Diazoxide improves hormonal counterregulatory responses to acute hypoglycemia in long-standing Type 1 Diabetes

Short Title; Diazoxide improves hypoglycemia responses

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ABSTRACT

Individuals with long-standing type 1 diabetes (T1D) are at increased risk of severe hypoglycemia secondary to impairments in normal glucose counterregulatory responses (CRR). Strategies to prevent hypoglycemia are often ineffective highlighting the need for novel therapies. ATP-sensitive potassium (K\textsubscript{ATP}) channels within the hypothalamus are thought to be integral to hypoglycemia detection and initiation of CRRs, however, to date this has not been confirmed in human subjects. In this study, we examined whether the K\textsubscript{ATP}-channel activator Diazoxide was able to amplify the CRR to hypoglycemia in T1D subjects with long-duration diabetes. A randomized, double-blind, placebo-controlled cross-over trial using a stepped hyperinsulinemic hypoglycemia clamp was performed in 12 T1D subjects with prior ingestion of Diazoxide (7mg/kg) or placebo. Diazoxide resulted in 37% increase in plasma levels of epinephrine and a 44% increase in plasma norepinephrine during hypoglycemia compared with placebo. In addition, a subgroup analysis revealed that participants with E23K polymorphism in the K\textsubscript{ATP} channel had a blunted response to oral diazoxide. This study has therefore shown for the first time the potential utility of K\textsubscript{ATP} channel activators to improve counterregulatory responses to hypoglycemia in individuals with T1D, and moreover that it may be possible to stratify therapeutic approaches by genotype.
Introduction

The goal of insulin therapy in Type 1 Diabetes (T1D) is ultimately to restore glucose levels to the non-diabetic physiological range in order to prevent the development of micro- and macroangiopathy. Intensive insulin therapy (IIT), using multi-injection regimens or continuous subcutaneous insulin infusion goes some way towards achieving this, but is associated with a rapidly increasing rate of severe hypoglycemia as glycemic targets are achieved [1]. The high rates of severe hypoglycemia with IIT reflect the limitations of current insulin replacement therapy as well as the presence in almost all individuals with T1D of defects in the normal homeostatic (counterregulatory) response to hypoglycemia [2]. Defective counterregulation results primarily from a failure to suppress endogenous insulin secretion, an inability to stimulate alpha-cell glucagon release, and suppression of the catecholaminergic response to hypoglycemia [2]. The latter defect is associated with impaired awareness of hypoglycemia (IAH) and together markedly increases (25-fold) an individual’s risk of severe hypoglycemia [3]. Given that dysregulation of alpha-cell glucagon release is thought to result in the main from an intra-islet defect secondary to beta-cell destruction [2] research efforts have focused on understanding the mechanisms that contribute to the suppression of catecholaminergic and symptom responses to hypoglycemia in T1D in the hope that this would lead to novel therapeutic strategies or interventions.

The seminal work by Heller and Cryer [4] established that even a single episode of hypoglycemia resulted in reduced symptom and catecholaminergic counterregulatory responses (CRR) during an equivalent episode of hypoglycemia induced within 24 hours. Recurrent exposure to hypoglycemia is thought to therefore underlie the pronounced suppression of symptom and catecholaminergic responses seen following IIT [5], a hypothesis supported by the observation in animals of greater suppression of hypoglycemic counterregulation following more frequent hypoglycemia [6] and conversely, that strict
avoidance of hypoglycemia can, at least in part, restore symptom and catecholaminergic responses to hypoglycemia in T1D [7]. Unfortunately, hypoglycemia avoidance in clinical practice is difficult to achieve and despite the positive benefits seen in RCTs from structured educational programs [8, 9], continuous glucose monitoring [10] and sensor augmented strategies [11] the frequency of SH in routine clinical practice remains relatively unchanged in the last 2 decades [12, 13]. This highlights the need for novel approaches to the prevention of severe hypoglycemia and restoration of IAH.

One potential target for therapeutic intervention is the ATP-sensitive potassium (K\textsubscript{ATP}) channel. The K\textsubscript{ATP} channel is a ligand-gated ion channel composed of 4 inward-rectifier potassium ion channels and 4 sulphonylurea receptor subunits (SUR-1, SUR 2-A and SUR 2-B) and plays a critical role in transducing changes in cellular energy status into changes in action potential firing. Glucose sensing hypothalamic neurons important to the detection of hypoglycemia express SUR-1 containing K\textsubscript{ATP} channels [14], and mice lacking functional K\textsubscript{ATP} channels display abnormal glucose sensing [15]. In rats, direct \textit{in vivo} local application of SUR-1 activators to the ventromedial hypothalamus (VMH) amplifies [16] while local SUR-1 inhibition suppresses [17] the counterregulatory response to acute hypoglycemia in rodents. Moreover, systemic delivery of a SUR-1 selective activator amplified counterregulatory responses during hyperinsulinaemic-hypoglycemic clamp studies in normal and recurrently hypoglycemic rodents, an effect that could be reversed by VMH K\textsubscript{ATP} channel inhibition [16, 18]. While these studies in animal models provide robust support for a role K\textsubscript{ATP} channels in the detection of hypoglycemia, it has not as yet been convincingly shown that K\textsubscript{ATP} channels are also important to the detection of hypoglycemia in human subjects and therefore a potential target for therapeutic intervention. Thus this study was designed to specifically test the hypothesis that K\textsubscript{ATP} channels were also integral to the detection of hypoglycemia in individuals with established T1D.
Research design and methods

This was a single centre, double-blinded, placebo-controlled randomized controlled trial. Ethical approval was obtained from an independent research ethics committee and the Medicines Healthcare Products Regulatory Agency (MHRA). The study was carried out in accordance with the Declaration of Helsinki, and written informed consent obtained from all participants before inclusion in the study. Inclusion criteria were patients with T1DM, aged 18 to 55 with tightly controlled glycaemia (HbA1c<6.4 mmol/L or 8% DCCT), with greater than 5 years disease duration. Reasons for exclusion were history of significant cardiac, hepatic, renal or neurological disease, pregnant and breast feeding mothers and already on any medications which may interact with diazoxide. All participants were identified using the Scottish Diabetes Research Network (SDRN), and the study took place at the Clinical Research Centre, Ninewells Hospital, Dundee.

After initial screening which included collection of demographic information, each subject attended the clinical research centre on 4 separate occasions. On two of these visits, separated by at least 2 weeks, the subject was given oral diazoxide or placebo before undergoing a hyperinsulinemic hypoglycemic clamp study. Diazoxide and placebo were given in capsule form that were identical in appearance and supplied by independent pharmacists according to a computer-generated randomization. Both the participants and investigators were blinded to allocation of treatment. The other 2 visits were to fit each participant with a continuous glucose monitor (RT-CGM), with low-glucose suspend (set at 4.5 mmol/l) where applicable, for 48 hours prior to each clamp study, to ensure the absence of significant hypoglycemia prior to the clamp procedure.

Experimental hypoglycemia. The evening prior to attendance, each participant was advised to reduce their night time long acting insulin by approximately 20% and advised to fast for at
least 8 hours prior to coming to the Clinical Research Centre at 0800AM. On the morning of
the clamp, a cannula was inserted into a dorsal hand vein of the non-dominant hand in a
retrograde fashion and then placed in a heated box at 55°C to arterialise venous blood [19].
This line was used for blood sampling during the clamp study. In the contralateral arm, the
ante-cubital vein was cannulated for insulin and glucose infusions.

Participants were given either oral diazoxide (7mg/kg) or placebo 2 hours before the start of
the euglycemic plateau. The timing of oral Diazoxide ingestion was based on the available
literature indicating that its hypotensive and anti-hypoglycemic effect lasted on average ≈ 3-
12 hours with a peak action at ≈ 5 hrs [20]. Participants were subsequently started on
50ml/hr of insulin for priming purposes until the blood glucose dropped to below 7mmol/L,
after which a rate of 1.5mU/kg/min was maintained for the duration of the clamp. A variable
20% dextrose infusion (Braun, Infusomat Space), was adjusted every 5 minutes based on
bed-side glucose measures. Euglycemia (glucose 4-5 mmol/L) was achieved and maintained
over the first 2 hours of the clamps and subsequently, blood glucose reduced by 0.5mmol/L
every 40 minutes to a final glucose level of 2.5mmol/L. This was maintained for 40 minutes
before glucose levels then allowed to return to the euglycemic range.

*Physiological measurements.* Blood pressure and pulse rate were measured every 10 minutes
using an Accutor Plus Monitor (Datascope Corp., Mahwah, New Jersey, USA).

*Counter-regulatory hormones.* Arterialised blood for insulin and counter-regulatory
hormones (epinephrine, norepinephrine, glucagon) was taken at midpoint and end of each
plateau.

*Symptoms.* Subjects rated symptoms at the mid point of every glucose plateau. Symptoms
were scored on a validated questionnaire, the Edinburgh Hypoglycemia Scale [21], scoring
from 1 (not at all) to 7(very severe) on a visual analogue scale.
**Cognitive function tests.** A battery of psychometric tests known to be sensitive to hypoglycemia were applied in the same order starting at the midpoint of each plateau - Trail making B (TMB) [22], Digit span backward (Dig-B) [23], Digit symbol substitution test (DSS) [24] and Four choice Reaction time (4CRT) [25].

**Laboratory assays.** Whole blood was measured at the bedside by a glucose oxidase method (Analox GM9D (Analox instruments, London, UK)). Samples were centrifuged to separate the plasma within 2 hours, and then stored at -80°C prior to assay. Hormones (Insulin-RIA-Diasorin; CV inter -6.7%, intra -5.8%), (Glucagon-RIA-MilliporeUK; CV inter 4.9%, intra 8.8%), (Epinephrine-EIA-Alpco; CV inter 22%, intra 16%), (Norepinephrine-EIA-Alpco; CV inter 16%, intra 22%) were measured by ELISA, and samples were analysed in duplicate according to the manufacturer’s instructions. Genomic DNA was prepared from whole blood using a Autopure DNA preparation robot (Qiagen). Genotyping of rs5219 was performed by TaqMan based allelic discrimination (Thermo-Life Technologies) according to manufacturers instructions.

**Data and statistical analysis.** The pre-specified primary endpoint was the magnitude of epinephrine responses at a glucose level of 2.5mmol/L. Secondary outcomes examined whether oral Diazoxide would affect glucose thresholds, defined using published protocols [26, 27], for activation of hormonal, symptomatic and cognitive responses or result in significant changes in heart rate or blood pressure. Data are presented as mean (SE). For the primary endpoint, normally distributed data were compared using paired samples t tests, while non-normally distributed data were compared using the Wilcoxon signed rank test. Statistical analyses were conducted using Graphpad Prism 6 and p<0.05 was considered statistically significant. Repeated measures ANOVA was used to determine differences in other parameters measured over time, with t-testing used to localize effects where indicated.
Results

Participant characteristics. Recruitment was from Jan 2012 to September 2012. Of the 24 participants screened, 6 did not meet the inclusion criteria and 4 withdrew consent; 14 subjects were randomized; 2 subsequently withdrew after the first clamp study. 12 participants (6 male and 6 female) completed all stages of the study (2 clamps). The median age for this group was 43 (range 18-52). Median (range) duration of diabetes was 24(6-40) years and median HbA1c was 7.6%/60mmol/mol [6.9-8% (52-64mmol/mol)]. There were an equal number of subjects on multiple daily injections (MDI) and those on continuous subcutaneous insulin infusion (CSII) therapy. 5 out of 12 participants had Impaired Awareness of Hypoglycaemia as classified by the Gold criteria (≥4)[28].

Hyperinsulinemic hypoglycemic clamp studies. Mean (SEM) baseline blood glucose levels prior to ingestion of Diazoxide (D) and Placebo (P) did not differ significantly between the two study days (10.6 (0.7) vs. 11.8 (1.0) D vs. P p=0.90). Glucose levels during the two clamp procedures were also well matched (see Figure 1). As expected, plasma glucose dropped with time over the stepped clamp (main effect of time F(16,187) =37.60 p<0.05). This drop was comparable in the two treatment groups (main effect of treatment F(1,187) =0.2882 p=0.59, with no time X treatment interaction (time x treatment F(16,187)=0.4403 p=0.97). We maintained a mean insulin level of 79 (3.0) vs. 76 (2.8) mU/L (D vs. P) throughout the clamp period (p=ns).

$K_{ATP}$ channel activation with diazoxide amplifies the counterregulatory response to hypoglycemia. We found that following oral administration of Diazoxide, there was a 37%
increase in the pre-specified primary outcome mean (SEM) epinephrine responses (0.40 (0.06) vs. 0.29 (0.05) ng/ml, D vs. P; p<0.05) and a 44% increase in mean (SEM) norepinephrine (0.85 (0.07) vs. P; 0.59 (0.06) ng/ml, D vs. P; p<0.05) at plasma glucose of 2.5mmol/L. (See Figure 2a and 2b). Glucagon levels remained, as expected, suppressed during hypoglycemia, with no significant differences found between groups (57.8 (11) vs. 50.0 (7.1) ng/l, D vs. P; p=0.21). Consistent with the amplified catecholaminergic response to hypoglycemia glucose infusion rates (GIR) required to maintain the hypoglycemic plateau were significantly lower different at 2.5mmol/L following oral Diazoxide (71.6 ± 1.8 vs 77.5 ± 2.1, D vs. P; p<0.05).

Despite the improved counterregulatory hormone response to hypoglycemia participants experienced similar total symptom scores at an arterialized plasma glucose of 2.5 mmol/l following administration of Diazoxide (22 (3) vs. 19 (3), D vs. P; p=0.32). Similarly, the overall increase in autonomic symptoms following Diazoxide was not significant (10 (1) vs 9 (1) p= 0.26). Cognitive performance of participants during the 2.5 mmol/l step was mixed with no significant impact of Diazoxide on Trail making B (30 (4) vs. 33 (5) s, D. vs. P; p=0.65), Digit symbol backward (6 (1) vs. 7 (1), D. vs. P; p=0.38), or on 4 choice reaction time (547 (21) vs. 543 (18), D. vs. P; p=0.82), and a significant deterioration in Digit symbol substitution following Diazoxide (70 (9) vs. 81 (8); D. vs. P; p<0.05) (see Online appendix Figure 1a, b and c).

K$_{ATP}$ Channel activation with diazoxide did not lower thresholds for counterregulatory responses to hypoglycemia. Secondary outcomes in this study were to determine whether diazoxide would lower (higher glucose for initiation) the glucose thresholds for onset of hormone responses. Thresholds were defined as the time of onset of a sustained ($\geq$2 successive time points) increase in hormone concentrations $\geq$ 2 SDs above the mean of the two baseline measurements for that hormone. Although the glucose threshold for generation
of both epinephrine and norepinephrine responses to hypoglycemia were lower following administration of diazoxide these did not reach statistical significance (see Online Appendix Table 1).

The E23K polymorphism in K\textsubscript{ATP} channels predict response to diazoxide during hypoglycemia. The E23K polymorphism in the K\textsubscript{ATP} channel results in an increase in the likelihood of the K\textsubscript{ATP} channel being open in the resting state[29, 30] and influences individual responses to sulphonylureas[31]. To determine whether the E23K polymorphism might influence individual responses to Diazoxide we genotyped all 12 participants in the study and divided the cohort into diazoxide-responders and diazoxide-non-responders. A diazoxide-responder was defined as an individual who had a greater than double the standard error of the mean increase in epinephrine response at 2.5mmol/L following diazoxide. In our study cohort, 7 of the 12 participants (58%) carried the K23 allele (2-KK, 5-EK), while the rest were of the wild type homozygous E23. Intriguingly, participants who expressed only the wild type E23 allele were all diazoxide-responders, while those hetero- or homozygous for the K23 allele were significantly less likely to respond to Diazoxide (Pearson’s chi squared, $\chi^2=6.12; p=0.013$) (See figure 3a and b). Those who expressed the wild type E23 allele also showed greater magnitude of epinephrine response particular as the blood glucose dropped down to 2.5mmol/L (see online appendix Figure 3).

Adverse events. Systolic BP was comparable between the two groups, with no effect of treatment (main effect of treatment F (1,22)=0.001228 p=0.97). Similarly there was no effect of treatment on Diastolic BP (main effect of treatment F (1,22) =0.4602 p=0.50) or on pulse rate (main effect of treatment F(1,22) =2. 893 p=0.10) . (Online appendix Figure 2a,b, and c)
1 participant had nausea and vomiting which was short lived in the recovery phase of both D and P studies, and reported nausea as being one of her usual symptoms during hypoglycemia. 1 participant had nausea and 1 bout of vomiting in the recovery stage after receiving Diazoxide. There were no serious adverse events/reactions.

**Discussion**

It is now generally accepted that the brain, and particularly specialized neuronal populations within the hypothalamus, plays a major role in both the detection of hypoglycemia and the development of IAH [2]. The importance of $K_{ATP}$ channels to hypothalamic glucose sensing was first proposed by Mayer [32], and the critical role of $K_{ATP}$ channels in hypothalamic glucose sensing has since been demonstrated in cell culture models [33], *ex-vivo* hypothalamic slices [14], transgenic mouse models [15] and rodent *in vivo* pharmacological studies [16-18]. In the current study, we now extend these findings by demonstrating for the first time in individuals with type 1 diabetes of long duration that oral diazoxide (7mg/kg) given prior to acute hypoglycemia can significantly increase the magnitude of both epinephrine and norepinephrine counterregulatory responses. Moreover, we make the novel observation that the E23K polymorphism in the Kir6.2 subunit of the $K_{ATP}$ channel predicts response to diazoxide therapy during hypoglycemia in T1D.

The mechanism by which diazoxide improves the neuroendocrine response remains unclear. While the *in vitro* and *in vivo* rodent literature support a central action of $K_{ATP}$ activators, all glucose-sensing cells both centrally and peripherally have been shown to contain Kir 6.2 and Sur-1 components of the $K_{ATP}$ channel. It therefore possible that oral diazoxide has acted primarily through peripheral $K_{ATP}$ channels, such as those in the hepatoporal veins [34]. The comparatively ‘slow-fall’ in glucose with the multi-step clamp would also be consistent with activation through hepatoporal sensors [34]. In contrast, studies of
direct hypothalamic modulation of $K_{\text{ATP}}$ channels in rodents during hypoglycemia [16, 17] support a central action of diazoxide, as do the findings in both our own study and that of Bingham et al. [27] of an effect of diazoxide on tests of psychomotor speed in human subjects. In a related study, Kishore et al. [35] demonstrated in the rodent model that the extra-pancreatic action of diazoxide to suppress hepatic glucose production could be reversed through ICV delivery of the $K_{\text{ATP}}$ channel blocker, glibenclamide. Moreover, they were able to detect diazoxide in the CSF of rodents after oral ingestion reaching levels of 0.26±0.06 µg/ml 1 hour after gavage and 0.78 ±0.03 µg/ml by 4 hours, providing convincing evidence that diazoxide penetrates the blood-brain-barrier (BBB) [35]. Species differences may effect BBB permeability to Diazoxide, however Diazoxide contains an ionisable sulphonyl group making it extremely lipid soluble and therefore able to partition into the lipid bilayer for penetration through the BBB [36]. These studies support the hypothesis that Diazoxide acts to amplify the counterregulatory response to acute hypoglycemia through a direct action in the brain.

Our findings contrast with those of Bingham et al. [27] and Raju et al. [37] who failed to see significant effects of oral diazoxide on counterregulatory responses to hypoglycemia in non-diabetic subjects. However in the study by, Bingham et al. [27] did report that hypoglycemia-induced peak epinephrine levels were higher following diazoxide (adrenaline 7.37±1.89 vs. 6.18±2.28 nmol/l, respectively, p=0.055). Similarly, although Raju et al. do not provide actual values to compare epinephrine and norepinephrine responses during the latter stages of the mild hypoglycemic challenge (3.0mmol/L), these appear greater in those subjects given diazoxide [37]. Both studies used lower doses of diazoxide (5 and 6 mg/kg, respectively), which may contribute to their failure to see a significant effect. In addition, it is possible that $K_{\text{ATP}}$ channel opening in hypothalamic glucose sensing neurons during moderate hypoglycaemia in non-diabetic individuals may already be near maximal. By comparison,
subjects with type 1 diabetes and IAH have impaired glucose sensing and the $K_{ATP}$ channel is therefore, less likely to be in the open-state and more likely to respond to $K_{ATP}$ channel activators.

In our study oral Diazoxide was able to augment the counterregulatory response sufficient to significantly reduce requirements for exogenous glucose during the clamp procedure suggestive of a real impact on whole body responses. Despite this we did not see a statistically significant change in glucose thresholds for counterregulation or for overall symptom responses. However, the subjects in this study had diabetes of relatively long duration and despite only 5 of the 12 participants having IAH as defined by Gold criteria, 11 out of 12 participants had an autonomic symptom threshold below or equal to 3mmol/L during the clamp studies. Thus, the subjects all had a profound defects in symptom and hormonal counterregulatory responses to hypoglycemia. It is likely that our study would only have detected large effect sizes in these secondary outcomes. In addition, a limitation of our study is that we calculated thresholds based on the euglycemic period prior to the induction of hypoglycemia as reported by others [38]. An additional euglycemic control arm to the study would have both reduced baseline variability and controlled for effects of time dependent changes.

An interesting further finding in the current study was the effect of Diazoxide on the DSS task. This psychomotor task is often used in hypoglycemia studies and provides a robust and sensitive measure of cognition during hypoglycemia [39]. Bingham et al, [27] reported that non-diabetic subjects showed a significant prolongation on the 4-choice reaction time (4CRT) task during hypoglycemia following diazoxide, but no effect was seen on Stroop and finger tapping tasks. In the present study, we did not see a significant effect on 4CRT however the findings of Bingham et al. [27] are convincing in that diazoxide and glibenclamide were shown to have the opposite effect on 4CRT performance during hypoglycemia. Therefore,
drugs that effect the $K_{\text{ATP}}$ channel may have widespread effects on brain function and at least under hypoglycemia conditions, KATP channel openers lead to an overall reversal of hypoglycemia-induced adaptations in brain glucose sensing and psychomotor performance.

In this study we also made the interesting observation that the presence of the E23K polymorphism predicted to a large extent whether an individual would respond to diazoxide during hypoglycemia. The exact prevalence of the E23K polymorphism has not been studied in the T1D population. Single nucleotide polymorphisms (SNPs) at codon 23 (E23K, rs5219) in Kir 6.2 (which is encoded by the $KCNJ11$, gene) is associated with Type 2 Diabetes [40] and also with better response to sulfonylureas [31]. The K23 variant of the $K_{\text{ATP}}$ channel results in a 60% increase in the likelihood of the $K_{\text{ATP}}$ channel being open in the resting phase compared to the wild type E23 form, and although this variant is in the pore-forming Kir6.2 channel, it demonstrates strong allelic association with a coding variant (A1369S) in the neighboring SUR1 gene thus predicting response to sulfonylureas [41, 42]. In our small cohort of 12 participants with well-established T1D, we found 58% carried the K23 variant. This is comparable with the prevalence of 51% (41% hetero- and 10% homozygote) for the E23K polymorphism reported in participants with pre-diabetes in the Diabetes Prevention Program [41] and with 63% and 59% respectively of type 2 diabetic subjects in the UKPDS and normoglycemic control subjects [43]. Although the small size of our study cohort limits the conclusions we can reliably draw from this analysis, our data suggest that the E23K polymorphism may identify individuals requiring a greater dose of diazoxide to amplify the counterregulatory response to hypoglycemia, allowing for a more stratified approach to intervention in the future. Further studies will be required to address this hypothesis.

In summary, we have shown for the first time in human subjects, that the $K_{\text{ATP}}$ channels are integral to hypoglycemia detection and in the generation of an adequate CRR to acute hypoglycemia. We report that, the $K_{\text{ATP}}$ channel opener diazoxide, when given orally prior
to a hypoglycemic stimulus to subjects with long-standing T1D and IAH, results in a 37-44% increase in the magnitude of the catecholaminergic counterregulatory hormonal response. Moreover, our data suggest more widespread central actions of diazoxide on neuronal populations involved in psychomotor responses and symptom generation. Finally, we have made the novel observation that the E23K polymorphism in the Kir6.2 subunit of the $K_{\text{ATP}}$ channel predicts response to diazoxide therapy during hypoglycemia in T1D. Taken together, we believe clinical trials of longer-term diazoxide therapy in T1D subjects with IAH recruited by genotype are warranted to explore the potential utility of this novel approach to improve hypoglycemia awareness and reduce frequency of severe hypoglycemia.
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Prof Rory McCrimmon 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.

P.G conducted the trial and researched data and wrote manuscript, CP and RT contributed to the genetic analysis and edited the manuscript. R.J.M conceived study wrote/edited the manuscript.

Conflicts of interest;

Priya S George; None,

Roger Tavendale; None

Colin Palmer; None

Rory McCrimmon; None
References


Figure Legends

FIGURE 1. Glucose profiles during stepped hyperinsulinaemic hypoglycemic clamp studies. The hyperinsulinaemic hypoglycemic clamp technique was utilised to slowly drop the blood glucose from euglycemia (4.0mmol/L) to hypoglycemia (3.5, 3.0, 2.5mmol/L). The drug (diazoxide or placebo) was given at 0mins, and after 120minutes, blood sugars were slowly dropped to euglycemia during that time. Following which the clamp was commenced. Each nadir was achieved over 20mins, and then
maintained for 20mins. Average blood glucose achieved at each of the desired steps during both the diazoxide and placebo clamp studies is shown in the bar chart below.

**FIGURE 2.** Diazoxide amplifies catecholaminergic responses during acute hypoglycemia in long standing Type 1 Diabetes. (a) Plasma epinephrine and (b) plasma norepinephrine levels during baseline and each hypoglycemic plateau. Placebo group shown as open bars, Diazoxide as closed bars. Values shown as mean (±SEM). *p<0.05.

**FIGURE 3.** E23K polymorphism in Kir 6.2 predicts response to Diazoxide during acute hypoglycemia. This figure shows the magnitude of the epinephrine response during acute hypoglycemia (2.5mmol/L) following placebo or diazoxide in individuals who expressed (a) Wild type (EE) or (b) Homo or heterozygous (KK,EK) for this E23K polymorphism for the Kir 6.2 channels. Results for each individual under the two conditions are shown.

**Online appendix FIGURE 1;** Of the 4 cognitive function tests performed, only Digit symbol substitution showed significant deterioration of performance with oral Diazoxide. (a)Trail making (b) Digit symbol substitution (c) Digit span backwards score (d) 4 choice reaction time scores are shown at baseline and at each hypoglycemic plateau. Values shown as mean (±SEM). *p<0.05.

**Online appendix FIGURE 2;** There was no significant change in any of the haemodynamic parameters during the clamp studies. The figure shows (a)systolic BP (b)diastolic BP(c)pulse rate from time 0 (drug given) to the conclusion of the clamp studies. Open circles represent the placebo arm, and closed circles are the diazoxide arm.

**Online appendix FIGURE 3;** This shows that there was fairly minimal epinephrine responses up to 250mins (achieving 3.0mmol/L plasma glucose), but responses diverge as the blood glucose drops down to 2.5mmol/L. Closed circles represent those with the wild type (WT)homozygous E23, and closed boxes are those with the K23 allele.

**Online appendix TABLE 1;** Glucose thresholds for the key counter-regulatory hormones and symptoms.

The glucose threshold defined as the glucose level at which there is a sustained (>2 successive Time Points) increase in hormone concentrations or symptom scores above the mean of the two baseline measurements for that hormone or symptom. Values reported as mean ±SEM.
Online appendix TABLE 1: Glucose thresholds for the key counter-regulatory hormones and symptoms.

The glucose threshold defined as the glucose level at which there is a sustained (>2 successive Time Points) increase in hormone concentrations or symptom scores above the mean of the two baseline measurements for that hormone or symptom. Values reported as mean ±SEM.

<table>
<thead>
<tr>
<th>Glucose Thresholds (mmol/L)</th>
<th>Placebo</th>
<th>Diazoxide</th>
<th>P value</th>
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<tr>
<td>Epinephrine</td>
<td>3.1±0.4</td>
<td>3.2±0.3</td>
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