Correcting Postprandial Hyperglycemia in Zucker Diabetic Fatty Rats with a SGLT2 Inhibitor Restores Glucose Effectiveness in Liver and Reduces Insulin Resistance in Skeletal Muscle

Tracy P. O’Brien,1 Erin C. Jenkins,1 Shanea K. Estes,1 Antonio V. Castaneda1, Kiichiro Ueta1, Tiffany D. Farmer2, Allison E. Puglisi2, Larry L. Swift3, Richard L. Printz,1 and Masakazu Shiota1, 2

1Department of Molecular Physiology and Biophysics, 2Diabetes Research Training Center, 3Department of Pathology, Vanderbilt University School of Medicine, Nashville, TN.
[A] Supplementary Table 1. Primers for real-time PCR analysis of gene expression.

| Gene  | Primer              |
|-------|---------------------|
| GK    | Forward 5'-GGAGCAGAAGGGAACACCATCG-3' |
|       | Reverse 5'-CCTCACATTTGGCGGTCTTTACAG-3' |
| GKRP  | Forward 5'-AGAGTGTTGTCATAGGACATC-3' |
|       | Reverse 5'-GCTGTGTTTATCCATGAGTAGTC-3' |
| PEPCK | Forward 5'-GACGGTTCATCATATTACCAAGAGC-3' |
|       | Reverse 5'-CGGGAACCTTGCGGTGAATG-3' |
| RPL13A| Forward 5'-TACTCTGGAGAGAAACGGAAG-3' |
|       | Reverse 5'-GCCTGTTTTCCTTAGCCTCAA-3' |

GK, glucokinase; PEPCK, phosphoenolpyruvate carboxykinase; RPL13A, ribosomal protein L3a.

[B] Immunohistochemical and Western blot analyses of GK and GKRP

Quantitative image analysis of GK and GKRP immunofluorescence, in the nucleus and the cytoplasm of hepatocytes, was performed using a Zeiss LSM510 confocal laser scanning microscope. The internal He/Ne laser and external argon-krypton laser at 543, 647, and 488 nm were used to optimally excite Cy3, Cy5, and YoPro-1 fluorescence, respectively. After the transfer of image files to a computer imaging workstation, the image files were converted to TIFF format and individual cells within each image were quantified using nuclear/cytoplasmic pixel density rationing with Metamorph Offline-Meta Series Imaging Environment (Molecular Devices Corporation, Sunnyvale, CA). Serial sections were incubated with either sheep anti-rat GST-GK serum or rabbit anti-rat GST-GKRP serum and YoPro-1 or the combination of preimmune serums of the sheep or rabbit and YoPro-1. GK and GKRP are not expressed homogenously in all parenchymal cells within the liver. GK is expressed from periportal to perivenous areas with an increasing gradient of expression in normal [1] and ZDF rats (data not shown). Even in perivenous areas, the intensities of immunofluorescence of GK and GKRP vary among hepatocytes [2]. Furthermore, it is possible that the extent of GK translocation in response to increased plasma glucose differs throughout the liver lobule. To avoid intentional selection and to obtain results reflecting changes in the whole liver, six microscopic areas were randomly selected from each section for image analysis.

The area of the nucleus in each cell was determined as an immunoreactive area with YoPro-1. We selected cells with a higher immunofluorescence intensity of Cy5 (GKRP) in the nucleus compared with controls stained with preimmune serum. To identify Cy3 (GK)-positive cells among these selected cells, we selected cells with a higher immunofluorescence intensity of Cy5 (GKRP) in the nucleus compared with controls stained with pre-immune serum. We did not detect any cells in which GK was present in the nucleus in the absence of GKRP.

Ten to twenty cells were Cy5-positive in each microscopic field, and thus the total number of chosen cells was 60-120 cells for each section. For selected cells, we measured the immunofluorescence intensity of Cy3 and Cy5 in the whole nucleus and cytosolic areas. Nuclear-to-cytoplasm pixel density ratios of GK and GKRP were determined by digital image analysis, using LSM5 Image Browser. The ratios of nuclear to cytoplasmic fluorescence of GK and GKRP were averaged for each animal, and the average value was normalized to that of a standard liver sample, stained on the same day. The results for each group were expressed as the mean ± SE of the normalized value in six animals.

[C] Calculations of glucose kinetics and liver intermediate fluxes.

Efficiency of detritiation of [2-3H]-G-6-P by exchange of [3H] of [2-3H]-G-6-P with [H+] of bulk water mediated by hexose isomerase.

Assuming that glycogen synthesis occurred at a constant rate during the test period, then fractional detritiation of [2-3H]-G-6-P (D[2-3H]), which occurs by exchange of [3H] of [2-3H]-G-6-P with [H+] of bulk water mediated by hexose isomerase should be stable during the test period and the ratio of [2-3H] to [3-3H]-glucose incorporated into glycogen would then approximate that of the G-6-P pool. The efficiency of detritiation was thereby
SUPPLEMENTARY DATA

calculated as the ratio of \([2-3H]\)-glucose to \([3-3H]\)-glucose incorporated into glycogen.

Incorporation of \([2-3H]\)- and \([3-3H]\)-glucose into hepatic and skeletal muscle glycogen

To estimate the amounts of incorporated \([2-3H]\)- and \([3-3H]\)-glucose into hepatic and skeletal muscle glycogen (GLY-[2-3H]-PG and GLY-[3-3H]-PG, respectively) during the test period were calculated as follows:

\[
\text{GLY-[2-3H]-PG} = \frac{[2-3H] \text{ radioactivity in hepatic glycogen (dpm/g liver)}}{([2-3H]SA-PG30+[2-3H]SA-PG60+[2-3H]SA-PG75+[2-3H]SA-PG90+[2-3H]SA-PG105+[2-3H]SA-PG120)/6}
\]

\[
\text{GLY-[3-3H]-PG} = \text{[3-3H] radioactivity in hepatic glycogen (dpm/g liver)}
\]

Where \(([2-3H]SA-PG30+[2-3H]SA-PG60+[2-3H]SA-PG75+[2-3H]SA-PG90+[2-3H]SA-PG105+[2-3H]SA-PG120)/6\) and \(([3-3H]SA-PG30+[3-3H]SA-PG60+[3-3H]SA-PG75+[3-3H]SA-PG90+[3-3H]SA-PG105+[3-3H]SA-PG120)/6\) are the averages of \([2-3H]\)SA-PG and \([3-3H]\)SA-PG of plasma glucose at 30, 60, 75, 90, 105 and 120 min, respectively. The amounts of incorporated \([2-3H]\)- and \([3-3H]\)-glucose were measured at the end of the clamp period.

A fractional detritiation of \([2-3H]\)G-6-P (D[2-3H]) by exchange of \([3H]\) of \([2-3H]\)G-6-P with \([H^+]\) of bulk water mediated by hexose isomerase was calculated as

\[
D_{[2-3H]} = \frac{\text{GLY-[2-3H]-glucose}}{\text{GLY-[3-3H]-glucose}}
\]

This calculation is based on the assumption that the ratio of \([2-3H]\) to \([3-3H]\)-glucose incorporated into glycogen approximates that of the G-6-P pool.

Rates of glucose appearance, total glucose disappearance and endogenous glucose production

\([2-3H]\)- and \([3-3H]\)-glucose determined rates of unlabeled glucose appearance (\([2-3H]\)Ra and \([3-3H]\)Ra) were calculated using Steele’s equation [3] which is based on a one-pool model and on the initial assumption of instant mixing of glucose in its entire space. Proietto et al. [4] reported that the one-pool model gave the best results given the effective volume of distribution of glucose was 50% of glucose distribution volume in obese \(fa/fa\) rats and their lean littermates. It can be written as follows:

\[
[2-3H]\text{Ra} = \frac{[2-3H]GI^* - \{0.5 \cdot V_D \cdot (PG_1 + PG_2)/2 \cdot ([2-3H]SA-PG_2 - [2-3H]SA-PG_1)/(t_2 - t_1)\}}{([2-3H]SA-PG_2 + [2-3H]SA-PG_1)/2}
\]

\[
[3-3H]\text{Ra} = \frac{[3-3H]GI^* - \{0.5 \cdot V_D \cdot (PG_1 + PG_2)/2 \cdot ([3-3H]SA-PG_2 - [3-3H]SA-PG_1)/(t_2 - t_1)\}}{([3-3H]SA-PG_2 + [3-3H]SA-PG_1)/2}
\]

where \([2-3H]GI^*\) and \([3-3H]GI^*\) equal the infusion rate of \([2-3H]\)-glucose and \([3-3H]\)-glucose, respectively, \(PG_1\) and \(PG_2\) equal plasma glucose concentration at time \(t_1\) and \(t_2\), \([2-3H]SA-PG_1\) and \([3-3H]SA-PG_2\) equal the \([2-3H]\) and \([3-3H]\) specific activities of plasma glucose at times \(t_1\) and \(t_2\). \(V_D\) is glucose distribution volume (ml/kg) and \(\{0.5 \cdot V_D \cdot (PG_1 + PG_2)/2\}\) is the effective fraction of glucose pool. We used 275 ml/kg as \(V_D\) in ZDF rats that was measured in our previous study [5].

\([2-3H]\)- and \([3-3H]\)-glucose determined rates of unlabeled glucose disappearance (\([2-3H]\)Rd and \([3-3H]\)Rd,
respectively) according to Steele’s equation [3]:

\[
[2-^3H]Rd = [2-^3H]Ra - 0.5\cdot VD \cdot \left(\frac{PG2 - PG1}{t2 - t1}\right)
\]

\[
[3-^3H]Rd = [3-^3H]Ra - 0.5\cdot VD \cdot \left(\frac{PG2 - PG1}{t2 - t1}\right)
\]

Endogenous glucose production rate was determined as the difference between [3-^3H]Ra and exogenous glucose infusion rates.

**Endogenous glucose disposal rate**

Amounts of glucose excreted into urine (UG) during the test period were assessed as the sum of the amount of glucose in urine urinated during the test period and collected from bladder at the end of the clamp period. Endogenous glucose disposal rate (E-Rd) could theoretically be estimated as the difference between measured Rd and UG. Since UG could not be measured at each time point as blood was collected, E-Rd was estimated as the difference between AUC of Rd and cumulated GU during the test period.

**Glucose cycling.**

Glucose cycling is defined as input of extracellular glucose into the G-6-P pool followed by exit of plasma-derived G-6-P back into the extracellular pool. Assuming 100% exchange of D[2-3H], the minimum estimation of unlabelled glucose cycling was assessed as the difference between [2-^3H]Rd and [3-^3H]Rd. The maximum estimation of unlabelled glucose cycling was assessed by dividing the minimal estimation by the efficiency of detritiation of [2-3H] G-6-P (D[2-3H]).

**Glucose-6-phosphatase flux.**

The in vivo flux through glucose-6-phosphatase (G-6-Pase) was assessed as the sum of glucose cycling and endogenous glucose production rates.

**Rates of glycogen synthesis via the direct pathways.**

The amount of newly synthesized glycogen from plasma glucose via the direct pathway (GLY[3-^3H]: glucose → G-6-P → glycogen) during the test period was calculated as the division of GLY-[3-^3H]-PG by the length of the test period (120 min).

**A negative value for endogenous glucose production**

During hyperinsulinemic-hyperglycemic clamps in ZCL rats, we obtained a negative value for endogenous glucose production and as a result, a lower rate of G-6-Pase flux relative to glucose cycling. The discrepancy is due to the paradox that tracer-derived glucose disposal rates are less than the exogenous glucose infusion rates. The tracer-derived glucose disposal rate can be equal to but never less than the exogenous glucose infusion rate because the tracer-derived glucose disposal rate represents total glucose flux. This discrepancy, however, has been reported by numerous investigators and the potential mechanism was extensively discussed by Argoud GM et al. [6] and Bell PM et al. [7]. The precise mechanism has yet to be determined. If the paradox occurs with [3-^3H]-glucose but not with [2-^3H]-glucose, then the glucose cycling rate calculated as the difference between [2-^3H]Rd and [3-^3H]Rd would be over-estimated.
SUPPLEMENTARY DATA

References

1. Eilers F, Bartels H, Jungermann K. Zonal expression of the glucokinase gene in rat liver. Dynamics during daily feeding rhythm and starvation-refeeding cycle demonstrated by in situ hybridization. *Histochemistry* 99: 133-140, 1993.

2. Fujimoto Y, Donahue EP, Shiota M. Defect in glucokinase translocation in Zucker diabetic fatty rats. *Am J Physiol Endocrinol Metab* 287: E414-E423, 2004.

3. Steele R: Influences of glucose loading and of injected insulin on hepatic glucose output. *Ann N Y Acad Sci* 82:420-430, 1959

4. Proietto J, Rohner-Jeanrenaud F, Ionescu E, Terretaz J, Sauter JF, Jeanrenaud B: Non-steady-state measurement of glucose turnover in rats by using a one-compartment model. *Am J Physiol* 252:E77-84, 1987

5. Torres TP, Fujimoto Y, Donahue EP, Prontz RL, Houseknecht KL, Treadway JL, Shiota M. Defective glycogenesis contributes toward the inability to suppress hepatic glucose production in response to hyperglycemia and hyperinsulinemia in Zucker diabetic fatty rats. Diabetes 2011; 60: 2225-2233.

6. Argoud GM, Schade DS, Eaton RP: Underestimation of hepatic glucose production by radioactive and stable tracers. *Am J Physiol* 252:E606-615, 1987

7. Bell PM, Firth RG, Rizza RA: Assessment of insulin action in insulin-dependent diabetes mellitus using [6(14)C]glucose, [3(3)H]glucose, and [2(3)H]glucose. Differences in the apparent pattern of insulin resistance depending on the isotope used. *J Clin Invest* 78:1479-1486, 1986

[D] Protocols for measurement of effect of SGLT2 inhibitor on postprandial metabolic flux.

A) 4 hours treatment protocol

| DAY-0 | ~ | DAY-5 |
|-------|---|-------|
| No treatment |

| 6 AM | 8 AM | 12 PM | 12:30 PM | 1:30 PM |
|-------|-------|-------|----------|---------|
| Fast  |       |       |          |         |
| SGLT2-I (10 mg/kg) |       |       |          |         |
| Mixed Meal |

B) 7 days treatment protocol

| DAY-0 | ~ | DAY-6 |
|-------|---|-------|
| Daily treatment at 4 pm of SGLT2-I (10 mg/kg) or vehicle |

| 6 AM | 12 PM | 12:30 PM | 1:30 PM |
|-------|-------|----------|---------|
| Fast  |       |          |         |
| Mixed Meal |

Supplementary Figure S1. Protocols for measurement of effects of acute and chronic treatment with SGLT2 inhibitor on postprandial metabolic flux. Two weeks prior to each study (at 8 weeks of age), rats underwent surgery to place catheters in the ileal vein, left carotid artery and right jugular vein. To assess the acute effect of SGLT2 inhibitor (Protocol A), 10-week-old ZDF rats and their lean littermates were fasted from 6 am and dosed once with either SGLT2 inhibitor (Canagliflozin, Mitsubishi Tanabe Pharma Corporation, Saitama, Japan) at 10 mg/kg within a 5 ml/kg volume or vehicle (0.2% carboxymethyl cellulose containing 0.2% Tween® 80) at 5
SUPPLEMENTARY DATA

ml/kg at 8 am. Likewise, age-matched ZCL rats were dosed once with vehicle. A mixed-meal tolerance test (MTT) was performed from 12:00 pm. Each MTT consisted of a 30-min control/basal period (-30 to 0 min) and a 60-min test period (0 to 60 min). At 0 min (12:30 pm), animals were given by gavage a liquid mixed meal (5 ml/kg) containing (per 5 ml) 1.55 g glucose (1.65 g of Polycose, ROSS nutrition, Columbus, OH), 0.75 g protein (0.87 g of Beneprotein, Novartis medical nutrition, Minneapolis, MN) and 0.21 g lipid (0.42 ml of Microlipid, First Option Medical, Cheyenne, WY). To examine the effect of a more chronic treatment with SGLT2 inhibitor (Protocol B), rats were given at 4:00 pm daily from 9 weeks of age for 7 days (from DAY-0 to DAY-6) either vehicle or SGLT2 inhibitor. On Day-7, animals were fasted from 6 am and a mixed-meal tolerance test (MTT) was performed from 12:00 pm.

[E] Protocol for measurement of the daily rhythm of energy metabolism.

Supplementary Figure S2. Protocols for measurement of effects of chronic treatment with SGLT2 inhibitor on the daily rhythm of energy metabolism. Two weeks prior to each study (at 8 weeks of age), ZDF rats and their lean littermates underwent surgery to place catheters in the ileal vein, left carotid artery and right jugular vein. Animals were given at 4:00 pm daily from 9 weeks of age for 8 days (from DAY-0 to DAY-7) either vehicle or SGLT2 inhibitor. Animals were housed in an Oxymax-CLAMS (Columbus Instruments, Columbus, OH) from DAY-5. The daily rhythm of energy metabolism; oxygen consumption, carbon dioxide production and food intake, were measured from 6 am on DAY-7 to 6 am on DAY-8, and then immediately followed by measurement of body composition using an EchoMRI 700 (Echo Medical System, Houston, YX). Right after measurement of body composition, animals were anesthetized by an intraperitoneal injection of pentobarbital sodium (60 mg·kg⁻¹), and a laparotomy was performed. Blood was collected from the vena cava. The left lobe of the liver and skeletal muscles (vastus lateralis, gastrocnemius-plantalis and soleus) were frozen using Wollenberg tongs pre-cooled in liquid nitrogen.

©2017 American Diabetes Association. Published online at http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db16-1410/-/DC1
Protocol for measurement of glucose flux.

Supplementary Figure S3. Protocols for measurement of effects of chronic treatment with SGLT2 inhibitor on glucose flux. Two weeks prior to each study (at 8 weeks of age), ZDF rats and their lean littermates underwent surgery to place catheters in the ileal vein, left carotid artery and right jugular vein. Animals were given at 4:00 pm daily from 9 weeks of age for 7 days (from DAY-0 to DAY-6) either vehicle or SGLT2 inhibitor. On DAY-7, animals were fasted from 6 am and the clamp studies were performed between 11:00 am and 3:30 pm. Each study consisted of a 90 min tracer equilibrium period (-150 to -60 min), a 1-hour basal period (-60 to 0 min), and a 2-hour test period (0 to 120 min). At -150 min (11 am), both [2-3H] and [3-3H] glucose were given at 60 µCi in a bolus followed by continuous infusion at 0.6 µCi·min⁻¹ into systemic circulation through the jugular vein catheter. During the test period, in basal (no clamp) study [Protocol- Basal (no clamp)], saline was infused at 20 µl/kg/min into the systemic circulation through the jugular vein catheter and into the hepatic portal circulation via the ileal vein catheter. During the test period of all pancreatic hormone and glycemic clamp studies, somatostatin was infused into systemic circulation at 5 µg·kg⁻¹·min⁻¹ to inhibit endogenous insulin and glucagon secretion. Recombinant glucagon (GlucaGen, Bedford Laboratories, Bedford, OH) was infused into the hepatic portal system through the ileal catheter at 1.8 ng·kg⁻¹·min⁻¹ to maintain basal plasma glucagon levels. During the hyperinsulinemic-hyperglycemic clamp (Protocol- HI-HG), human recombinant insulin (Novolin R, Novo Nordisk Inc., Plainsboro, NJ) was infused into the portal vein at 16 mU·kg⁻¹·min⁻¹. Plasma glucose levels were maintained at ~13 mM by infusing 50% dextrose solution (D50, Hospira, Inc., Lake Forest, IL) into the systemic circulation. During hyperinsulinemic-basal glucose clamps (Protocol- HI-Basal G), human recombinant insulin was infused into the portal vein at 16 mU·kg⁻¹·min⁻¹. Plasma glucose levels were maintained at basal levels of each animal by infusing 50% dextrose solution. During basal insulnemic-hyperglycemic clamps (Protocol- Basal
SUPPLEMENTARY DATA

I-HG), human recombinant insulin was infused into the portal vein at 4 mU·kg⁻¹·min⁻¹ to maintain basal plasma insulin levels (~4 ng/ml). Plasma glucose levels were maintained at ~13 mM by infusing 50% dextrose solution. Blood samples were taken from the arterial catheter.