

Adaptation of β -cell mass to substrate oversupply: enhanced function with normal gene expression

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Steil, Garry M., Nitin Trivedi, Jean-Christophe Jonas, Wendy M. Hasenkamp, Arun Sharma, Susan Bonner-Weir, and Gordon C. Weir. Adaptation of β -cell mass to substrate oversupply: enhanced function with normal gene expression. *Am J Physiol Endocrinol Metab* 280: E788–E796, 2001.—Although type 2 diabetes mellitus is associated with insulin resistance, many individuals compensate by increasing insulin secretion. Putative mechanisms underlying this compensation were assessed in the present study by use of 4-day glucose (GLC; 35% Glc, 2 ml/h) and lipid (LIH; 10% Intralipid + 20 U/ml heparin; 2 ml/h) infusions to rats. Within 2 days of beginning the infusion of either lipid or glucose, plasma glucose profiles were normalized (relative to saline-infused control rats; SAL; 0.45% 2 ml/h). During glucose infusion, plasma glucose was maintained in the normal range by an approximately twofold increase in plasma insulin and an ~80% increase in β -cell mass. During LIH infusion, glucose profiles were also maintained in the normal range. Plasma insulin responses during feeding were doubled, and β -cell mass increased 54%. For both groups, the increase in β -cell mass was associated with increased β -cell proliferation (98% increase during GLC and 125% increase during LIH). At the end of the 4-day infusions, no significant changes were observed in islet-specific gene transcription (i.e., the expression of islet hormone genes, glucose metabolism genes, and insulin transcription factors were unaffected). Two days after termination of the infusions, the glucose-stimulated plasma insulin response was increased ~67% in glucose-infused animals. No sustained effect on insulin secretory capacity was observed in the LIH animals. The increase in plasma insulin response after glucose infusion was achieved in the absence of any change in insulin clearance. We conclude that, in rats, an increase in insulin demand after an increase in glucose appearance or free fatty acid leads to an increase in β -cell mass, mediated in part by an increase in β -cell proliferation, and that these compensatory changes lead to increased insulin secretion, normal plasma glucose levels, and the maintenance of normal islet gene expression.

insulin resistance; insulin secretion; β -cell; mitosis; maturity onset diabetes of the young

ALTHOUGH TYPE 2 DIABETES MELLITUS is associated with insulin resistance (11), not all insulin-resistant subjects develop diabetes; rather, many individuals com-

pensate for the resistance by increasing insulin secretion (22, 23, 36). Although the increased insulin response protects individuals from developing diabetes, very little is known about the cellular or molecular mechanisms underlying the adaptive change. An increase in insulin secretory capacity can result from either an increase in the ability of existing β -cell mass to manufacture and secrete insulin or from an increase in the β -cell mass per se (6). An increase in the secretory capacity of the existing β -cell mass most likely involves changes in the rates of key steps in the β -cell stimulus/secretion pathway. An increase in β -cell mass can result from either hypertrophy or hyperplasia, with hyperplasia resulting from an increase in β -cell replication (mitosis), a decrease in β -cell death (apoptosis), or the formation of new β -cells from precursor cells (neogenesis) that reside in pancreatic ducts (14).

One reason for our lack of understanding of the adaptive β -cell response is the lack of an established animal model in which to investigate in vivo the temporal relationships between changes in insulin secretion, expansion of β -cell mass, and alterations in islet-specific gene expression. Possible animal models include the glucose- or lipid-infused rat. It has long been known that β -cell mass increases during glucose infusion (49) and that the increase is due to both hypertrophy and hyperplasia (7). However, although glucose infusion increases β -cell mass, it is unclear whether the increase leads to an increase in insulin secretory capacity. During glucose infusion, plasma insulin levels increase within 1–2 days to reestablish euglycemia (7). The increase in insulin secretion at the renormalized glucose level is thought to be due to an increase in the catalytic rate of glucokinase (GK) (9), GK being rate limiting for glucose entry into the β -cell (38). However, despite the increase in both β -cell mass and GK activity, studies in the in vitro perfused pancreas have consistently indicated that the glucose-stimulated insulin response is decreased, rather than increased, after glucose infusion (28), leading to the belief that the model represents β -cell exhaustion or glucotoxicity. Nonetheless, although the in vitro insu-

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lin response is reduced, one study has reported an increase in insulin secretion when the response is assessed in vivo (25), suggesting that the model may be appropriate for the study of β -cell adaptation.

Free fatty acids (FFA) have also been implicated in altering β -cell mass and insulin secretion. In vitro, short-term elevations in FFA increase β -cell proliferation in islets isolated from Wistar rats (39) but not in islets isolated from Zucker diabetic fatty rats (19). However, whereas an elevation in FFA may initiate a hyperplasia response, prolonged exposure has been shown to increase β -cell apoptosis (43). Thus the net effect on in vivo β -cell mass, which is determined by a balance of mitosis, apoptosis, and islet neogenesis (14), is unclear. Functionally, short-term increases in FFA augment in vivo glucose-stimulated insulin secretion (12, 44), whereas, with one exception (34), longer-term elevations decrease the response (33, 37). Thus it is also unclear whether elevations in FFA lead to a compensatory increase in β -cell mass or function (adaptation) or to a failure of the existing β -cells (lipotoxicity).

Ultimately, β -cell adaptation, be it a change in β -cell mass or secretory capacity per unit mass, is likely to be under the control of key regulatory genes, of which there are many candidates. All known genes leading to maturity-onset diabetes of the young [the "MODY" genes: hepatic nuclear factors (HNF)4- α , HNF1- α , and HNF1- β , pancreatic duodenum homeobox protein (PDX)-1, and GK] have been implicated in defective insulin secretion, and at least two of these (HNF4- α and PDX-1) have been reported to be affected by FFA (16, 18). Knockout studies in mice have shown that PDX-1 is essential for pancreas development (21) and that the basic helix-loop-helix factor NeuroD/BETA2 is required for β -cell development (41). PDX-1, NeuroD/BETA2, and NeuroD/BETA2's dimeric partner pan1 all play a postdevelopmental role in regulating insulin gene transcription (17). The link between these genes and β -cell development and/or function suggests that any or all of them may be instrumental during β -cell adaptation.

The present study was conducted to determine whether rat β -cells can adapt to the increase in insulin demand during glucose or lipid infusion, and if so, to determine what physiological and molecular mechanisms underlie the adaptation. Results indicated that within 1–2 days of either glucose or lipid infusion, rats renormalize their plasma glucose profiles by dramatically increasing their plasma insulin responses. The increased insulin responses were associated with increases in β -cell mass, which were paralleled by increased rates of β -cell proliferation. At the end of the 4-day substrate infusions, islet gene expression was completely normal. Two days after the infusions were concluded, insulin secretory capacity remained elevated in glucose-infused animals but returned to normal in lipid-infused animals.

METHODS

Substrate infusions. Male Sprague-Dawley rats (~250 g) with indwelling jugular vein and carotid artery catheters

were obtained from Taconic Farms (Germantown, NY). After arrival at the animal facility, rats were observed for ≥ 3 days to ensure general well-being as assessed by weight gain and appearance. Rats were then randomly divided into four groups: a glucose infusion group (GLC; 35% hydrated dextrose; McGaw, Irvine, CA), a lipid infusion group (LIH; 10% Intralipid + 20 U/ml heparin; Baxter iv), a saline-infused control group (SAL; 0.45%), and a noninfused nonrestrained control group (NON). Substrates were infused into the carotid artery at 2 ml/h for 96 h beginning at ~9:30 AM. The infusion system consisted of a swivel and saddle (Lomir Biomedical, Malone, NY) designed to allow the animal unrestricted mobility. Blood samples (~0.1 ml) were drawn from the jugular vein at -0.5, 0 (before start of infusions), 0.1, 0.5, 1, 4, 6, 12, 24, 36, 48, 60, 72, 84, and 96 h, placed in heparin-coated tubes, and immediately centrifuged. The samples at 12, 36, 60, and 84 h were obtained during the time rats normally eat (~10 o'clock in the evening). For all samples, a portion of the supernatant (10 μ l) was immediately assessed for glucose (Beckman Glucose Analyzer II; Beckman, Brea, CA), and the remainder (~40 μ l) was frozen and stored at -20°C for later determination of plasma insulin concentration. Additional samples (~0.1 ml) were taken at -0.5, 1, 24, 48, 72, and 96 h for the determination of plasma FFA levels; these samples were taken in tubes coated with EDTA (diethyl *p*-nitrophenyl phosphate) and paraoxon to prevent lipoprotein lipase-stimulated breakdown of triglycerides (50). Rats were allowed ad libitum food and water during the 4-day infusions, and a standard (12:12-h light-dark) cycle was maintained. At the end of the infusion period, animals were removed from the restraint system, returned to their cages, and eventually euthanized for morphological assessment of β -cell mass and proliferation or for islet isolation and semiquantitative RT-PCR analysis of islet gene expression. After return to their cages, animals were given 2 days to renormalize plasma glucose and insulin levels; then the animals were used to assess postinfusion glucose-stimulated plasma insulin responses (hyperglycemic clamp) or peripheral insulin clearance (euglycemic-hyperinsulinemic clamp). Infusions for each of the four measures (β -cell mass, islet gene expression, insulin response, and insulin clearance) were randomized by group (NON, SAL, GLC, LIH). Rats removed from the study due to technical problems were replaced, and the infusion process was rerandomized; the rerandomization resulted in some groups having a higher number of experiments. Because the number of infusions was four times greater than the number of postinfusion tests (assessment of β -cell mass, islet gene expression, insulin secretion, and insulin clearance each requires a separately infused animal), the insulin and FFA levels during the infusions were not assessed in all animals (the total number of infused animals in each group was $n = 32$, SAL; $n = 37$, GLC, and $n = 33$, LIH) but rather in a random subset of 6–9 animals, as reported in the text and Figs. 1–4 of RESULTS.

Hyperglycemic clamps. Hyperglycemic clamps ($n = 7$ NON; $n = 6$ SAL; $n = 9$ GLC; $n = 6$ LIH) were performed in conscious unrestrained fed animals. For each clamp, two basal blood samples (~0.1 ml each; at -30 and -5 min) were taken in heparin-coated tubes and immediately centrifuged. As described in *Substrate infusions*, 10 μ l of supernatant were assessed for plasma glucose, with the remainder immediately frozen and stored at -20°C for later determination of insulin and C-peptide concentrations. A priming bolus of 20% hydrated glucose was then calculated on the basis of the measured basal glucose level, an assumed glucose distribution space of 25% body weight, and a desired clamp set point of 250 mg/dl. The priming bolus was given at 0 min into the

carotid artery, together with an initial exogenous glucose infusion of 6 mg/min. Subsequent blood samples (each ~0.1 ml) were then taken at 1, 3, 5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, and 90 min, and the exogenous glucose infusion was adjusted in proportion to the difference between the measured glucose and the set point and the rate of change of this difference (integrated proportional-derivative control). Insulin secretory capacity was assessed as the average plasma insulin concentration during the final 0.5 h of the clamp (60–90 min). Hematocrit was assessed at the beginning and end of clamps in a subset of animals and typically decreased from ~35 to ~32%.

Hyperinsulinemic clamps. Increased plasma insulin levels can be due to either an increase in insulin secretion or a decrease in insulin clearance. Both glucose (26) and lipid infusions (48) have been shown to decrease insulin clearance. Although C-peptide might be considered a surrogate of insulin secretion, the clearance of this peptide has not been shown to be constant during chronic infusion studies. As well, the rat has two insulin/C-peptide genes, preventing the assessment of prehepatic insulin secretion with traditional C-peptide deconvolution. Because C-peptide could not be used to correct for changes in insulin clearance, the clearance of insulin was directly assessed with euglycemic-hyperinsulinemic clamps. These clamps ($n = 6$ for all groups) were performed in an identical manner to the hyperglycemic clamps, with the exception that glucose was clamped at basal with a variable glucose (20% hydrated glucose) infusion that was co-infused with human insulin (Eli Lilly, Indianapolis, IN; $4.2 \text{ mU} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$, 0.032 ml/min) through a small Y-connector. The variable glucose infusion was also calculated on the difference, and rate of change of the difference, between plasma glucose and the set point, in this case basal. Peripheral insulin clearance was calculated as the insulin infusion rate ($\mu\text{U} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$) divided by the average insulin concentration during the final 0.5 h of the clamp (60–90 min). Changes in hematocrit were similar to those observed during hyperglycemic clamps.

Quantification of β -cell mass and mitotic rate. At ~6 AM on the 4th day of substrate infusion, rats ($n = 6$ for all groups) were lightly anesthetized with pentobarbital sodium (Nembutal; ~0.05 ml, 50 mg/ml into the jugular vein) and given an intraperitoneal injection of BrdU (100 mg/kg crystalline grade 5-bromo-2'-deoxyuridine, diluted in PBS to 10 mg/ml). Rats typically recovered from the anesthesia/BrdU injection within 5–10 min. Heparin was discontinued for ~8 h before the BrdU injection to prevent internal bleeding at the injection site and was restarted immediately after the injection. Exactly 6 h after the BrdU injection, a second nonlethal dose of pentobarbital sodium (0.1–0.2 ml, 50 mg/ml into the jugular vein) was administered, and the pancreas was rapidly removed. The animal was then killed by pentobarbital sodium overdose. The pancreas was cleaned of lymph nodes and fat, divided into head and tail portions, weighed, and fixed in Bouin's solution. The head and tail portions of the pancreas were embedded in paraffin, sectioned, and double-stained for BrdU (cell proliferation kit; Amersham; Arlington Heights, IL) and non- β -endocrine cells (by use of antibodies against glucagon, somatostatin, and pancreatic polypeptide as described in Ref. 40). Total β -cell mass and percent β -cell proliferation were then quantified on systematically chosen fields within a single section spanning a complete cross section of the head and tail (one section each). Mass was quantified by point-counting, as originally described by Weibel (47) and applied by Bonner-Weir et al. (7) to the endocrine pancreas. To minimize variance and

prevent bias, all quantification was performed by one observer (N. Trivedi), who was unaware of the infusion group.

Quantification of mRNA levels (gene expression). Islet mRNA level was measured by semiquantitative radioactive multiplex RT-PCR. On the morning of the 4th day of infusion, rats were removed from the restraint system under Nembutal anesthesia, and the pancreas was digested with collagenase ($n = 7$ NON; $n = 5$ SAL, $n = 7$ GLC; $n = 5$ LIH). After digestion, islets were isolated and hand picked as previously described (20). The mRNA oligonucleotide primers, appropriate control genes, multiplex RT-PCR conditions, and validation and test of linearity have also previously been reported in detail (20).

Plasma insulin and FFA assays. Rat insulin was assayed using radioimmunoassay kits (Linco Research, St. Charles, MO). Kits were modified for small blood samples by reducing the manufacturer's recommended sample and diluent volumes by two (from 100 $\mu\text{l/sample}$ to 50 $\mu\text{l/sample}$). Samples for insulin were diluted 1:5 (basal) or 1:10 (stimulated) such that determinations were performed in duplicate with only 5–10 μl of plasma. For the euglycemic-hyperinsulinemic clamps in which human insulin was infused, the plasma immunoreactive insulin level was assayed using a porcine standard. Plasma FFAs were measured using a colorimetric assay (Wako Chemicals, Neuss, Germany) modified for small samples by reducing the manufacturer's recommended sample and diluent volumes (factor of 2.5).

Data analysis and statistics. Results are presented as means \pm SE. Differences among groups (SAL, GLC, LIH, and NON) were assessed with one-way ANOVA followed by Dunnett's test, with saline as the control group ($P < 0.05$ was considered significant). Differences between the saline-infused controls and noninfused animals (SAL and NON) were evaluated by unpaired t -tests [this was considered a priori or planned test (24)]. Differences between time points within an animal (for example, pre- vs. postinfusion body wt) were evaluated with paired t -tests. In one case, Grubb's outlier test (2) was used to remove a data point ($P < 0.05$). Statistical tests were performed using Graphpad software (Graphpad, Irvine, CA). All animal procedures were approved by the Joslin Diabetes Center Institutional Animal Care Committee.

RESULTS

Substrate infusions. During saline infusion, the morning fed glucose level fell from $125 \pm 2 \text{ mg/dl}$ on *day 1* to $117 \pm 3 \text{ mg/dl}$ on *day 4* (Fig. 1; $P < 0.05$; paired t -test, $n = 32$), whereas FFA [0.32 ± 0.06 vs. $0.35 \pm 0.13 \text{ mM}$; not significant (NS); $n = 6$] and insulin (1.7 ± 0.2 vs. $1.6 \pm 0.2 \text{ ng/ml}$; NS; $n = 13$) levels were unchanged. Animals maintained, but did not gain, body weight during the infusion (278 ± 8 vs. $278 \pm 7 \text{ g}$; $n = 32$; NS).

During glucose infusion, plasma glucose increased from an initial basal level of $129 \pm 2 \text{ mg/dl}$ to a peak level of $214 \pm 7.6 \text{ mg/dl}$ and then slowly returned to near basal levels by ~36 h (Fig. 1A). The morning glucose level was slightly lower on *day 4* compared with *day 1* (121 ± 3 vs. $129 \pm 2 \text{ mg/dl}$; $P < 0.01$; $n = 37$). During *days 3* and *4*, glucose levels were maintained near basal and were similar to the values observed in saline-infused controls (Fig. 1B). FFA levels tended to be lowest in the glucose-infused rats (Fig. 1C), but no significant differences were observed be-

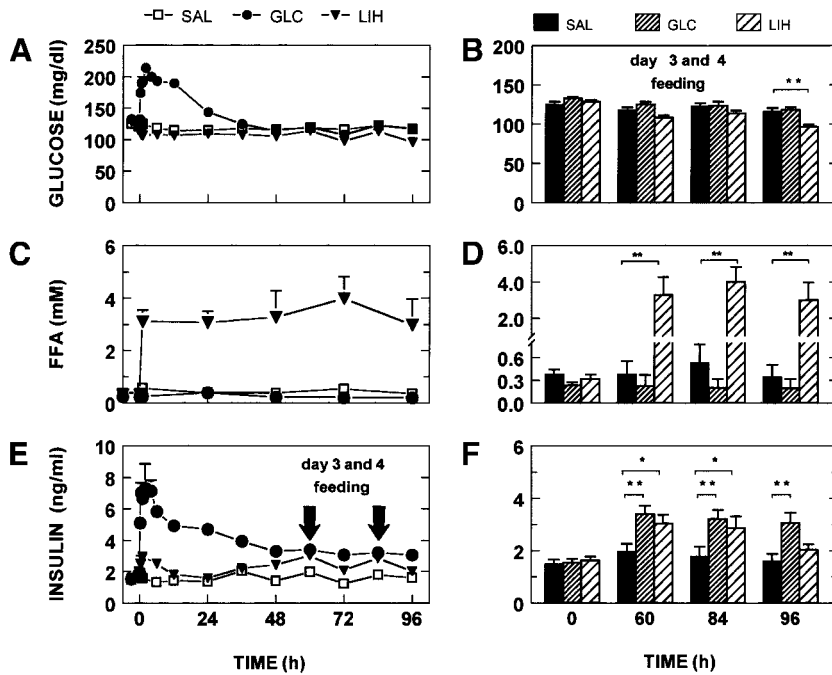


Fig. 1. A: plasma glucose during 4-day infusions of saline (SAL; $n = 32$; \square), glucose (GLC; $n = 37$; \bullet), and lipid [Intralipid + heparin (LIH); $n = 33$; \blacktriangledown]. B: comparison of morning glucose levels on *days 1* and *4* and glucose levels during feeding on *days 3* and *4*. C: plasma free fatty acids (FFA) measured in a random subset of infused animals ($n = 6$ all groups). D: morning plasma FFA levels on *days 1* and *4* and during feeding on *days 3* and *4* (broken axis, 60- and 80-h FFA values interpolated). E: plasma insulin measured in a subset of infused animals ($n = 13$ saline; $n = 21$ glucose; $n = 10$ lipid). F: comparison of morning insulin levels on *days 1* and *4* and glucose levels during feeding on *days 3* and *4*. ** $P < 0.01$; * $P < 0.05$ by ANOVA with Dunnett's test vs. saline control, and data pooled for feeding on *days 3* and *4*.

tween saline- and glucose-infused rats during feeding on *days 3* and *4* (60 and 84 h) or on the morning of the 4th day (Fig. 1D, ANOVA; $n = 6$). Morning FFA levels on *days 1* and *4* were also similar (0.23 ± 0.04 vs. 0.19 ± 0.12 mM; NS; $n = 6$). By *day 4*, an approximately twofold increase in basal insulin was needed to maintain morning euglycemia (1.6 ± 0.1 to 3.0 ± 0.3 ng/ml; $P < 0.05$ by paired *t*-test; $n = 21$). Compared with saline-infused controls, insulin levels were doubled during feeding on *days 3* and *4* (Fig. 1, E and F; ANOVA; $P < 0.01$). As with saline infusion, animals maintained, but did not gain, body weight over the 4-day infusions (271 ± 9 vs. 267 ± 11 g; $n = 37$; NS).

During lipid infusion, plasma glucose levels were slightly suppressed at all time points measured. The morning glucose level on *day 4* (99 ± 2 mg/dl) was significantly lower than the value on *day 1* (120 ± 2 mg/dl; $P < 0.05$; paired *t*-test, $n = 33$) and lower than that observed in saline-infused controls (Fig. 1B; ANOVA; $P < 0.01$). FFA levels were elevated ~ 10 -fold for the entire infusion period (0.32 ± 0.05 vs. 3.0 ± 0.97 mM, basal vs. 96 h; Fig. 1, C and D; $n = 6$; $P < 0.01$). During *day 2*, plasma insulin levels were similar in the saline- and lipid-infused groups (1.4 ± 0.3 vs. 1.6 ± 0.2 ng/ml at 24 h and 2.0 ± 0.4 vs. 2.2 ± 0.3 ng/ml at 36 h; NS) but were significantly elevated during feeding on *days 3* and *4* (Fig. 1, E and F, $P < 0.05$ by ANOVA), suggesting that the insulin resistance induced by the high FFA level was being compensated for by an increase in insulin secretion in the absence of any change in plasma glucose (glucose levels were similar during *days 3* and *4*; Fig. 1, A and B; NS, ANOVA). By *day 4*, a small but significant increase in basal insulin was needed to maintain morning euglycemia (2.0 ± 0.5 vs. 1.7 ± 0.3 ng/ml; $P < 0.05$ by paired *t*-test; $n = 10$). Again, animals maintained, but did not gain, body

weight during the infusion (292 ± 5 vs. 289 ± 5 g; $n = 33$; NS).

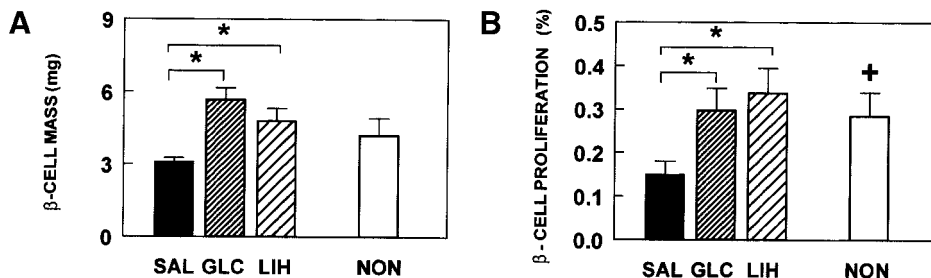
Islet gene expression. With the exception of a small change in islet amyloid polypeptide (IAPP), islet gene expression assessed at the end of the 4-day infusions was identical among the groups (Table 1). Although IAPP significantly increased compared with saline-

Table 1. Islet gene expression after 4 days of infusion

	SAL ($n = 5$)	GLC ($n = 7$)	LIH ($n = 5$)	NON ($n = 9$)
Islet hormones				
Insulin	100 ± 8	127 ± 11	127 ± 21	120 ± 2
IAPP	100 ± 7	$133 \pm 8^*$	105 ± 5	$128 \pm 2^*$
Glucagon	100 ± 20	82 ± 15	168 ± 57	86 ± 6
Somatostatin	100 ± 12	114 ± 14	108 ± 9	111 ± 3
Metabolism				
GLUT-2	100 ± 6	117 ± 11	100 ± 3	106 ± 1
GK	100 ± 15	96 ± 9	84 ± 7	91 ± 3
HK	100 ± 10	86 ± 14	111 ± 17	104 ± 7
LDH	100 ± 12	89 ± 22	120 ± 21	93 ± 8
mGPDH	100 ± 17	95 ± 10	80 ± 6	95 ± 2
Transcription factors				
PDX-1	100 ± 4	97 ± 11	94 ± 8	101 ± 3
NeuroD/BETA2	100 ± 9	88 ± 8	107 ± 12	105 ± 3
pan1	100 ± 3	91 ± 7	87 ± 4	81 ± 11
HNF1- α	100 ± 14	79 ± 7	82 ± 7	91 ± 2
HNF4- α 1	100 ± 9	82 ± 7	91 ± 4	87 ± 3
HNF4- α 2/5	100 ± 7	87 ± 9	95 ± 4	87 ± 3

Islet gene expression (means \pm SE) after 4 days of saline (SAL), glucose (GLC), lipid (LIH), or no infusion (NON). IAPP, islet amyloid polypeptide; GK, glucokinase; HK, hexokinase; LDH, lactic dehydrogenase; mGPDH, mitochondrial glycerol-3-phosphate dehydrogenase; PDX-1, pancreatic duodenum homeobox protein; NeuroD/BETA2 and pan1, a basic helix-loop-helix factor and its dimeric partner; HNF, hepatic nuclear factors. Gene expression was first normalized to an internal control gene (see Ref. 20) and then expressed as a % of SAL. * $P < 0.05$ vs. SAL with Dunnett's multiple comparison test.

Fig. 2. A: total β -cell mass in noninfused rats (NON; $n = 6$) and at the end of 4 days of saline (SAL; $n = 5$), glucose (GLC; $n = 6$), and LIH ($n = 6$) infusion. B: β -cell proliferation in noninfused rats (NON; $n = 4$) and at the end of 4 days of saline (SAL; $n = 5$), glucose (GLC; $n = 5$), and LIH ($n = 6$) infusion. * $P < 0.05$ by ANOVA with Dunnett's test vs. saline control; + $P < 0.05$ by t -test between saline and noninfused controls.



infused controls, this increase was not significant compared with that of noninfused animals.

Quantification of β -cell mass and mitotic rate. Once sectioning and staining were completed, sections were evaluated for BrdU incorporation; sections in which BrdU staining could not be adequately detected were excluded (two noninfused animals and one glucose-infused animal could not be quantified because of poor BrdU staining). As well, at the completion of the β -cell mass calculations, one saline-infused animal was observed to have a mass that was >4 SD from the mean; following outlier analysis ($P < 0.05$, Grubb's outlier test), this animal was also excluded. Thus the final number of animals used for assessment of β -cell mass was 5 in the SAL group and 6 in all of the other groups, and the final number of animals used for quantification of β -cell proliferation was 4 in the NON group, 5 in the SAL and GLC groups, and 6 in the LIH group. In these animals, β -cell mass was increased 80% by glucose infusion (Fig. 2A; $P < 0.01$; compared with SAL) and 54% by lipid infusion (Fig. 2A; $P < 0.05$; compared with SAL), and both glucose and lipid increased β -cell proliferation (Fig. 2B; $P < 0.01$; both comparisons relative to SAL). However, compared with noninfused controls, saline infusion lowered β -cell proliferation ($P < 0.05$, t -test), and the animals tended to end the infusion with lower β -cell mass ($P = 0.23$; t -test), suggesting that part of the differences observed between the saline- and substrate-infused animals may have been due to an effect of the restraint or fluid loading (saline infusion controls for these effects, because all infused animals have the same restraint and the same level of fluid loading).

Assessment of postinfusion β -cell function. During hyperglycemic clamps, plasma glucose was rapidly elevated to 250 mg/dl (Fig. 3A). The glucose infusion rate required to maintain this level of glycemia was similar in NON, SAL, and LIH animals but was significantly elevated in the GLC animals (Fig. 3B; $P < 0.01$). The high glucose infusion rate was associated with a significantly higher plasma insulin response (Fig. 3C; $P < 0.05$).

Assessment of postinfusion peripheral insulin clearance. To assess whether the increase in plasma insulin observed during the postglucose infused hyperglycemic clamp (Fig. 3C) was due to a decrease in peripheral insulin clearance, euglycemic hyperinsulinemic clamps were performed in a separate set of rats undergoing similar infusions. For these clamps, the glucose infusion rate was similar in NON, SAL, and LIH animals

but was increased $\sim 20\%$ in the GLC group ($P < 0.05$; Fig. 4B). Steady-state plasma insulin levels tended to be lower in the LIH-infused animals (Fig. 4C), suggesting that insulin clearance may have been elevated in this group (22% relative to saline-infused controls, Fig. 4D); however, this difference did not achieve statistical significance ($P = 0.17$).

DISCUSSION

The present study demonstrated that, during chronic substrate oversupply, rats undergo compensatory β -cell adaptations leading to increased plasma insulin responses and normal glucose levels. During glucose infusion, euglycemia was maintained by an approximately twofold increase in plasma insulin (Fig. 1), and after 4 days, β -cell mass was increased $\sim 80\%$ relative to saline-infused animals (Fig. 2A). After these

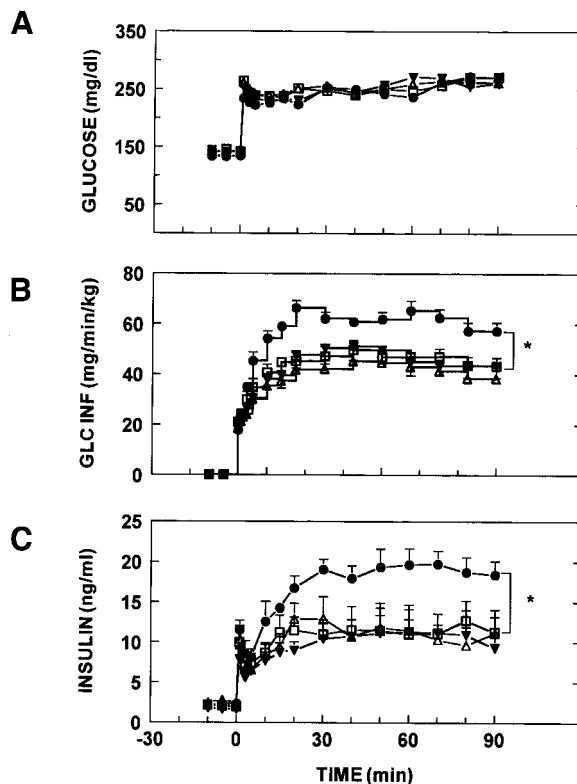


Fig. 3. Plasma glucose (A), exogenous glucose infusion (B), and plasma insulin (C) during hyperglycemic clamps performed in noninfused (NON; Δ ; $n = 7$) rats and 2 days after saline (SAL; \square ; $n = 6$), glucose (GLC; \bullet ; $n = 9$), and LIH infusion (\blacktriangledown ; $n = 6$). * $P < 0.05$ by ANOVA.

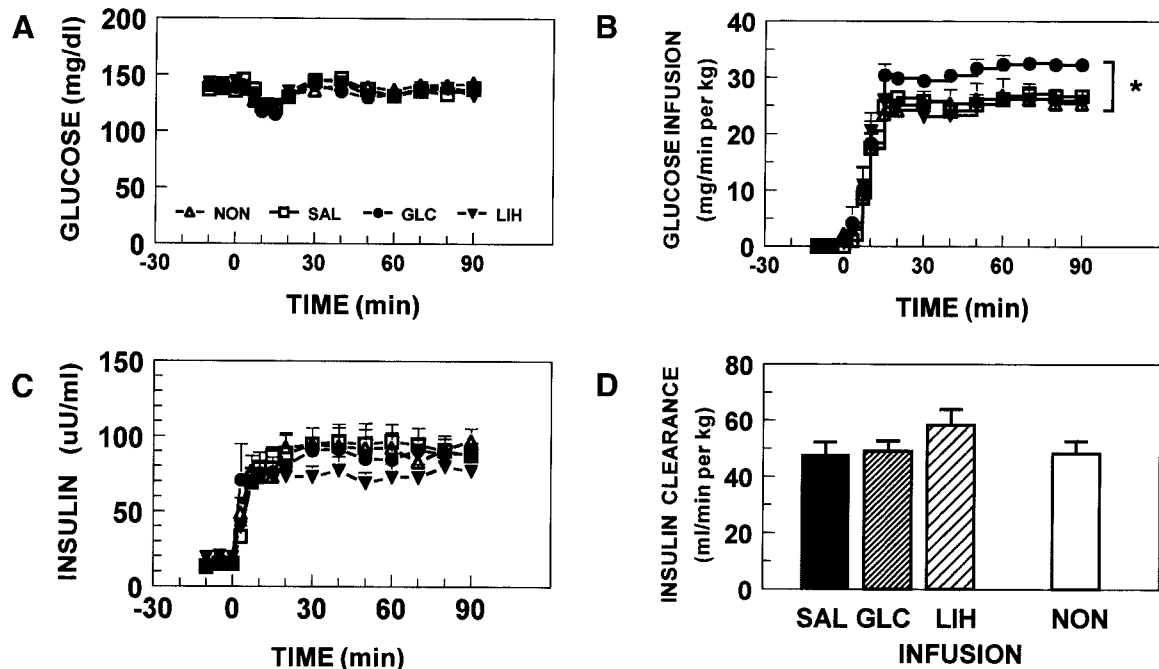


Fig. 4. Plasma glucose (A), exogenous glucose infusion (B), plasma insulin (C), and insulin clearance (D) during euglycemic-hyperinsulinemic clamps performed in noninfused rats (NON; Δ) and 2 days after saline (SAL; \square), glucose (GLC; \bullet), and LIH (\blacktriangledown) infusion; $n = 6$ for all groups. * $P < 0.05$ by ANOVA.

changes, gene expression was unchanged for islet hormones, genes involved in β -cell glucose uptake and metabolism, and transcription factors linked to insulin gene expression and β -cell development (Table 1). Compensatory increases in plasma insulin with normal glucose levels were also seen with lipid infusion, particularly during feeding (Fig. 1F). After 4 days of lipid infusion, β -cell mass increased $\sim 54\%$ (Fig. 2A) and, with the exception of a small change in IAPP, islet gene expression was again unchanged (Table 1). Saline infusion resulted in the lowest plasma insulin levels of any infused group and the lowest rate of β -cell proliferation (Fig. 2B). At the end of the saline infusion, β -cell mass tended to be lower in saline-infused animals compared with noninfused, nonrestrained animals. Of all the changes in β -cell mass, only the increase with glucose infusion yielded a sustained (2-day) increase in the plasma insulin response to glucose (Fig. 3C). This increase in plasma response was not due to any change in peripheral insulin clearance (Fig. 4D), indicating that the insulin secretory capacity per se was increased.

The lack of significant changes in islet gene expression after prolonged glucose or lipid oversupply is surprising. During glucose infusion, plasma insulin levels were doubled, but there was no change in the expression of the insulin gene per se or of genes regulating the entry into and metabolism of glucose in the β -cell (GLUT-2, GK, hexokinase and mitochondrial glycerol-3-phosphate dehydrogenase, and lactic dehydrogenase; Table 1). However, at the time gene expression was assessed (end of the 4-day infusions), glucose had been normal for >2 days (Fig. 1A) and β -cell mass was

significantly increased (Fig. 2A). The increase in β -cell mass may have allowed individual β -cells to secrete at a normal basal level, obviating the need for an increase in the insulin gene transcription or genes required for glucose metabolism in the β -cell.

Genes known to be affected by FFA in vitro were also unchanged in the present study despite an ~ 10 -fold increase in plasma FFA levels (Table 1). Elevated FFA has been shown to decrease PDX-1, GLUT-2, GK, and insulin expression in vitro (16). The difference in the results presented here and in Ref. 16 can likely be attributed to different antecedent glucose levels. In the present study, glucose levels were normal or elevated for the 4 days preceding the islet isolation, whereas in Ref. 16, the glucose level was decreased to 2.8 mM and then increased to 18 mM. Antecedent hypoglycemia has been shown to alter β -cell gene expression (15, 42). In addition to PDX-1, FFA has also been proposed to act as a natural ligand of HNF4- α (18) and potentially regulate its transcriptional activity on target genes. However, GLUT-2, which is a target of HNF4- α (45), was not affected in the present study. Finally, high FFA levels have been reported to reduce insulin gene translation in vitro (5). Although we cannot rule out an effect of FFA on the translation of insulin mRNA, we can rule out an acute consequence of such an effect, in that, in the present study, the β -cell was able to maintain normal glucose levels and to dramatically increase insulin secretion during feeding despite the increase in FFA. One possible reason for the discrepancies between the in vivo results reported here and many of the in vitro results may be related to the high FFA-to-BSA ratios used in vitro; high FFA-to-BSA ratios result in

unphysiological levels of unbound FFA (46). The lack of a detrimental effect of FFA on gene expression or insulin secretory capacity *in vivo* makes many of the reported *in vitro* effects difficult to interpret.

Although neither glucose nor lipid infusion altered the transcription of the genes measured in the present study, we have recently shown that many of these genes are markedly altered in the 90% pancreatectomized (Px) rat (20). Islet isolation, RNA preparation, and PCR conditions were identical in the two studies (the same personnel performed all procedures relating to the PCR). The important difference between the two studies is that, here, the β -cells were able to maintain euglycemia, whereas in the 90% Px rat, graded levels of hyperglycemia were observed that likely triggered a loss of β -cell differentiation (20). Together, the two studies clearly indicate that failure of β -cells to adapt to an increase in insulin demands results in hyperglycemia and altered gene expression, whereas successful β -cell adaptation leads to normal glucose levels and normal gene expression.

The increase in β -cell mass observed in the present study with glucose infusion is comparable to the increase observed in our earlier glucose infusion studies (7). However, in the present study, a second control group that was neither infused nor restrained was also studied. Compared with this group, saline infusion tended to decrease the β -cell mass and significantly lowered the β -cell proliferation rate. Reasons for the decrease in proliferation and mass are not entirely clear; however, during all of the substrate infusions, the animals did not gain body weight, whereas noninfused nonrestrained animals gain body weight at ~ 5 – 10 g per day. Thus it is possible that the stress of the infusion led to a decrease in food intake (not measured), leading to a lower insulin requirement and a subsequent reduction in β -cell mass. Use of saline-infused animals as the control group, as was done in the present study and in most studies of this type, controls for this effect; together, the two controls demonstrate that β -cell proliferation and mass are tightly coupled to insulin demand. That is, during periods of reduced insulin demand (saline infusion), β -cell mass and proliferation fall, whereas during periods of increased insulin demand (glucose infusion), mass and proliferation increase.

Consistent with the link between insulin demand and changes in β -cell mass, lipid infusion increased both the mass and proliferation rate (Fig. 2). It is known that lipid infusion leads to insulin resistance in the rat within ~ 5 h (8). The increase in proliferation observed here is consistent with results obtained *in vitro* (39). Interestingly, the increase in β -cell mass observed with lipid infusion was smaller than that observed during glucose infusion despite similar increases in β -cell proliferation. This might suggest that the elevation in FFA also increased apoptosis, as has been shown to occur *in vitro* (43); however, this is speculative, given that changes in islet neogenesis and hypertrophy were not measured. In any case, what is clear is that, during the lipid infusion, β -cell mass was

maintained at a level sufficient to meet the demands of the animal.

Of all the changes in β -cell mass observed in the present study, only the change with glucose infusion resulted in a sustained increase in insulin secretory capacity. In this group, during hyperglycemic clamps that were performed 2 days after the chronic glucose infusion was increased $\sim 70\%$ relative to all of the other groups (NON, SAL, and LIH animals were all within 8% of each other; Fig. 3C). This result, combined with our earlier finding that the increase in β -cell mass is stable for ≥ 1 wk (7), indicates that the β -cell mass formed during glucose infusion is functional. This conclusion can be contrasted to numerous *in vitro* studies demonstrating that the insulin response is reduced immediately after glucose infusion (27, 28, 30–32). Although it is possible that a glucotoxicity effect may have dissipated in the 2 days between the infusion and the clamp, a study by Laury et al. (25) demonstrated that the insulin response to a hyperglycemic clamp is increased, even if the clamp is performed immediately after the glucose infusion. The inconsistency between results obtained with the *in vivo* hyperglycemic clamp and the *in vitro* isolated perfused pancreas has been suggested to be due to the loss of neural stimulation during *in vitro* perfusion (1). That the *in vivo* insulin response is increased after glucose infusion is also supported by a recent study in streptozotocin-diabetic rats indicating that β -cell mass and function are both increased after glucose infusion in nondiabetic control rats (3). Thus the present study, the Laury study (25), and the control rats in Ref. 3 all indicate that, during chronic glucose infusion, rats undergo compensatory β -cell adaptations leading to an increased ability to secrete insulin.

The change in plasma insulin response observed with glucose infusion cannot by itself be used to infer an increase in insulin secretion, because a decrease in insulin clearance will also appear as an increase in plasma insulin. Insulin clearance has been shown to be decreased during chronic glucose infusion (26). Although C-peptide has been used as a surrogate marker of clearance, no direct evidence exists to indicate that the clearance of this peptide is unaffected by chronic infusion. Furthermore, the rat has two insulin/C-peptide genes; this prevents the assessment of insulin secretion using traditional C-peptide deconvolution (13). In light of these issues, peripheral insulin clearance was evaluated by euglycemic-hyperinsulinemic clamp (Fig. 4) and determined to be normal. The normal clearance at this time point can be compared with the 50% reduction observed 1 h after glucose infusion (26). Together, the two studies suggest that insulin clearance may also be tightly linked to insulin demand.

The ability of the β -cell to adapt to elevated FFA is controversial. The two most recent studies using lipid-infused rats have concluded, in one case, that the glucose-stimulated insulin response is increased (34), and in the second case that it is decreased (37). In the present study, lipid infusion did not result in a postinfusion increase in insulin secretion, but compensatory

increases in plasma insulin responses during feeding were observed during the infusion period (Fig. 1F). Although there are methodological differences in the three studies (in Ref. 37 hyperglycemic clamps were performed while the lipid infusions were in progress, in Ref. 34 intravenous glucose tolerance tests were performed immediately after the infusions, and in the present study hyperglycemic clamps were performed 2 days after the infusions), several important observations can nonetheless be made. First, the increased insulin response seen in Ref. 34 supports the contention that rat β -cells compensate during lipid infusion. Interestingly, no change in mass was observed in Ref. 34 after 2 days of lipid infusion, whereas in the present study a 54% increase in mass was observed after 4 days. This implies that the adaptation in mass occurred on *days 3 and 4*. In contrast to the increased insulin response in Ref. 34, a decreased response was observed in Ref. 37 that the authors speculate may have been due to altered gene expression or reduced β -cell mass. In this regard, we did not find any alterations in gene expression at *day 4*, and the mass was elevated on *day 4*. Thus the increased insulin response observed in Ref. 34 and results of the present study both support the contention that rat β -cells are able to adapt to the increased need for insulin brought about by high FFA levels.

Although the elevation in FFA generated a clear adaptive response during the infusion period, no effect on the plasma insulin response was observed 2 days after the lipid infusion (Fig. 3C). Note that it is possible that secretion was elevated at this time and that the elevation was masked by an increase in insulin clearance [clearance was increased 22% at this time point (Fig. 4D), but this increase was not statistically significant; $P = 0.17$]. However, it is also possible that the normalized plasma insulin response was simply a result of insulin sensitivity having returned to a normal level. In this regard, one can argue that the elevated insulin response after glucose infusion was inappropriate given that the insulin sensitivity had also normalized in this group. Additional time points assessing insulin sensitivity, secretion, and clearance will need to be conducted to fully address this issue.

Finally, it is interesting to compare characteristics of the substrate-infused rat with those of developing type 2 diabetes in humans. In particular, it is noteworthy that the animals do not gain body weight during the infusion period. Thus the model is not one of caloric overconsumption or obesity-induced insulin resistance. However, lipid infusion clearly results in peripheral insulin resistance (8) and an inability of insulin to suppress hepatic glucose output (4). Regarding the latter, overproduction of glucose by the liver is a well known characteristic of developing type 2 diabetes (10, 11, 35), and this overproduction is clearly mimicked by continuous glucose infusion. In the present study, both the insulin resistance induced by lipid infusion and the elevated rate of glucose appearance with glucose infusion were rapidly compensated for by the β -cell. This compensation included an increase in β -cell mass, a

functional increase in insulin secretion, and the normal expression of key islet genes. Understanding the cellular and molecular mechanisms of this adaptive response has important implications for understanding why some individuals can adapt to increases in insulin demand while others go on to develop diabetes.

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