SUPPLEMENTAL DATA

METHODS

Human subject recruitment*:* All procedures were approved by Einstein's Institutional Review Board. The purpose, nature, risks and benefits of the study were explained to all potential subjects, 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), and baseline EKG. A 2 hour oral glucose tolerance test was performed to ensure normal glucose tolerance.

Human infusion studies: Each subject underwent two euglycemic pancreatic clamp studies separated by 4-6 weeks. Two hours prior to onset of the clamp studies, a primed continuous infusion of [6,6-$^2H_2$]glucose was initiated. Glucose metabolism was quantified during the final hour of the studies, once "steady state" conditions were achieved. Given the potential for diazoxide at high doses to lower blood pressure, vital signs were recorded hourly. An 18-gauge catheter was inserted in an antecubital vein for infusions and a contralateral hand vein was cannulated for blood sampling (1). To obtain arterialized venous blood, this hand was kept in a warming blanket maintained at 55°C.

Based on pilot studies in which optimal metabolic effects of diazoxide were observed 6 hours after oral administration (2), our experimental protocols lasted 7 hours and consisted of an initial 3 hour waiting period after the administration of the study agent, followed by a 4 hour euglycemic pancreatic clamp study (Figure 1A). Subjects received either oral diazoxide 4 mg/kg or matched placebo (similar in taste and appearance) in a randomized, double-blinded fashion at t=-180 minutes.

To measure glucose fluxes, a primed continuous infusion of [6,6-$^2H_2$]glucose was initiated at t=-120 minutes (primed infusion of 5.32 mg/kg/min for 5 minutes, then continued at 0.038 mg/kg/min for the duration of the study). The calculations to obtain the bolus and continuous

infusion rates for [6,6-$^{2}H_2$]glucose were obtained from the estimated volume of distribution of glucose and glucose kinetics based on the mathematical models of Finegood and Steele (3, 4).

The 'euglycemic pancreatic clamp study' began at t=0 minutes. All experiments consisted of 240 minutes of insulin/somatostatin (250 µg/hr) infusions with replacement of glucoregulatory hormones (glucagon 1 ng/kg/min; growth hormone 3 ng/kg/min). Plasma glucose concentrations were measured at 5 to 10 minute intervals during the 240 minutes of the study. From t=0 to t=180 minutes, optimal insulin infusion rates were selected in each individual by making frequent adjustments (~ every 25 minutes) to the insulin infusion rates in order to establish rates required to maintain euglycemia (90 mg/dl) without the requirement for exogenous glucose infusion. The individual's optimal insulin infusion rate was then kept constant for the remainder of the study. All infusions were stopped at t=240 minutes.

From t=0 to t=240 minutes, blood samples were obtained for determinations of plasma glucose, insulin, glucagon, C-peptide, cortisol, growth hormone, free fatty acids, glycerol, lactate, catecholamines, and [6,6-$^{2}H_2$]glucose. Data for glucose turnover represent the mean values during the final 60 minutes of the studies (t=180-240 minutes). Rates of glucose appearance (Ra) and disappearance (Rd) and other indices of glucose turnover were estimated using Steele equations (4), using the assumption that Ra=Rd for steady state., and using the following calculation: Rd= (Basal [6,6-$^{2}H_2$]glucose infusion rate + D20/[6,6-$^{2}H_2$]glucose infusion rate)/APE fraction/ wt (kg). Endogenous glucose production (EGP) was determined by subtracting the rates of glucose infusion from the tracer-derived Ra. For the [6,6-$^{2}H_2$]glucose determinations, GC/electron impact-mass spectrometry analysis were performed in the CTSA Analytic Core Laboratory (5). Plasma samples for Gas Chromatography-Mass Spectrometry (GC-MS) were derivatized after protein precipitation to the aldehyde penacetate with hydroxykamine hydrochloride-acetic anhydride.

Rats: Twenty-two 5-7 week-old male Sprague Dawley rats (Charles River Breeding Laboratories) with an average weight of 306.4 ± 2.0 g were studied under the following conditions:

1. Oral (gavage) saline control (S, n=6)
2. Oral (gavage) diazoxide (D, n=6)
3. Oral (gavage) diazoxide with ICV infusion of the K_{ATP} channel blocker glibenclamide (D+G, n=5)
4. Oral (gavage) saline with ICV glibenclamide (G, n=5)

Rats matched for age and weight received oral saline (S) or diazoxide (D) by gavage two hours prior to a four-hour pancreatic clamp study (Figure 3A). Additional groups of rats received the same dose of oral diazoxide together with an ICV infusion of the K_{ATP} channel blocker glibenclamide (D+G) or ICV glibenclamide alone (G), commencing two hours prior to the pancreatic clamp study, to determine whether the effects of oral diazoxide on EGP are mediated through central mechanisms. Rats were prepared for the *in vivo* experiments as previously described (6) with implantation of an ICV catheter under anesthesia two weeks prior to the study and implantation of carotid and internal jugular catheters one week prior.

Rat infusion studies: Each infusion study lasted 240 minutes. 120 minutes *prior* to the infusion study (t=-120 minutes), rats were anesthetized with isofluorane and either saline or diazoxide (100 mg/kg) was administered by oral gavage. For the remainder of the studies, the rats were conscious and unrestrained. An ICV infusion of glibenclamide (0.006 µl/min; n=5 rats) or saline was started at t=-120 minutes. At t=0 minutes (2 hours after saline or diazoxide gavage), a primed continuous IV infusion of [3-^{3}H]-glucose (1600 µl bolus, 40 µl/min thereafter, Perkin Elmer) was begun and maintained throughout the study to assess glucose kinetics in vivo. Blood samples were obtained at 10 minute intervals from 180-240 min to assess [3-^{3}H]-glucose specific activity. A peripheral basal insulin (1 mU/kg/min) pancreatic-euglycemic clamp was performed for the final two hours of the infusion study (t=120-240 minutes) as previously

described (7). At the onset of the clamp studies (t=120 min), plasma glucagon levels were comparable among groups (S=56.1+2.8, D=51.9+4.4, D+G=55.1+1.8, S+G=50.4+4.9 pg/ml; *P*>0.05 for all groups). Plasma insulin levels at the onset of the clamp were undetectable in 4 of the rats in the diazoxide group, but did not differ in the other groups (S=0.60+0.09, D+G=0.62+0.09, S+G=0.62+0.09 ng/ml; *P*>0.05 for all groups). The data for glucose infusion rate, glucose production, and glucose uptake are presented as an average of values obtained from the final 60 minutes (t = 180–240 minutes) of the infusion studies. Following the study, rats were anesthetized with ketamine (150 mg/kg). CSF samples were obtained by ventricular puncture and liver tissue samples were obtained by freeze clamping. An additional n=13 rats underwent time course studies in which oral diazoxide was administered by gavage at t=0, with CSF samples obtained by ventricular puncture under anesthesia at t=1,3,5 or 6 hours. CSF was analyzed for diazoxide content in the laboratory of Nancy Brown at Vanderbilt University using high-pressure liquid chromatography as previously described (8). All animal studies were approved by Einstein's Institutional Animal Care and Use Committee.

Western blot analysis: Rat liver tissue (10mg) was homogenized in RIPA lysis buffer containing 1mM sodium orthovanadate and complete protease inhibitor cocktail (Santa Cruz Biotechnology, Santa Cruz, CA). Samples were run three times on separate gels for quantification purposes. Each membrane was cut into two pieces for target proteins and housekeeping protein as loading control, which are as follows: phospho-STAT3 (Tyr705), total STAT3, phospho-Akt (Ser473), total Akt and HRP conjugated GAPDH (Cell Signaling Biotechnology, Inc., Beverly, MA), PEPCK and G6Pase (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The primary antibodies were incubated overnight at 4^{o}C. HRP-conjugated anti-rabbit (Zymed) secondary antibody was applied to the membrane about 1 hour at room temperature. Membranes from phospho-STAT3 (Tyr705) and phospho-Akt (Ser473) were stripped with the stripping agent Restore (Pierce, Rockford, IL) according to the protocol of the manufacturer and reprobed with total STAT3 and total Akt antibodies, respectively. All the reactions were detected

using Pierce® ECL reagent (Pierce, Rockford, IL). Image Reader LAS3000 (Fujifilm) was used for the immunoreactive band visualization. Quantification of band intensity was performed using a digital image system (Multi Gauge V3.1, Fujifilm). Each p-Stat3 and p-Akt densitometric value was normalized to the corresponding total-Stat3 and total-Akt value. For PEPCK and G6Pase, relative protein levels were calculated relative to the levels of the housekeeping protein GAPDH.

Real-time Reverse Transcriptase Polymerase Chain Reaction (rt-PCR): Real-time rt-PCR was performed to examine the expression of PEPCK and G6Pase in rat liver, using a Roche LightCycler and SYBR Green I (Qiagen), as previously described (9). Expression of the 'housekeeping genes' CPHN2, B2M, and RPL-19 was also examined. The primer sequences are provided in Table S3. Relative gene expression was calculated as the ratio of target gene divided by the geometric mean of the expression of housekeeping genes (10).

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Table S1:

Hormone concentrations and EGP measurements at baseline, during the equilibration and steady state phases of the human clamp studies.

A

Time (min)	Insulin (μU/ml)		C-peptide (ng/ml)		FFA (μmol/L)		Cortisol (μg/dl)		Glucagon (pg/ml)	
	Plc	DZX	Plc	DZX	Plc	DZX	Plc	DZX	Plc	DZX
0	11.36± 2.34	7.41±1.50*	1.52±0.31	1.19±0.23	495±55	533±69	9.66±1.40	6.57±0.76	50.39± 4.68	46.56±3.56
60-120	26.47±3.62	21.59±3.48	0.28±0.05	0.27±0.03	208±28	216±36	8.81±1.38	8.02±0.48	65.70± 3.66	63.06±2.68
180-240	23.40± 2.39	18.31±3.05	0.17±0.02	0.18± 0.03	141±33	171±39	8.54±1.16	7.31±0.21	63.94± 3.86	59.89±2.99

B

Time (min)	IIR (mU/kg/min)		Glucose (mg/dl)		GIR (mg/kg/min)		EGP (mg/kg/min)	
	Plc	DZX	Plc	DZX	Plc	DZX	Plc	DZX
0	0.23±0.03	0.24±0.04	92.90±1.81	96.80±2.27	0	0	1.76±0.09	1.73±0.08
60-120	0.27±0.04	0.26±0.05	97.56±2.41	97.56±2.48	0.24±0.14	0.44±0.19	1.99±0.13	1.75±0.16
180-240	0.26± 0.04	0.23±0.05	93.98±1.52	91.20±0.73	0.93±0.30	1.19±0.21	1.63±0.17	1.15±0.13*

Time 0 min = Baseline, Time (60-120) = Equilibration phase, Time (180-240) = steady state.
Plc = Placebo, Dzx = Diazoxide.
*P<0.05.

Table S2:

Rat hormone concentrations and EGP measurements during the steady state phase of the clamp studies.

Group	Insulin (ng/ml)	C-peptide (nmol/L)	Glucagon (pg/ml)	FFA (mmol/L)	Glucose (mg/dl)	EGP (mg/kg/min)	%EGP change from Basal
Saline	1.14±0.79	0.11±0.05	39.6±4.7	0.35±0.33	139.05±2.76	4.85±0.42	31.17±8.12
Diazoxide	1.01±0.27	0.11±0.03	38.4±5.1	0.32±0.04	146.31±1.06	2.23±0.28*	72.82±2.20*
D+G	1.18±0.10	0.10±0.04	38.3±4.4	0.33±0.02	142.89±1.70	4.64±0.41	44.25±3.64
S+G	1.11±0.08	0.08±0.03	35.7±2.6	0.28±0.03	143.51±2.99	5.87±0.42	19.17±4.68

Table S3:

Primer sequences for PEPCK, glucose-6-phosphatase, and housekeeping genes.

Genes	Forward Sequence	Reverse Sequence
Rat PEPCK	ATACGGTGGGAACTCACTGC	TGCCTTCGGGGTTAGTTATG
Rat G6Pase	TGCTGCATCTCTTTGACTCG	TTGTGTGTCTGTCCCAGGAG
Rat CPHN2	AGTGACCTTTGGACTCTTTGGA	ATTGGTGTCTTTGCCTGCATT
Rat B2M	CTGCTACGTGTCTCAGTTCCAC	TGCAAGCATATACATCGGTCTC
Rat RPL-19	GAAGAGGAAGGGTACTGCCAAC	TTTTTGAACACATTCCCTTTGA

Figure S1

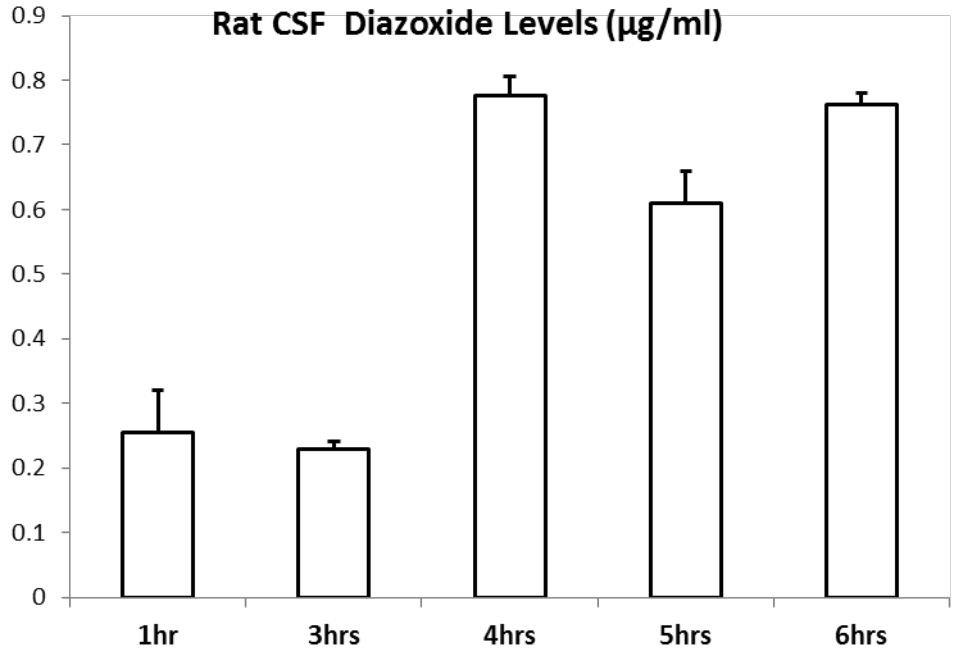

Figure S1. Diazoxide levels in rat CSF sampled at designated time points after gavage.