Supporting Information

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SI Materials and Methods

The Joslin Diabetes Center Human Subject Committee approved the studies. Informed consent was obtained. Subjects were healthy, had no first-degree relative with diabetes, and were on no prescription medication other than oral contraception (Table S1). Studies were performed sequentially. Two subjects participated in all three protocols, one in the GSIS and insulin clearance protocols only and one in the GSIS and C-peptide clearance only. Subjects consumed a ≥250-g carbohydrate diet for 3 days before infusion visits and were studied in the postabsorptive state after a 10- to 12-h fast.

Insulin Effect on GSIS. To test whether insulin could enhance the β-cell insulin secretory response to glucose, subjects were evaluated during paired studies, conducted in a single-mask design, of either 4 h of saline (sham clamp/low insulin) or isoglycemic-hyperinsulinemia (high insulin) immediately followed by glucose administration (Fig. S1*A*). Intravenous catheters were inserted for infusion of test substances and phlebotomy of arterialized venous blood (1). During the first visit, subjects received a 4-h infusion of saline (sham clamp), as a time and volume control using the hypothetical rate for dextrose needed for a healthy subject receiving 14 pM/kg/min insulin (2.0 mU insulin/kg per min) (2), followed by i.v. dextrose to promote insulin secretion administered over the next 80 min at the rate of 8 and 10 mg/kg per min for 40 min at each step.

On a subsequent day ∼4 weeks later, subjects underwent an isoglycemic-hyperinsulinemic clamp for 4 h (2), using a two-step primed (56 pmol/kg per min followed by 28 pmol/kg per min each for 5 min), continuous insulin infusion [14 pM/kg per min (2.0 mU/kg per min)], using B28-Asp insulin (Novolog; NovoNordisk) with relative receptor binding and in vitro potency similar to that of Regular insulin (3) but distinguishable from endogenous insulin immunologically. Plasma glucose was clamped at the subject's individual glucose concentrations observed in the saline/sham condition; isoglycemia was maintained by adjusting a variable rate infusion of 20% dextrose. The steady-state glucose infusion rate to maintain isoglycemia was determined over the fourth hour of the clamp with additional glucose infused to stimulate endogenous insulin secretion over the following 80 min, maintaining the same glycemia observed in the subject during glucose stimulation following preexposure to saline. Potassium chloride (KCl) was administered at 10 mEq/h to prevent hypokalemia during all clamp protocols.

Insulin Effect on Insulin Clearance. To determine whether the exogenous insulin would saturate insulin clearance, another set of paired studies was performed in a subset of four participants (Fig. S1*B*). A sham clamp was again performed with saline infused for 4 h as control, followed by a bolus of Human Insulin Regular (Novolin R, NovoNordisk) 0.03 units/kg to determine the insulin clearance rate. On a subsequent day ∼4 weeks later, an isoglycemic-hyperinsulinemic clamp was performed with B28-Asp insulin infused as a primed, continuous infusion as before, with dextrose 20% to maintain glycemia. At 4 h, a bolus of Human Insulin Regular (0.03 units/kg) was likewise administered to determine whether insulin clearance was altered by the preexposure to higher insulin.

Insulin Clearance Model. A linear two-compartment kinetic model of B28-Asp insulin disappearance kinetics was used to calculate the clearance rates of insulin in the basal state and during hyperinsulinemia. This model assumes instantaneous mixing of the exogenous insulin bolus. The distribution volume for insulin is fixed over the time course of the experiment; the rate of insulin loss from the circulation is negligible and is therefore assumed to be zero. In addition, the first compartment was assumed to be the circulation and the second compartment the interstitium. The mathematical representation of the two compartment linear model is as follows:

$$\frac{dF(1)}{dt} = -L(2,1)\cdot F(1) + L(1,2)\cdot F(2)$$
$$\frac{dF(2)}{dt} = L(2,1)\cdot F(1) - (L(0,2) + L(1,2))\cdot F(2)$$

where F(1) is the amount of particles in compartment 1, and L(1,2) is the fractional rate of transfer from compartment 1 to compartment 2. This analysis has irreversible loss from the interstitial compartment, and the analysis was repeated with a model in which irreversible loss is from the plasma compartment. The kinetic modeling was conducted using the mathematical modeling computer package WinSAAM (4).

Insulin Effects on C-peptide Clearance. Another group of participants underwent assessment of C-peptide clearance during saline/sham and hyperinsulinemic clamp. During the first visit, saline was given as a time and volume control over 4 h (sham clamp); ∼4 weeks later, an isoglycemic-hyperinsulinemic clamp was performed as above. However, in this subset of participants, during both studies, ^{13}C-C-peptide was infused as a primed (33 pmol/kg over 2 min) continuous infusion at 1.5 pmol/kg/min.

C-Peptide Metabolic Clearance. C-peptide metabolic clearance rate (MCR; in mL/kg per min) was calculated by dividing the ^{13}C-C-peptide infusion rate (pmol/kg/min) by the achieved steady state plasma ^{13}C-C-peptide concentration (pmol/mL).

Preparation of Human-Sequence Isotope-Labeled ^{13}C-peptide. Human-sequence isotope-labeled ^{13}C-peptide was synthesized and quantified as previously described (5). Briefly, C-peptide of molecular weight 3031 containing ^{13}C labels was synthesized using an ABI 433A peptide synthesizer by solid-phase chemistry at the Laboratory for Macromolecular Analysis and Proteomics of Albert Einstein College of Medicine. Peptides were purified by preparative reverse-phase (RP) HPLC, validated by amino acid analysis, molecular weight by matrix assisted laser desorption ionization mass spectrometry (MALDI) (Voyager DE STR, Applied Biosystems), and peptide content was quantitated by nitrogen analysis (Galbraith Labs). Final aliquots of m+^{11}C-peptide tracer were lyophilized and documented sterile and pyrogen free before use.

A 100-μL quantity of m+30 (m.w. 3050) C-peptide (3.25 nmol/L) was added to 300 μL plasma, and C-peptide extracted by solid phase extraction as previously described (5). ^{13}C tracer (m+11), internal standard (m+30), and endogenous C-peptides were measured via two-dimensional liquid chromatography–mass spectrometry with an 1100 series 2D LC system (Agilent) equipped with binary and capillary pumps and a column compartment with two-position Rheodyne thermostabilized valves. LC/MS analysis was performed on a API 4000 triple-quadrupole mass spectrometer (Applied Biosystems) equipped with a Turboionspray source. Ions *m/z* 1007.7, 1011.0, and 1017.7 were monitored in the +3 charge state.

Assays. Glycohemoglobin was assessed by HPLC (Tosho 2.2; Tosho Bioscience), and potassium, total cholesterol, HDL, and triglycerides were measured in Joslin's clinical laboratory (Beckman Synchron CX9). Serum glucoses were measured using the glucose oxidase method (YSI 2300 STAT).

Immunoassays were performed in duplicate in Joslin's special assay core facility by commercial assays for total insulin, measuring both endogenous (secreted) and exogenous (administered) insulin, and C-peptide (RIA; Diagnostic Systems Laboratories) with endogenous serum insulin assayed using an ELISA that would not detect the administered B28-Asp insulin (DAKO Insulin; DakoCytomation). Additional immunoassays included serum FFAs (NEFA FFA ELISA, Wako Chemicals), serum cortisol (Cortisol RIA; INSTAR Corporation), and plasma glucagon (Glucagon RIA; Linco Research). Epinephrine and norepinephrine were measured using HPLC (HPLC) by the General Clinical Research Center assay core.

Statistical Analysis. Data are presented as mean ± SE. Simple regression was used for correlation analysis, Student's paired *t* test was used for point comparison, and analysis of variance for repeated measures within individual on saline (sham) or insulin clamp days. Results are considered significant with two-tailed *P* values <0.05. Analyses were done with StatView (SAS Institute) except the mean fractional transfer rates for insulin clearance, which were analyzed with STATA (StatCorp).

1. McGuire EA, Helderman JH, Tobin JD, Andres R, Berman M (1976) Effects of arterial versus venous sampling on analysis of glucose kinetics in man. *J Appl Physiol* 41: 565–573.
2. Rizza RA, Mandarino LJ, Gerich JE (1981) Mechanisms of insulin resistance in man. Assessment using the insulin dose-response curve in conjunction with insulin-receptor binding. *Am J Med* 70:169–176.
3. Brange J, Owens DR, Kang S, Vølund A (1990) Monomeric insulins and their experimental and clinical implications. *Diabetes Care* 13:923–954.
4. Stefanovski D, Moate PJ, Boston RC (2003) WinSAAM: A Windows-based compartmental modeling system. *Metabolism* 52:1153–1166.
5. Rogatsky E, et al. (2006) Sensitive quantitative analysis of C-peptide in human plasma by 2-dimensional liquid chromatography-mass spectrometry isotope-dilution assay. *Clin Chem* 52:872–879.

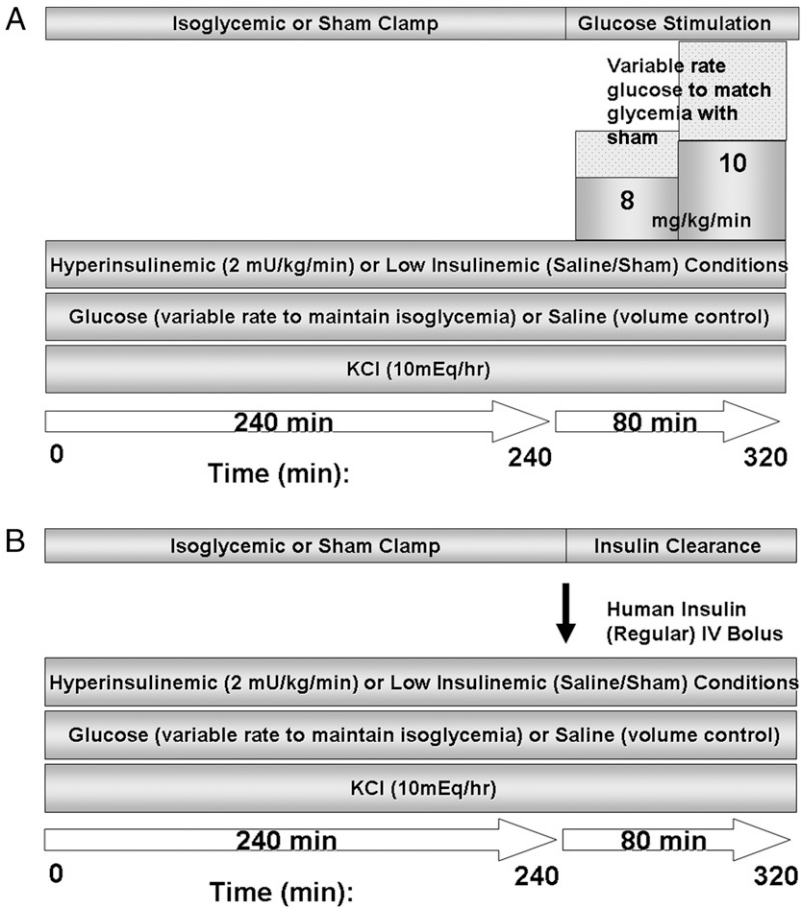

Fig. S1. Study protocol: Participants underwent paired isoglycemic clamp studies under high insulin (B28-Asp insulin 2 mU/kg per min) and low insulin (saline-sham) conditions. Glucose (20% dextrose) was administered during insulin clamp to maintain isoglycemia, and saline during sham clamp as a time and volume control. Potassium chloride (KCl) was administered during all clamps. At the start of the fourth hour of the clamp, additional glucose was administered to investigate glucose-stimulated insulin secretion. During the sham clamp, the rate was 8 and 10 mg/kg/min for 40-min each step (dark filled bar); on the hyperinsulinic day rates were variable to match the glycemic challenge achieved during the sham clamp conditions (dotted bar) (*A*). To assess insulin clearance, participants underwent paired isoglycemic clamp studies under high insulin (B28-Asp insulin 2 mU/kg/min) (light filled bar) and low insulin (saline-sham) conditions (dotted bar). At the start of the fourth hour of the clamp, a bolus of human insulin (Regular) was administered and the rate of insulin clearance was calculated. Glucose (20% dextrose) was administered during insulin clamp to maintain isoglycemia, and saline during sham clamp as a time and volume control. Potassium chloride (KCl) was administered during both clamps. (*B*) The final 10 subjects received ^{13}C-C-peptide during paired, 4-h isoglycemic and sham clamps.

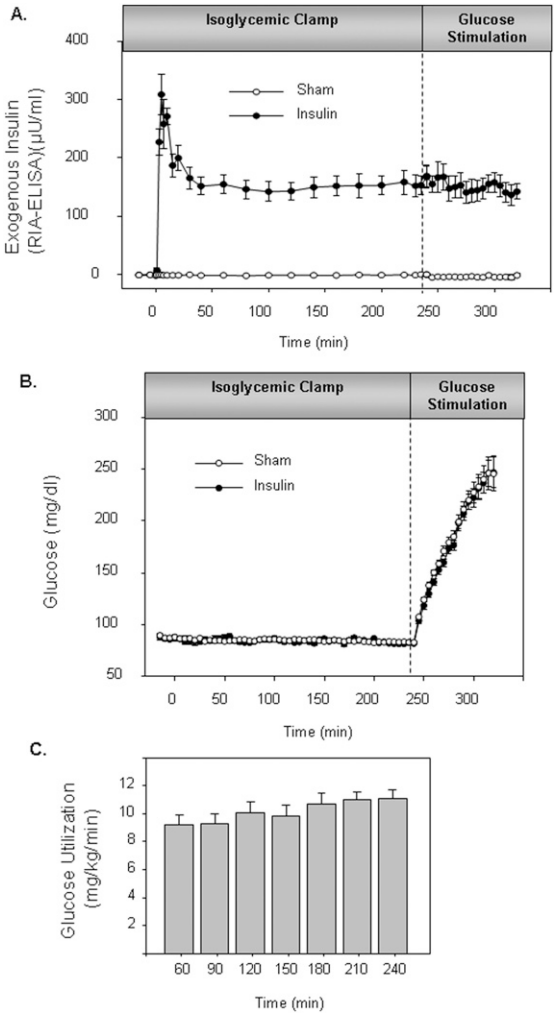

Fig. S2. Isoglycemic-hyperinsulinemic and sham clamp conditions. β-Cell exposure to exogenous insulin differed on the 2 study days and were in the high physiologic range during isoglycemic-hyperinsulinemic clamp. Exogenous insulin was calculated by subtracting endogenous (ELISA) from total (RIA) measured insulin concentrations (*A*). Glucose concentrations were similar on the 2 study days, achieving isoglycemia during the preexposure to hyperinsulinemic conditions and during the GSIS portion of the study (*B*). Glucose use was at steady state over the 4 h of the isoglycemic-hyperinsulinemic clamp (*C*). Isoglycemic-hyperinsulinemic clamp (●); sham clamp (○).

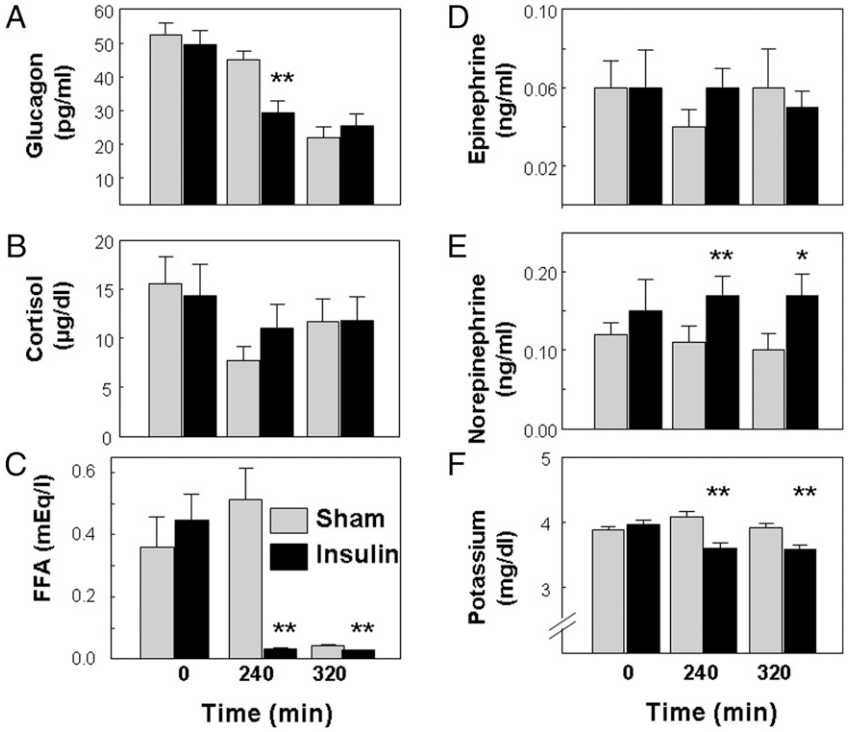

Fig. S3. Hormone and metabolites during isoglycemic-hyperinsulinemic and sham clamps and GSIS are shown for glucagon (*A*), cortisol (*B*), FFA (*C*), epinephrine (*D*), norepinephrine (*E*), and potassium (*F*) at baseline (time = 0 min) and following insulin (black filled bars) or sham (light filled bars) clamp, before (time = 240 min) or after (time = 320 min) glucose-induced insulin secretion. (*$P < 0.05$, **$P < 0.009$).

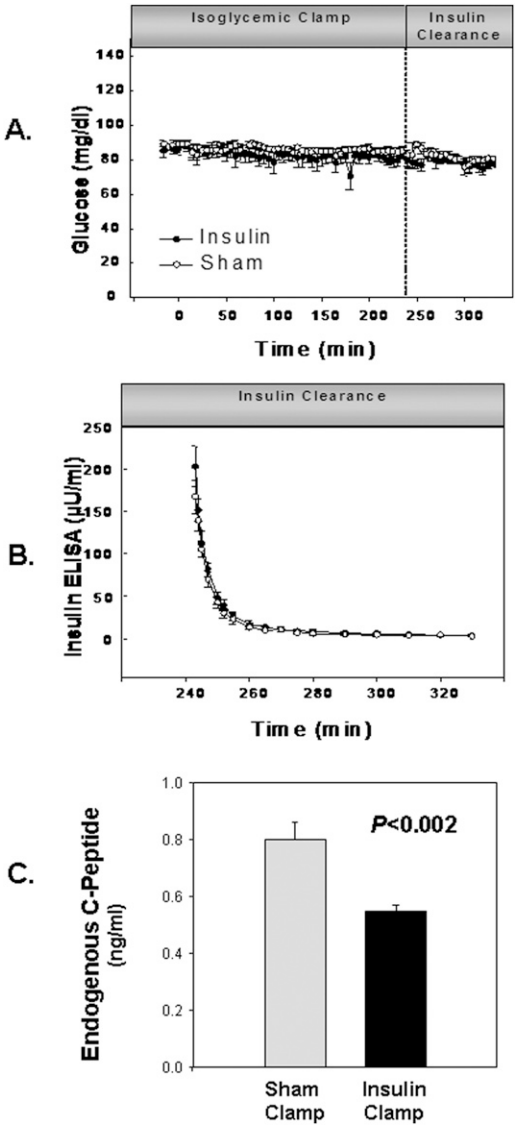

Fig. S4. Insulin clearance was not different following preexposure to isoglycemic-hyperinsulinemic conditions, whereas C-peptide clearance increased during isoglycemic-hyperinsulinemic clamp. Glucose concentrations were similar on the 2 study days evaluating insulin clearance, achieving isoglycemia during the preexposure to hyperinsulinemic conditions compared with sham clamp and following bolus of Regular human insulin administered at 240 min to assess insulin clearance (*A*). Insulin concentrations declined similarly following preexposure to high physiologic insulin concentrations during isoglycemic clamp or sham clamp (*B*). Isoglycemic-hyperinsulinemic clamp (●), sham clamp (○). Isoglycemic-hyperinsulinemic clamp conditions induce lower endogenous C-peptide concentrations ($P < 0.002$) (*C*). Insulin (dark filled bar) or sham (light filled bar) clamp.

Table S1. Subject characteristics

Group	Glucose-stimulated insulin secretion	Insulin clearance	C-peptide clearance
No. of subjects	8	4	10
Age (yr)	25 ± 9	26 ± 5	26 ± 5
Gender	3M/5F	2M/2F	4M/6F
Body mass index (kg/m^2)	22.6 ± 1.8	22.4 ± 3.7	24.5 ± 3.1
Systolic blood pressure (mmHg)	113 ± 6	116 ± 13	110 ± 6
Diastolic blood pressure (mmHg)	76 ± 8	72 ± 6	71 ± 7
HbA1c (%)	5.0 ± 0.3	5.2 ± 0.2	5.1 ± 0.2
Fasting glucose (mg/dL)	87.9 ± 5.9	84.7 ± 4.0	88.6 ± 3.5
Fasting insulin (μU/mL)	3.8 ± 1.3	5.8 ± 4.3	5.5 ± 2.4
Cholesterol (mg/dL)	149 ± 21	151 ± 21	177 ± 26
LDL (mg/dL)	90 ± 15	80 ± 26	116 ± 37
HDL (mg/dL)	49 ± 9	56 ± 10	50 ± 11
Triglycerides (mg/dL)	52 ± 20	65 ± 28	85 ± 48
Glucose utilization (mg·kg^{-1} min^{-1})	11.1 ± 2.3	10.6 ± 1.4	9.6 ± 2.5

Demographic information is provided for the three groups studied including assessment of the effect of preexposure to insulin on GSIS, on insulin clearance, or on C-peptide clearance. Two subjects participated in all three protocols; one participated in the GSIS and insulin clearance protocols only, and one subject in the GSIS and C-peptide clearance only. There were no significant differences in baseline characteristics between the groups enrolled in the three protocols. Data are mean ± SD. Glucose utilization (M) calculated at clamp steady state 180–240 min.