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Chronic Exposure of βTC-6 Cells to Supraphysiologic Concentrations of Glucose Decreases Binding of the RIPE3b1 Insulin Gene Transcription Activator

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Abstract
We have shown previously that chronic exposure of HIT-T15 cells to supraphysiologic glucose concentrations causes decreased insulin gene transcription and decreased binding activities of two β-cell specific transcription factors, STF-1 and the RIPE3b1 activator, and have suggested that these events may provide a mechanism for glucose toxicity on β-cell function. However, this contention can be criticized because it is not clear whether these observations are unique to the HIT-T15 cell or generalizable to other β-cell lines and the islet. Therefore, we cultured βTC-6 cells for up to 41 wk in either 11.1 or 0.8 mM glucose. We observed a passage-dependent decrease in insulin content and insulin mRNA levels in βTC-6 cells chronically cultured in 11.1 mM glucose. Cells chronically cultured in 0.8 mM glucose had higher insulin mRNA levels than cells chronically cultured in 11.1 mM glucose. The relative activity of a chloramphenicol acetyl transferase (CAT) reporter gene controlled by the 5′ regulatory region of the human insulin gene was decreased in late passage βTC-6 cells chronically cultured in 11.1 mM glucose, but was preserved in late passages of cells chronically cultured in 0.8 mM glucose. Electromobility shift assays demonstrated that binding of a specific nuclear protein that recognizes the RIPE3b1 binding site of the insulin gene was markedly diminished in late passage cells chronically exposed to 11.1 mM glucose, whereas binding activities of STF-1 and ICE activators were unchanged. RIPE3b1 binding activity was preserved in late passage cells chronically exposed to 0.8 mM glucose. Mutation of the RIPE3b1 binding site almost completely abolished insulin gene transcription as well as binding activity. We conclude that chronic exposure of βTC-6 cells to high glucose concentrations paradoxically decreases insulin gene transcription, in part, by decreasing activity of the trans-activating factor which binds to the RIPE3b1 sequence. This study uniquely demonstrates that altered binding to the RIPE3b1 sequence mediates glucose toxicity in βTC-6 cells, thus reinforcing the importance of this cis-acting element in the regulation of insulin gene transcription. We conclude that the phenomenon of glucose toxicity decreasing binding of transcription factors and thereby reducing insulin gene expression is not a feature solely of HIT-T15 cells and may be demonstrable generally in β-cell lines. (J. Clin. Invest. 1996. 97:1041–1046.) Key words: glucose toxicity • insulin gene • transcription factor • RIPE3b1 • STF-1

Introduction
Glucose is the major physiologic stimulator of insulin secretion and biosynthesis, stimulates insulin gene transcription, and stabilizes insulin mRNA levels (reviewed in reference 1). However, we have observed that chronic exposure to supraphysiologic glucose concentrations can paradoxically and irreversibly decrease insulin gene transcription in HIT-T15 cells, a phenomenon more suggestive of glucose toxicity than glucose desensitization because the latter occurs more quickly and is reversible (2). HIT-T15 cells, an insulin-secreting cell line derived from SV40-transformed Syrian hamster islets of Langhans, cultured in 11.1 mM glucose for 25 passages lose their ability to secrete insulin in response to glucose and show decreased insulin content, insulin mRNA levels and insulin gene promotor activity (3–5). These changes are prevented by chronically culturing the cells in 0.8 mM glucose (3–5). The decrease in insulin gene transcription is associated with diminished binding of a glucose-sensitive transcription factor, known as STF-1, to the insulin promoter (5, 6). STF-1 is a homeoprotein specifically expressed in the pancreas and the duodenum (7). STF-1 binds to the CT motifs (CTAATG) located at −215/−210 (CT2 motif) and −82/−77 (CT1 motif) in the human insulin gene promotor (8), and its expression is required for normal pancreas development (9). Although the role of STF-1 in insulin gene regulation by glucose is still controversial, it is thought to be an important regulator of insulin gene transcription in vivo (10). We also demonstrated that loss of insulin gene transcription in HIT-T15 cells chronically cultured in high glucose concentrations is associated with decreased binding of a β-cell specific trans-activating factor to the RIPE3b1 cis-acting element of the rat insulin II gene (11). The RIPE3b1 sequence plays a key role in tissue specific expression of the insulin gene (12) and regulation of insulin gene transcription by glucose (13, 14). The trans-activating factors that interact with the RIPE3b1 element have not been isolated, and will be referred to as RIPE3b1-Act. In contrast, binding activity of the ICE/E1 activators (ICE/E1-Act), which are essential for tissue-specific and glucose-stimulated expression of the insulin gene (14), is not affected in HIT-T15 cells chronically cultured in 11.1 mM glucose (11). To evaluate whether these events might provide a general mechanism for glucose toxicity on β-cell function, it is necessary to ascertain whether our observations made in the HIT-
cell are or are not unique to this cell line. It is also essential to
determine whether inactivity of one or both transcription fac-
tors, STF-1 and RIPE3b1-Act, is sufficient and necessary to
determine whether inactivity of one or both transcription fac-
cell are or are not unique to this cell line. It is also essential to
(17), to address three questions: (a) Does long term culture of
βTC-6 cells in supraphysiologic glucose concentrations lead to
decreased insulin gene expression? If so, (b) does long term culture of βTC-6 cells in low glucose concentrations preserve
insulin gene expression, and, if so, (c) are these changes associ-
ated with decreased insulin gene transcription and binding of
STF-1 and/or RIPE3b1-Act?

Methods

βTC-6 cells culture. The original culture of βTC-6 cells, developed by
Efrat et al. (16), was provided by Norman Fleischer (Albert Einstein
College of Medicine, Bronx, NY). Cells were grown in RPMI-1640
culture medium containing 10% FBS, in 5% CO₂/95% air at 37°C.
Medium was changed every 48 h and cells were passaged once weekly
after detachment using trypsin-EDTA. Beginning at passage 35,
βTC-6 cells were continuously cultured either in 0.8 mM glucose, a
slightly stimulatory concentration, or 11.1 mM glucose, a maximal
stimulatory concentration (17). Cell population doubling times were
determined as reported previously (4).

Secretion studies. Static insulin secretion in response to increas-
ing glucose concentrations, perfusion experiments, and determina-
tion of intracellular insulin content were performed as previously de-
deribided for 16 h with 32P-labeled Syrian hamster preproinsulin
cDNA probe (19) in the same solution. The membrane was then
was washed three times at room temperature in 2× SSC and 0.1% SDS,
then twice at 60°C in 0.2× SSC and 0.1% SDS, then exposed to x-ray
films (Kodak X-omat AR; Eastman Kodak Co., Rochester, NY) for
4–12 h. Under the hybridization conditions used, the probe hybrid-
ized with a single 0.5-kb band on agarose gel fractionation of total
βTC-6 cell RNA, consistent with βTC cell insulin mRNA (16). For
slot-blot analysis, total RNA (2.5–5 µg) was loaded on a nylon hy-
bridization membrane using the bio-dot apparatus (Bio Rad, Her-
cules, CA) and the membrane was prehybridized and hybridized as
described above. Membranes were stripped for 2 h at 75°C in 1 mM
Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0 and 0.1× Denhardt’s, and
rehybridized with 32P-labeled human β-actin cDNA probe (20) in order
to control for variations in the amount of total RNA. Insulin and
β-actin mRNAs were quantitated by scanning densitometry of autora-
diographs, and data are expressed as the density ratio of insulin
mRNA to β-actin mRNA.

Rates of degradation of insulin and β-actin mRNA in βTC-6 cells
chronically cultured in 0.8 or 11.1 mM glucose were compared by in-
cubating the cells in the presence of 5 µg/ml actinomycin D and har-
vesting samples for mRNA analysis 0, 6, 12, and 24 h after the begin-
ing of the incubation.

Cell transfections, CAT assay and luciferase assay. The plasmid
INSCAT contains the human insulin gene sequences –326 to +30
linked to the chloramphenicol acetyl transferase (CAT) reporter
gene (5). Insulin-luciferase constructs contain rat insulin II gene se-
quences spanning the region from –238 to +2 hp linked to the lu-
ciferase (LUC) reporter gene. The wild type (~238 WT LUC)(21)
and the RIPE3b1 mutant (~238 b1m LUC)(14) were gifts from Dr.
Roland Stein (Vanderbilt University, Nashville, TN).

βTC-6 cells were subcultured for 48 h in RPMI-1640 containing
11.1 mM glucose at a density of 3 × 10⁶ cells per well in six-well
plates. Duplicate wells were transfected with 2 µg of an insulin re-
porter construct or 0.5 µg of RSVCAT or RSVLUC DNA by a li-
poseome-mediated method (Lipofectin; BRL, Gaithersburg, MD). All
transfections and subsequent incubations were performed in 11.1 mM
glucose. Cells were rinsed with FBS-free RPMI-1640, then 1 ml of
FBS-free RPMI-1640 containing 1:4 (wt/wt) plasmid DNA to lipofec-
tin was added to each well. Cells were incubated for 4 h at 37°C, after
which the transfection media was replaced by 4 ml of RPMI 1640, 30 h

1. Abbreviations used in this paper: CAT, chloramphenicol acetyl
transferase; EMSA, electromobility shift assay; LUC, luciferase.
Insulin secretion and gene expression in βTC-6 cells chronically cultured in 11.1 mM glucose. βTC-6 cells were chronically cultured in RPMI-1640 containing 11.1 mM glucose from passage 35 to 76. Insulin secretion was serially examined by static incubation at times 0, 6, 12, and 24 h. Insulin and β-actin mRNA levels were serially assessed over the culture period. Data are expressed as relative insulin content from 5 experiments performed at passage 55–62. Data are expressed as mean±SE relative density of insulin mRNA/β-actin mRNA. Cells chronically cultured in 0.8 mM glucose had more insulin mRNA than cells chronically cultured in 11.1 mM glucose. *P < 0.05.

Results

Insulin secretion and gene expression in βTC-6 cells chronically cultured in 11.1 mM glucose. βTC-6 cells were chronically cultured in RPMI-1640 containing 11.1 mM glucose from passage 35 to 76. Insulin secretion was serially examined by static incubation with increasing glucose concentrations. Both basal and glucose-stimulated insulin release gradually decreased as the passage number increased (Fig. 1 A). Similarly, intracellular insulin content from βTC-6 cells chronically cultured in 11.1 mM glucose gradually decreased over time (Fig. 1 B).

Insulin mRNA levels in βTC-6 cells chronically cultured in 11.1 mM glucose were serially assessed over the culture period by slot blot analysis. Insulin mRNA was readily detectable at passage 35, and was markedly diminished at passage 50 and higher (Fig. 2).

Insulin gene expression in βTC-6 cells chronically cultured in 0.8 vs. 11.1 mM glucose. To determine whether the observed decrease insulin gene expression was related to the glucose concentration in the media, βTC-6 cells were chronically cultured from passage 35 to 71 in RPMI-1640 containing either 0.8 or 11.1 mM glucose. Cells chronically cultured in 0.8 or 11.1 mM glucose had similar population doubling times (26.2±1.6 vs. 25.5±1.6 h respectively, n = 2). Insulin mRNA levels in βTC-6 cells chronically cultured in low and high glucose were compared by Northern blot or slot blot analysis. Insulin mRNA was more abundant in cells cultured in 0.8 mM glucose than cells cultured in 11.1 mM glucose (Fig. 3). To determine whether this was due to differences in mRNA stability, we compared insulin mRNA degradation rates by incubating the cells in the presence of 5 μg/ml actinomycin D and by measuring insulin mRNA levels 0, 6, 12, and 24 h after the beginning of the incubation. βTC-6 cells from passage 43, passage 62 chronically cultured in high glucose and passage 62 chronically cultured in low glucose had indistinguishable mRNA degradation rates under these conditions (Fig. 4).
insulin gene (Fig. 5). In early passages of βTC-6 cells the relative expression of INSCAT to RSVCAT was 1.00±0.12. In late passages of βTC-6 cells cultured in 11.1 mM glucose the relative INSCAT/RSVCAT expression was 0.59±0.14 (P = 0.001, n = 5). In contrast, relative INSCAT/RSVCAT expression in late passage βTC-6 cells cultured in 0.8 mM glucose was 1.02±0.23 (P < 0.01 vs. cells chronically cultured in 11.1 mM glucose; n = 5). These data indicate that insulin promoter activity is decreased by ~40% in βTC-6 cells chronically cultured in 11.1 mM glucose compared to both early passage cells and late passage cells cultured in 0.8 mM glucose.

Chronic exposure of βTC-6 cells to high glucose also led to a marked decrease in the activity of the rat insulin II promoter as determined by transient transfection with −238WTLUC (see below).

Insulin gene 5′-regulatory region binding proteins in βTC-6 cells chronically cultured in 0.8 vs. 11.1 mM glucose. To determine whether the decreased promoter activity was associated with altered interaction between transcription factors and regulatory sequences of the insulin gene, nuclear proteins were extracted from early passage (P 42) and late passage (P 71) βTC-6 cells either in 0.8 or in 11.1 mM glucose. Formation of specific DNA-protein complexes was detected by EMSA using oligodeoxynucleotide probes containing the RIPE3b1, CT2 and ICE/E1 elements. With the oligodeoxynucleotide probe containing the STF-1 binding sequence (CT2), a major complex was detected in early passage βTC-6 cells, late passage βTC-6 cells cultured in high glucose, and late passage βTC-6 cells cultured in low glucose (Fig. 6 A, lanes 1–3), and the intensity of the binding was similar in the three cell extracts. Similarly, nuclear extracts from early passage βTC-6 cells and late passage βTC-6 cultured in low and high glucose shifted the oligodeoxynucleotide probe containing the ICE/E1 element to similar extent (Fig. 6 A, lanes 4–6). The oligodeoxynucleotide probe containing the RIPE3b1 sequence formed a single complex in early passages βTC-6 cells (Fig. 6 A, lane 7).

In contrast to STF-1 and ICE/E1-Act, the RIPE3b1 complex was markedly reduced in late passage βTC-6 cells cultured in 11.1 mM glucose (Fig. 6 A, lane 8), but was preserved in late passage βTC-6 cells chronically cultured in 0.8 mM glucose (Fig. 6 A, lane 9). Similar results were obtained in a replicate experiment performed with nuclear extracts from βTC-6 cells at passage 36 and 67 cultured in 0.8 or 11.1 mM glucose. The specificity of the binding to the RIPE3b1 sequence was analyzed in competition experiments using excess unlabeled wild type or mutant oligodeoxynucleotide to compete for binding (Fig. 6 B, lanes 4–9). The protein-DNA complex was competed by the unlabeled wild-type sequence (Fig. 6 B, lanes 4–6), but not by the mutant sequence (Fig. 6 B, lanes 7–9). The mutant RIPE3b1 sequence containing a 2-bp substitution has been shown to eliminate its activity both in vitro and in vivo (13). These results indicate that binding to the RIPE3b1, but not to the CT2 and ICE/E1, element is reduced in late-passage βTC-6 cells cultured in 11.1 mM glucose.

Expression of −238WTLUC and −238b1mLUC constructs in βTC-6 cells. To determine whether the decreased binding of RIPE3b1-Act could account for the diminished promoter activity, cells were transiently transfected with either −238WTLUC or a −238b1mLUC construct containing the mutation described above (Fig. 7). Expression of −238WTLUC was reduced in late passage βTC-6 cells cultured in 11.1 mM glucose as compared to early passage cells, and was preserved in
late passage cells cultured in 0.8 mM glucose. These observations, using a rat insulin II promoter, confirm the results obtained with the human insulin promoter INSCAT (see above). Expression of −238b1mLUC was markedly decreased in early passage βTC-6 cells as well as in late passage βTC-6 cells cultured in either 0.8 or 11.1 mM glucose. These data indicate that loss of RIPE3b1 binding activity can lead to the observed decrease in insulin gene promoter activity.

**Discussion**

This study was designed to determine whether chronic exposure of βTC-6 cells to supraphysiologic glucose concentrations would adversely affect insulin gene expression. We found that βTC-6 cells chronically cultured in 11.1 mM glucose have decreased insulin secretion, insulin content and insulin mRNA levels, and that cells chronically cultured in 0.8 mM glucose have higher levels of insulin mRNA than cells chronically cultured in 11.1 mM glucose. The decrease in insulin mRNA levels in cells chronically cultured in 11.1 mM glucose is associated with reduced insulin gene transcription and diminished binding to the RIPE3b1 cis-acting element. Both are preserved by culturing the cells in 0.8 mM glucose.

These results agree with those we previously reported in the HIT-T15 cell (4), and reinforce our contention that chronic exposure of β-cells to a supraphysiologic glucose concentration adversely affects insulin gene expression. βTC-6 cells chronically cultured in the presence of 0.8 mM glucose plus 10.3 mM mannitol have similar levels of insulin mRNA compared to cells cultured in 0.8 mM glucose only (data not shown), indicating that deleterious effects of supraphysiologic glucose are not due to differences in osmolarity of the culture media. The rate of degradation of insulin mRNA is similar in early passage βTC-6 cells, late passage cells cultured in low glucose, and late passage cells cultured in high glucose, suggesting that the observed decrease in insulin mRNA levels in late passage cells cultured in high glucose reflects a diminution in the rate of transcription. Accordingly, expression of human insulin and rat insulin II promoters is diminished by ~40% in these cells, and is preserved by culturing the cells in low glucose concentration. These observations are also in accordance with those previously reported in HIT cells (5).

STF-1, RIPE3b1-Act and ICE/E1-Act have been shown to play important roles in the control of insulin gene transcription. We have previously demonstrated that chronic culture of HIT cells in high glucose concentrations is associated with decreased binding of STF-1 and RIPE3b1-Act, whereas ICE/E1-Act binding is unchanged (5, 6, 11). The results we now report indicate that continuous culture of βTC-6 cells in high glucose concentrations leads to decreased binding activity of the RIPE3b1-Act to its cognate sequence with no alteration in STF-1 binding activity. In addition, mutation of the RIPE3b1 sequence almost abolishes insulin gene transcription in βTC-6 cells. It is therefore reasonable to conclude that decreased binding of RIPE3b1-Act is responsible for the decrease in insulin promoter activity observed in βTC-6 cells chronically cultured in high glucose concentrations. Further culture of βTC-6 cells in high glucose for an additional 8 passages does not lead to decreased STF-1 binding (data not shown). This preserved binding of STF-1 in βTC-6 cells prompted us to question whether decreased RIPE3b1-Act binding activity precedes the loss of STF-1 binding activity in HIT cells. Nuclear extracts from various passages of HIT cells chronically cultured in 0.8 or 11.1 mM glucose were isolated, and EMSAs were performed using oligodeoxynucleotide probes to the RIPE3b1 and CT2 sequences. Both binding activities decreased simultaneously (data not shown). Consequently, this study uniquely provides a model for glucose toxicity in which RIPE3b1-Act, but not STF1, binding activity is affected, and reinforces the importance of reduced RIPE3b1-Act binding activity in mediating toxic effects of glucose on the insulin gene. Elucidation of the mechanism by which RIPE3b1-Act binding activity is altered awaits the isolation and characterization of this activator.

Chronic hyperglycemia is thought to play a secondary role in the pathogenesis of Type II diabetes (reviewed in references 26 and 27). Studies in Type II diabetic patients have demonstrated that defective glucose-induced insulin release can be partially reversed by normalization of blood glucose levels (28, 29). Eizirik et al. (30) observed diminished insulin content from human islets cultured in high glucose concentrations for 7 d. These changes were partially reversed by subsequent culture in lower glucose concentrations. Orland and Permutt (31) observed a diminution in both insulin content and mRNA levels in db/db mice after several weeks of hyperglycemia. However, in vitro studies utilizing primary β-cells can only be conducted over relatively limited periods of time, and experiments using animals do not allow for harvesting large amounts of material needed for investigation of insulin gene transcription. Alternatively, the use of insulin-secreting cell lines permits an assessment of the effects of chronic exposure to high glucose on insulin gene transcription. Our previous studies in the HIT-T15 cell have demonstrated that chronic exposure to supraphysiologic glucose concentrations is associated with decreased insulin gene transcription and decreased binding activities of both transcription factors STF-1 and RIPE3b1-Act. We now report that insulin gene expression and transcription are also diminished in βTC-6 cells chronically exposed to supraphysiologic glucose concentrations. These alterations are associated with, and likely to be caused by, decreased ability to form a
specific protein/DNA complex with the RIPE3b1 cis-acting element. These observations suggest that reduced binding activity of RIPE3b1-Act by itself can mediate glucose toxicity on the insulin gene, and may provide insights into the mechanism by which hyperglycemia contributes to β-cell dysfunction in Type II diabetes.

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