypoglycemic agents and a GLP-1 analogue, and two were on oral hypoglycemic agents and basal insulin. Baseline prothrombotic and inflammatory markers are shown in supplementary table S3. Subjects with diabetes had higher platelet reactivity to ADP but no significant differences were detected in fibrinogen, PAI-1, C3 and hsCRP plasma levels (supplementary table S3).

Blood glucose and counter-regulatory hormones

Blood glucose levels were similar at end of morning euglycemic and hypoglycemic clamps in both groups (supplementary table S4). Glucose targets were reached during afternoon clamps within each arm. During day 1, blood glucose was higher following hypoglycemic clamps

compared with euglycemia in the diabetes group only. Blood glucose levels were similar between glycemic arms at day 7. subjects with diabetes did not report symptomatic hypoglycemia or capillary glucose values below 3mmol/l in the week following each clamp.

Median (IQR) free insulin levels at 120 minutes were 576 (468-627) pmol/L during euglycemia and 689 (477-1076) pmol/L during hypoglycemia in the diabetes group. In those without diabetes, these were 865 (509-952) pmol/L in the euglycemic arm and 665 (468-967) pmol/L in the hypoglycemic arm. Insulin levels between groups during both euglycemia and hypoglycemia were not different ($p = 0.23$).

Counter-regulatory hormones were unchanged during euglycemia (supplementary table S4). During acute hypoglycemia, epinephrine, norepinephrine and cortisol increased significantly (all $p<0.05$ versus baseline) similarly in both groups (supplementary table S4). Plasma epinephrine and cortisol returned to baseline at day 1 and day 7 following both arms in both subject groups. However, in the non-diabetes group, plasma norepinephrine levels increased significantly at day 1 after both clamps.

Fibrin clot properties

Clot lysis time

In individuals without diabetes, clot lysis times decreased following euglycemia (from 729±216 to 611±159s, - Δ146±110, $p=0.001$ versus baseline) but returned to baseline at day 1 (Fig 2a). There were no changes in clot lysis times in the hypoglycemic arm in the non-diabetes group. In individuals with diabetes, clot lysis times decreased at the end of euglycemic clamp (Δ-81±86s) but were prolonged at the end of hypoglycemia, with further increases at day 1 (Δ71±153s) and day 7 (Δ67±107s). Changes in clot lysis times were

significantly different between euglycemic and hypoglycemic arms in individuals with diabetes (p=0.001, Fig 2a).

Clot maximum absorbance

In controls, clot MA decreased at the end of both clamps, but to a lesser extent during hypoglycemia (Δ-0.02±0.05AU) compared with euglycemia (Δ-0.05±0.05 AU) (p=0.02 for glycemic arm) (Fig 2b). In individuals with diabetes, clot MA decreased during euglycemia and during recovery. There was a non-significant increase in clot MA at the end of hypoglycemia (Δ0.02±0.05 AU), which resolved at recovery, followed by gradual increases at day 1 and 7 (Fig. 2b). There were significant differences in clot MA between glycemic arms in the diabetes group (p=0.002) and interaction between time and glycemic arm (p=0.02).

Scanning Electron Microscopy

Fibrin Diameter

Fibrin diameter did not change significantly during euglycemia or hypoglycemia in the non-diabetes group (Fig 3a). In diabetes individuals, there were no changes in fibrin fiber diameter during euglycemia, which increased at day 1 and day 7 following hypoglycemia ($p<0.01$ versus euglycemia at equivalent timepoints) (Fig 3a). There was a significant difference between glycemic arms ($p<0.0001$) and in the interaction between time and glycemic arm ($p<0.0001$).

Fibrin Network Density

In the non-diabetes group, fiber network density decreased at day 1 after euglycemia compared to no change following hypoglycemia ($p <0.001$ for glycemic arm). The fibrin network density

returned to baseline at day 7 following euglycemia but increased at day 7 following hypoglycemia ($p<0.01$ for glycemic arm) (Fig 3b).

In the diabetes group, fiber network density decreased at day 7 following euglycemia, whilst following hypoglycemia, there was an increase in fiber network density at day 1 and day 7 (both $p<0.01$ for glycemic arm) (Fig 3b). There were significant differences between the glycemic arms and the interaction between glycemic arm and time in both the diabetes and non-DM groups (both $p<0.001$). Representative examples of scanning electron micrographs of pooled fibrin clots are shown in Fig. 3c.

Coagulation proteins

In the non-diabetes group, fibrinogen tended to decrease at the end of the clamp and recover similarly during both euglycemia and hypoglycemia. However, in the diabetes group, fibrinogen levels did not change during euglycemia but increased $\Delta 0.20 \pm 0.10$mg/mL at day 1 and $\Delta 0.85 \pm 0.69$mg/mL at day 7 following hypoglycemia ($p=0.05$ for glycemic arm) (supplementary Fig S5a).

PAI-1 falls in both diabetes and control subjects were similar during euglycemia and hypoglycemia (supplementary Fig. S5b). PAI-1 decreased in the controls during euglycemia and hypoglycemia ($p=0.15$ and $p=0.005$ for time respectively) with no significant differences between the arms ($p=0.56$). In type 2 diabetes subjects, PAI-1 decreased from baseline to Δ -811±204pg/mL at the end of euglycemia ($p=0.02$ versus baseline) and remained lower at day 7. PAI-1 decreased to a similar extent during hypoglycemia (Δ-888±256, $p=0.02$ versus baseline) with no differences detected between glycemic arms ($p=0.85$).

Change in clot MA correlated with changes in fibrinogen (r=0.98, p<0.001), PAI-1 (r=0.99, p<0.001) and C3 (r = 0.99, p<0.001) across both groups. Change in clot lysis time correlated with changes in fibrinogen (r=0.98, p<0.001), PAI-1 (r=0.99, p<0.001) and C3 (r = 0.98, p<0.00). There was no correlation between epinephrine, norepinephrine and clot MA or lysis times.

Inflammatory markers

In the non-diabetes group, C3 levels were similar during euglycemia and hypoglycemia and did not change significantly (supplementary Fig S5c). C3 levels did not change during euglycemia in the diabetes group but tended to rise at day 7 following hypoglycemia (from 99±5 at hypoglycemic baseline to 108±4 mg/mL at day 7).

In non-diabetes individuals, hsCRP increased similarly at day 1 following euglycemia and hypoglycemia, in both glycemic arms (supplementary Fig. S5d). In diabetes individuals, hsCRP decreased following euglycemia at day 7 (log hsCRP - Δ0.11±0.01, p=0.009 versus baseline) compared with no change following hypoglycemia (log hsCRP Δ0.06±0.01 at day 7). There was a significant interaction between glycemic arm and time (p= 0.04).

In the non-diabetes group, IL-6 was higher at day 1 following euglycemia but did not change following hypoglycemia (data not shown). In the diabetes group, IL-6 did not change during the euglycemic arm, and trended towards an increase in IL-6 following hypoglycemia at day 7.

Platelet reactivity and activation

Collagen- and ADP-induced platelet aggregation

In non-diabetes individuals, there was a non-significant change in collagen-induced platelet aggregation during euglycemia (Fig 4a). During hypoglycemia, collagen-induced platelet aggregation increased significantly higher compared to euglycemia, which resolved at recovery. The overall difference in platelet aggregation response to collagen between arms was significant ($p=0.04$).

In diabetes individuals, collagen-induced platelet aggregation tended to decrease during acute euglycemia (Δ-15±23 U, $p=0.11$ versus baseline) in contrast to an increase following hypoglycemia ($p<0.01$ between glycemic arms), persisting to the recovery period but not beyond. There was a significant interaction between time and glycemic arm ($p=0.03$).

ADP-induced platelet aggregation decreased in diabetes subjects during euglycemia compared to an increase at the end of hypoglycemia ($p=0.03$ between arms). In the non-diabetes group, secondary rises in platelet aggregation induced by both collagen and ADP at day 1 occurred following both hypoglycemia and euglycemia (Fig 4a and b).

There were no significant changes in platelet reactivity to 5HT during euglycemia in both groups (Fig 4c). There were trends towards increased platelet reactivity following hypoglycemia in both groups, resolving at recovery. In non-diabetes subjects, no significant changes in platelet activation occurred during euglycemia. Platelet activation tended to increase following hypoglycemia and at day 1 with no overall difference between arms (Fig 4d). In diabetes individuals, platelet activation, as measured by unstimulated P-selectin expression, decreased following euglycemia, maximally at day 1 but increased immediately following hypoglycemia ($p=0.01$ for glycemic arm) (Fig 4d).

Conclusions

To our knowledge, this is the first study investigating the effects of hypoglycemia on both cellular and protein arms of the thrombosis pathway in the period beyond a hypoglycemic challenge. The novel findings include: i) hypoglycemia was associated with early and late prothrombotic changes in the fibrin network in the diabetes group but less evident in healthy controls; ii) a rise in fibrinogen and C3 levels that may contribute to late prothrombotic changes in fibrin network properties; and iii) hypoglycemia enhanced platelet reactivity in individuals with and without diabetes, lasting less than 24 hours following the event.

The anti-aggregatory, anti-inflammatory and profibrinolytic responses to euglycemic hyperinsulinemia are consistent with previous human studies (20; 21). In the present work, a striking observation was the difference in clot lysis time between euglycemia and hypoglycemia in the diabetes group. Initially, the decrease in clot lysis time during euglycemia may be explained by a reduction in PAI-1. Insulin-mediated suppression of fibrinogen synthesis may also result in enhanced clot lysis, but is unlikely to contribute to early changes given the long half-life of the protein, unlike PAI-1. On the other hand, early prolongation in clot lysis in the diabetes group does not appear to be PAI-1-mediated as protein levels were reduced following hypoglycemic clamps. In a previous study, PAI-1 was decreased during both hyperinsulinemic hypoglycemia and euglycemia in type 1 diabetes individuals; however, the authors found increased PAI-1 following hypoglycemia in healthy controls (8).

A novel and intriguing observation is the persistent effect of hypoglycemia on clot density and impaired fibrinolysis, at least 7 days following hypoglycemia in the diabetes group but not in controls. This finding may be important clinically, particularly in individuals at higher

vascular risk. The mechanisms for the late impairment in lysis are not entirely clear, although the rise in fibrinogen and complement C3 plasma levels provide plausible explanations. Increased levels of fibrinogen are associated with denser clots, as we have previously shown (18), which may impair fibrinolysis. This is supported by an observational study in type 2 diabetes; low fasting glucose was associated with denser *ex-vivo* fibrin clots as compared with fasting glucose in the normal range (22). We have demonstrated that C3 modulates clot lysis independently of PAI-1 and this effect is particularly pronounced in individuals with diabetes (17; 23). Taken together, it is possible that repeated hypoglycemic episodes lead to a state of chronic low-grade inflammation, resulting in elevated fibrinogen and C3 levels, which in turn compromises fibrinolysis. However, we cannot exclude the possibility that other plasma components may also have an effect. Gogtidize-Joy and colleagues demonstrated that nitric oxide-mediated endothelial dysfunction, which creates an inflammatory environment, was exaggerated by repeated experimental hypoglycemia 24 hours later, but effects beyond 48 hours were not reported (9).

We also showed that hypoglycemia modulates platelet reactivity in type 2 diabetes, probably mediated by catecholamine release, but the effects are short-lived. Our results are consistent with previous reports of individuals with type 1 diabetes demonstrating increased platelet reactivity during acute hypoglycemia (24). The hypoglycemia-mediated increase in platelet reactivity can be abolished by α-receptor blockade, implicating α_2 adrenoreceptors in mediating this effect (6). In contrast to fibrin clot studies, we did not observe consistent differences between platelet responses to acute hypoglycemia in the groups studied.

Our data identify a mechanism whereby hypoglycemia could contribute to increased CV mortality by opposing the benefits of intensive glycemic control. In a post-hoc analysis of

the NICE-SUGAR study, which reported excess mortality in patients treated to tight glucose levels, the median time to death from a hypoglycemic event was 7 days for moderate hypoglycemia and 8 days for severe (25). The time course matches our findings of a worsening atherothrombotic risk profile 1 week after hypoglycemia. In the ADVANCE and VADT trials, the increased CV risk extended for many months after a severe hypoglycemic event and it is unlikely that the changes we observed would persist over this period. On the other hand, less severe episodes (equivalent to those induced in this study) were not measured consistently in these trials. They are arguably occurring more frequently in those experiencing severe events and if recurrent, might contribute to thrombotic events, particularly in those at increased CV risk.

Our study had the following relevant limitations. First, the relatively small number of participants, while adequate to demonstrate changes in fibrin clot properties, may have left us short of statistical power to identify subtle differences between type 2 diabetes and groups without diabetes. However, recruiting individuals into experimental studies is challenging and this remains one of the largest studies investigating both type 2 diabetes and controls with combined platelet and fibrin network analyses. Second, the experimental model requires supra-physiological doses of insulin to maintain stable hypoglycemia, higher than that observed during routine clinical care. This might artefactually diminish prothrombotic and proinflammatory changes during experimental hypoglycemia. Third, two subjects were on aspirin treatment, which can affect collagen-induced platelet aggregation and clot lysis (26). However, these subjects remained on aspirin throughout the study and displayed similar patterns of collagen-induced platelet aggregation to other participants (data not shown). In these studies, we induced hypoglycemia on two occasions during day 0 and we cannot be

certain that similar persistent prothrombotic changes would have been observed following a single or shorter hypoglycemic episodes. Future studies should be designed to address this. For obvious ethical reasons, we did not induce hypoglycemia in individuals with known CV disease. It is not known whether the deleterious atherothrombotic effects of hypoglycemia would be exaggerated in high-CV-risk patients and the protective effects of insulin attenuated, compared with our study group.

In conclusion, we have shown that two episodes of moderate hypoglycemia have acute prothrombotic effects in both non-diabetic and type 2 diabetes subjects. We have also demonstrated that the impact of moderate, short-lived hypoglycemia is maintained for at least 7 days after the event, with adverse effects on fibrin clot properties, fibrinolysis and subclinical inflammation. These effects were more prominent among individuals with type 2 diabetes compared to a non-DM control group. We have identified potential mechanisms whereby hypoglycemia could increase the risk of CV events during and after an episode and so oppose the benefits of intensive glycemic control. The precise clinical relevance of these findings remains to be established by further studies. Nevertheless, we believe that clinicians should be consider our results when addressing vascular health in people with type 2 diabetes and choose approaches that minimise hypoglycemia when optimising glycemic management.

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EC is the guarantor of this work and as such had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. EC designed the study, collected, analyzed the data and wrote the manuscript. AI, EW and FP helped to collect the data, conduct clotting and platelet assays and reviewed the manuscript.

IAM analyzed the catecholamine data and reviewed the manuscript. RFS, RA and SRH designed the study, reviewed the data and edited and redrafted the manuscript.

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This study was presented in part at the American Diabetes Association 73rd Scientific Sessions 2013.

Figures legends

Fig. 1 Study design

Each type 2 diabetes or non-DM subject participated in euglycemic and hypoglycemic clamp visits separated by 4 to 8 weeks. Euglycemic clamp preceded hypoglycemic clamps. In the euglycemic clamp visit, blood glucose was maintained at 6mmol/L for 60 minutes in the morning and 60 minutes in the afternoon. In the hypoglycemic clamp visit, blood glucose was maintained at 2.5mmol/L for 60 minutes in the morning and 60 minutes in the afternoon. Blood samples for platelet function, clotting assays, coagulation proteins and counterregulatory hormones were collected at baseline, end of clamp at 60 minutes, recovery from morning clamp, and then on the morning of day 1 and day 7 after the euglycemic and hypoglycemic clamp visits respectively. EU euglycemic, HYPO hypoglycemic

Fig. 2 Effect of euglycemia and hypoglycemia on a) clot lysis time and b) clot density by turbidimetric and lysis assay

type 2 diabetes euglycemia (EU)- open square, type 2 diabetes hypoglycemia (HYPO)- closed square, non-DM euglycemia- open circle, non-DM hypoglycemia - closed circle. EU arm preceded HYPO arm. † $p<0.05$ †† $p<0.01$ euglycemia versus hypoglycemia at equivalent time points * $p<0.05$ 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: AU arbitrary units, MA maximum absorbance.

Fig. 3 Fibrin network properties and scanning electron micrographs of fibrin clots following euglycemia versus hypoglycemia in DM and non-DM subjects

a) Fiber network density and b) fibrin fiber thickness following euglycemia (EU) (black) versus hypoglycemia (HYPO) grey. Fiber thickness was measured for a total number of 160 fibers (40 fibers measured from 4 different clot areas at each time point) * $p<0.01$, ** $p<0.01$, ****$p<0.0001$ between euglycemia versus hypoglycemia. Data mean are (SE) c) Visualisation of *ex-vivo* fibrin clots from pooled plasma samples in type 2 diabetes (n=10) and non-DM (n=10) subjects. There is a decrease in the fiber network following euglycemia in both groups as opposed to an increase in the network density following hypoglycemia at day 7

Fig. 4 Effect of euglycemia and hypoglycemia on platelet reactivity and platelet activation

Data represent change in value from baseline. Type 2 diabetes euglycemia (EU)- open square, type 2 diabetes hypoglycemia (HYPO)- closed square, Non-DM euglycemia- open circle, non-DM hypoglycemia closed circle. EU arm preceded HYPO arm. † $p<0.05$ †† $p<0.01$ euglycemia versus hypoglycemia at equivalent timepoint * $p<0.05$ versus baseline, ** $p<0.01$ versus baseline. Differences between glycemic arm and interaction between glycemic arm and

time by mixed model with repeated measures are shown. Data mean(SE). Abbreviations: AUC area under the curve, MFI median fluorescence intensity

References

1. Patel A, MacMahon S, Chalmers J, Neal B, Billot L, Woodward M, Marre M, Cooper M, Glasziou P, Grobbee D, Hamet P, Harrap S, Heller S, Liu L, Mancia G, Mogensen CE, Pan C, Poulter N, Rodgers A, Williams B, Bompoint S, de Galan BE, Joshi R, Travert F: Intensive blood glucose control and vascular outcomes in patients with type 2 diabetes. N Engl J Med 2008;358:2560-2572
2. Duckworth W, Abraira C, Moritz T, Reda D, Emanuele N, Reaven PD, Zieve FJ, Marks J, Davis SN, Hayward R, Warren SR, Goldman S, McCarren M, Vitek ME, Henderson WG, Huang GD: Glucose control and vascular complications in veterans with type 2 diabetes. N Engl J Med 2009;360:129-139
3. Gerstein HC, Miller ME, Byington RP, Goff DC, Jr., Bigger JT, Buse JB, Cushman WC, Genuth S, Ismail-Beigi F, Grimm RH, Jr., Probstfield JL, Simons-Morton DG, Friedewald WT: Effects of intensive glucose lowering in type 2 diabetes. N Engl J Med 2008;358:2545-2559
4. Zoungas S, Patel A, Chalmers J, de Galan BE, Li Q, Billot L, Woodward M, Ninomiya T, Neal B, MacMahon S, Grobbee DE, Kengne AP, Marre M, Heller S: Severe hypoglycemia and risks of vascular events and death. N Engl J Med 2010;363:1410-1418
5. Bridge KI, Philippou H, Ariens R: Clot properties and cardiovascular disease. Thromb Haemost 2014;112:901-908
6. Trovati M, Anfossi G, Cavalot F, Vitali S, Massucco P, Mularoni E, Schinco P, Tamponi G, Emanuelli G: Studies on mechanisms involved in hypoglycemia-induced platelet activation. Diabetes 1986;35:818-825
7. Wright RJ, Newby DE, Stirling D, Ludlam CA, Macdonald IA, Frier BM: Effects of Acute Insulin-Induced Hypoglycemia on Indices of Inflammation: Putative mechanism for aggravating vascular disease in diabetes. Diabetes Care 2010;33:1591-1597
8. Gogitidze Joy N, Hedrington MS, Briscoe VJ, Tate DB, Ertl AC, Davis SN: Effects of acute hypoglycemia on inflammatory and pro-atherothrombotic biomarkers in individuals with type 1 diabetes and healthy individuals. Diabetes Care 2010;33:1529-1535
9. Joy NG, Tate DB, Younk LM, Davis SN: Effects of Acute and Antecedent Hypoglycemia on Endothelial Function and Markers of Atherothrombotic Balance in Healthy Humans. Diabetes 2015;64:2571-2580
10. Grant PJ, Stickland MH, Wiles PG, Davies JA, Wales JK, Prentice CR: Hormonal control of haemostasis during hypoglycaemia in diabetes mellitus. Thromb Haemost 1987;57:341-344
11. Robinson AM, Parkin HM, Macdonald IA, Tattersall RB: Antecedent hypoglycaemia in non-diabetic subjects reduces the adrenaline response for 6 days but does not affect the catecholamine response to other stimuli. Clin Sci (Lond) 1995;89:359-366
12. Adler GK, Bonyhay I, Failing H, Waring E, Dotson S, Freeman R: Antecedent hypoglycemia impairs autonomic cardiovascular function: implications for rigorous glycemic control. Diabetes 2009;58:360-366

13. Tracey KJ: Physiology and immunology of the cholinergic antiinflammatory pathway. In *J Clin Invest*, 2007, p. 289-296
14. Chow E, Bernjak A, Walkinshaw E, Lubina-Solomon A, Freeman J, Macdonald IA, Sheridan PJ, Heller SR: Cardiac Autonomic Regulation and Repolarization During Acute Experimental Hypoglycemia in Type 2 Diabetes. Diabetes 2017;66:1322-1333
15. Tesfaye S, Boulton AJ, Dyck PJ, Freeman R, Horowitz M, Kempler P, Lauria G, Malik RA, Spallone V, Vinik A, Bernardi L, Valensi P: Diabetic neuropathies: update on definitions, diagnostic criteria, estimation of severity, and treatments. Diabetes Care 2010;33:2285-2293
16. Carter AM, Cymbalista CM, Spector TD, Grant PJ: Heritability of clot formation, morphology, and lysis: the EuroCLOT study. Arterioscler Thromb Vasc Biol 2007;27:2783-2789
17. Hess K, Alzahrani SH, Mathai M, Schroeder V, Carter AM, Howell G, Koko T, Strachan MW, Price JF, Smith KA, Grant PJ, Ajjan RA: A novel mechanism for hypofibrinolysis in diabetes: the role of complement C3. Diabetologia 2012;55:1103-1113
18. Hooper JM, Stuijver DJ, Orme SM, van Zaane B, Hess K, Gerdes VE, Phoenix F, Rice P, Smith KA, Alzahrani SH, Standeven KF, Ajjan RA: Thyroid dysfunction and fibrin network structure: a mechanism for increased thrombotic risk in hyperthyroid individuals. J Clin Endocrinol Metab 2012;97:1463-1473
19. Bland JM, Altman DG: Calculating correlation coefficients with repeated observations: Part 1--Correlation within subjects. BMJ 1995;310:446
20. Chaudhuri A, Janicke D, Wilson MF, Tripathy D, Garg R, Bandyopadhyay A, Calieri J, Hoffmeyer D, Syed T, Ghanim H, Aljada A, Dandona P: Anti-inflammatory and profibrinolytic effect of insulin in acute ST-segment-elevation myocardial infarction. Circulation 2004;109:849-854
21. Dandona P, Ghanim H, Bandyopadhyay A, Korzeniewski K, Ling Sia C, Dhindsa S, Chaudhuri A: Insulin Suppresses Endotoxin-Induced Oxidative, Nitrosative, and Inflammatory Stress in Humans. Diabetes Care 2010;33:2416-2423
22. Gajos G, Konieczynska M, Zalewski J, Undas A: Low fasting glucose is associated with enhanced thrombin generation and unfavorable fibrin clot properties in type 2 diabetic patients with high cardiovascular risk. In *Cardiovasc Diabetol*, 2015
23. Hess K, Alzahrani SH, Price JF, Strachan MW, Oxley N, King R, Gamlen T, Schroeder V, Baxter PD, Ajjan RA: Hypofibrinolysis in type 2 diabetes: the role of the inflammatory pathway and complement C3. Diabetologia 2014;57:1737-1741
24. Dalsgaard-Nielsen J, Madsbad S, Hilsted J: Changes in platelet function, blood coagulation and fibrinolysis during insulin-induced hypoglycaemia in juvenile diabetics and normal subjects. Thromb Haemost 1982;47:254-258
25. NICE-SUGAR Study Investigators: Hypoglycemia and risk of death in critically ill patients. N Engl J Med 2012;367:1108-1118
26. Ajjan RA, Standeven KF, Khanbhai M, Phoenix F, Gersh KC, Weisel JW, Kearney MT, Ariens RA, Grant PJ: Effects of aspirin on clot structure and fibrinolysis using a novel in vitro cellular system. Arterioscler Thromb Vasc Biol 2009;29:712-717