Supporting Online Material

Materials and Methods

**GK Assay:** Briefly, the final incubation mixture contained 25 mM HEPES (pH, 7.1), 25 mM KCl, 2 mM MgCl₂, 1 mM ATP, 1 mM DTT, 1 mM NAD, 0.1% BSA, 5% DMSO, 5 U/ml glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides*, 5 mM glucose (unless indicated otherwise), and 0.7 ug/ml human liver GST-GK fusion protein in a volume of 0.12 ml. Recombinant GST-GK was generated using principles and practical procedures as previously described (1). Human GKRP was added to a final concentration of 15 ug/ml and 1.5 uM sorbitol 6-phosphate where indicated. Assays were performed using a Molecular Device SpectraMax 250 96-well plate reader (Molecular Devices, Sunnyvale CA).

**Purification of Human Liver GKRP by DEAE Chromatography:** Approximately 500g of stable transfected CHO cells were harvested, pelleted, resuspended in lysis buffer (25 mM HEPES, pH 7.1, 100 mM KCl, 1 mM EDTA and 1 mM DTT) and homogenized. The homogenate was cleared by centrifugation, precipitated using 40% (NH₄)₂SO₄, resuspended and dialyzed to a final concentration of 25 mM Tris-HCl, pH 8.0, 10 mM KCl and 1 mM DTT. The concentrated supernatant was loaded onto a Q-Sepharose column and the proteins eluted with a linear gradient from 10 mM KCl to 200 mM KCl. Fractions containing GKRP were pooled, and precipitated using 65% (NH₄)₂SO₄ and resuspended with 20 mM HEPES, pH 7.4, 0.6 M (NH₄)₂SO₄ and 1 mM DTT. The concentrated supernatant was loaded onto a octyl Sepharose column and the proteins eluted with a linear gradient from 20 mM HEPES, pH 7.4, 0.6 M (NH₄)₂SO₄ and 1 mM DTT.
DTT to 20 mM HEPES, pH 7.4 and 1 mM DTT. Purity of GKRP was estimated to be 50% by SDS-PAGE analysis.

**GSIR in rat islets:** The perifusion solution was Krebs-Ringer bicarbonate buffer containing 2.2 mM Ca^{2+}, 0.25% of bovine serum albumin and equilibrated with 95% O_2, 5% CO_2, pH 7.4 and contained 0.5% DMSO to solubilize the test agent.

**Pancreatic clamp surgical procedures:** Rats were anesthetized with pentobarbital (40-60 mg/kg, i.p.) and a sterile cannula (PE10) inserted into the jugular vein and passed to the level of the superior vena cava. The cannula was fixed in place by a suture around the vessel and then attached to the muscle. A second sterile cannula (PE50) was inserted into the carotid artery to the level of the aorta arch by the same procedure. The cannulae were filled with heparinized saline (100 U heparin/ml), sealed with a stainless steel pin, and exteriorized via subcutaneous tunneling with a 16 gauge needle to the back of the neck. The cannulae are held in place and upright with small flexible tubing. The incisions were closed and the animals observed until they recover from the anesthesia. Buprenorphine (0.05 mg/kg, subcutaneously) was administered as needed. All animal procedures were approved by the Institutional Animal Care and Use Committee.

**Text**

**GKRP:** Hepatic GK is regulated by binding to GKRP, an inhibitor that inhibits GK competitively with respect to glucose. GKRP exists in 2 conformations, one bound to fructose 6-phosphate sequestering inactivated GK to the nucleus and another conformation bound to fructose 1-phosphate releasing active GK into the cytoplasm. Data
from GKRP knock-out mice suggest both a regulatory and a stabilizing role for GKRP in maintaining adequate glucokinase in the liver (2-5).

**Supplementary Figures**

Fig S1. Hill plot in the absence (filled circles) and presence of RO-28-1675 (open circles, 1 uM; filled squares, 3 uM; open squares, 10 to uM; filled triangles, 20 uM). For reasons of clarity, data is shown for the control and 1 uM, 3 uM, 10 uM and 20 uM RO-28-1675.
Fig S2. Acute effects of RO-28-1675 on basal blood glucose levels in rodents. (A) Blood glucose levels in 5-week old female ob/ob mice (Jackson Labs) treated with a single oral dose of vehicle (n=11/time point) (filled circles) or 15 mg/kg RO-28-1675 (n=11/time point) (open circles). (B) Glucose levels in 7-week old male KK/Upj-Ay/J mice (Jackson Labs) treated with a single oral dose of vehicle (n=6/time point) (filled circles) or 50 mg/kg RO-28-1675 (n=6/time point) (open circles). (C) Glucose lowering effects in 7-week old male Wistar rats (Charles River Laboratories) (circles) and Goto-
Kakizaki rats (Charles River Laboratories) (squares) orally administered vehicle (n=5-6/time point) (filled symbols) or RO-28-1675 (n=5-6/time point) (open symbols).

Experimental details are described in Fig. 3. All results are reported as the mean ± SEM. A Student’s t test was used to test for statistical significance (*, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.005$).

Fig S3. Effects of orally administered RO-28-1675 on a glucose tolerance test in rodents. (A) Oral glucose tolerance test in eight-week old male C57Bl/6J mice administered a single oral dose of vehicle (filled circles) or 50 mg/kg RO-28-1675 (open circles) 120min prior to glucose administration (2 g/kg). Mice (n=6/treatment group) were fasted overnight, administered test compounds and glucose given two hours post-dose. (B) Oral glucose tolerance test in 8-week old male Wistar rats (Charles River Laboratories) (circles) and Goto-Kakizaki rats (Charles River Laboratories) (squares) orally
administered vehicle (n=4-5/time point) (filled symbols) or 50 mg/kg RO-28-1675 (n=4-5/time point) (open symbols) as described above. A Student’s t test was used to test for statistical significance (*, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.005$).

![Test Period Table]

<table>
<thead>
<tr>
<th>Test Period</th>
<th>-120 (min)</th>
<th>0 (min)</th>
<th>120 (min)</th>
<th>240 (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose vehicle or RO-28-1675 (30 mg/kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyperglycemic pancreatic clamp. Arterial blood glucose at 180 mg/dl.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Euglycemic control: $^3$H-glucose (100 µCi)</td>
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<td></td>
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</tbody>
</table>

**Fig S4.** Schematic diagram showing the experimental design of the pancreatic clamp experiment. Pancreatic clamps were conducted in conscious 12-week old male Sprague Dawley rats (Charles Rivers Laboratories). The study had a 2h control and 4h test periods. At the beginning of the control period, somatostatin (Sigma) was infused to inhibit endogenous insulin and glucagon secretion. This step was necessary to block the insulin release effects of RO-28-1675 so that extrapancreatic effects can be evaluated in both treatment groups with the same level of insulin. During this control period, glucagon (Eli Lilly and Company) and insulin (Eli Lilly and Company) were infused at basal rates in order to maintain the rats at basal glucose levels. The glucagon infusion
rate was fixed throughout the study, while insulin infusion rate was increased during the
test period to mimic the physiological postprandial situation. During the test period,
vehicle or RO-28-1675 (30 mg/kg) were administered orally and glucose infused to
bring blood glucose to postprandial levels (~180 mg/dl). Glucose disposal rates and
hepatic glucose production were measured using HPLC purified [3-\(^3\)H]glucose
(Amersham Life Science) as the metabolic tracer. EGP and utilization was determined
using the method of isotope dilution during a constant rate infusion of radioactive glucose
([3-\(^3\)H] glucose) according to the equation of modified Steele’s model (6). Blood glucose
and plasma insulin were measured as previously described in Fig. 3.
Fig. S5. Hepatic glucose metabolite levels following pancreatic clamp in vehicle and RO-28-1675 treated groups. Following the conclusion of the 4-hour hyperglycemic test period, livers were removed and flash frozen in liquid nitrogen. Glucose-6-phosphate and fructose-6-phosphate was measured as previously described (7). Hepatic glycogen (8) and lactate (9) levels were analyzed by standard enzymatic assays. Values are the means ± SEM (n = 8 per treatment group). * p < 0.05 when compared with hyperglycemic vehicle group.
Fig. S6. Location of the GKA binding site on GK. Of the amino acids that comprise the GKA, three mutations V455M (10), A456V (11) and Y214A (12), increase the catalytic activity of GK. The V455M and A456V are naturally occurring mutations associated with persistent hyperinsulinemic hypoglycemia of infancy, whereas the Y214A activating mutant was identified by site-directed mutagenesis. The methods used to obtain a GKA co-crystal are described in a non-provisional patent application ("Crystals of Glucokinase and Methods of Growing Them") filed on December 19, 2002 having the serial number 10/318,308.
**Supplementary Table**

**Table S1.** Effects of RO-28-1675 on metabolic parameters in 18-hour-fasted rats maintained on a pancreatic clamp during the test period

<table>
<thead>
<tr>
<th></th>
<th>Euglycemic Control</th>
<th>Hyperglycemic Vehicle</th>
<th>Hyperglycemic RO-28-1675</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arterial blood glucose (mg/dl)</td>
<td>84 ± 9</td>
<td>183 ± 10(^a)</td>
<td>188 ± 12(^a)</td>
</tr>
<tr>
<td>Plasma Insulin (ng/ml)</td>
<td>1.0 ± 0.1</td>
<td>3.6 ± 0.3(^a)</td>
<td>3.7 ± 0.6(^a)</td>
</tr>
<tr>
<td>Glucose disposal rate (mg/kg·min)</td>
<td>9.8 ± 0.9</td>
<td>24.9 ± 3.1(^a)</td>
<td>34.0 ± 2.0(^{ab})</td>
</tr>
<tr>
<td>Endogenous glucose production (mg/kg·min)</td>
<td>10 ± 2</td>
<td>3 ± 1(^a)</td>
<td>-9 ± 2(^{ab})</td>
</tr>
</tbody>
</table>

Values are the means ± SEM (n = 8 per treatment group). \(^a\) p < 0.05 when compared with euglycemic control group. \(^b\) p < 0.05 when compared with hyperglycemic vehicle group.

**References and Notes**


13. We thank P. Dunten, A. Swain, U. Kammlott, R. Crowther, C. Lukacs, L. Reik and W. Levin for GK-GKA co-crystallization studies.

**Supporting Online Material**

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Materials and Methods

Figs. S1, S2, S3, S4, S5, S6

Table S1