Central regulation of glucose production may be impaired in type 2 diabetes mellitus

Yonah B. Esterson*, Michelle Carey*, Laura Boucai, Akankasha Goyal, Pooja Raghavan, Kehao Zhang, Deeksha Mehta, Daorong Feng, Licheng Wu, Sylvia Kehlenbrink, Sudha Koppaka, Preeti Kishore, Meredith Hawkins.

*authors contributed equally

1 Diabetes Research and Training Center, Division of Endocrinology, Department of Medicine, Albert Einstein College of Medicine, Bronx, NY

Correspondence:
Meredith Hawkins, MD
1300 Morris Park Avenue
Belfer 709
Bronx, NY 10461
Tel: 718-430-8562
Fax: 718-430-8557
meredith.hawkins@einstein.yu.edu

Running title: Central regulation of glucose production in diabetes
ABSTRACT

The challenges of achieving optimal glycemic control in type 2 diabetes highlight the need for new therapies. Inappropriately elevated endogenous glucose production (EGP) is the main source of hyperglycemia in type 2 diabetes. Since activation of central K\textsubscript{ATP} channels suppresses EGP in non-diabetic rodents and humans, this study examined whether type 2 diabetic humans and rodents retain central regulation of EGP. The K\textsubscript{ATP} channel activator diazoxide was administered in a randomized, placebo-controlled crossover design to eight type 2 diabetes subjects and seven age- and BMI-matched healthy controls. Comprehensive measures of glucose turnover and insulin sensitivity were performed during euglycemic ‘pancreatic’ clamp studies following diazoxide and placebo administration. Complementary rodent clamp studies were performed in Zucker Diabetic Fatty rats. In type 2 diabetes subjects, extrapancreatic K\textsubscript{ATP} channel activation with diazoxide under fixed hormonal conditions failed to suppress EGP, while matched controls demonstrated a 27\% reduction in EGP (p=0.002) with diazoxide. Diazoxide also failed to suppress EGP in diabetic rats. These results suggest that suppression of EGP by central K\textsubscript{ATP} channel activation may be lost in type 2 diabetes. Restoration of central regulation of glucose metabolism could be a promising therapeutic target to reduce hyperglycemia in type 2 diabetes.
INTRODUCTION

Substantial evidence indicates that optimal glycemic control is associated with better clinical outcomes in type 2 diabetes (1,2). However, despite a sizeable therapeutic armamentarium that targets pathways in liver, muscle, and pancreas, 50% of patients with type 2 diabetes are unable to achieve adequate glycemic control (3). Therefore, new approaches to improve glucose homeostasis are urgently needed. Increased endogenous glucose production (EGP) is the major source of both fasting and post-absorptive hyperglycemia in type 2 diabetes (4,5). Although EGP is suppressed by both insulin and glucose in non-diabetic humans, this effect is considerably impaired in individuals with type 2 diabetes (4). Therefore, this inappropriately elevated EGP is an important target for intervention in type 2 diabetes.

Importantly, evidence for regulation of glucose homeostasis by the central nervous system (CNS) has been accumulating in both rodents and humans. Several rodent studies have demonstrated that the CNS is involved in the regulation of glucose metabolism through its detection of nutrients and hormones, subsequent signaling through hypothalamic ATP-sensitive potassium (K$_{\text{ATP}}$) channels, and transduction of those signals to the liver via vagal efferent fibers (6-14). We recently reported that oral administration of diazoxide, a K$_{\text{ATP}}$ channel activator, significantly reduces EGP in non-diabetic humans under fixed hormonal conditions (15). Additionally, we presented supporting evidence in rats that diazoxide’s suppressive effects on EGP are abolished with central administration of the K$_{\text{ATP}}$ channel blocker glibenclamide, suggesting that these effects are centrally mediated (15). Furthermore, intranasal administration of insulin at doses previously shown to increase CSF insulin concentrations ~2-fold suppressed glucose production to a similar extent and over a similar time course, likely through activation of central K$_{\text{ATP}}$ channels (16). Collectively, these studies in rodents and humans suggest a role for hypothalamic K$_{\text{ATP}}$ channels in regulation of glucose metabolism.
Of note, a number of studies in obese or diabetic rodents have indicated that central sensing mechanisms are ineffective at maintaining glucose homeostasis in these models (17-20). In fact, it has been hypothesized that dysregulated CNS circuits may contribute to impaired glucose homeostasis in type 2 diabetes (21). Therefore, it is essential to establish whether central regulation of glucose homeostasis remains intact in humans with type 2 diabetes. If so, hypothalamic $K_{\text{ATP}}$ channels represent a potential therapeutic target to counteract excessive EGP and improve hyperglycemia in type 2 diabetes. Conversely, if central regulation of EGP is lost in type 2 diabetes, future therapies could be directed at restoring these pathways.

Given the evidence supporting regulation of EGP by a brain-liver pathway in non-diabetic humans (15), the current randomized, placebo-controlled crossover study was designed to determine whether activation of central $K_{\text{ATP}}$ channels would suppress EGP in individuals with type 2 diabetes. Importantly, we conducted parallel studies in a group of specifically recruited age- and BMI-matched non-diabetic controls. To exclude any effects of diazoxide on insulin secretion, these studies were performed under euglycemic ‘pancreatic clamp’ conditions. Complementary studies in Zucker Diabetic Fatty (ZDF) rats were also performed to assess diazoxide’s ability to cross the blood brain barrier and to suppress EGP in this animal model of type 2 diabetes.

**RESEARCH DESIGN AND METHODS**

**Human Studies.** Eight subjects with moderately-to-poorly controlled type 2 diabetes were studied (Table 1). Eligible subjects were diagnosed with type 2 diabetes within the past 10 years, and were otherwise in good health. Seven healthy age- and BMI-matched non-diabetic control subjects were also studied (Table 1). The purpose, nature, risks and benefits of the study were explained to all subjects in the Clinical Research Center (CRC) prior to their enrollment in the study, and their voluntary, informed, written consent was obtained. All subjects had an initial
screening visit to allow for a clinical evaluation which included history, physical examination, hematologic, lipid, and chemistry screening (including fasting glucose levels), baseline EKG, and consent procedures. A 2-hour oral glucose tolerance test was performed to ensure normal glucose tolerance in non-diabetic controls. Each subject received the experimental agents in random order, and the agents were identical in appearance.

**Euglycemic pancreatic clamp procedures.** All experiments consisted of basal insulin and somatostatin (250 µg/hr) infusions with replacement of glucoregulatory hormones (glucagon 0.6 ng/kg/min; growth hormone 3 ng/kg/min) starting at t=-120 minutes. From t=-120 to t=0 minutes, insulin infusion rates were adjusted every 20-25 mins to determine optimal insulin infusion rates to maintain euglycemia. Finer calibration of insulin infusion rates were performed from t=0 to t=120 mins, to establish individualized basal insulin infusion rates by 120 mins (15). In eight of the thirty studies (n=1 non-diabetic placebo, n=2 non-diabetic diazoxide, n=2 diabetic placebo, n=3 diabetic diazoxide), minor changes to the insulin infusion rates were made from t=120 to t=170 minutes to prevent hypoglycemia. There were no significant changes in either insulin infusion rates or in plasma insulin concentrations between 0 and 240 mins, emphasizing the fact that any changes were minor. This careful approach to attaining individualized, basal insulin infusion rates, avoiding the need for virtually any exogenous glucose infusion, permits highly sensitive measures of glucose production without over-insulinization (15; 23). Plasma glucose concentrations were measured at 5 minute intervals during the 240 minutes of the study and maintained at normal fasting concentrations (~90 mg/dl), employing low infusion rates of dextrose 20% if needed. All infusions were stopped at t=240 minutes, and subjects received a standard meal with subsequent plasma glucose monitoring for 60 minutes after the completion of the study before being discharged from the CRC. Data for glucose turnover represent the mean values during the final 60 minutes of the studies (t=180-240 minutes).
Each subject underwent two paired euglycemic pancreatic clamp studies separated by 4-6 weeks. After an overnight fast, non-diabetic subjects were admitted to the CRC on the morning of the study. Subjects with type 2 diabetes were admitted to the CRC the night prior to the clamp study for gradual lowering of plasma glucose levels with intravenous insulin infusions (22,23) and were also fasted overnight prior to the study. Since an algorithm was used for insulin infusion rates, subjects received progressively lower rates of insulin infusion as their glucose levels dropped. Sulfonylurea agents and metformin were discontinued for 72 hours prior to all admissions and thiazolidinediones were held for 8 days prior to admission. Long or intermediate acting preparations of insulin were discontinued prior to admission such that subjects received no long acting insulin for 24 hours prior to the study and no intermediate acting insulin for at least 12 hours. An 18-gauge catheter was inserted in an antecubital vein for infusions and a contralateral hand vein was cannulated in a retrograde fashion for arterialized venous blood sampling. To obtain arterialized venous blood, the hand was kept in a warming pad maintained at 55°C. During initial pilot studies (24), it was determined that optimal metabolic effects of diazoxide were observed approximately 6-7 hours following drug administration.

At t=-180 minutes, the subjects were administered either oral diazoxide 4-6 mg/kg or placebo in a double blinded fashion (Figure 1). Vital signs were recorded at t=-180 minutes and hourly thereafter. Primed continuous infusions of 6-6 glucose (D2G) tracer were initiated at t=-120 minutes (10.4 ml/min bolus, then 3.903 mg/min), to measure glucose fluxes under ‘pancreatic clamp’ conditions (15). From t=0 to t=240 minutes, blood samples were obtained for determinations of plasma glucose, insulin, glucagon, C-peptide, cortisol, free fatty acids, glycerol, lactate, and 6-6 glucose determinations. Each subject returned to the CRC for a second study (either placebo or diazoxide) after 4-6 weeks had elapsed.
**Plasma hormone and substrate determinations.** Plasma glucose was measured at the bedside with a Beckman glucose analyzer (Fullerton, CA) by use of the glucose oxidase method. Measurements of plasma insulin, C-peptide, and glucagon were undertaken in order to evaluate the inhibitory effects of somatostatin on insulin secretion and the consistency of hormone replacement. Plasma insulin, C-peptide, glucagon, and cortisol concentrations were measured by radioimmunoassay in the Diabetes Research Center Hormone Assay Core (25). Plasma lactate, free fatty acids, and glycerol were measured using spectrophotometric techniques (26-28). 6-6 glucose concentrations were measured by gas chromatography mass spectrometry (GCMS), as previously described (29,30). The rates of EGP were compared during euglycemic clamp studies following diazoxide administration versus following placebo in each subject. Rates of glucose appearance (Ra) and disappearance (Rd) and other indices of glucose turnover were estimated by using Steele equations (31), using the assumption that Ra=Rd for steady state, and using the following equation: Rd=(Basal [6,6-\textsuperscript{2}H\textsubscript{2}]glucose infusion rate +D20/[6,6-\textsuperscript{2}H\textsubscript{2}] glucose infusion rate/APE fraction/wt (kg), with data averaged over 60-minute segments of each experiment. EGP was determined by subtracting the rates of glucose infusion from the tracer-derived Ra.

**Rat studies:** Eleven-week old male Zucker Diabetic Fatty rats (n=12) (Charles River Laboratories; Wilmington, MA), with an average weight of 354.5 ± 10.6 g, were studied under the following conditions: (a) oral (gavage) saline control (n=6); (b) oral (gavage) diazoxide (n=6) (Figure 5A). The night before infusion studies were performed, each animal received Neutral Protamine Hagedorn (NPH) insulin (3-5 U/kg) to slowly correct hyperglycemia prior to the study. Each infusion study lasted 240 minutes. 120 minutes prior to infusion studies, rats were anesthetized with isofluorane, and either saline or diazoxide (100mg/kg) was administered by oral gavage. For the remainder of the studies, rats were conscious and unrestrained. Insulin infusion (3-6mU/kg/min) was then initiated to slowly lower blood glucose to ~140-150mg/dL.
prior to initiation of the study. At t=0 minutes, a primed continuous i.v. infusion of [3-3H]-
glucose was begun and maintained for 4 hours to assess glucose kinetics (40µCi bolus followed
by 0.4µCi/min infusion, Perkin Elmer). Blood samples were obtained at 10-minute intervals
during the final hour of the clamp to assess [3-3H]-glucose specific activity. A peripheral basal
insulin pancreatic-euglycemic clamp was performed for the final 2 hours of the infusion study (t
= 120-240 min), as previously described, using continuous i.v. somatostatin infusion (15). This
specific protocol was followed, without glucagon infusion, in order to replicate previous studies
examining central regulation of glucose production (12, 15).

Rats were prepared for the in vivo experiments with implantation of carotid and internal jugular
catheters one week prior to the study. Following the study, rats were anesthetized with ketamine
(150mg/kg). CSF samples were obtained by ventricular puncture and liver tissue samples were
obtained by freeze clamping. CSF was analyzed for diazoxide content by NMS Labs (Willow
Grove, PA) using liquid chromatography tandem mass spectrometry (LC-MS). Real-time reverse
transcriptase polymerase chain reaction (rt-PCR) was performed to examine gene expression and
protein levels of phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase
(G6Pase) in rat liver, using a Roche LightCycler abd SYBR Green I (Qiagen). Relative gene
expression was calculated as the ratio of target gene divided by the geometric mean of
housekeeping genes.

**Statistical Analysis.** Comparison of EGP, rate of glucose disappearance, glucose infusion rate,
and hormone and substrate levels during diazoxide versus placebo studies within each group
were performed using paired Student’s t-tests. Unpaired Student’s t-tests were used to compare
the percent change in EGP (diazoxide vs. placebo studies) and subject characteristics between
the two groups. Repeated measures analysis of variance (ANOVA) was used to assess the
stability of average percent enrichment of 6-6 glucose in the plasma during the last two hours of
the clamp studies. Student’s t-tests and ANOVA were performed under the assumptions of
equality of variances and normality. Equality of variances was met when the standard deviations of the variables differed by less than an order of magnitude. Normality of data could not be fully assessed given that the classical tests of normality are not robust to smaller sample sizes. Therefore, the significance or non-significance of each parametric test result was confirmed with the alternative non-parametric test (Wilcoxon matched pairs signed rank sum test for comparison of medians of paired data, Mann-Whitney tests for comparison of medians of two independent groups, and Kruskall-Wallis test for comparison of medians of three or more independent groups). Alpha was set at 0.05 for all tests. Data are reported as mean±standard error unless otherwise noted.

**Study Approval.** All procedures were approved by the Institutional Review Board of Albert Einstein College of Medicine.

**RESULTS**

**Human Subject Characteristics.** Eight diabetic subjects and seven non-diabetic controls were frequency matched for age and BMI. Additional subject characteristics are presented in Table 1. Since a number of rodent studies suggest that central regulation of metabolic homeostasis is impaired with both obesity and aging (18-20), we specifically recruited an age- and BMI-matched non-diabetic control group for these studies. All subjects with type 2 diabetes who completed both placebo and diazoxide clamps were males. We included only male control subjects to maintain consistency between groups. Of note, analysis of our previously published data reveals that the decrease in EGP seen with diazoxide administration relative to placebo administration in healthy subjects was not significantly different in females versus males (p=0.22) (15). Furthermore, since study recruitment aimed to be representative of the ethnic composition of the Bronx, both groups were racially/ethnically heterogeneous, although there were more Hispanic subjects in the diabetic vs. non-diabetic group (4 vs. 1, respectively). While
the effects of diazoxide were comparable among all subjects in both groups, this study was not sufficiently powered for a subgroup analysis to discern ethnic differences.

**Human Clamp Conditions.** Over the interval of measurement, average percent enrichment of 6-6 glucose in the plasma during diazoxide studies remained stable in both diabetic subjects (p=0.42) and non-diabetic controls (p=0.96). Similarly, APE during placebo studies remained stable in both diabetic subjects (p=0.99) and non-diabetic controls (p=0.89). Plasma hormone levels were measured to confirm that the clamp conditions prevented pancreatic hormone secretion. There were no statistically significant differences in plasma levels of insulin, glucagon, cortisol, free fatty acids, C-peptide, glycerol, or lactate in response to diazoxide versus placebo in diabetic subjects (Table 2) or in non-diabetic controls (Table 3). While this likely reflects a lack of difference, we cannot rule out Type II error in light of the relatively small sample sizes common to all resource-intensive physiologic studies. Of note, baseline C-peptide levels were suppressed following overnight insulin infusion in the diabetic subjects. Average insulin infusion rates were similar during the final hour of the clamp under both experimental conditions in diabetic subjects (0.35 ± 0.08 mU/m²/min with diazoxide versus 0.32 ± 0.07 mU/m²/min with placebo, P=0.52) and in non-diabetic controls (0.21 ± 0.04 mU/m²/min with diazoxide versus 0.16 ± 0.02 mU/m²/min with placebo, P=0.09). Although diazoxide has the potential to lower blood pressure at high doses, there were no differences in mean systolic blood pressure (p=0.36 in diabetic subjects, p=0.69 in controls), mean diastolic blood pressure (p=0.17 in diabetic subjects, p=0.17 in controls), or mean heart rate (p=0.39 in diabetic subjects, p=0.44 in controls) with diazoxide versus placebo versus baseline.

**Glucose Fluxes.** We previously reported that oral administration of diazoxide caused a 29% decrease in EGP in healthy human subjects (15). In the current study, we determined that oral administration of diazoxide to diabetic subjects under fixed hormonal conditions did not affect EGP (1.55 ± 0.08 mg kg⁻¹ min⁻¹ with diazoxide versus 1.59 ± 0.08 mg kg⁻¹ min⁻¹ with placebo,
P=0.74) (Figure 2A and Figure 3A). This is in contrast to the 27.4% decrease in EGP after diazoxide administration in age- and BMI-matched non-diabetic controls (1.14 ± 0.07 mg kg\(^{-1}\) min\(^{-1}\) with diazoxide versus 1.61 ± 0.14 mg kg\(^{-1}\) min\(^{-1}\) with placebo, P=0.002) (Figure 2A and Figure 3B). Relative to basal rates of EGP, there was a 0.4±8.4% increase in EGP in the placebo studies and a 26.4±3.0% decrease in the diazoxide studies between time -120 and 240 mins in the non-diabetic subjects. The diazoxide-induced suppression of EGP in the non-diabetic controls thus differed significantly from the complete lack of response in the diabetic subjects (P=0.01) (Figure 2B). Of note, our intent in designing the current studies was to avoid over-insulinization of the liver, and in fact it should be noted that rates of EGP remained unsuppressed throughout the placebo studies relative to basal EGP at time -120 min, prior to the onset of the clamp. Furthermore, among subjects in whom insulin infusion rates were unchanged after 120 mins, there was a 32% suppression of EGP by diazoxide in the nondiabetic group and no change in the diabetic group. However, it would be interesting to know whether diazoxide exerts an effect on EGP in the presence of hepatic hyperinsulinemia in humans.

Diazoxide did not alter the rate of glucose disappearance in diabetic subjects (1.91 ± 0.14 mg kg\(^{-1}\) min\(^{-1}\) with diazoxide versus 1.96 ± 0.17 mg kg\(^{-1}\) min\(^{-1}\) with placebo, P= 0.77) or in non-diabetic controls (2.24 ± 0.38 mg kg\(^{-1}\) min\(^{-1}\) with diazoxide versus 2.29 ± 0.29 mg kg\(^{-1}\) min\(^{-1}\) with placebo, P=0.81) (Figure 4A). Of note, since these studies were conducted under basal insulin conditions, glucose infusion rates required to maintain euglycemia were minimal in all groups (diabetic subjects: 0.43 ± 0.17 mg kg\(^{-1}\) min\(^{-1}\) with diazoxide versus 0.41 ± 0.08 mg kg\(^{-1}\) min\(^{-1}\) with placebo, P= 0.94; non-diabetic controls: 1.02 ± 0.36 mg kg\(^{-1}\) min\(^{-1}\) with diazoxide versus 0.65 ± 0.20 mg kg\(^{-1}\) min\(^{-1}\) with placebo, P=0.24) (Figure 4B).

**Rat Studies:** Complementary rodent studies were performed in n=12 Zucker Diabetic Fatty rats (Average weight= 354.5 ± 10.6 g). Due to limited sample volume from each rodent, pooled CSF collected at the end of the clamp studies was analyzed and demonstrated measurable levels of
diazoxide 6 hours after administration (1µg/mL, with a reporting limit of 0.5µg/mL) by LCMS, comparable with CSF diazoxide levels reported in our studies in Sprague Dawley rats (15). Tail stick blood glucose levels the night before the clamp studies were elevated in both groups of ZDF rats prior to treatment with NPH insulin (blood glucose= 409.5 ± 50.3 mg/dL in the diazoxide group and 474.2 ± 44.3 mg/dL in the saline group, p=NS). During the steady state phase of the clamp studies, average plasma glucose levels were similar between the diazoxide and saline groups (143.1 ± 1.3 mg/dL with diazoxide vs. 145.9 ± 1.0 mg/dL with saline, p=NS). Insulin levels were also similar for the two groups during the steady state phase of the clamp (143.0 ± 21.7 uU/mL with diazoxide vs. 130.41 ± 35.87 uU/mL with saline, p=NS). Consistent with our findings in human subjects with diabetes, there was no significant difference in average rates of EGP following administration of diazoxide compared with saline gavage (3.5± 0.9 mg kg^{-1} min^{-1} with diazoxide versus 2.8± 0.8 mg kg^{-1} min^{-1} with saline, p=0.52) (Figure 5B). Intriguingly, while these rates of EGP are lower than previously published clamp results in ZDF rats (32), clamp studies performed in the absence of glucagon infusion were associated with EGP rates that were similar to those observed in the current studies (M. Shiota, personal correspondence). The reason for performing these clamp studies without glucagon infusion was to reproduce study conditions previously used to examine the impact of diazoxide on central regulation of EGP (12,15).

Gene expression of hepatic gluconeogenic enzymes PEPCK and G6Pase also showed no significant differences following diazoxide versus saline administration (relative PEPCK gene expression: 1.12 ± 0.12 with diazoxide vs. 1.04 ± 0.15 with saline, p=0.68; relative G6Pase gene expression: 0.10 ±0.01 with diazoxide vs. 0.09 ± 0.02 with saline, p=0.72; Figure 5C).
Endogenous glucose production (EGP) is a critical component of the homeostatic mechanisms that maintain blood glucose at appropriate levels, and its dysregulation in type 2 diabetes contributes importantly to hyperglycemia. Given that activation of extrapancreatic $K_{\text{ATP}}$ channels is able to suppress EGP in both animal models and in healthy humans (12,15), the current study examined the ability of the $K_{\text{ATP}}$ channel activator diazoxide to regulate EGP in ZDF rats and in humans with moderately-to-poorly controlled diabetes under fixed hormonal conditions. Our results indicate that central regulation of EGP is impaired in both rats and humans with type 2 diabetes.

Given the potential that age and obesity might impact central regulation of glucose metabolism (33), we specifically recruited a group of age- and BMI-matched non-diabetic subjects as a comparison group for this study. Of note, the EGP response to diazoxide in these overweight, middle-aged subjects was consistent with our previous observations in younger, leaner subjects (15). An additional methodologic point pertains to diazoxide’s ability to activate $K_{\text{ATP}}$ channels in the plasma membrane of pancreatic $\beta$-cells, thereby inhibiting insulin secretion (34,35). Therefore, the current study utilized somatostatin, known to suppress insulin secretion via G-protein coupled somatostatin receptors and inhibit intracellular calcium ion translocation (36). The absence of any differences in plasma hormone levels confirms the adequacy of the ‘pancreatic clamp’ technique to allow us to isolate diazoxide’s extrapancreatic effects.

Furthermore, since hyperglycemia would be expected to suppress EGP in the diabetic subjects, it was important to correct hyperglycemia prior to the onset of the clamp studies. Indeed, the overnight insulin infusions in the diabetic group were designed to attain comparable basal rates of EGP and plasma glucose levels in the two groups. Additionally, insulin requirements progressively fell with correction of glucose toxicity overnight, such that insulin
infusion rates averaged 0.24±0.07 mU/kg/min by the final hour prior to the clamp studies (Supplementary Figure 1) and rates of EGP were similarly unsuppressed at the onset of the studies in both groups. Although we cannot exclude the possibility that some suppression of basal EGP by overnight insulin might have attenuated the effect of diazoxide on EGP in the diabetic rats and humans, since hepatic hyperinsulinemia may mask CNS effects on the liver (37-39), it is important to note that insulin infusion rates were no greater than basal for more than 6 hours prior to the study interval when EGP was calculated. Future studies using an SGLT2 inhibitor to lower glucose levels prior to the clamp could further address this question. Furthermore, activation and deactivation of $K_{ATP}$ channels are very rapid phenomena (40), making residual activating effects of insulin on $K_{ATP}$ channels unlikely.

Of note, plasma insulin infusion rates were approximately doubled in the diabetic vs. non-diabetic subjects in the placebo studies. This reflected mild insulin resistance despite correction of glucose toxicity in the diabetic subjects. The comparable rates of EGP in both subject groups demonstrated that we were successful in selecting appropriate insulin infusion rates to study both groups under basal EGP conditions. Furthermore, attaining individualized, basal insulin infusion rates during the clamp studies avoided the need for virtually any exogenous glucose infusion and permitted highly sensitive measures of glucose production without over-insulinization (15, 23).

The results of these studies in humans and rats are consistent with prior literature in rodents, suggesting that metabolic disturbances including those present in obesity and type 2 diabetes disrupt central regulation of glucose homeostasis. Inhibiting insulin action in the arcuate nucleus by a number of experimental approaches including insulin antibodies and inhibition of phosphatidylinositol 3-kinase (PI3K) results in a diminished ability of insulin to suppress EGP (41,42). Furthermore, insulin is unable to activate central $K_{ATP}$ channels in obese rats (20), consistent with our findings in ZDF rats. Indeed, high fat feeding of even short duration activates
hypothalamic S6 kinase, a putative mediator of insulin resistance, which in turn impairs the ability of circulating insulin to suppress EGP (14). Additionally, hypothalamic signaling via the insulin receptor substrate-phosphatidylinositol 3-kinase (IRS-PI3K) pathway, an important mediator of insulin action, is impaired in rats with streptozotocin-induced diabetes (17). Of note, it is likely that poor metabolic control contributes to the lack of EGP response in our diabetic subjects, since diabetic subjects in comparably poor control showed a lack of suppression of EGP by hyperglycemia (likely mediated at least in part by central K$_{ATP}$ channels), while diabetic subjects in good control showed normal suppression of EGP (43). This is also consistent with the observation that centrally-administered diazoxide was able to modulate EGP in high-fat fed yet normoglycemic rats (41). Furthermore, while intranasal insulin (presumably via central mechanisms) increased hepatic energy metabolism and reduced lipid storage in healthy humans, this effect was absent in patients with type 2 diabetes (44).

Collectively, these findings highlight the need for interventions to restore central signaling mechanisms. Various molecular targets have been proposed that could restore the brain’s sensitivity to nutrients in type 2 diabetes. These targets include regulators of insulin and leptin action (such as protein tyrosine phosphatase 1B, c-Jun-N terminal kinase, and SRC homology 2B) as well as peripheral modulators of glucose metabolism (such as glucagon-like peptide-1) (42). A critical challenge lies in developing medications that specifically target the brain without acting peripherally. One potential model is the established treatment for Parkinson’s disease, L-dopa, which is delivered with a peripheral decarboxylase inhibitor to prevent it from being metabolized prior to passing through the blood-brain barrier (45). In fact, even short-term improvement in an individual’s metabolic state may restore the integrity of the brain-liver pathway in type 2 diabetes (43). It will also be important to identify the critical stage(s) at which therapies targeting restoration of the brain-liver pathway would be most beneficial. Although delineating whether central or hepatic mechanisms are responsible for the
lack of response in diabetes was not the goal of the current studies, we hope to perform future studies specifically designed to delineate the central and/or peripheral site(s) at which the response is altered.

While it has long been accepted that type 2 diabetes is associated with malfunctions of the beta cell, muscle, and liver, growing evidence suggests that the brain also plays a key role in the pathogenesis of type 2 diabetes. The results of the current study are also relevant to recent work demonstrating that neurologic disorders such as Alzheimer’s disease, major depressive disorder, Parkinson’s disease, Huntington’s disease, and vascular dementia are all associated with metabolic derangements. Specifically, these diseases feature impaired systemic glucose metabolism and insulin resistance, as well as poor cerebral glucose utilization, cerebral insulin resistance, cerebral insulin deficiency, and abnormal expression of genes that are typically regulated by insulin (46-49). Indeed, a great deal of evidence supports a role for the brain in metabolic disease and, conversely, for metabolic derangements in the pathophysiology of neuropsychiatric disorders. This work also highlights a potential concern with the use of sulfonylurea agents in the longer treatment of type 2 diabetes, especially in patients with waning beta cell reserve. Inhibition of central \( \text{K}_\text{ATP} \) channels by these agents could increase EGP and hence contribute to deterioration of glycemic control in these individuals (50).

Thus, we present the first study in humans to show that regulation of EGP through activation of extrapancreatic \( \text{K}_\text{ATP} \) channels is impaired in type 2 diabetes, providing further insight into the CNS basis for the pathogenesis of metabolic disorders. Complementary rodent studies in a rat model of type 2 diabetes support our findings in humans. Given that unrestrained EGP is the chief source of hyperglycemia in type 2 diabetes, restoring the brain’s sensitivity to nutrient signals would be a promising therapeutic target.
AUTHOR CONTRIBUTIONS

Y.B.E. and M.C. wrote the manuscript, collected data, and assisted with running clamp studies. L.B., P.K. and A.G. contributed to the manuscript, collected data, and assisted with running clamp studies. D.M., S.Ke., P.R., S.Ko., D.F. and L.W. assisted with running clamp studies in humans and rats and/or performing laboratory assays. K.Z. oversaw the rodent experiments in collaboration with the Animal Physiology Core. M.H. designed the study, researched data, and wrote the manuscript. M.H. 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 and the analysis.

ACKNOWLEDGEMENTS

The authors Y.B.E., M.C., L.B., P.R., K.Z., D.M., S.Ke., S.Ko., A.G., P.K. and M.H. have no conflict of interests. The authors thank Kevin Jordan, Amelia Starr, Sarah Reda, Laura Clintoc, Stephen Marsh, and the staff of the Albert Einstein College of Medicine CRC and Hormone Assay Core of Einstein’s Diabetes Research Center (P60-DK20541), Dr. Gary Schwartz of the DRC’s Animal Physiology Core, and Drs. Jeffrey Pessin, Nir Barzilai and Richard Kitsis for helpful discussions. This work was supported by grants from the National Institutes of Health (DK069861 and DK48321) and the American Diabetes Association, and by the CTSA Grant UL1 RR025750 and KL2 RR025749 and TL1 RR025748 from the National Center for Research Resources (NCRR). Its contents are solely the responsibility of the authors and do not necessarily represent the official view of the NCRR or NIH.
REFERENCES

1. Turner R, Cull C, Holman R: United Kingdom Prospective Diabetes Study 17: a 9-year update of a randomized, controlled trial on the effect of improved metabolic control on complications in non-insulin-dependent diabetes mellitus. Ann Intern Med 1996;124:136-145

2. Gaster B, Hirsch IB: The effects of improved glycemic control on complications in type 2 diabetes. Arch Intern Med 1998;158:134-140

3. Suh DC, Choi IS, Plauschinat C, Kwon J, Baron M: Impact of comorbid conditions and race/ethnicity on glycemic control among the US population with type 2 diabetes, 1988-1994 to 1999-2004. J Diabetes Complications 2010;24:382-391

4. Campbell PJ, Mandarino LJ, Gerich JE: Quantification of the relative impairment in actions of insulin on hepatic glucose production and peripheral glucose uptake in non-insulin-dependent diabetes mellitus. Metabolism: clinical and experimental 1988;37:15-21

5. Consoli A: Role of liver in pathophysiology of NIDDM. Diabetes care 1992;15:430-441

6. Carey M, Kehlenbrink S, Hawkins M: Evidence for central regulation of glucose metabolism. J Biol Chem 2013;288:34981-34988

7. Kokorovic A, Cheung GW, Rossetti L, Lam TK: Hypothalamic sensing of circulating lactate regulates glucose production. J Cell Mol Med 2009;13:4403-4408

8. Lam CK, Chari M, Lam TK: CNS regulation of glucose homeostasis. Physiology (Bethesda, Md) 2009;24:159-170

9. Lam TK, Gutierrez-Juarez R, Pocai A, Rossetti L: Regulation of blood glucose by hypothalamic pyruvate metabolism. Science (New York, NY) 2005;309:943-947

10. Liu L, Karkanias GB, Morales JC, Hawkins M, Barzilai N, Wang J, Rossetti L: Intracerebroventricular leptin regulates hepatic but not peripheral glucose fluxes. The Journal of biological chemistry 1998;273:31160-31167

11. Obici S, Zhang BB, Karkanias G, Rossetti L: Hypothalamic insulin signaling is required for inhibition of glucose production. Nat Med 2002;8:1376-1382

12. Pocai A, Lam TK, Gutierrez-Juarez R, Obici S, Schwartz GJ, Bryan J, Aguilar-Bryan L, Rossetti L: Hypothalamic K(ATP) channels control hepatic glucose production. Nature 2005;434:1026-1031

13. Pocai A, Obici S, Schwartz GJ, Rossetti L: A brain-liver circuit regulates glucose homeostasis. Cell metabolism 2005;1:53-61

14. Ross R, Wang PY, Chari M, Lam CK, Caspi L, Ono H, Muse ED, Li X, Gutierrez-Juarez R, Light PE, Schwartz GJ, Rossetti L, Lam TK: Hypothalamic protein kinase C regulates glucose production. Diabetes 2008;57:2061-2065

15. Kishore P, Boucai L, Zhang K, Li W, Koppaka S, Kehlenbrink S, Schiwek A, Esterson YB, Mehta D, Bursheh S, Su Y, Gutierrez-Juarez R, Muzumdar R, Schwartz GJ, Hawkins M: Activation of K(ATP) channels suppresses glucose production in humans. J Clin Invest 2011;121:4916-4920

16. Dash S, Xiao C, Morgantini C, Koulajian K, Lewis GF: Intranasal insulin suppresses endogenous glucose production in humans compared with placebo in the presence of similar venous insulin concentrations. Diabetes 2015;64:766-774

17. Gelling RW, Morton GJ, Morrison CD, Niswender KD, Myers MG, Jr., Rhodes CJ, Schwartz MW: Insulin action in the brain contributes to glucose lowering during insulin treatment of diabetes. Cell metabolism 2006;3:67-73

18. Ikeda H, West DB, Pustek JJ, Figlewicz DP, Greenwood MR, Porte D, Jr., Woods SC: Intraventricular insulin reduces food intake and body weight of lean but not obese Zucker rats. Appetite 1986;7:381-386
19. Parton LE, Ye CP, Coppari R, Enriori PJ, Choi B, Zhang CY, Xu C, Vianna CR, Balthasar N, Lee CE, Elmquist JK, Cowley MA, Lowell BB: Glucose sensing by POMC neurons regulates glucose homeostasis and is impaired in obesity. Nature 2007;449:228-232
20. Spanswick D, Smith MA, Mirshamsi S, Routh VH, Ashford ML: Insulin activates ATP-sensitive K+ channels in hypothalamic neurons of lean, but not obese rats. Nat Neurosci 2000;3:757-758
21. Sandoval DA, Obici S, Seeley RJ: Targeting the CNS to treat type 2 diabetes. Nat Rev Drug Discov 2009;8:386-398
22. Esterson YB, Zhang K, Koppaka S, Kehlenbrink S, Kishore P, Raghavan P, Maginley SR, Carey M, Hawkins M: Insulin sensitizing and anti-inflammatory effects of thiazolidinediones are heightened in obese patients. J Investig Med 2013;61:1152-1160
23. Koppaka S, Kehlenbrink S, Carey M, Li W, Sanchez E, Lee DE, Lee H, Chen J, Carrasco E, Kishore P, Zhang K, Hawkins M: Reduced adipose tissue macrophage content is associated with improved insulin sensitivity in thiazolidinedione-treated diabetic humans. Diabetes 2013;62:1843-1854
24. Schiwek A, Lee DE, Saper M, Rossetti L, Kishore P, Hawkins M: Diazoxide suppresses endogenous glucose production in humans (Abstract). Diabetes 2007;56:1538
25. Sotsky MJ, Shilo S, Shamon H: Regulation of counterregulatory hormone secretion in man during exercise and hypoglycemia. J Clin Endocrinol Metab 1989;68:9-16
26. Novak M: COLORIMETRIC ULTRAMICRO METHOD FOR THE DETERMINATION OF FREE FATTY ACIDS. Journal of lipid research 1965;6:431-433
27. Pinter JK, Hayashi JA, Watson JA: Enzymic assay of glycerol, dihydroxyacetone, and glyceraldehyde. Archives of biochemistry and biophysics 1967;121:404-414
28. Williamson JR, Corkey BE: [65] Assays of intermediates of the citric acid cycle and related compounds by fluorometric enzyme methods. In Methods Enzymol John ML, Ed., Academic Press, 1969, p. 434-513
29. Hovorka R, Jayatillake H, Rogatsky E, Tomuta V, Hovorka T, Stein DT: Calculating glucose fluxes during meal tolerance test: a new computational approach. American journal of physiology Endocrinology and metabolism 2007;293:E610-619
30. Kehlenbrink S, Koppaka S, Martin M, Relwani R, Cui MH, Hwang JH, Li Y, Basu R, Hawkins M, Kishore P: Elevated NEFA levels impair glucose effectiveness by increasing net hepatic glycogenolysis. Diabologia 2012;55:3021-3028
31. Steele R: Influences of glucose loading and of injected insulin on hepatic glucose output. Ann N Y Acad Sci 1959;82:420-430
32. Torres TP, Catlin RL, Chan R, Fujimoto Y, Sasaki N, Printz RL, Newgard CB, Shiota M: Restoration of hepatic glucokinase expression corrects hepatic glucose flux and normalizes plasma glucose in zucker diabetic fatty rats. Diabetes 2009;58:78-86
33. Gong Z, Muzumdar RH: Pancreatic function, type 2 diabetes, and metabolism in aging. International journal of endocrinology 2012;2012:320482
34. Fabiano de Bruno L, Karabatas L, Cresto JC, Aparicio M, Basabe JC: A comparative study of two insulin secretion inhibitors: somatostatin and diazoxide. Horm Metab Res 1982;14:351-356
35. Giddings AE: Diagnosis and management of insulinoma. Proc R Soc Med 1974;67:833-836
36. Schwetz TA, Ustione A, Piston DW: Neuropeptide Y and somatostatin inhibit insulin secretion through different mechanisms. American journal of physiology Endocrinology and metabolism 2013;304:E211-221
37. Edgerton DS, Cherrington AD: Is brain insulin action relevant to the control of plasma glucose in humans? Diabetes 2015;64:696-699
38. Ott V, Lehnert H, Staub J, Wonne K, Born J, Hallschmid M: Central nervous insulin administration does not potentiate the acute glucoregulatory impact of concurrent mild hyperinsulinemia. Diabetes 2015;64:760-765
39. Ramnanan CJ, Kraft G, Smith MS, Farmer B, Neal D, Williams PE, Lautz M, Farmer T, Donahue EP, Cherrington AD, Edgerton DS: Interaction between the central and peripheral effects of insulin in controlling hepatic glucose metabolism in the conscious dog. Diabetes 2013;62:74-84
40. Shyng S, Ferrigni T, Nichols CG: Regulation of KATP channel activity by diazoxide and MgADP. Distinct functions of the two nucleotide binding folds of the sulfonylurea receptor. J Gen Physiol 1997;110:643-654
41. Filippi BM, Yang CS, Tang C, Lam TK: Insulin activates Erk1/2 signaling in the dorsal vagal complex to inhibit glucose production. Cell metabolism 2012;16:500-510
42. Prodi E, Obici S: Minireview: the brain as a molecular target for diabetic therapy. Endocrinology 2006;147:2664-2669
43. Hawkins M, Gabriely I, Wozniak R, Reddy K, Rossetti L, Shamoon H: Glycemic control determines hepatic and peripheral glucose effectiveness in type 2 diabetic subjects. Diabetes 2002;51:2179-2189
44. Gancheva S, Koliaki C, Bierwagen A, Nowotny P, Heni M, Fritsche A, Haring HU, Szendroedi J, Roden M: Effects of intranasal insulin on hepatic fat accumulation and energy metabolism in humans. Diabetes 2015;9
45. Koller WC, Rueda MG: Mechanism of action of dopaminergic agents in Parkinson's disease. Neurology 1998;50:S11-14; discussion S44-18
46. Bruning JC, Gautam D, Burks DJ, Gillette J, Schubert M, Orban PC, Klein R, Krone W, Muller-Wieland D, Kahn CR: Role of brain insulin receptor in control of body weight and reproduction. Science (New York, NY) 2000;289:2122-2125
47. Campayo A, de Jonge P, Roy JF, Saz P, de la Camara C, Quintanilla MA, Marcos G, Santabarbara J, Lobo A: Depressive disorder and incident diabetes mellitus: the effect of characteristics of depression. The American journal of psychiatry 2010;167:580-588
48. de la Monte SM: Brain insulin resistance and deficiency as therapeutic targets in Alzheimer's disease. Current Alzheimer research 2012;9:35-66
49. McIntyre RS, Soczynska JK, Konarski JZ, Woldeyohannes HO, Law CW, Miranda A, Fulgos D, Kennedy SH: Should Depressive Syndromes Be Reclassified as "Metabolic Syndrome Type II"? Annals of clinical psychiatry : official journal of the American Academy of Clinical Psychiatrists 2007;19:257-264
50. Gribble FM: Metabolism: a higher power for insulin. Nature 2005;434:965-966
| Continuous Variables                      | DIABETIC (N=8) | NON-DIABETIC (N=7) | P-value |
|------------------------------------------|----------------|-------------------|---------|
| **Age, years**                           | 49.75 (1.39)   | 47.14 (2.57)      | 0.37    |
| **BMI, kg/m\(^2\)**                     | 31.04 (0.54)   | 30.28 (0.98)      | 0.49    |
| **Weight, kg**                           | 95.64 (3.40)   | 97.20 (3.47)      | 0.75    |
| **HbA1c, %**                             | 9.15 (0.44)    | 5.26 (0.14)       | <0.0001 |
| **HbA1c, mmol/mol**                      | 76.62 (4.81)   | 34.14 (1.58)      | <0.0001 |
| **Fasting plasma glucose, mg/dL**        | 199.57 (17.74) | 95.6 (7.10)       | <0.001  |
| **Fasting insulin, uU/mL**               | 42.61 (12.30)  | 12.54 (2.33)      | 0.04    |
| **Fasting C-peptide, ng/mL**             | 0.31 (0.10)    | 1.41 (0.54)       | 0.04    |
| **Baseline Systolic Blood Pressure, mmHg**| 138.9 (6.47)   | 139.0 (7.90)      | 0.99    |
| **Baseline Diastolic Blood Pressure, mmHg**| 83.63 (4.77)   | 81.71 (3.74)      | 0.76    |
| **Baseline Heart Rate, bpm**             | 66.63 (3.71)   | 73.00 (3.51)      | 0.24    |

| Categorical Variables | N(%) | N(%) |
|-----------------------|------|------|
| **Race**              |      |      |
| Black                 | 3 (37.5) | 3 (42.9) |
| Hispanic              | 4 (50.0) | 1 (14.3) |
| Ethnicity | Count | Percentage |
|-----------|-------|------------|
| White     | 0 (0) | 2 (28.6)   |
| Other     | 1 (12.5) | 1 (14.3) |

| Sex       | Count | Percentage |
|-----------|-------|------------|
| Male      | 8 (100.0) | 7 (100.0) |
| Female    | 0 (0)   | 0 (0)      |
TABLE 2: Plasma hormone and substrate levels in diabetic subjects from t=180-240 min during the clamp studies (N=8).

|                  | DIAZOXIDE     | PLACEBO       | P-value |
|------------------|---------------|---------------|---------|
| Insulin, µU/ml   | 35.0 (5.6)    | 41.1 (8.4)    | 0.26    |
| Glucagon, pg/ml  | 61.2 (5.0)    | 73.6 (6.4)    | 0.10    |
| Lactate, mmol/L  | 0.8 (0.1)     | 0.8 (0.1)     | 0.73    |
| Glycerol, µmol/L | 32.2 (4.5)    | 28.8 (4.8)    | 0.63    |
| Free Fatty Acid, µmol/L | 385.8 (64.7) | 329.7 (70.1) | 0.58    |
| Cortisol, µg/dl  | 11.1 (1.9)    | 7.2 (1.1)     | 0.12    |
| C-peptide, nmol/L| 0.07 (0.01)   | 0.05 (0.01)   | 0.07    |
**TABLE 3:** Plasma hormone and substrate levels in non-diabetic controls from t=180-240 min during the clamp studies (N=7).

|                  | DIAZOXIDE | PLACEBO | P-value |
|------------------|-----------|---------|---------|
| Insulin, µU/ml   | 21.0 (3.3)| 17.4 (2.5)| 0.14    |
| Glucagon, pg/ml  | 76.8 (8.6)| 77.3 (6.3)| 0.90    |
| Lactate, mmol/L  | 0.5 (0.06)| 0.5 (0.04)| 0.74    |
| Glycerol, µmol/L | 19.8 (3.6)| 18.6 (5.0)| 0.82    |
| Free Fatty Acid, µmol/L | 134.2 (29.7) | 163.2 (41.4) | 0.26    |
| Cortisol, µg/dl  | 9.3 (0.7) | 10.1 (1.5) | 0.60    |
| C-peptide, nmol/L| 0.08 (0.02)| 0.08 (0.01)| 0.96    |
Figure 1

1A

Variable insulin infusion rates  Basal insulin infusion rates

Somatostatin, Glucagon, GH

6,6-D₂-Glucose Tracer

Glucose 90 mg/dl

Diazoxide or Placebo

Time Point (mins)

1B

Plasma Glucose (mg/dL)

- Non-diabetics w/ placebo
- Non-diabetics w/ diazoxide
- Diabetics w/ placebo
- Diabetics w/ diazoxide

Time (mins)
Figure 3

3A

Rate of EGP (mg/L/kg/min) in Diabetic Subjects

0 60 120 135 150 165 180 195 210 225 240
Time (mins)

Placebo
Diazoxide

3B

Rate of EGP (mg/L/kg/min) in Non-Diabetic Subjects

0 0.5 1 1.5 2 2.5 3

Placebo
Diazoxide

0 60 120 135 150 165 180 195 210 225 240
Time (mins)
Figure 5

A

Insulin infusion to slowly lower glucose to ~140mg/dL

Basal insulin with variable glucose infusion

Tracer

Somatostatin

Glucose ~140 mg/dL

Diazoxide or Saline Gavage

Pancreatic Clamp Begins

NPH Insulin 3-5 U/kg ~10 hours before the clamp study

Time Point (mins)

B

Average EGP during t=180-240 min of clamp (mg/kg/min)

Saline

Diazoxide
**Figure 1:** (A) Euglycemic pancreatic clamp protocol scheme. (B) Plasma glucose levels for each study group throughout the clamp studies. (C) Average insulin infusion rates for each study group throughout the clamp studies.

**Figure 2:** (A) The average rate of EGP during the final hour of clamp studies was significantly suppressed with diazoxide administration relative to placebo administration in non-diabetic controls (*p=0.002) but not in diabetic subjects. (B) The percent suppression of EGP by diazoxide was significantly impaired in diabetic subjects relative to non-diabetic controls (*p=0.01).

**Figure 3:** Time course of EGP during the clamp studies in (A) diabetic subjects and in (B) non-diabetic controls (*p<0.05).

**Figure 4:** (A) The average rate of glucose disappearance during the final hour of clamp studies did not significantly differ between placebo and diazoxide studies in either diabetic subjects or non-diabetic controls. (B) The average glucose infusion rate during the final hour of clamp studies did not significantly differ between placebo and diazoxide studies in either diabetic subjects or non-diabetic controls.

**Figure 5:** (A) ZDF rat euglycemic pancreatic clamp protocol scheme. (B) The average rate of EGP during the final hour (t=180-240 min) of the study. EGP was not significantly suppressed in ZDF rats after diazoxide administration relative to saline control. (C) Hepatic PEPCK and G6Pase gene expression levels in diazoxide-treated and saline-treated ZDF rats.
Supplementary Figure Legend:

**Supplementary Figure 1:** (A) Overnight capillary blood glucose levels for diabetic subjects prior to onset of the clamp studies. (B) Overnight insulin infusion rates for diabetic subjects prior to onset of the clamp studies.