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**Cardiac autonomic regulation and repolarization during acute experimental
hypoglycemia in Type 2 diabetes**

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Abstract

Hypoglycemia is associated with increased cardiovascular mortality in trials of intensive therapy in type 2 diabetes (T2DM). We previously observed an increase in arrhythmias during spontaneous prolonged hypoglycemia in T2DM patients. Our aim was to examine changes in cardiac autonomic function and repolarization during sustained experimental hypoglycemia.

Twelve adults with T2DM and eleven age, BMI-matched nondiabetic controls underwent paired hyperinsulinemic clamps separated by 4 weeks. Glucose was maintained at euglycemia (6.0mmol/L) or hypoglycemia (2.5mmol/L) for one hour. Heart rate, blood pressure, heart rate variability were assessed every thirty minutes and corrected QT (QTc) and T wave morphology every 60 minutes.

Heart rate initially increased in T2DM participants but then fell towards baseline despite maintained hypoglycemia at 1 hour, accompanied by reactivation of vagal tone. In nondiabetic participants, vagal tone remained depressed during sustained hypoglycemia. Diabetic participants exhibited greater heterogeneity of repolarization during hypoglycemia as demonstrated by T wave symmetry and Principal Component Analysis (PCA) ratio compared with the nondiabetic group. Epinephrine levels during hypoglycemia were similar between groups.

Cardiac autonomic regulation during hypoglycemia appears time-dependent. T2DM individuals demonstrate greater repolarization abnormalities for a given hypoglycemic stimulus despite comparable sympathoadrenal responses. These mechanisms could contribute to arrhythmias during clinical hypoglycemic episodes.

Glossary

BRS - baroreceptor sensitivity

BMI – body mass index

CVD - cardiovascular disease

ECG - electrocardiogram

HRV - heart rate variability

HF - high frequency (power of heart rate variability)

LF - low frequency (power of heart rate variability)

LFnorm - normalized low frequency (power of heart rate variability)

NN - normal-to-normal RR intervals

PCA ratio – principal component analysis ratio

RMSSD - root mean square of successive differences in NN

SDNN - standard deviation of NN

T2DM- type 2 diabetes mellitus

TCRT – total cosine R to T

TWLD – T wave loop dispersion

Recent large trials of intensive glycemic control involving individuals with type 2 diabetes (T2DM) have not reduced cardiovascular death in type 2 diabetes patients at high cardiovascular risk (1; 2) and in one trial was associated with increased mortality (3). Severe hypoglycemia was increased several fold in the intensive control arm in all three trials and was a strong independent predictor of mortality in post hoc analyses (4). Hypoglycemia is a plausible explanation for the observed excess mortality but evidence for a direct mechanistic link remains unclear. Recent studies of insulin therapy in T2DM have shown a higher risk of fatal arrhythmic death associated with severe hypoglycemia (5). One mechanism by which hypoglycemia could promote arrhythmias is through changes in cardiac autonomic activity and repolarization.

In a recent study examining ECG responses to spontaneous hypoglycemia in type 2 diabetes patients using ambulatory glucose and Holter monitoring (6), we observed an increase in bradycardia and atrial ectopic activity during hypoglycemia when compared to euglycemia. During nocturnal hypoglycemic episodes when glucose was generally lower and episodes more prolonged, we observed a phased response whereby initial increases in heart rate were followed by a relative bradycardia. We speculated that this may reflect diurnal differences in the depth and duration of hypoglycemia, leading to differential sympathetic and parasympathetic stimulation varying over time. However, in spontaneous clinical episodes neither the depth or duration can be controlled and it is not possible to measure circulating catecholamines or electrolytes which are relevant to these responses.

In the same study, we observed a higher rate of ventricular ectopic activity during hypoglycemia compared with euglycemia; other studies have also reported higher rates of ventricular tachyarrhythmias during spontaneous hypoglycemic episodes (7). This could be related to abnormal cardiac repolarization which we have previously demonstrated during experimental and spontaneous hypoglycemia (8). Individuals with type 2 diabetes (with

varying degrees of autonomic dysfunction) exhibit longer baseline QT intervals than non-diabetic individuals which is associated with increased mortality (9). In our recent ambulatory study, we observed QT prolongation in excess of 500ms with gross morphological changes in T waves in some individuals (6).

Our primary aim was to examine changes in cardiac autonomic regulation and repolarization during controlled sustained experimental hypoglycemia as potential mechanisms that may provoke cardiac arrhythmias. We hypothesized that there are phasic changes in cardiac autonomic response that may occur during sustained hypoglycemia. Responses in T2DM individuals were compared against nondiabetic individuals as a control group.

Research Design and Methods

Participants

Twelve individuals with type 2 diabetes and no known cardiovascular disease (CVD) were recruited from Sheffield Teaching Hospitals diabetes outpatient clinics. Patients had been prescribed one or more oral hypoglycemic agents and/or glucagon-like-peptide -1 (GLP-1) analogue or insulin for less than two years. Eleven nondiabetic, age, BMI matched subjects were recruited from staff at the University of Sheffield and Sheffield Teaching Hospitals. Nondiabetic participants had fasting plasma glucose (<7mmol/L) and HbA1c <6.5% (<48mmol/mol) as measured using ion exchange high performance liquid chromatography. Participants taking beta-blocking agents or QT prolonging medications were excluded. Written informed consent was obtained from all participants and the study had received local ethics approval.

Baseline assessment

Cardiovascular autonomic reflex tests were performed as previously described (10) following a recent consensus statement (11). All patients were euglycemic at the time of autonomic function testing. Individuals with two or more abnormal cardiovagal tests were regarded as having definite cardiac autonomic neuropathy and excluded. Spontaneous cardiovagal baroreceptor sensitivity (BRS) was obtained using a Portapres (Finapres Medical Systems, Amsterdam, The Netherlands). BRS analysis was performed using dedicated software based on the sequence method (Nevrokard v5.1.3, Intellectual Services, Slovenia) (12). All participants had a normal 12 lead electrocardiogram at baseline.

Hyperinsulinemic clamp protocol

All subjects participated in paired hyperinsulinemic euglycemic and hypoglycemic studies separated by at least 4 weeks. Participants were fasted from midnight and were instructed to avoid caffeine and vigorous exercise 24 hours prior. No symptomatic hypoglycemia or a capillary blood glucose $<3.0\text{mmol/L}$ occurred in the previous 24 hours in all participants.

Participants attended the Clinical Research Facility at 8am after an overnight fast. In the diabetic group, the blood glucose was initially stabilized between 6-7mmol/L using a variable low dose intravenous insulin infusion. An intravenous cannula was inserted into the antecubital fossa of the non-dominant arm for insulin and dextrose infusion. A retrograde cannula was inserted in the non-dominant hand in a warming chamber at 55°C for blood glucose and catecholamine sampling. During euglycemic and hypoglycemic clamps in both groups, a primed continuous intravenous insulin infusion was administered at $120\text{mU/m}^2/\text{min}$ along with 20% dextrose at a variable rate, 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 2300STAT, Ohio, USA). In the hypoglycemic clamp glucose was lowered from euglycemia to 2.5mmol/L over 60 minutes (T0 to T60), thereafter maintained at 2.5mmol/L for a further 60 minutes (T60-

T120). In the euglycemic clamp, arterialized whole blood glucose was maintained at 6mmol/L for the duration of the study (120 minutes). Participants were blinded to blood glucose values.

Heart rate variability and blood pressure

ECG signals were obtained using a 3 lead ECG monitor (Ivy Cardiac Trigger Monitor 3000, Ivy Biomedical Systems Inc., Branford CT, USA), digitized at a sampling frequency of 200Hz) and recorded using WR-TestWorks™ software (version 2.4.0, WR Medical Electronics Co.). ECG recordings for HRV were performed at 30 minute intervals during the clamp study (Baseline, T30, T60, T90, T120). Heart rate variability was determined from 5 minute resting recordings with the participant supine and free breathing. Manual editing of RR intervals was performed along with visual inspection of QRS complexes to exclude any ectopic beats and artifacts. Normal RR intervals (NN) were extracted in the time domain and frequency domain heart rate variability analysis was performed in accordance with recommendations of the Taskforce on Heart Rate Variability (13). Fast Fourier Transformation was applied to 5-minute segments of RR intervals for frequency domain analysis and the power of heart rate variability was calculated within the low frequency (LF) (0.04-0.15 Hz) and high frequency (HF) bands (0.15-0.4 Hz) (13). The power in the HF band reflects parasympathetic activity. The ratio between the LF power and total power (LF power + HF power) was calculated (LFnorm), which indicates the level of sympathetic modulation in heart rate variability (14; 15).

Blood pressure was measured every 30 minutes using an automated oscillometric sphygmomanometer (DINAMAP ®GE Medical Systems Information technologies, Inc.) after at least 5 minutes in a supine position. Pulse pressure was calculated as systolic blood pressure (SBP) minus diastolic blood pressure (DBP).

Cardiac repolarization

To assess cardiac repolarization, high resolution 12 lead ECGs were recorded for five minutes at the onset (T60) and end of hypoglycaemia or euglycemia (T120) in a Mason-Likar (16) configuration with the subject lying supine. Signals were sampled at 1200 Hz and amplified using g®.USBamp amplifier (g.tec Medical Engineering, GmbH, Austria) and recorded with g®Recorder software (g.tec Medical Engineering, GmbH, Austria). All pre-processing and data analysis was performed using custom built software in Matlab (The Mathworks, Inc.). The ECG signals were bandpass filtered between 0.2 and 40 Hz. Beat averaging was then performed on 5 minute segments using template matching to improve the signal to noise ratio. The repolarization analysis was based on a composite wave, calculated from averaged beats from leads I, II and V5 (17).

Measurement of the QT interval, from Q onset to T end, was based on the tangent method and Bazett correction for heart rate was applied. As the Bazett correction may overcorrect at higher heart rates, QT was also corrected according to the nomogram method (18) which has been validated for heart rates between 40 to 120 beats per minute. In addition to QT which represents duration of repolarisation, conventional measures of T wave morphology (T wave symmetry and T wave amplitude) were also calculated based on the composite waveform as a measure of heterogeneity of repolarization. T wave symmetry was defined as the area under the T wave from T onset to T peak divided by the T wave area between T peak to T end (19). The median T wave symmetry in normal individuals is 1.5 and T symmetry close to 1.0 is abnormal and associated with increasing risk of arrhythmias (20). The normalised T wave amplitude was calculated as the ratio of the T wave amplitude during clamp at each timepoint relative to the T wave amplitude at baseline.

Additional measures of T wave morphology were calculated using the principal component analysis (PCA), derived from averaged beats from 8 ECG leads: lead I, lead II

and leads V1 to V6 as previously described (21). These provide more complex information on cardiac repolarisation that are unaffected by heart rate. PCA ratio was calculated as a ratio between the height and width of the T wave loop. An increased PCA ratio indicates a fatter T wave loop and a more complex T wave morphology which is predictive of all-cause and CV mortality in the general population (22; 23), after myocardial infarction (MI) (24; 25) and in patients with diabetes (26). The T-wave loop dispersion (TWLD) represents the length of the loop and describes the temporal variation of interlead relationships during cardiac repolarisation. The total cosine R-to-T (TCRT) was calculated as the global angle between the main QRS and T wave vectors and describes the difference between the depolarisation and repolarisation wavefronts. Decreased TWLD and TCRT have been shown to be predictive of cardiac death post MI (24) and associated with arrhythmias post MI (25).

Recordings were made at baseline, T60 and T120 minutes. Analysis of recordings was blinded to the glucose concentration.

Biochemical analysis

For measurement of catecholamines, 6ml whole blood was collected into chilled lithium heparin tubes containing 50 microlitres of EGTA/glutathione preservative and centrifuged at 4 °C, 3000 rpm for 10 minutes. The resulting supernatant was stored at -80 °C until assayed by high performance liquid chromatography. Plasma free insulin was analysed by an immunometric assay (Invitron Insulin ELISA, Invitron Ltd, Monmouth, UK) following precipitation with polyethyleneglycol. Serum potassium was analysed using an automated system (Roche Cobas, Roche Diagnostics, United Kingdom), using the direct ion selective electrode method. Biochemical parameters were measured at baseline and 120 minutes during all clamps.

Statistical analysis

Data that followed an approximate normal distribution were summarised using mean±SE unless otherwise stated, whilst skewed data were summarised using the median (interquartile range).

Within each participant group, autonomic and repolarization parameters were analyzed using two way repeated measures ANOVA where both time and glycemic arm were specified as repeated factors. The Greenhouse Geisser correction was applied where sphericity was violated. Planned contrasts were made versus baseline and also between euglycemia and hypoglycemia at equivalent time points with Sidak's correction for multiple comparisons. To compare changes in repolarization in diabetic versus nondiabetic subjects, two way repeated ANOVA was performed with glycemic arm as a repeated factor. Planned contrasts were made for the effect of group and glycemic arm with Sidak's correction for multiple comparisons. Catecholamines, glucose and potassium at T120 were compared under euglycemic and hypoglycaemic conditions using a two way ANOVA with planned contrasts for the effect of the group (diabetes versus nondiabetic) and glycemic condition with Sidak's correction for multiple comparisons. A nonparametric Kruskal-Wallis test was used to compare free insulin levels at T120 under euglycaemia versus hypoglycaemia and between groups. Missing data were dealt with using casewise deletion. Analysis was performed using SPSS (version 20.0, IBM, Chicago, Illinois) and GraphPad Prism (version 6.04, Graphpad Prism Inc). A p value of <0.05 was deemed statistically significant.

Results

Participant characteristics

Participant characteristics are shown in Table 1. Patients with Type 2 diabetes were similar in age and BMI compared to the nondiabetic group. Five patients were taking oral

hypoglycemic agents only, 5 were taking a combination of oral hypoglycemic agents and a GLP-1 analogue and two had been taking oral hypoglycemic agent and basal insulin for less than two years. Two diabetic patients were on ACE inhibitors and remained on them throughout the study. Diabetes patients tended to have higher baseline heart rates, blood pressure and lower HRV and BRS. Parameters of T wave repolarisation, including T wave amplitude, TWLD, PCA ratio and TCRT tended to be lower in the diabetic group at baseline compared with nondiabetic subjects; however, other measures, including QTc and T wave symmetry, were similar.

Hyperinsulinemic clamp

Target arterialized blood glucose levels are shown in Fig 1. Blood glucose concentrations were 5.81 ± 0.29 mmol/L and 5.96 ± 0.18 mmol/L at the end of euglycemic clamp in diabetic and nondiabetic groups respectively with no significant differences between groups (mean difference -0.15, 95% CI -0.85 to 0.55 mmol/L, $p = 0.86$). At the end of hypoglycemia these were 2.56 ± 0.22 mmol/L and 2.56 ± 0.09 mmol in diabetic and nondiabetic groups respectively (mean difference 0.0, 95% CI -0.70 to 0.70 mmol/L, $p > 0.99$). Free insulin levels at 120 minutes were Median (IQR) 576 (468-627) pmol/L during euglycemia and 689 (477-1076) pmol/L during hypoglycemia in the diabetic group and 865 (509-952) pmol/L in the euglycemic and 665 (468-967) pmol/L in the hypoglycemic arms of the nondiabetic group respectively which were comparable across all four conditions ($p = 0.23$).

Cardiac autonomic function

Heart rate

Baseline heart rates were higher among diabetic subjects. There were no significant changes in heart rate during euglycemia in either group (Fig 2 top panel, Table 2). In nondiabetic participants, heart rate increased from T60 and up to the end of the hypoglycemic clamp. Diabetic individuals showed a delayed maximal increase in heart rate at T90, after thirty

minutes at hypoglycemic levels. However, there was a subsequent fall in heart rate towards baseline at T120, after sustained hypoglycemia of 1 hour (Fig 2 top panel).

Heart rate variability

In nondiabetic individuals, the frequency domain measure of vagal activity, log HF, decreased from T60, coincident with the rise in heart rate, and remained decreased till the end of hypoglycemic clamp (Fig 2 middle panel). In diabetic subjects, during hypoglycemia, log HF decreased maximally at T90. However, at T120 log HF returned to baseline levels suggesting reactivation of vagal tone coincident with a decrease of heart rate towards baseline. This did not occur in nondiabetic participants (Fig 2 middle panel, Table 2). Similar trends were observed by time domain analyses. RMSSD, a time-domain measure of vagal activity decreased at T90 following hypoglycemia but returned to baseline levels at T120 in diabetic subjects (Supplementary material, Table 1). In nondiabetic participants, normalized low frequency power, a marker of relative sympathetic contribution, increased at T30 during hypoglycemia compared with a decrease in the euglycemic clamp. In diabetic participants LFnorm did not change significantly over time in either glycaemic condition (Fig 2 bottom panel).

Blood pressure

Systolic, diastolic blood pressures and pulse pressure did not change significantly during euglycemia in either group (Fig 3 top panel, Table 2). SBP did not change during hypoglycemia in nondiabetic participants but tended rise in SBP in diabetic participants (Table 2). There was a smaller decline in DBP among T2DM as compared with nondiabetic individuals (maximum change $\Delta-6\pm 10$ mmHg versus $\Delta-11.3\pm 5.93$ mmHg respectively, Fig 3 middle panel). DBP continued to decline till the end of the hypoglycemic clamp in nondiabetic participants, with the minimum DBP occurring at median (IQR) 120 (105-120) minutes compared to 90 (82.5-90) minutes in diabetic group. During hypoglycemia, there

was a significant increase in pulse pressure in diabetic participants ($p=0.02$ for interaction between time and glycemc arm) which was more abrupt than in nondiabetic individuals (Fig 3 bottom panel).

Cardiac repolarization

Cardiac repolarization data are presented for 10 diabetic subjects and 9 nondiabetic subjects (Table 3 and Figure 4). In two diabetic and two nondiabetic subjects, repolarization analyses could not be performed at T120 due to technical issues relating to the ECG data which occurred at random.

QTc

There were significant, relatively small increases in corrected QT duration by Bazett's formula (QTcB) in both diabetic and nondiabetic participants during euglycemic clamp at T120 (Table 3 and Figure 4). Compared with euglycemia, QTcB increased significantly more during hypoglycemia in both groups (Fig 4). QTc B increased by $\Delta 57\pm 5$ ms in nondiabetic participants and $\Delta 76 \pm 20$ ms in diabetic participants during hypoglycemia. Although QTcB tended to increase to a larger extent in diabetic subjects, the difference did not reach statistical significance (mean difference 19, 95% CI -22 to 59 ms, $p= 0.50$). The maximum increase occurred at T120 in both groups. QT corrected by the normogram method (QTcN) exhibited similar trends, increasing by $\Delta 12\pm 8$ versus $\Delta 68\pm 21$ ms during euglycemia and hypoglycemia respectively among diabetic subjects ($p = 0.003$ for glycemc arm). QTcN increased by $\Delta 18\pm 11$ versus $\Delta 56\pm 13$ ms during euglycemia and hypoglycemia respectively in nondiabetic subjects ($p=0.06$ for glycemc arm), with no significant differences between groups in euglycemic or hypoglycemic responses.

T wave morphology

T wave symmetry, an index of T wave morphology fell by a similar extent in nondiabetic subjects during euglycemia and hypoglycemia (Fig. 4 middle panel). However, in diabetic subjects T symmetry decreased significantly more during hypoglycemia compared with euglycemia, resulting in more abnormally shaped, symmetrical T waves. There was a significant difference in the change in T symmetry during hypoglycemia between diabetic and nondiabetic groups (mean difference $\Delta - 0.33$, 95% CI -0.62 to -0.03, $p= 0.03$). The amplitude of the T waves, normalised to the baseline (Tamp norm) fell during both euglycemia and hypoglycemia in both groups by a similar extent (Table 3). Repolarization and wavefront propagation parameters derived from principle component analysis are shown in Table 3. The PCA ratio, which describes the complexity of the T wave morphology across the 12 ECG leads, did not change during euglycemia in either group. The PCA ratio significantly increased during hypoglycemia in diabetic participants ($\Delta 0.16\pm 0.05$), indicating higher complexity, compared with no change in nondiabetic participants ($\Delta 0.01\pm 0.05$) ($p=0.03$ for difference between groups). The T wave loop dispersion (TWLD), which indicates temporal variation in interlead relationships, decreased similarly across glycaemic conditions in both groups. No significant changes occurred for the wavefront direction descriptor (TCRT) in either glycaemic arm for both groups.

Biochemical measurements

Baseline levels of catecholamines were similar between diabetic and nondiabetic subjects ($p=0.99$). Catecholamines were significantly higher at the end of hypoglycemia versus euglycemia in both groups (all $p<0.01$) (Table 4). Epinephrine levels at end of hypoglycemia were 3.05 ± 0.71 nmol/L and 3.83 ± 0.85 nmol/L in diabetic and nondiabetic individuals respectively, a difference which was not statistically significant ($p=0.54$). Peak norepinephrine at the end of hypoglycemia were 2.45 ± 0.23 nmol/L and 2.69 ± 0.44 nmol/L in diabetic and nondiabetic subjects respectively, a difference between the two groups which

was not statistically significant (mean difference -0.24, 95% CI -0.11 to 0.58 nmol/L, $p=0.75$).

In the nondiabetic group, potassium levels were not significantly different during hypoglycemia versus euglycemia (3.48 ± 0.11 mmol/L versus 3.69 ± 0.14 mmol/L respectively, $p = 0.40$). However, in the diabetic group, potassium was significantly lower at the end of hypoglycemic clamp compared with euglycemia (3.27 ± 0.09 mmol/L versus 3.72 ± 0.13 mmol/L, $p=0.02$). Potassium levels at the end of hypoglycemic clamp tended to be lower in diabetic subjects but the difference did not reach statistical significance (mean difference -0.21, 95% CI -0.60 to 0.17 mmol/L, $p = 0.39$).

Discussion

The main findings of this study are as follows. 1) In individuals with type 2 diabetes, hypoglycemia resulted in transient increases in heart rate with coincident vagal withdrawal, followed by a relative decrease in heart rate during more sustained hypoglycemia of one hour accompanied by vagal reactivation (shown by increased HF power and RMSSD). In nondiabetic subjects this did not occur and there was continued vagal inhibition throughout hypoglycemia. 2) There were greater repolarization abnormalities as shown by QTc and T wave morphology measures in type 2 diabetes subjects despite similar levels of hypoglycemia.

Diabetic participants exhibited impaired baseline autonomic function compared with nondiabetic subjects, with higher resting heart rates and lower HRV although none had frank autonomic neuropathy by formal testing of cardiovascular reflexes. In the nondiabetic group, subjects demonstrated continued vagal withdrawal as evidenced by decreased high frequency HRV throughout hypoglycemia. This is consistent with previous studies of experimental hypoglycaemia in healthy and type 1 diabetic participants (27; 28). In contrast, following

initial increases in heart rate and vagal withdrawal, we observed a slowing of heart rate in diabetic subjects after 60 minutes of hypoglycemia (T120) with vagal reactivation. We observed a similar phasic response in heart rate during spontaneous prolonged hypoglycemia in type 2 diabetes patients in our previous ambulatory study with some patients demonstrating profound bradycardia accompanied by ectopic activity (6). Reasons for the differences between the diabetic and non-diabetic groups in this study are unclear. The arterial baroreflex is a negative feedback reflex that regulates arterial pressure around an operating point. Baroreceptor sensitivity describes the degree to which the heart rate increases or decreases in response to a given change in mean arterial pressure as a consequence of the baroreflex. In detailed experimental studies of nondiabetic and type 1 diabetic participants, baroreceptor sensitivity has been shown to fall during experimental hypoglycemia along with resetting of the working range to higher heart rates (29; 30). It is possible that in diabetic subjects, a failure of baroreceptors to reset to higher heart rates could lead to an increase in vagal restraint in the face of sustained sympathetic stimulation, rising SBP and pulse pressure as the operating point remains at baseline levels. Impaired acute resetting of the baroreceptor operating point has also been reported among hypertensive individuals (31). Future studies could examine this hypothesis with concurrent measurements of baroreceptor function during experimental hypoglycaemia in type 2 diabetic patients.

During hypoglycaemia, maximal changes in heart rate and decline in high frequency HRV occurred later in diabetic individuals at T90 as compared with nondiabetic individuals in which they occurred at T30. Delayed increments in heart rate and cardiac output responses during hypoglycaemia have previously been reported in intensively treated type 1 diabetic individuals, and may reflect blunted counterregulatory hormonal responses (32). However, in this study epinephrine and norepinephrine levels during hypoglycaemia were similar between type 2 diabetic and nondiabetic groups.

We have also shown greater abnormalities in duration and heterogeneity of repolarization during hypoglycemia in diabetic participants. Changes in the morphology of the T wave, both symmetry (T wave symmetry) and complexity across the leads (PCA ratio) were greater during hypoglycemia in diabetic subjects. This suggests greater dispersion of repolarisation, which is proarrhythmic and has been linked with increased cardiovascular risk (22; 23; 33). These T wave changes during hypoglycaemia have not been previously described in the type 2 diabetic population and the differences compared to non-diabetic individuals are noteworthy.

A stronger sympathoadrenal response is unlikely to be the primary explanation as the peak epinephrine levels were similar in both groups but might be the result of greater declines in serum potassium, which we observed during hypoglycemia in diabetic subjects. It is possible that autonomic dysfunction in individuals with diabetes might, through denervation metabolic adrenergic hypersensitivity lead to a greater beta-adrenoreceptor mediated fall in potassium. This would be analogous to those observed in other responses, such as free fatty acid production during experimental hypoglycaemia among subjects with type 1 diabetes and autonomic dysfunction (34). This mechanism could be explored in future experimental studies involving potassium clamping or adrenergic blockade.

Previous studies have shown a relationship between potassium and T wave amplitude during hyperinsulinemic hypoglycaemia (35; 36). Our group has previously shown that replacement of potassium during hyperinsulinemic clamps reversed increases in QT dispersion but not QTc duration during hypoglycemia in nondiabetic individuals (37). However, we cannot exclude the possibility that this could also be related to intrinsic abnormalities in repolarisation substrate among diabetic participants that are exacerbated by hypoglycemia. Interestingly, differences have also been reported in animal models of experimental hypoglycemia where diabetic mice were at greater risk of arrhythmias than

nondiabetic counterparts (38). The underlying mechanisms require further testing in experimental studies.

One strength of the present study is that cardiovascular variables were measured at multiple time points during hypoglycemia. Previous studies have shown increases (39), decreases (35) or no change (36) in vagal power during hyperinsulinemic hypoglycaemia in type 1 diabetic and nondiabetic individuals. These discrepant data may reflect measurements at a single, often variable timepoint, an explanation supported by the present study, indicating that changes in cardiac autonomic tone appear phasic and depend on duration of hypoglycemia.

Our study had some limitations. It proved challenging to control for the depth and frequency of breathing during HRV recordings which may affect the HF component of HRV, an index of vagal activity (14). In the original protocol, participants were asked to pace their breathing at 12 breaths/minute (0.2 Hz) by following a timed visual display, but failed to comply consistently during hypoglycemia, perhaps due to cognitive impairment. However, the mean spontaneous respiratory frequencies across five minutes of recording were consistently situated in the middle of the HF frequency band, around 0.25 Hz. Differences between experimental conditions were small and unlikely to significantly affect the HRV spectra and the estimation of vagal activity and its changes during the protocol (Supplementary material Table 2).

There are relevant differences between our experimental model of sustained hyperinsulinemic hypoglycaemia and spontaneous clinical episodes. In this study, although heart rate tended to fall towards baseline during sustained hypoglycemia with vagal reactivation, we did not observe profound bradycardia as we previously reported during spontaneous prolonged nocturnal episodes in an observational clinical study (6). These

differences could be related to circadian variation as experiments were conducted during the day. During the night, background vagal tone tends to be greater and sympatho-adrenal responses to hypoglycaemia are suppressed during sleep which may lead to greater vagal predominance (40). Spontaneous nocturnal episodes were generally more prolonged and reached lower glucose values than those induced during the current study (which might also explain the relatively modest changes in heart rate). We limited the duration (60 minutes) and depth (2.5 mmol/L) of experimental hypoglycemia for ethical reasons and it is possible that duration and depth of hypoglycaemia may also play a role. Experimental hypoglycemia is associated with greater decline in potassium and catecholamine flux which could lead to exaggerated repolarisation abnormalities. However, the magnitude of QT prolongation observed here, has been reported in clinical settings. In a retrospective cohort study of type 2 diabetes patients presenting to the emergency room with severe hypoglycemia, 14% had QTc in excess of 500ms on presentation and up to a third had hypokalemia (41).

The present study suggests that abnormal repolarization and autonomic tone interact differentially depending upon duration of hypoglycemia. Thus autonomic dysfunction in the patients with diabetes may contribute to the different time-course in vagal tone between the two groups as well as the more pronounced abnormalities in some measures of cardiac repolarization. This interaction might also explain the different patterns of arrhythmias we previously observed during our ambulatory clinical study (6). Using a rodent model of experimental hypoglycemia, Reno and colleagues have observed arrhythmias ranging from QT prolongation, ventricular ectopy to heart block that was dependent on duration and severity of experimental hypoglycemia in mice (38). In the present study, sustained experimental hypoglycemia was associated with later reactivation of vagal tone in diabetic subjects, despite sustained increased in circulating catecholamines. Of note this occurred when QT prolongation and T wave changes were at their maximal.

In conclusion, our data indicate that cardiac autonomic regulation during hypoglycemia appears to be time-dependent and different between those with type 2 diabetes and nondiabetic subjects. This may be explained by differential hemodynamic effects in response to hypoglycemia. In type 2 diabetes, the initial heart rate increment to hypoglycemia was delayed and there was reactivation of vagal activity during sustained hypoglycemia. Individuals with type 2 diabetes also exhibited greater repolarization abnormalities compared to those without diabetes, despite a similar hypoglycemic stimulus and comparable catecholamine levels. These mechanisms could contribute to arrhythmias that have been reported during clinical hypoglycemic episodes. Our data provide further evidence supporting a possible relationship between hypoglycemia and increased cardiovascular mortality in type 2 diabetes and highlight the potential contribution of autonomic dysfunction.

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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. AB developed the ECG related methodology and software, analyzed the data, helped to collect the data and edited the manuscript. EW and ALS helped to collect the data and edited the manuscript. IAM analyzed the catecholamine data and reviewed the manuscript. JF advised on statistical analysis and reviewed the manuscript. PS and SRH designed the study, reviewed the data and edited and redrafted the manuscript. This study was funded by the National Institute of Health Research, UK. We would like to thank the Diabetes department and the Clinical Research Facility in Sheffield Teaching Hospitals for their assistance, and all patients who have kindly given their time towards this study. SRH has served as a Consultant for Sanofi Aventis and Boeringher Ingelheim; has served as an advisory board panel member for Eli Lilly & Co, Novo Nordisk A/S, Lifescan Inc. and Takeda; and has attended Speakers' Bureaux for Astra Zeneca, Novo Nordisk, Eli Lilly & Co and MSD. The other authors have no conflicts of interest to declare.

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Table 1 Participant characteristics

Data are mean \pm SE except age as median (interquartile range). HbA1c – glycated hemoglobin SBP - systolic blood pressure; DBP - diastolic blood pressure; HR - heart rate; BMI - body mass index; QTcB – corrected QT by Bazett's formula; QTcN – corrected QT by nomogram method; TWLD - T wave loop dispersion; TCRT – total cosine R to T; PCA ratio – principal component analysis ratio; SDNN – standard deviation of normal RR intervals; RMSSD - root mean square of the difference of successive R-R intervals; log HF - log high frequency power; LFnorm- normalized low frequency power; BRS – baroreceptor sensitivity

	nonDM n=11	T2DM n=12
Age (years)	52 (34-63)	53.5 (37-64)
Male (M/F)	5/6	9/3
BMI (kg/m ²)	31 \pm 2	34 \pm 1
Duration of diabetes (years)	n/a	11 \pm 2
HbA1c (%)	5.5 \pm 0.3	7.8 \pm 0.4
(mmol/mol IFCC)	34 \pm 3	62 \pm 4
SBP (mmHg)	131 \pm 5	135 \pm 4
DBP (mmHg)	72 \pm 3	82 \pm 3
HR (bpm)	64 \pm 2	78 \pm 2
Repolarization		
QT (ms)	406 \pm 9	381 \pm 8
QTcB (ms)	412 \pm 8	417 \pm 6
QTcN (ms)	410 \pm 7	406 \pm 4
T wave amplitude (μ V)	466 \pm 84	399 \pm 35
T wave symmetry	1.44 \pm 0.06	1.55 \pm 0.06
TWLD	1415 \pm 257	1279 \pm 75
TCRT	0.78 \pm 0.06	0.69 \pm 0.08
PCA ratio	0.10 \pm 0.03	0.08 \pm 0.02
Heart rate variability		
SDNN (ms)	48.2 \pm 4.4	27.4 \pm 3.4
RMSSD (ms)	32.9 \pm 4.6	15.5 \pm 7.94
Log Total power (ms ²)	2.90 \pm 0.11	2.34 \pm 0.10
Log HF (ms ²)	2.32 \pm 0.14	1.85 \pm 0.11
LFnorm	0.70 \pm 0.04	0.65 \pm 0.04
BRS (ms/mmHg)	11.51 \pm 2.31	8.59 \pm 1.18

Table 2 Heart rate variability and blood pressure during euglycemia and hypoglycemia in type 2 diabetes and nondiabetic subjects

euglycemia (EU) hypoglycemia (HYPO) HR - heart rate; bpm - beats per minute; log HF - high frequency power; SBP systolic blood pressure; DBP diastolic blood pressure. Data Mean \pm SE

Timepoint	nonDM		DM	
	EU	HYPO	EU	HYPO
HR (bpm)				
0	62 \pm 3	62 \pm 3	73 \pm 3	72 \pm 3
30	63 \pm 3	62 \pm 3	75 \pm 3	73 \pm 3
60	61 \pm 3	67 \pm 4	76 \pm 3	75 \pm 3
90	64 \pm 3	64 \pm 4	74 \pm 3	77 \pm 3
120	63 \pm 3	64 \pm 3	73 \pm 3	71 \pm 2
Log HF				
0	2.18 \pm 0.16	2.36 \pm 0.13	1.87 \pm 0.14	1.89 \pm 0.18
30	2.33 \pm 0.14	2.29 \pm 0.13	1.71 \pm 0.17	1.86 \pm 0.17
60	2.30 \pm 0.13	2.14 \pm 0.15	1.75 \pm 0.15	1.65 \pm 0.19
90	2.20 \pm 0.13	2.15 \pm 0.15	1.78 \pm 0.15	1.56 \pm 0.18
120	2.22 \pm 0.12	2.15 \pm 0.15	1.82 \pm 0.14	1.79 \pm 0.23
SBP (mmHg)				
0	128 \pm 6	121 \pm 4	136 \pm 5	136 \pm 5
30	129 \pm 8	125 \pm 6	134 \pm 4	135 \pm 5
60	126 \pm 5	126 \pm 6	138 \pm 6	141 \pm 5
90	128 \pm 5	128 \pm 7	139 \pm 5	144 \pm 7
120	126 \pm 5	124 \pm 7	138 \pm 6	134 \pm 4
DBP (mmHg)				
0	75 \pm 4	74 \pm 3	77 \pm 2	77 \pm 2
30	75 \pm 4	69 \pm 4	75 \pm 3	77 \pm 2
60	75 \pm 3	68 \pm 3	77 \pm 3	73 \pm 3
90	73 \pm 3	67 \pm 3	76 \pm 2	71 \pm 4
120	73 \pm 3	62 \pm 3	78 \pm 4	72 \pm 3

Table 3: Change in cardiac repolarization among diabetic and nondiabetic individuals during euglycemia and hypoglycemia

Change in measures of repolarisation at time 120 as compared with baseline. Two way repeated measures ANOVA with multiple comparisons between glyceamic arms (euglycemia versus hypoglycaemia) and between subjects groups; * indicates significant difference between diabetic and nondiabetic participants within each glyceamic arm. Data from 9 nondiabetic (nonDM) and 10 diabetic (DM) subjects. Data (Mean \pm SE). nonDM – nondiabetic; DM – diabetic; QTc- corrected QT duration; Tamp norm- normalised T wave amplitude; TWLD - T wave loop dispersion; PCA ratio – principle component analysis ratio; TCRT - total cosine R to T

	Change from baseline		EU vs HYPO
	EU	HYPO	Adjusted p value
QTc (ms)			
nonDM	$\Delta 21 \pm 4$	$\Delta 57 \pm 5$	0.08
DM	$\Delta 14 \pm 4$	$\Delta 76 \pm 20$	0.0009
T symmetry			
nonDM	$\Delta -0.22 \pm 0.04$	$\Delta -0.20 \pm 0.06$	0.98
DM	$\Delta -0.21 \pm 0.06$	$\Delta -0.52 \pm 0.14^*$	0.09
Tamp norm			
nonDM	$\Delta -0.41 \pm 0.06$	$\Delta -0.32 \pm 0.18$	0.74
DM	$\Delta -0.43 \pm 0.04$	$\Delta -0.57 \pm 0.06$	0.42
TWLD			
nonDM	$\Delta -476 \pm 64$	$\Delta -404 \pm 243$	0.90
DM	$\Delta -452 \pm 41$	$\Delta -576 \pm 53$	0.70
PCA ratio			
nonDM	$\Delta 0.03 \pm 0.02$	$\Delta 0.01 \pm 0.05$	0.90
DM	$\Delta 0.04 \pm 0.02$	$\Delta 0.16 \pm 0.05^*$	0.03
TCRT			
nonDM	$\Delta -0.01 \pm 0.04$	$\Delta -0.002 \pm 0.08$	0.93
DM	$\Delta -0.05 \pm 0.17$	$\Delta 0.02 \pm 0.15$	0.99

Table 4: Biochemical parameters at end of clamp in diabetic and nondiabetic individuals

Data (Mean \pm SE). p value comparison between EU (euglycemia) and HYPO (hypoglycemia) by two way ANOVA with multiple comparisons between glyceamic arms and

between subject groups. There were no significant differences between nondiabetic (nonDM) and diabetic (DM) groups under either glycemic condition.

	EU	HYPO	EU vs HYPO Adjusted p value
Potassium (mmol/L)			
nonDM	3.69±0.14	3.48±0.11	0.40
DM	3.72±0.13	3.27±0.09	0.02
Epinephrine (nmol/L)			
nonDM	0.14±0.02	3.83±0.85	0.0002
DM	0.16±0.05	3.05±0.71	0.003
Norepinephrine (nmol/L)			
nonDM	1.56±0.12	2.69±0.44	0.002
DM	1.27±0.09	2.45±0.23	0.009

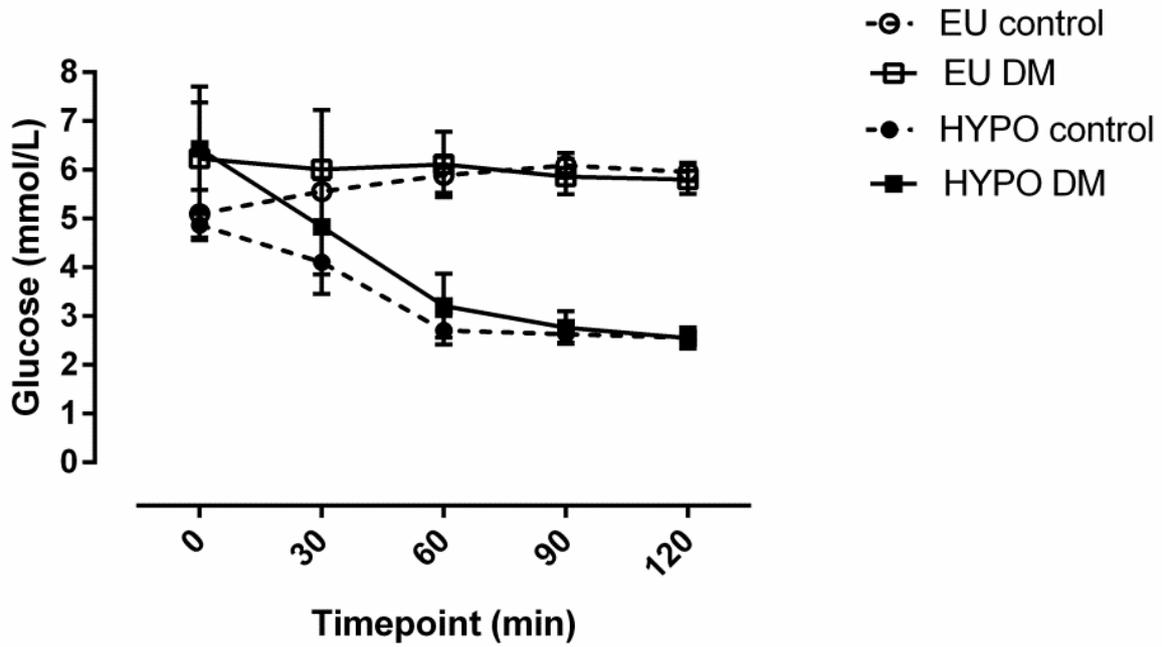


Figure 1: Arterialised blood glucose during hyperinsulinemic euglycemic and hypoglycemic clamps

Open circle - Euglycemic clamp nondiabetic group; open square euglycaemic clamp diabetic group; closed circle – hypoglycaemic clamp nondiabetic group; closed square hypoglycaemic clamp diabetic group; Data mean (SE)

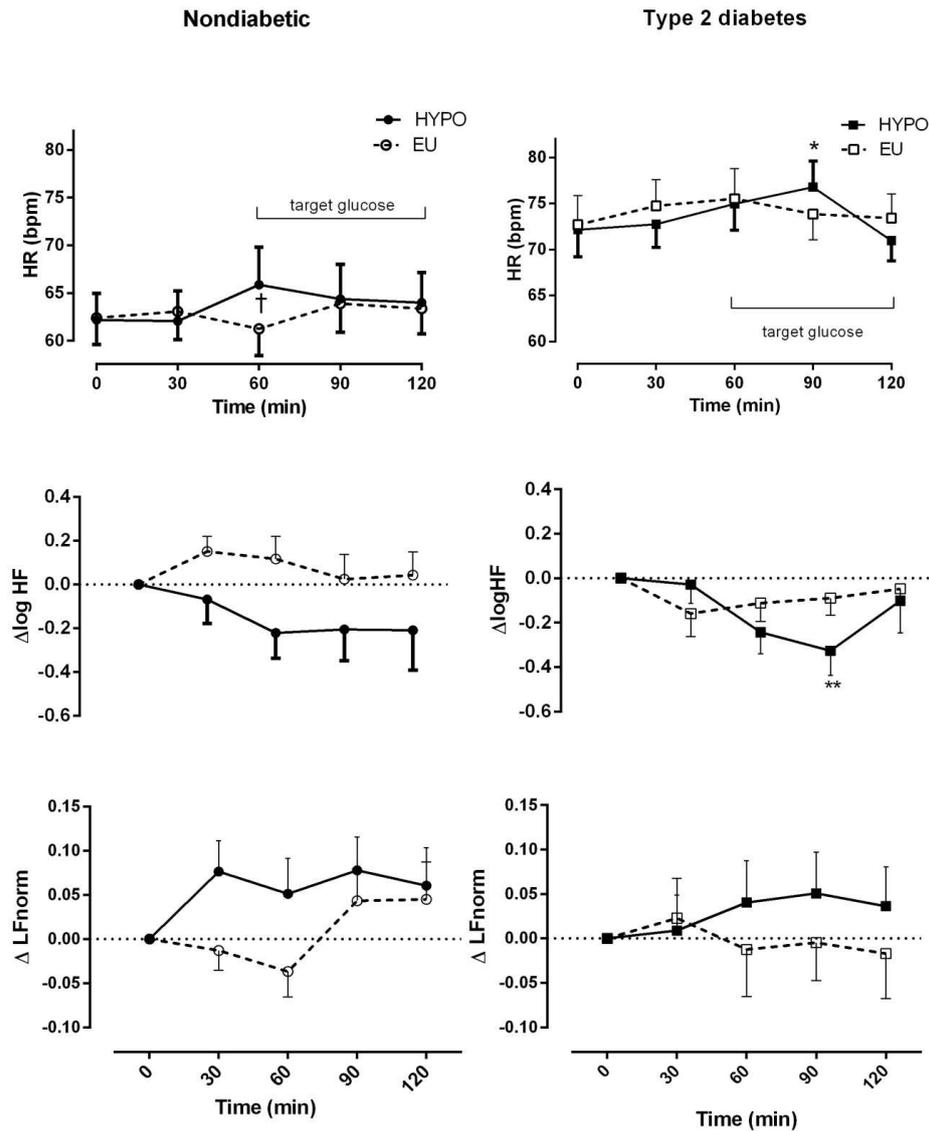


Figure 2: Heart rate and change in heart rate variability during hypoglycemia in type 2 diabetes and nondiabetic participants

* $p < 0.05$ ** < 0.01 compared with baseline † $p < 0.05$ euglycemia (EU) vs hypoglycemia

(HYPO); HR - heart rate; bpm - beats per minute; log HF - high frequency power, index of parasympathetic activity; LFnorm - normalized low frequency power, index of sympathetic modulation; Data Mean (SE). Open circle - Euglycemic clamp nondiabetic group; open square euglycaemic clamp diabetic group; closed circle – hypoglycaemic clamp nondiabetic group; closed square - hypoglycaemic clamp diabetic group

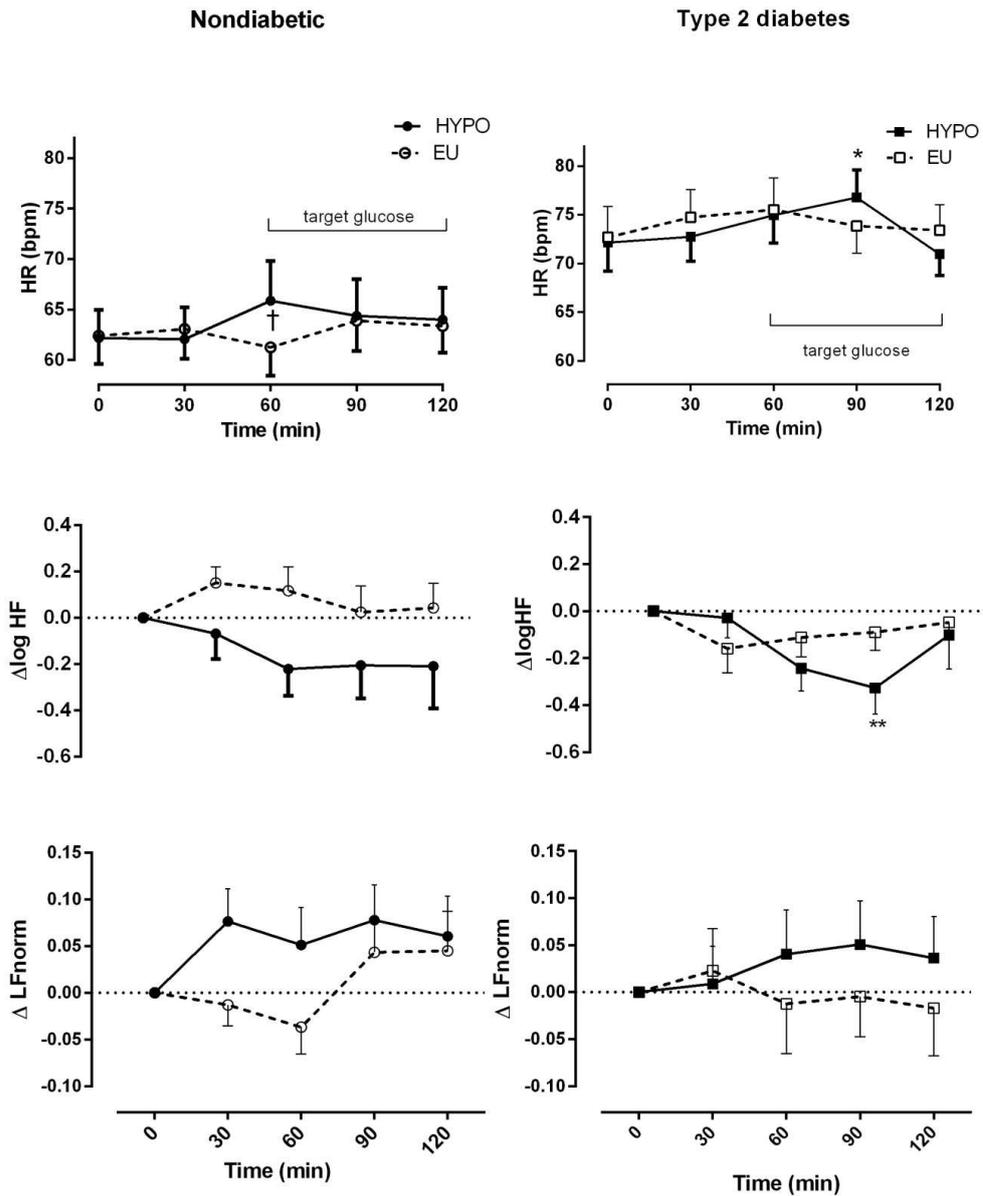


Figure 3: Blood pressure during hypoglycemia and euglycemia in type 2 diabetes and nondiabetic participants

Data Mean (SE). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ compared with baseline.

† $p < 0.05$, †† $p < 0.01$, †††† $p < 0.0001$ euglycemia (EU) vs hypoglycemia (HYPO) at equivalent

timepoint. SBP - systolic blood pressure; DBP - diastolic blood pressure; MAP - mean

arterial pressure. Data are mean (SE). Open circle - Euglycemic clamp nondiabetic group;

open square euglycaemic clamp diabetic group; closed circle – hypoglycaemic clamp

nondiabetic group; closed square - hypoglycaemic clamp diabetic group

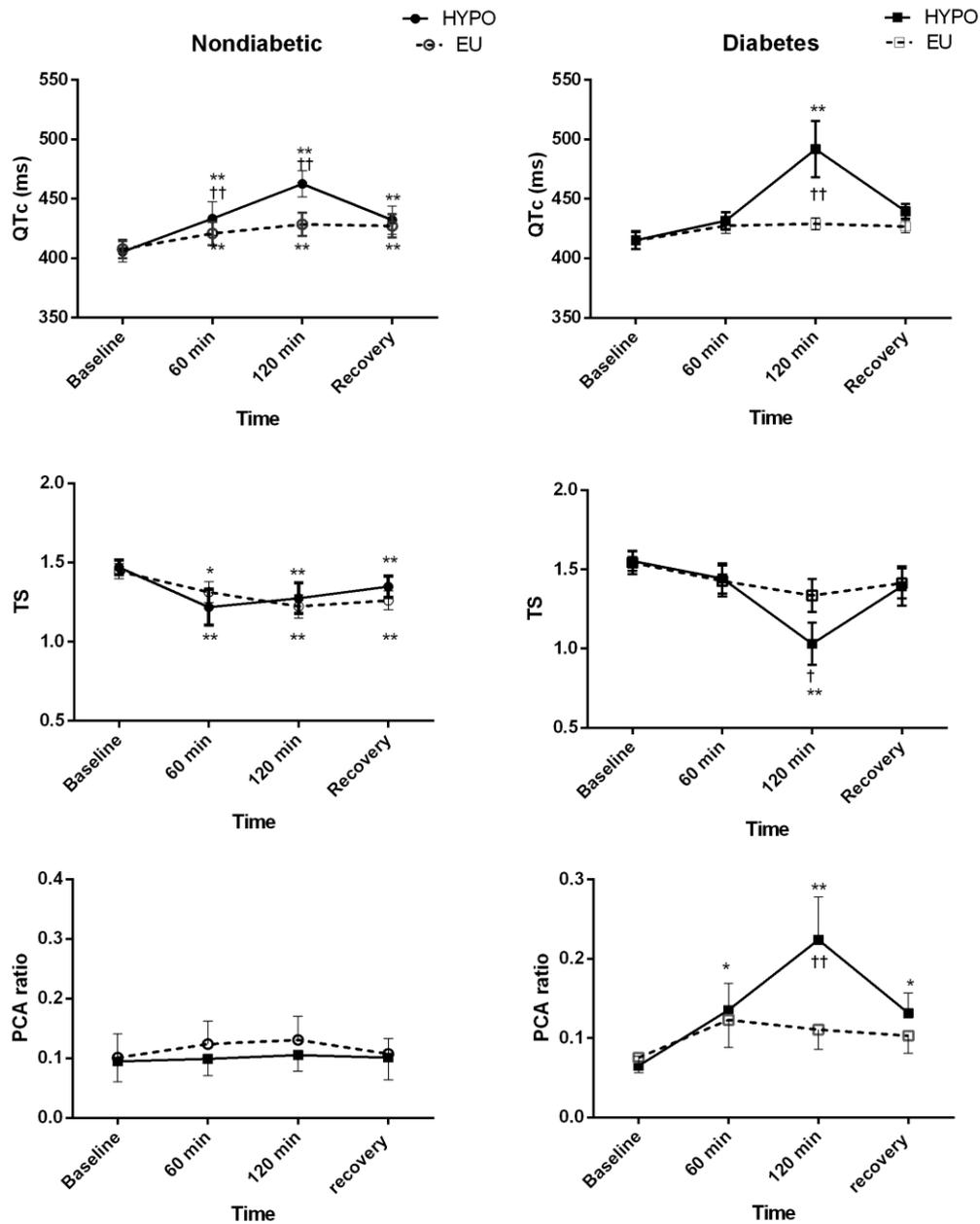


Figure 4: Changes in cardiac repolarisation during hypoglycaemic and euglycaemic clamps in diabetic and nondiabetic subjects

Data Mean (SE). * $p < 0.05$, ** $p < 0.01$ compared with baseline. † $p < 0.05$, †† $p < 0.01$

euglycemia (EU) vs hypoglycemia (HYPO) at equivalent timepoint. Data from 9 nondiabetic and 10 diabetic participants. QTcB – corrected QT duration using Bazett’s correction; TS – T wave symmetry, index of T wave morphology; PCA ratio – Principal component analysis ratio, index of complexity of the T wave morphology between the 12 leads.