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Determinants of glucose toxicity and its reversibility in the pancreatic islet β-cell line, HIT-T15

CATHERINE E. GLEASON, MICHAEL GONZALEZ,
JAMIE S. HARMON, AND R. PAUL ROBERTSON
Pacific Northwest Research Institute, and Departments of Pharmacology and Medicine, University of Washington, Seattle, Washington 98122

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Gleason, Catherine E., Michael Gonzalez, Jamie S. Harmon, and R. Paul Robertson. Determinants of glucose toxicity and its reversibility in the pancreatic islet β-cell line, HIT-T15. *Am J Physiol Endocrinol Metab* 279: E997–E1002, 2000.—HIT-T15 cells, a clonal β-cell line, were cultured and passaged weekly for 6 mo in RPMI 1640 media containing various concentrations of glucose. Insulin content decreased in the intermediate- and late-passage cells as a continuous rather than a threshold glucose concentration effect. In a second series of experiments, cells were grown in media containing either 0.8 or 16.0 mM glucose from *passages 76* through *105*. Subcultures of *passages 86, 92,* and *99* that had been grown in media containing 16.0 mM glucose were switched to media containing 0.8 mM glucose and also carried forward to *passage 105*. Dramatic increases in insulin content and secretion and insulin gene expression were observed when the switches were made at *passages 86* and *92* but not when the switch was made at *passage 99*. These findings suggest that glucose toxicity of insulin-secreting cells is a continuous rather than a threshold function of glucose concentration and that the shorter the period of antecedent glucose toxicity, the more likely that full recovery of cell function will occur.

insulin gene expression; PDX-1 binding

THE RELATIONSHIP BETWEEN fasting plasma glucose level and the magnitude of glucose-induced insulin secretion is reciprocal (4), suggesting that elevated glycemia might adversely affect β-cell function. Adverse (glucotoxic) effects of glucose on cells have been shown to occur when they are exposed chronically to supraphysiological concentrations of this hexose. A molecular mechanism of action for glucose toxicity has been identified in studies demonstrating decreased insulin gene expression (3, 13, 14, 24, 25, 27, 28, 30, 33, 36, 37). However, these mechanistic studies have not addressed two important functional issues. One is whether the adverse effects on insulin-secreting cells of chronic exposure to high glucose concentrations are glucose concentration related in a continuous manner or whether there exists a threshold that must be reached before glucose toxicity is expressed. The other is whether successful reversal of glucose toxicity is related to the length of previous exposure to supraphysiological glucose concentrations. The clinical relevance of these questions stems from the need to understand the intensity with which hyperglycemia should be treated in type 2 diabetes. It is often suggested that the more quickly and more completely glycemia is normalized through diet or drug treatment in diabetic patients, the more likely residual β-cell function will be sufficient to help maintain normoglycemia. Consequently, we designed experiments to answer two questions. *1*) Is induction of glucose toxicity of the HIT-T15 cell over a defined period of time a continuous or a threshold function of glucose concentration? *2*) Is successful reversal of glucose toxicity of the HIT-T15 cell inversely related to the duration of time over which the antecedent glucose toxic state exists?

RESEARCH MATERIALS AND METHODS

HIT-T15 cell culture. HIT-T15 cells were routinely grown in 5% CO_2-95% humidified air at 37°C, maintained in RPMI 1640 medium containing 10% fetal bovine serum, and passaged once weekly after trypsin-EDTA detachment (41).

Insulin content and secretion. Determination of intracellular insulin content and insulin secretion during static incubations in 0.2 or 5.0 mM glucose was determined as described previously (40).

Analysis of insulin mRNAs. The abundance of insulin mRNAs in HIT-T15 cells was determined by ribonuclease protection assay (RPA) using the Direct Protect RPA Kit (Ambion, Austin, TX) as previously described (13). Riboprobes were transcribed from template DNA using the MAXIscript In Vitro Transcription Kit (Ambion). Insulin DNA template was generated by inserting the 307-bp *Pst* I fragment of the human preproinsulin genomic DNA (phins 214, ATCC) into the pSport 1 vector. The insulin template was linearized with *Kpn* I and transcribed with T7 RNA polymerase to generate a 330-bp RNA fragment (a 186-bp protected fragment). β-Actin was used for the control. The β-actin template DNA was transcribed with T7 RNA polymerase from a linearized pTRIPLEscript plasmid containing a 250-bp mouse β-actin gene fragment to generate a 304-bp RNA fragment (a 245-bp protected fragment). The probes were gel purified on a 5% polyacrylamide-8 M urea gel and eluted overnight at 37°C in elution buffer (0.5 M NH_4OAc, 1

Address for reprints and other correspondence: R. Paul Robertson, Pacific Northwest Research Institute, 720 Broadway, Seattle, WA 98122 (E-mail: rpr@u.washington.edu).

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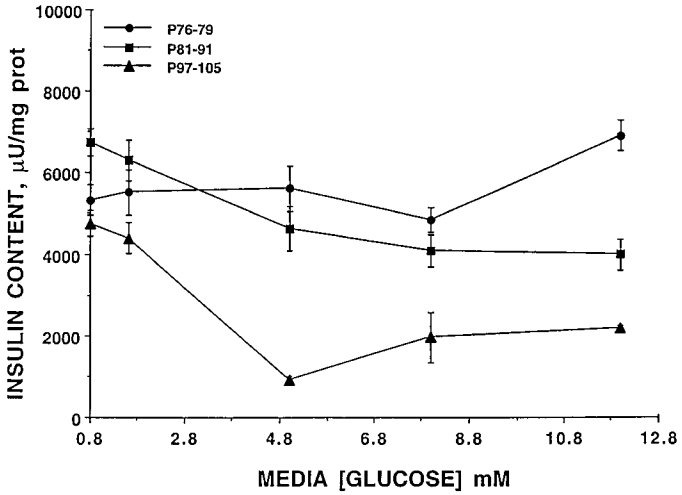

Fig. 1. Insulin content of HIT-T15 cells cultured in RPMI 1640 media containing increasing concentrations of glucose. Passages are grouped as early (*p 76–79*; $n = 8$), intermediate (*p 81–91*; $n = 11$) and late (*p 97–105*; $n = 8$). Early passage cells maintained insulin content regardless of the glucose concentration in the media, whereas intermediate and late passage cells maintained insulin content only when media with the lowest glucose concentration was used (intermediate cells = 6,738 ± 324 vs. 3,975 ± 366, $P < 0.001$; late cells = 4,750 ± 322 vs. 2,172 ± 50. Values are means ± SE, $P = <0.0001$; comparisons when using 0.8 vs. 12 mM glucose in media).

mM EDTA, 0.1% SDS). Probes were precipitated from the elution buffer with 10 μg of yeast tRNA and 1 ml of 100% ethanol on ice for 15 min and resuspended in an appropriate volume of RNase-free trypsin-EDTA buffer. The quantity of probe generated was determined by spectrometry. Riboprobes (0.5 μg) were labeled with Psoralen-biotin using the BrightStar Psoralen-Biotin Nonisotopic Labeling Kit (Ambion).

HIT-T15 cells were subcultured in RPMI 1640 medium containing either 0.8 or 16 mM glucose in 100-mm^2 dishes. The cells were grown to confluence and subcultured in medium containing 11.1 mM glucose for 48 h before harvesting. The cells were trypsinized, transferred to 15-ml tubes, and washed with PBS. Cells were resuspended in 1 ml lysis/denaturation solution and vortexed. Five microliters of the appropriate probe were hybridized to 10 μl of cell lysate and incubated overnight at 45°C. The unprotected mRNA fragments were digested with 200 U of RNase T1 for 30 min at 37°C. The protected fragments were precipitated with 0.5 ml of isopropanol and resuspended in 10 μl of gel loading buffer. The fragments were separated on a 5% polyacrylamide-8 M urea gel. RNA was transferred from the gel to a positively charged nylon membrane (Ambion's BrightStar-Plus Membrane) by electrotransfer in 1× Tris-borate-EDTA buffer and cross-linked by ultraviolet illumination. The mRNA fragments were detected with Ambion's Biodetect Nonisotopic Detection Kit, and the membrane was exposed to X-ray film. Density of the insulin band was divided by the density of the β-actin band within the same lane to correct for loading.

Analysis of transcription factor binding to the insulin promoter. The binding of PDX-1 and RIPE-3b1-activator proteins to the insulin promoter was analyzed by electrophoretic mobility shift assay (EMSA). HIT-T15 cells were subcultured for 48 h in RPMI 1640 medium containing 11.1 mM glucose. Nuclear extracts were prepared from these cells as previously described (27). Oligonucleotide probes consisting of the human insulin CT2 (−230 to −201) and rat insulin II RIPE-3b1 (−126 to −101) fragments were annealed and end-labeled with [^{32}P]dCTP using the Klenow fragment of *Escherichia coli* DNA polymerase I. The EMSA was performed as previously described (27), with binding reactions containing 10 or 20 μg of protein. Autoradiograms were analyzed and quantitated by densitometry. Protein concentrations were determined by bicinchoninic acid assay (Pierce, Rockford, IL).

Statistical analysis. Statistical analysis was performed by Student's test (Fig. 1), by Pearson product-moment correlations (Fig. 2), and by ANOVA with Bonferroni/Dunnett 2 × 2 comparisons (Figs. 3–5).

RESULTS

Glucose toxicity: a continuous or a threshold glucose concentration-related event? Experiments were conducted with HIT-T15 cells, beginning with *passage 76,* that were carried forward for 30 wk, during which the cells were split and passaged on a weekly basis until *passage 105* was reached. Cells were grown in RPMI 1640 containing one of five increasing (0.8, 1.6, 5.0, 8.0, 12.0 mM) concentrations of glucose. For purposes of comparison, cell passages were clustered into three groups: early passages (*p 76–79*); intermediate passages (*p 81–91*); and late passages (*p 97–105*). Insulin content in the early passages did not diminish, regardless of the glucose concentration in the cell culture media (Fig. 1). Similarly, insulin content in the intermediate and late passages did not decrease in cells cultured in media containing 0.8 or 1.6 mM glucose. However, insulin content decreased dramatically in the intermediate and late passage cells when 5.0, 8.0, or 12.0 mM glucose was used. Correlation analysis indicated a continuous rather than a threshold glucose concentration effect on insulin content in the intermediate and late passages (Fig. 2).

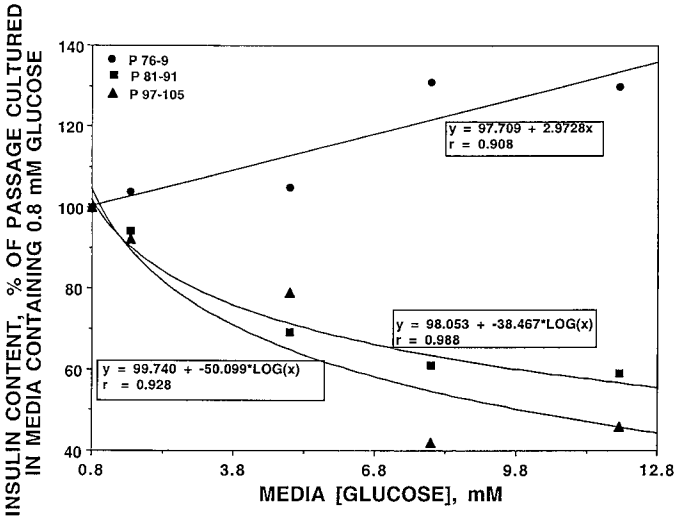

Fig. 2. Data shown in Fig. 1 from the early, intermediate, and late passage groups expressed as a percentage of the insulin content data obtained when media contained 0.8 mM glucose. Regression analysis using the best-fit equation of the line yielded highly statistically significant correlations, suggesting continuous relationships between declining insulin content and increasing glucose concentrations in the media for the intermediate and late passage groups.

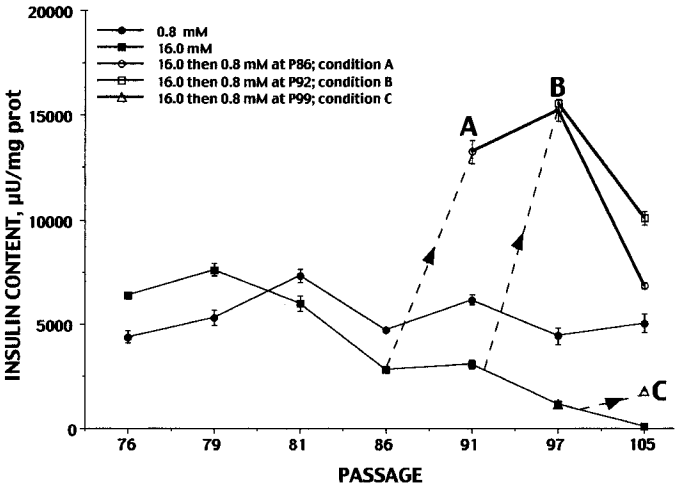

Fig. 3. Insulin content in HIT-T15 cells grown in 0.8 or 16 mM glucose grown from *passages 76* though *105*. At *passages 86* (*condition A*), *92* (*condition B*), and *99* (*condition C*), subcultures of cells previously grown in 16 mM glucose were introduced into media containing 0.8 mM glucose and continued to *passage 105*. Cells grown in media containing 0.8 mM showed no significant decrease in insulin content between *passages 76* and *105*. In contrast, cells grown in 16 mM glucose showed a progressive decline in content (6,341 ± 63 vs. 93 ± 28, $P < 0.0001$). Cells whose media were changed at *passages 86* and *92* from 16 to 0.8 mM glucose showed a dramatic increase in insulin content by *passage 97* (*condition A* = 15,210 ± 531 vs. 4,434 ± 398; *condition B* = 15,555 ± 187 vs. 4,434 ± 398; both $P < 0.0001$ compared with cells grown in media containing 0.8 mM glucose) that was sustained through *passage 105*. In contrast, insulin content did not increase dramatically when the switch in glucose concentration was made at *passage 99* (*condition C* = 1,809 ± 23 vs. 4,434 ± 398). All $n = 4$ for each data point.

Duration of exposure to toxic concentrations of glucose as a determinant of reversal of glucose toxicity. To ascertain whether duration of antecedent exposure to supraphysiological glucose concentrations is a determinant of the degree of reversibility of glucose toxicity, experiments were conducted in which HIT-T15 cells were grown in RPMI 1640 media containing either 0.8 or 16.0 mM glucose. Cells were passaged weekly for 6 mo from *passages 76* through *105*. Insulin content in the later passages did not decrease in cells cultured in media containing 0.8 mM glucose, whereas a progressive decline in insulin content was observed when cells were passaged in the presence of 16 mM glucose (Fig. 3). Subcultures of *passages 86, 92,* and *99* that were being grown in media containing 16.0 mM glucose were switched to media containing 0.8 mM glucose and carried forward to *passage 105*. A dramatic increase in insulin content was observed five passages beyond the switch when the glucose concentrations were changed at *passages 86* and *92*. However, this large increase in insulin content was not observed when the switch to the lower glucose concentration was made at *passage 99* (Fig. 3).

Basal and glucose-stimulated insulin release from subcultures of the cells cultured in either 0.8 mM or 16.0 mM glucose or those switched from 16.0 to 0.8 mM glucose at various passages provided results consistent with the insulin content measurements. Increases in basal insulin release during static incubations in 0.2 mM glucose were observed from the cells that were switched at *passages 86* and *92*; however, basal insulin secretion from passages switched at *passage 99* did not change impressively (Fig. 4). Subcultures of the cells were also exposed to stimulatory concentrations of glucose during static incubations in buffer containing 5.0 mM glucose, a concentration that is maximal for stimulation of insulin release from HIT-T15 cells. Glucose-induced insulin responses were improved when cells were switched from media containing 16.0 to 0.8 mM glucose at *passages 86* and *92* (Fig. 5). In contrast, no similar increase was observed when the cells were switched at *passage 99*. These data indicated that recovery of insulin content, basal insulin secretion, and glucose-stimulated insulin secretion are heavily influenced by the duration of exposure of the cells to supraphysiological glucose concentrations in the media in which they are grown.

To determine whether recovery of insulin content might be related to recovery of insulin gene expression, levels of insulin mRNA were determined in subcultures of cells that were grown in either 0.8 mM or 16.0 mM glucose and in cells that were switched from 16 mM glucose to 0.8 mM glucose at *passages 86*, *92*, and *99*. In two of two experiments, recovery of insulin mRNA was detected in cells that were switched to media containing the lower glucose concentration when the switches were made at *passages 86* and *92*,

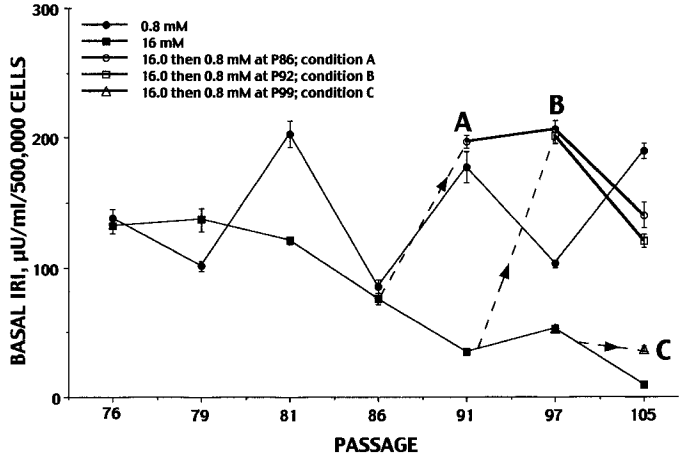

Fig. 4. Basal insulin secretion (IRI) during static incubations in 0.2 mM glucose for 2 h from HIT-T15 cells grown in 0.8 or 16 mM glucose from *passages 76* through *105*. At *passages 86, 92,* and *99*, subcultures of cells previously grown in 16 mM glucose were introduced into media containing 0.8 mM glucose and continued to *passage 105* as in Fig. 3. Cells grown in media containing 0.8 mM glucose showed no significant decrease in basal IRI between *passages 76* and *105*. In contrast, cells grown in 16 mM glucose showed a progressive decline in basal IRI (132 ± 6 vs. 9 ± 2, $P < 0.0001$). Cells whose media were changed at *passages 86* and *92* from 16 to 0.8 mM glucose showed a dramatic increase in basal insulin release by *passage 97* (*condition A* = 206 ± 7 vs. 103 ± 3; *condition B* = 201 ± 6 vs. 103 ± 3; both $P <$ 0.0001 compared with cells grown in media containing 0.8 mM glucose) that was sustained through *passage 105*. In contrast, basal IRI did not increase dramatically when the switch in glucose concentration was made at *passage 99* (*condition C* = 37 ± 2 vs. 103 ± 3). All $n = 4$ for each data point.

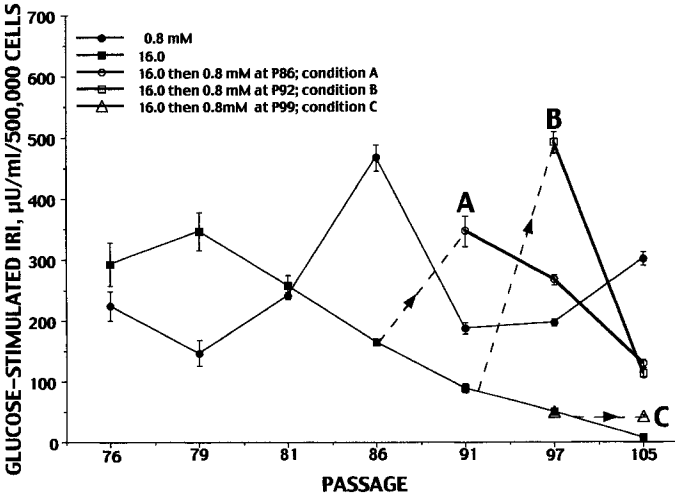

Fig. 5. Glucose-stimulated IRI during static incubations containing 5.0 mM glucose for 2 h from HIT-T15 cells grown in 0.8 or 16 mM glucose grown from *passages 76* through *105*. At *passages 86, 92,* and *99*, subcultures of cells previously grown in 16 mM glucose were introduced into media containing 0.8 mM glucose as in Fig. 3. Cells grown in media containing 0.8 mM glucose showed no significant decrease in glucose-stimulated IRI between *passages 76* and *105*. In contrast, cells grown in 16 mM glucose showed a progressive decline in glucose-stimulated IRI (292 ± 35 vs. 8 ± 2, $P < 0.0002$). Cells whose media were changed at *passages 86* and *92* from 16 to 0.8 mM glucose showed a dramatic increase in glucose-stimulated IRI by *passages 91* and *97*, respectively (*condition A* = 346 ± 25 vs. 187 ± 9; *condition B* = 490 ± 17 vs. 196 ± 5; both $P < 0.0001$ compared with cells grown in media containing 0.8 mM glucose). In contrast, glucose-stimulated IRI did not increase dramatically when the switch in glucose concentration was made at *passage 99* (*condition C* = 43 ± 2 vs. 300 ± 12). All $n = 4$ for each data point.

but not *99* (Fig. 6). EMSAs revealed recovery of PDX-1 and RIPE-3b1-activator protein binding to the insulin promoter when the switches were made at all three passages (Figs. 7 and 8).

DISCUSSION

These studies were designed to assess two hypotheses: first, that the development of glucose toxicity in insulin-secreting cells over a finite period of time is a

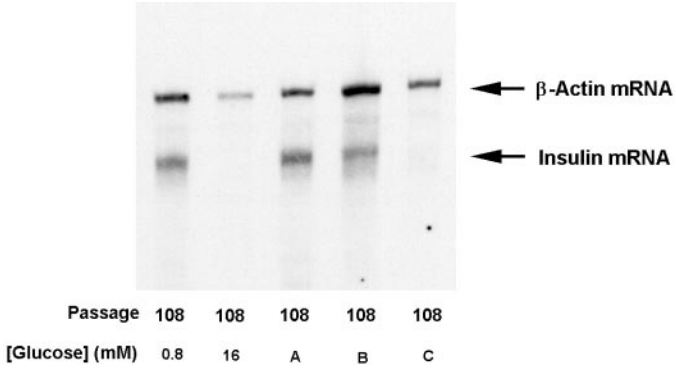

Fig. 6. Ribonuclease protection assay (RPA) of insulin mRNA in HIT-T15 cells. Photoimage of RPA for insulin mRNA. Cells had been grown in media containing 16.0 mM glucose and then switched to 0.8 mM glucose at *passages 86* (*A*), *92* (*B*), or *99* (*C*). Insulin/β-actin = 0.58, 0.00, 0.84, 0.26, and 0.02 for *lanes 1–5*, respectively. Data representative of 2 separate experiments.

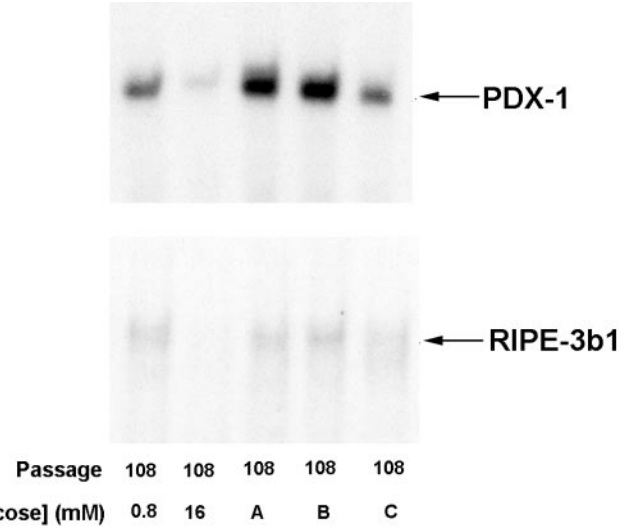

Fig. 7. Electrophoretic mobility shift assay for PDX-1 binding and RIPE-3b1-activator binding to the insulin promoter region. Experimental conditions were as indicated in legend for Fig. 6. Data representative of 2 separate experiments.

continuous rather than a threshold function of the glucose concentration in the environment of the β-cell; second, that the magnitude of recovery from glucose toxicity is inversely related to the length of antecedent exposure to supraphysiological glucose concentrations. Consistent with our previous observations (14, 27, 28, 30, 33, 37), we found no evidence of diminished insulin content or defective insulin secretion when cells were cultured in any of the glucose concentrations for several weeks only. However, chronic exposure of the cells for up to 30 wk to glucose concentrations >1.6 mM resulted in progressive declines in insulin content and insulin secretion. This deterioration appeared to be a continuous function of increasing glucose concentrations rather than being related to a threshold glucose concentration. For interpretation of these findings, it is important to note that the EC_{50} for glucose is ~1.0 mM and its maximal effect is ~2.0 mM for insulin secretion in HIT-T15 cells (40). Pancreatic islets typically have an EC_{50} of 7.0–8.0 mM glucose. To assess the second hypothesis, we chronically cultured HIT-T15 cells in media containing either 0.8 or 16.0 mM glucose for 30 wk. In this paradigm, we intervened by switching subcultures of the cells grown in the presence of 16.0 mM glucose to media containing 0.8 mM glucose at three intervals into the long-term culture. We observed that the earlier the switch to the lower glucose concentration was made, the greater the recovery of insulin content and secretion. Unexpectedly, within five passages of switching to media containing the lower glucose concentration, insulin content of the cells increased to greater levels than the level observed in the control cells being chronically grown in media containing 0.8 mM glucose. This was associated with increased insulin gene expression and increases in PDX-1 and RIPE-3b1-activator binding to the insulin promoter. The cells switched at the latest passage failed to have

such a strong recovery of insulin content and insulin secretion. These findings indicate that an early, finite window of time exists during which full recovery from glucose toxicity is possible. The recovery of PDX-1 and RIPE-3b1-activator binding, but not insulin mRNA in the passage switched latest, may indicate that levels of other transcription factors (24) not studied in these experiments had changed and had not normalized sufficiently to allow full insulin gene expression.

Although it would have been more desirable to conduct these studies using isolated islets, it is not possible to continuously culture islets for 30 wk. In this situation, use of β-cell lines provides a useful surrogate approach, even though one cannot assume that all our findings are necessarily pertinent to authentic β-cells within islets. Because we have previously demonstrated that no differences in population doubling times exist when HIT-T15 (33) or βTC-6 (30) cells are grown in media containing 0.8 or 11.1 mM glucose, these adverse effects of supraphysiological glucose concentrations cannot be explained by accelerated aging. We have also shown that other osmotically active moieties do not cause these changes. To assess whether the adverse effect of elevated glucose concentrations on hormone synthesis and secretion might be specific to β-cells, we performed control experiments using the glucagon secreting cell line αTC-1/9 (32). In these experiments, chronic exposure to media containing supraphysiological glucose concentrations did not cause defective glucagon secretion or defective glucagon gene expression. Olson et al. (26) demonstrated that supraphysiological glucose concentrations could decrease insulin gene expression in INS-1 cells, an insulin secreting cell line that secretes insulin in response to physiological glucose concentrations. However, this glucose-induced decrease in insulin gene expression was observed after only 48 h of exposure to elevated glucose concentrations and therefore is more consistent with glucose desensitization than with glucose toxicity. The associated diminished binding of PDX-1 and RIPE-3b1-activator to the insulin promoter was readily reversible when the cells were switched to media containing physiological glucose concentrations.

In summary, these findings indicate that glucose toxicity of the β-cell is a continuous rather than a threshold function of glucose concentration and that the shorter the period of antecedent glucose toxicity, the greater the degree of recovery. Findings from experiments such as these, in conjunction with many findings published by other researchers (1–3, 5–12, 15–24, 29, 31, 34–36, 38, 39), suggest that abnormally elevated glucose concentrations in the β-cells' environment can cause a spectrum of changes. With short-term exposure to high glucose concentrations, decreases in insulin secretion and insulin content can occur that are reversible upon return to normal glucose concentrations. The term glucose desensitization seems most apt to describe this sequence of events (16). On the other hand, a spectrum of pathophysiological changes may occur with more prolonged exposure of the β-cell to supraphysiological glucose concentrations. Using various experimental models, many researchers have described adverse effects of glucose on β-cell function by the term "β-cell exhaustion." The distinction between β-cell exhaustion and glucose toxicity is not always clear. We favor the concept that the two may be in a pathophysiological continuum (32). In this context, β-cell exhaustion might be earlier and more likely to be reversible, whereas glucose toxicity is later and less likely to be reversible. In this context, it seems likely that early, effective management by diet and drugs of hyperglycemia in type 2 diabetes is an important aspect of preserving residual β-cell function. The same argument for meticulous glycemic control can be made after pancreas or islet transplantation.

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