Chronic Exposure to Excess Nutrients Left-shifts the Concentration Dependence of Glucose-stimulated Insulin Secretion in Pancreatic \( \beta \)-Cells*

Received for publication, October 21, 2014, and in revised form, April 17, 2015 Published, JBC Papers in Press, May 1, 2015, DOI 10.1074/jbc.M114.620351

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**Background:** Fasting insulin secretion is increased in obesity and type 2 diabetes.

**Results:** Culture of \( \beta \)-cells in excess nutrients resulted in increased lipid stores and a left-shifted dose-response curve of glucose-stimulated insulin secretion due to an enhanced sensitivity of exocytosis to \( \text{Ca}^{2+} \).

**Conclusion:** Intracellular lipid modulates the dose response of glucose-stimulated insulin secretion.

**Significance:** A shift in \( \beta \)-cell glucose sensitivity may contribute to hyperinsulinemia.

Hyperinsulinemia (HI) is elevated plasma insulin at basal glucose. Impaired glucose tolerance is associated with HI, although the exact cause and effect relationship remains poorly defined. We tested the hypothesis that HI can result from an intrinsic response of the \( \beta \)-cell to chronic exposure to excess nutrients, involving a shift in the concentration dependence of glucose-stimulated insulin secretion. INS-1 (832/13) cells were cultured in either a physiological (4 mM) or high (11 mM) glucose concentration with or without concomitant exposure to oleate. Isolated rat islets were also cultured with or without oleate. A clear hypersensitivity to submaximal glucose concentrations was evident in INS-1 cells cultured in excess nutrients such that the 25% of maximal (\( S_{0.25} \)) glucose–stimulated insulin secretion was significantly reduced in cells cultured in 11 mM glucose (\( S_{0.25} = 3.5 \text{ mM} \)) and 4 mM glucose with oleate (\( S_{0.25} = 4.5 \text{ mM} \)) compared with 4 mM glucose alone (\( S_{0.25} = 5.7 \text{ mM} \)). The magnitude of the left shift was linearly correlated with intracellular lipid stores in INS-1 cells (\( r^2 = 0.97 \)). We observed no significant differences in the dose responses for glucose stimulation of respiration, NAD(P)H autofluorescence, or \( \text{Ca}^{2+} \) responses between left- and right-shifted \( \beta \)-cells. However, a left shift in the sensitivity of exocytosis to \( \text{Ca}^{2+} \) was documented in permeabilized INS-1 cells cultured in 11 versus 4 mM glucose (\( S_{0.25} = 1.1 \) and 1.7 \( \mu \text{M} \), respectively). Our results suggest that the sensitivity of exocytosis to triggering is modulated by a lipid component, the levels of which are influenced by the culture nutrient environment.

Obesity is associated with a marked elevation in insulin secretion under both fasting and nutrient-stimulated conditions even though glucose tolerance may be normal (1). This phenomenon manifests as hyperinsulinemia (HI)\textsuperscript{2} and has been classically ascribed a compensatory response by the pancreatic \( \beta \)-cells under conditions of systemic insulin resistance to maintain normal glucose homeostasis (2). Alternatively, the hypothesis that chronically elevated insulin may be a cause rather than a consequence in the progression of type 2 diabetes (T2D) has been suggested previously (3–5). Chronically elevated insulin has been shown to induce insulin resistance by promoting lipid accumulation in peripheral tissues (6) and down-regulating its own receptor, particularly in the liver (7, 8). Thus, understanding the mechanisms by which HI manifests in the context of obesity and nutrient excess will help to identify potential targets needed to test this alternative prequel of T2D.

Many hypotheses have been proposed to account for the HI observed in obesity, including reduced hepatic insulin extraction (9, 10) and increased \( \beta \)-cell number and/or mass (11). The observation that C-peptide concentrations are consistently found to be elevated in obesity signifies that although reduced hepatic extraction may contribute to HI increased secretion is clearly present (1). Increases in \( \beta \)-cell mass have been documented in both diet-induced and genetic models of obesity. However, it remains unclear how much this \( \beta \)-cell mass increase contributes to HI as islets from these animals also have reduced insulin content (12, 13). Interestingly, gastric bypass has been shown to quickly normalize HI in obese patients with and without T2D prior to significant changes in insulin sensitivity or substantial weight loss (14). Thus, because of the rapid nature of this reduction, functional changes seem likely to contribute to HI in addition to possible changes in \( \beta \)-cell mass.

The ability of \( \beta \)-cells to sense glucose is critical for normal \( \beta \)-cell function (15). The consensus pathway of glucose-stimulated insulin secretion (GSIS) includes both triggering and amplification (16). Increased glucose metabolism in the \( \beta \)-cell following a rise in glucose results in an elevation of the ATP/ADP ratio, closure of ATP-sensitive \( K^+ \) (\( K_{\text{ATP}} \)) channels, depolarization of the plasma membrane, and activation of voltage-dependent \( \text{Ca}^{2+} \) channels. The subsequent rise in intracellular \( \text{Ca}^{2+} \) concentration ([\( \text{Ca}^{2+} \)]) triggers fusion of insulin granules colipotocibility, [\( \text{Ca}^{2+} \)], intracellular calcium concentration; GSIS, glucose-stimulated insulin secretion; KREBS, Krebs-Ringer bicarbonate buffer; FA, fatty acid.

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* This work was supported, in whole or in part, by National Institutes of Health Grants R01 DK35914 (to B. E. C.) and T32 DK007201 (to K. A. E.). The authors declare that they have no conflicts of interest with the contents of this article.

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2 The abbreviations used are: HI, hyperinsulinemia; T2D, type 2 diabetes; DZ, diazoxide; \( K_{\text{ATP}} \), ATP-sensitive \( K^+ \); LC, long-chain; GK, glucokinase; GLT, gluco-
to the plasma membrane, resulting in exocytosis (16). Other metabolic signals derived from glucose amplify secretion when \([Ca^{2+}]\), is elevated. These signals include but are not limited to long-chain (LC)-CoA, ATP, diacylglycerol, monoacylglycerol, and cAMP (17).

Growing interest has been expressed in the concepts of gluco-toxicity (18) (chronically elevated glucose), lipotoxicity (19) (chronically elevated lipid), and glucolipotoxicity (GLT) (20, 21) (combination of elevated glucose and lipid) to explain the \(\beta\)-cell dysfunction observed in T2D. Most studies in this area have focused on the reduced GSIS observed following exposure of \(\beta\)-cells to these conditions (22–25). Whether the reduction in GSIS develops as a result of a defect in the pathway(s) leading to insulin secretion or a depletion of insulin stores is controversial. Normally, insulin synthesis and secretion are tightly coupled such that stores are maintained within a narrow range. Glucose and other secretagogues stimulate both insulin secretion and synthesis by enhancing the translation of insulin mRNA to proinsulin (26). In contrast, it is known that LC fatty acids can deplete insulin stores by promoting secretion of insulin while simultaneously inhibiting proinsulin synthesis (26, 27). Additionally, multiple reports have documented the ability of excess lipid to inhibit insulin gene transcription both \(\textit{in vitro}\) (28) and \(\textit{in vivo}\) (29). It has also been recognized that culture in excess nutrients enhances basal secretion, which has been difficult to reconcile with the reduced maximal secretion. Few studies have attempted to characterize insulin secretion at intermediate glucose concentrations under these conditions despite the fact that they are likely more physiologically relevant. Understanding how excess nutrients alter the dose response of GSIS may help to elucidate how \(\beta\)-cells adapt and ultimately fail in T2D.

Herein we have documented a relationship between \(\beta\)-cell culture conditions and subsequent glucose sensitivity. We found that culture in excess nutrients reduces insulin content, increases lipid stores, and left-shifts the concentration dependence of GSIS. This shift appears to be due to a difference in the sensitivity of exocytosis to the triggering Ca\(^{2+}\) signal.

**Experimental Procedures**

**Ethics Statement**—This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee at Boston University Medical Center (Boston University Medical Center Animal Welfare Assurance A-3316-01).

**Islet Isolation/Culture**—Islets were isolated from male Sprague-Dawley rats (150–250 g) as described previously (30). Islets were cultured overnight in 35-mm dishes in RPMI 1640 medium containing 11 mM glucose, 10% FBS, 50 IU/ml penicillin, and 50 \(\mu\)g/ml streptomycin in the absence of phenol red. Islets were then cultured in 35-mm dishes in RPMI 1640 medium with or without oleate for 48 h. Oleate (Sigma) was diluted in dimethyl sulfoxide and complexed to FBS at 57 °C while vortexing. The oleate/BSA ratio was ~3.5:1 for 0.1 mM oleate, 5:1 for 0.15 mM oleate, and 7:1 for 0.2 mM oleate.

**INS-1 Cell Culture**—INS-1 (832/13) cells (31) were cultured as described previously (32). Glucose was added to RPMI 1640 medium without glucose to achieve 4 mM glucose where indicated. Culture of cells for >2 weeks in a particular glucose concentration (4 or 11 mM) was considered “chronic.”

**Insulin Secretion**—INS-1 cells were grown in 48- and 96-well plates (Corning, Corning, NY) to an approximate density of 240,000 and 100,000 cells/well, respectively, after which insulin secretion in static incubations was assessed as described previously (32). The Krebs-Ringer bicarbonate buffer (KREBS) used in experiments contained 119 mM NaCl, 4.6 mM KCl, 5 mM NaHCO\(_3\), 2 mM CaCl\(_2\), 1 mM MgSO\(_4\), 0.15 mM Na\(_2\)HPO\(_4\), 0.4 mM KH\(_2\)PO\(_4\), 20 mM HEPES, 0.05% BSA, pH 7.4. Cells were lysed in cold PBS containing 0.1% Triton X-100 (Sigma) and 25 mM NaOH for insulin content. Islets were picked and spotted in Matrigel (BD Biosciences) at 30 islets/well in a 48-well plate. Islets were preincubated in KREBS containing 3 mM glucose for 30 min at 37 °C. After removal of the preincubation buffer, test solutions were added, and the islets were incubated for 1 h at 37 °C. Insulin was measured using the HTRF insulin assay (Cis-Bio, Bedford, MA). The software program GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA) was used to determine the half-maximal concentration (\(S_{0.5}\)) from which the quarter-maximal (\(S_{0.25}\)) and tenth-maximal (\(S_{0.1}\)) concentrations were derived. Islet perifusions were performed as described previously (30).

**Nile Red**—Nile red (Sigma) was reconstituted in dimethyl sulfoxide and added to cells at a final concentration of 3.1 \(\mu\)M to image the intracellular lipid stores (33). Following a 15-min incubation, the solution was removed, and cells were imaged in KREBS using a Nikon TE200 microscope (Nikon, Tokyo, Japan) with excitation and emission wavelengths of 488 and 515 nm, respectively, at 20× magnification. NIH ImageJ was used to analyze the images to quantify the fluorescence as a percentage of the total intracellular area.

**Glucokinase Measurement**—The method used to measure glucokinase (GK) activity from cell extracts has been described previously (34). Briefly, cells were sonicated and centrifuged at 4,500 \(\times\) g for 5 min. GK activity was measured in the supernatant as NADPH generated from the conversion of glucose 6-phosphate to 6-phosphogluconate and from the conversion of 6-phosphogluconate to ribulose 5-phosphate using an F-2000 fluorescence spectrophotometer (excitation, 340 nm; emission, 460 nm) (Hitachi, Tokyo, Japan). The GK reaction was initiated with glucose. Glucose was increased in a stepwise manner, and the slope was used to calculate the relative activity. Total protein in the supernatant was measured by the bicinchoninic acid method (Thermo Fisher Scientific, Waltham, MA), and absorbance (562 nm) was measured using a Tecan Infinite M1000 plate reader (Tecan, Männedorf, Switzerland).

**Oxygen Consumption**—\(O_2\) consumption was assessed in INS-1 cells using the Seahorse Extracellular Flux Analyzer XF24 machine (Seahorse Bioscience, Billerica, MA) as described previously (32). Briefly, cells were grown in Seahorse V.7 24-well culture plates to a density of ~120,000 cells/well using culture conditions described above. Cells were incubated prior to the experiment in the same manner as in insulin secretion experiments, and measurements were performed in

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**Center (Boston University Medical Center Animal Welfare Care and Use of Laboratory Animals by the National Institutes in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals**
KREBS. Oligomycin (5 μM), carbonyl cyanide 4-trifluoro-methoxyphenyldrazone (2.5 μM) and antimycin (10 μM) were injected while maintaining the glucose concentration at 4, 6, or 8 mM to calibrate.

Redox Measurement—INS-1 cells were plated in four-quadrant glass bottom dishes (Greiner Bio-One, Frickenhausen, Germany) and cultured as described above to a density of 30,000 cells/quadrant. A deconvolution wide field epifluorescence microscope (Nikon) was used to measure NAD(P)H autofluorescence in INS-1 cells. This method cannot distinguish between NADH and NADPH, hence the designation of NAD(P)H (35). Prior to measurement, cells were preincubated as described for insulin secretion. Next, 300 μL of KREBS containing 2 mM glucose but without BSA was added to the cells and equilibrated for 5 min at 37 °C. Autofluorescence (excitation, 340 nm; emission, 460 nm) was collected at 20× magnification. Then 150 μL of KREBS with concentrated glucose was added to each quadrant to bring the glucose to the desired concentration. NaCN (2 mM final) and concentrated glucose (12 mM final) were added to each quadrant to obtain maximum reduction. Because carbonyl cyanide 4-trifluoromethoxyphenyldrazone (2 μM) was found to interfere with the subsequent NaCN response, maximal and minimal fluorescence signals at basal glucose were obtained in separate traces. Images from each trace were analyzed by subtracting the background and creating regions of interest from a cell mask that were applied to the entire time course using ImageJ. Islets were spotted in Matrigel in four-quadrant dishes and treated similarly to INS-1 cells, although carbonyl cyanide 4-trifluoromethoxyphenyldrazone was not used because of differences in basal (3 mM glucose) fluorescence within islets of the same condition. This was likely in part due to size differences among islets in which anoxia can be observed in the core of larger islets and which anoxia can be observed in the core of larger islets and may contribute to artificially high baseline levels of NADH (36). Thus, the scale that was used to analyze islets was fluorescence at basal (3 mM) glucose (0%, minimum NAD(P)H) and NaCN (100%, maximum NAD(P)H).

Ca2+ Determination—[Ca2+]i, was measured using fura-2 AM (Invitrogen) as described previously (32). Briefly, INS-1 cells and whole islets were loaded with fura-2 (2 μM) for 30 min in KREBS with 0.1% pluronic acid (Thermo Fisher Scientific) on 35-mm glass bottom dishes (MatTek, Ashland, MA). A 15-min cleavage period was then initiated following removal of the fura-2. Imaging was performed on an Olympus DSU spinning disk confocal microscope at 20× magnification at 37 °C. Fluorescence images were captured every 10 s with wavelengths of 340/380 nm dual excitation and 510 nm emission. Data were analyzed using ImageJ. The oscillatory pattern was assessed using the software program Optimized Optimal Segments (OOPSEG) as described previously (37).

Permeabilized Cell Exocytosis—INS-1 cells were pretreated as in secretion experiments except without the 30-min KREBS incubation. Cells were washed once with a permeabilization buffer containing 220 mM mannitol, 70 mM sucrose, 10 mM KH2PO4, 5 mM MgCl2, 2 mM HEPES, 1 mM EGTA with 0.2% BSA. Cells were then incubated in the same buffer with 2 mM XF PMP permeabilizing agent (Seahorse Bioscience) at 37 °C for 5 min. Cells were then incubated in an ATP-regenerating buffer with different free [Ca2+]i for 15 min as described previously (38). Ca2+ concentrations were set using Theo Schoenmakers’ Chelator program and verified with a Ca2+-sensitive electrode (Orion, Boston, MA).

Statistics—The data are presented as the mean of independent experiments ± S.E. Statistical significance was determined by two-tailed Student’s t test or by a one-way analysis of variance followed by Bonferroni’s post hoc test where indicated. A p value of < 0.05 (*) was considered significant; # indicates a p value of < 0.01, and + indicates a p value of < 0.001.

Results

Culture of Pancreatic Islets in Excess Lipid Altered Both Insulin Content and Secretion—Fig. 1A shows that a 48-h incubation of isolated rat islets in standard culture medium (11 mM glucose) with 0.2 mM oleate reduced the total insulin content by ~80%. The depletion of insulin content was prevented by coin incubation with the KATP channel agonist diazoxide (DZ), which inhibits membrane depolarization and Ca2+ influx. This indicates that hypersecretion of insulin plays an important role in the reduction of insulin content under GLT (27), although we cannot rule out a contribution of DZ preventing β-cell apoptosis, a process known to be dependent on a rise in Ca2+.

To examine the effect of preserving the insulin content on insulin secretion under conditions of GLT, we performed a series of islet perfusion experiments (Fig. 1B). Control islets (blue) not incubated in oleate or DZ displayed very low basal secretion at 3 mM glucose. In response to a rise in the glucose concentration from 3 to 11 mM, a classic, biphasic pattern of GSIS was observed. Upon a further increase to 15 mM glucose, the islets exhibited greater insulin secretion. Islets cultured in DZ (green) but tested following DZ washout exhibited secretion identical to that of control islets (Fig. 1B), which suggested that sustained membrane polarization had no effect on β-cell secretory function.

Islets cultured in oleate (red) displayed elevated basal secretion compared with controls, had a blunted response to 11 mM glucose, and showed no further increase in secretion at 15 mM glucose, indicative of a left shift in GSIS (Fig. 1B). In islets cultured in oleate plus DZ (purple), which have normal insulin content, a much different pattern of secretion was observed. These islets displayed greatly enhanced basal secretion of insulin compared with both control islets and islets cultured in oleate alone. They responded to 11 mM glucose and demonstrated enhanced secretion compared with control islets. Similar to islets cultured in oleate alone, those co-cultured with oleate and DZ did not exhibit a further response when the glucose concentration was raised to 15 mM (Fig. 1B). When expressed as a percentage of content secreted, the secretion from islets treated with oleate alone was superimposable to that of islets treated with oleate plus DZ (Fig. 1C), suggesting that under GLT normalization to insulin content is critical for correctly assessing the rate of insulin exocytosis. The -fold GSIS, assessed by dividing first phase secretion (average between 16.1 and 17.2 min) by basal secretion (average from 0 to 12.6 min), was reduced in islets cultured in oleate (~4-fold) compared with control islets (~15-fold) (Fig. 1D). However, this result was due to increased basal secretion not reduced GSIS. These
results also indicated that islet insulin content was an important determinant of secretion but not glucose sensitivity.

**Rat Islets Cultured in a High Lipid Environment Exhibited Left-shifted GSIS**—We next wanted to examine whether chronic exposure to fatty acid induced a left shift in the concentration dependence of GSIS. To prevent a dramatic loss in insulin content as was observed with 0.2 mM oleate, we incubated islets in standard culture medium (11 mM glucose) with and without 0.1 mM oleate for 48 h. Insulin content in islets incubated with 0.1 mM oleate was not significantly different from that in control islets (p = 0.52; Fig. 2A). The disparity between this result and the large difference in insulin content observed with culture of islets in 0.2 mM oleate likely results from a substantial increase in free fatty acid concentration due to the rise in the FA/BSA ratio (3.5:1 and 7:1 for 0.1 and 0.2 mM oleate, respectively). Similar to our results in the perifusions, islets cultured in oleate displayed a left-shifted dose response for GSIS as demonstrated by increased responsiveness to submaximal glucose concentrations (6, 7, 8, and 11 mM) (Fig. 2B). Maximal secretion as a percentage of the total insulin content was similar under both conditions, plateauing near 15 mM glucose.

**Culture Medium Glucose Concentration Affected Intracellular Lipid, Insulin Content, and the Concentration Dependence of GSIS in INS-1 Cells**—To determine the underlying cause of the observed left-shifted GSIS, we used the clonal β-cell line INS-1 (832/13), which retains many of the characteristics of normal β-cells (31). INS-1 cells are traditionally cultured in standard culture medium (11 mM glucose) with or without 0.2 mM oleate plus or minus 0.4 mM DZ for 48 h. A, insulin content following exposure to these conditions. B, islets were perfused at basal (3 mM) glucose for 30 min, and a baseline was collected for 10.5 min. Islets were stimulated with 11 mM glucose and then brought to 15 mM glucose at 34 min. Lines are a two-point running average fit to data points. Error bars are displayed every sixth point for clarity. C, the same insulin secretion as in B but expressed as a percentage of total insulin content secreted. D, fold stimulation over basal insulin secretion by 11 mM glucose derived from B. Basal insulin secretion was the average of 0–12.6 min, and stimulated secretion was the average of 16.1–17.2 min. Data are derived from three independent experiments. Error bars represent ±S.E. (†, p < 0.05; ††, p < 0.001). Colors from A correspond to B, C, and D.

**Preserving insulin content during culture in high lipid concentration increases insulin secretion at basal and intermediate glucose in isolated rat islets.** Islets were cultured in standard culture medium (11 mM glucose) with or without 0.2 mM oleate plus or minus 0.4 mM DZ for 48 h. A, insulin content following exposure to these conditions. B, islets were perfused at basal (3 mM) glucose for 30 min, and a baseline was collected for 10.5 min. Islets were stimulated with 11 mM glucose and then brought to 15 mM glucose at 34 min. Lines are a two-point running average fit to data points. Error bars are displayed every sixth point for clarity. C, the same insulin secretion as in B but expressed as a percentage of total insulin content secreted. D, fold stimulation over basal insulin secretion by 11 mM glucose derived from B. Basal insulin secretion was the average of 0–12.6 min, and stimulated secretion was the average of 16.1–17.2 min. Data are derived from three independent experiments. Error bars represent ±S.E. (†, p < 0.05; ††, p < 0.001). Colors from A correspond to B, C, and D.

**Alteration of the Glucose Dose Response by Addition of Oleate to INS-1 Cells Cultured at 4 and 11 mM Glucose**—From the previous experiments, it was clear that 11G cells had more...
intracellular lipid and a left-shifted concentration dependence of GSIS compared with 4G cells. To determine whether the left shift was driven by or simply associated with the ability of high glucose to increase intracellular lipid, we supplemented the culture medium of 4G and 11G cells with the common monounsaturated LC fatty acid oleate. Culture of 4G cells with 0.15 mM oleate for 24 h increased lipid accumulation ~3.5-fold and decreased the total insulin content by one-third (Table 1). The concentration dependence of GSIS was left-shifted compared with 4G cells, consistent with the hypothesis that something closely associated with or derived from accumulated lipid influenced the concentration dependence of GSIS. A lowering of the threshold needed for stimulatory secretion from 5 mM glucose to 3 mM was observed without any significant differences in maximal secretion (Fig. 4B). This resulted in a marked reduction of the S0.25 in 4G cells supplemented with oleate to a value similar to that observed in 11G cells. The S0.25 and S0.5 were also decreased, although the latter was not statistically significant (Table 1).

Culture of 11G cells with 0.15 mM oleate for 48 h resulted in increased lipid accumulation without having any significant effect on the total insulin content (Table 1). A significant increase in secretion at 1 and 2 mM glucose and a reduction in maximal insulin secretion was documented when 11G cells were supplemented with oleate (Fig. 4C). This resulted in a decrease in the S0.1, S0.25, and S0.5 in 11G cells cultured in oleate compared with 11G cells (Table 1). Importantly, the reduction in maximal insulin secretion does not affect the indices of glucose sensitivity.

A clear inverse relationship existed between the amount of intracellular lipid and the S0.25 of the insulin secretion glucose dose response (Fig. 4D). Taken together, these results document the ability of excess nutrient cultures to induce glucose hypersensitivity in INS-1 cells, a phenomenon that appears to be closely tied to increased intracellular lipid.

**No Difference in GK Activity in 4G and 11G Cells**—Evidence exists to suggest that the glucose-sensing enzyme GK plays an important role in controlling glucose flux in β-cells (40). We therefore wanted to determine whether differences in GK activity existed between 4G and 11G cells, which are right- and left-shifted relative to one another. We used an *in vitro* GK activity assay in which incremental increases in glucose are coupled to production of NADPH. No significant differences were detected at any of the glucose concentrations tested, including those in the range of hexokinase I (0.01–1 mM) (Fig. 5A).

**Metabolic Changes in Response to Acute Glucose in β-Cells Cultured in Different Nutrient Environments**—Culture of INS-1 cells in high glucose has been reported to increase mRNA and protein levels of key glycolytic regulatory enzymes apart from GK, including phosphofructokinase-1, glyceraldehyde-3-phosphate dehydrogenase, and pyruvate kinase (41). To assess whether alterations in glucose metabolism distal to GK could account for the left shift, we assessed changes in NAD(P)H concentration (by autofluorescence) and respiration in response to relevant concentrations of glucose. We determined that both 4G and 11G cells were ~10% reduced at 2 mM glucose (Fig. 6A). Both 4G and 11G cells exhibited a similar increase in NAD(P)H autofluorescence upon a rise of the extracellular glucose concentration from 2 to 4 mM. A further increase was observed...
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TABLE 1
Effect of chronic culture of INS-1 cells in different nutrient environments on insulin content, lipid accumulation, and glucose sensitivity

|                | 4G (48 hrs) | 4G, 0.15 mm FA (24 h) | 4G, 0.15 mm FA (48 h) | p     | 11G | 11G, 0.15 mm FA (48 h) | p     |
|----------------|-------------|-----------------------|-----------------------|-------|-----|------------------------|-------|
| Insulin content (ng/10⁶ cells) | 898 ± 155 | 1159 ± 274 | N.S. | 576 ± 169 | <0.05 | 307 ± 37 | <0.001 | 293 ± 27 | <0.05 |
| Lipid (% cell area) | 2.0 ± 0.4 | 2.7 ± 0.6 | N.S. | 6.7 ± 1.3 | <0.001 | 2.9 ± 0.3 | <0.001 | 2.6 ± 0.2 | <0.001 |
| S₀,1 | 4.9 ± 0.2 | 0.25 ± 0.3 | <0.05 | 5.7 ± 0.2 | 45.5 | 3.5 ± 0.3 | <0.001 | 3.5 ± 0.3 | <0.001 |
| S₀,25 | 6.7 ± 0.2 | 0.5 ± 0.3 | N.S. | 6.0 ± 0.4 | N.S. | 5.0 ± 0.2 | <0.001 | 4.0 ± 0.3 | <0.001 |

FIGURE 4. INS-1 cells cultured in excess nutrients have a left-shifted insulin secretion glucose-dose response. A, transition of 11G cells to 4 mM glucose for 48 h. B, supplementation of 4G cells with 0.15 mM oleate (FA) for 24 h. C, incubation of 11G cells with 0.15 mM FA for 48 h (eight, six, and four independent experiments for 4G (48 h), 4G FA, and 11G FA, respectively). D, correlation analysis between intracellular lipid and the quarter-saturation level for GSIS derived from Table 1 (●, 4G cells; ●, 4 mM glucose for 48 h; ●, 4G cells with 0.15 mM oleate; ■, 11G cells; ■, 11G cells with 0.15 mM oleate). Data are expressed as the mean from independent experiments. Error bars represent ± S.E. (*, p < 0.05; #, p < 0.01; +, p < 0.001).

When the glucose concentration was raised to 6, 8, and 12 mM glucose (Fig. 6A). At these glucose concentrations, a small increase in NAD(P)H as a percentage of the full scale was seen in 11G cells compared with 4G cells, although only the difference at 12 mM glucose was significant (p < 0.01).

A similar procedure was performed to examine NAD(P)H concentration in response to relevant glucose concentrations in whole islets incubated with and without oleate. Upon an increase of the glucose from basal to 5 and 7 mM glucose, both islets cultured with and without oleate exhibited a similar increase in fluorescence (Fig. 6B). In response to 11 and 15 mM glucose, islets cultured in oleate had a tendency to become more reduced than islets not cultured in oleate (p = 0.07 and 0.06 for 11 and 15 mM glucose, respectively) (Fig. 6B).

We further examined these effects by gauging changes in the oxygen consumption rate in response to the same glucose concentrations used to assess redox. Oxygen consumption at 2 mM glucose was significantly higher in 11G cells (1535 ± 141 pmol/10⁶ cells/min) compared with 4G cells (1091 ± 91 pmol/10⁶ cells/min) (n = 4, p < 0.05). Following a 32-min baseline, the extracellular glucose was raised to 4, 6, or 8 mM glucose. The responses to each glucose concentration as a percent increase over basal (2 mM glucose) respiration were similar in both 4G and 11G cells (Fig. 7A). However, when compared on an absolute basis, 11G cells consumed more oxygen at 4 (Fig. 7B), 6 (Fig. 7C), and 8 mM glucose (Fig. 7D). Thus, at any given glucose concentration, increased rates of respiration were observed in 11G cells compared with 4G cells. However, this difference was primarily due to an increase in basal respiration rather than a shift in the glucose dose response.

Following 60 min at 4, 6, and 8 mM glucose, mitochondrial function was assessed. Injection of oligomycin, an ATP synthase inhibitor, indicated that more respiration was coupled to ATP synthesis in 11G cells at all glucose concentrations except 8 mM (Fig. 7E). Inhibition of all electron flow with antimycin A resulted in minimal respiration, which was identical in both 4G and 11G cells. The mitochondrial leak, defined as proton flow across the inner mitochondrial membrane not coupled to ATP synthesis (oligomycin-insensitive), was increased in INS-1 cells cultured at high glucose, which has been observed previously (Fig. 7F) (25). The increased leak accounted for approximately half of the elevated basal respiration in 11G cells. The
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FIGURE 5. Chronic culture in high glucose does not affect GK activity in INS-1 cells. GK activity was determined by the rate of NADPH appearance as described under “Experimental Procedures.” The slope of NADPH fluorescence was determined at different glucose concentrations and was normalized to the protein content of the soluble portion of the extract (n = 6 independent experiments). Data are presented as a mean of independent experiments. Error bars represent ±S.E. with no significant differences detected between 4G and 11G cells as determined by one-way analysis of variance. RFU, relative fluorescence units.

FIGURE 6. No difference in NAD(P)H concentration in β-cells with left- and right-shifted GSIS. A, the autofluorescence of NAD(P)H was assessed in 4G (black) and 11G (gray) cells both basally (2 mM glucose) and in response to glucose stimulation at 4, 6, 8, and 12 mM glucose. B, NAD(P)H production in response to glucose was assessed in whole isolated pancreatic islets cultured in 0 mM oleate (black) and 0.1 mM oleate (gray). Islets were brought from basal (3 mM glucose) to the concentrations indicated. NAD(P)H is expressed as a percentage of the full scale as described under “Experimental Procedures.” (n = 6–7 and 6 separate experiments for A and B, respectively. Each experiment consisted of four to six islets per condition in B. Data are presented as a mean from independent experiments. Error bars represent ±S.E. One-way analysis of variance was used for comparisons between conditions (#, p < 0.01).

FIGURE 7. Increased mitochondrial leak in 11G cells but no change in the glucose dose response of O2 consumption. A, increase in oxygen consumption as a percent increase over basal in 4G (black) and 11G (gray) cells. Oxygen consumption traces document the differences between 4G (♀) and 11G (♂) in response to acute stimulation with 4 (B), 6 (C), and 6 mM (D) glucose. E, oligomycin-sensitive respiration used to estimate ATP synthesis. F, oligomycin-insensitive respiration used to estimate mitochondrial leak. Figures are averages from four separate experiments. Error bars represent ±S.E. (n = 4 replicates per experiment *) (p < 0.05).

other half is likely a result of increased fatty acid oxidation, mitochondrial mass, or a combination thereof. Maximal respiration following injection of the uncoupler carboxyl cyanide 4-trifluoromethoxyphenylhydrazone was greater in 11G cells at every glucose concentration. This increase in maximal respiration indicated that mitochondrial mass was likely increased in 11G cells and contributed to the elevated basal respiration.

No Significant Differences in [Ca2+], in β-Cells with High or Low Intracellular Lipid—The absence of a left shift in redox and respiration linked to ATP synthesis prompted us to look at [Ca2+], in response to the same glucose concentrations. Additionally, a modification at the KATP channel or voltage-dependent Ca2+ channel could alter Ca2+ influx independently of altered metabolism and account for the left shift. We used the Ca2+ dye fura-2 to determine the changes in [Ca2+], in response to a rise in the extracellular glucose concentration. Interestingly, our results show that when the extracellular glucose was raised from 2 to 4 mM a substantial increase in [Ca2+], occurred in both 4G and 11G cells (Fig. 8, A and B). A similar increase in average Ca2+ was observed in response to 8 mM glucose (Fig. 8, C and D).

We found a similar result in islets cultured with and without oleate. Average [Ca2+], increased in response to 7 mM glucose in both groups (Fig. 9, A and B). Islets cultured with oleate displayed less of a lag in the Ca2+ rise upon an increase of the glucose to 7 mM. There was no significant rise in [Ca2+], when the glucose concentration was raised from 7 to 15 mM in either islet type (Fig. 9, A and B). This is consistent with previous reports that have suggested a discordance between the [Ca2+], and insulin secretion in the context of a glucose dose response (42–44). The majority of control islets (20 of 31; 65%) demonstrated Ca2+ oscillations with a period of ~3 min (179.7 ± 16.6 s; Fig. 9C) as shown previously (44). None of 26 separate islets cultured with oleate displayed [Ca2+] oscillations in
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FIGURE 8. Increase in [Ca^{2+}]i at 4 mM glucose in both 4G and 11G cells. 4G (A) and 11G (B) cells were brought from 2 mM glucose to 4 mM glucose at 4 min, and the change in average [Ca^{2+}]i was documented. The same procedure was carried out in response to 8 mM glucose in 4G (C) and 11G (D) cells. Experiments consisted of ~150 cells per trace. Data are expressed as the mean from four independent experiments. Error bars represent ± S.E. Error bars are shown every 10th measurement for clarity.

FIGURE 9. Decreased lag time but no change in [Ca^{2+}]i, glucose dose response in whole islets cultured with oleate. Whole islets were brought from a baseline of 3 mM glucose to 7 mM glucose at 3 min and then to 15 mM glucose at 15 min. Average responses from control islets (31 from five rats) cultured without oleate (A) and islets cultured for 48 h with 0.1 mM oleate (26 from five rats) (B) are shown. Error bars represent ± S.E. Error bars are shown every 10th measurement for clarity. A three-point running average of a representative islet from control culture (C) and 0.1 mM oleate culture (D) is shown.

response to glucose (Fig. 9D). Taken together, these data indicate that differences in either the glucose concentration at which Ca^{2+} influx occurs or the amount of Ca^{2+} influx are unlikely to drive the left-shifted insulin secretion in β-cells with elevated intracellular lipid.

Concentration Dependence of Amplification of Insulin Secretion by Glucose in 4G and 11G Cells—Although the exact mechanisms by which glucose amplifies insulin secretion remain unclear, it may in part have to do with its ability to enhance lipolysis and fatty acid cycling (45). Enhanced amplification at submaximal glucose concentrations could theoretically account for the left shift in β-cells cultured in excess nutrients. This was a particularly attractive explanation due to the clear relationship between lipid stores and the S_{0.25} of GSIS.

To test this, we assessed insulin secretion in both 4G and 11G cells at relevant glucose concentrations while in the presence of 30 mM KCl and 0.4 mM DZ. This maneuver causes [Ca^{2+}]i to be clamped at an elevated concentration independently of K_{ATP} channel closure by ATP (46). As a result, basal insulin secretion was greatly increased (Fig. 10 compared with Fig. 3D). No differences in amplification were detected. A glucose dose response was observed in both 4G and 11G cells with no significant differences at any glucose concentration tested in contrast to previous results obtained under normal conditions (Fig. 3D). The stimulation of insulin secretion by 4 mM glucose in 4G cells under KCl/DZ conditions indicates that these cells are capable of responding to low glucose concentrations if the Ca^{2+}i is elevated high enough.

Sensitivity of Exocytosis to Ca^{2+} in 4G and 11G Cells—The lack of insulin secretion above basal in response to acute exposure of 4 mM glucose in 4G cells despite a clear rise in [Ca^{2+}]i suggested the possibility of a change in Ca^{2+} sensitivity of exocytosis. We utilized a permeabilized cell system to test this directly in which the free Ca^{2+}i was set to the desired concentration by varying the Ca^{2+}/EGTA ratio. Nutrients were omitted from the buffer, and instead an ATP-regenerating system was used, reducing potential confounding differences in metabolism. Similar to GSIS, we found that 11G cells had a left-shifted dose-response curve for Ca^{2+}-induced exocytosis compared with 4G cells (Fig. 11). The S_{0.5} and S_{0.25} were both reduced by ~30% in 11G cells compared with 4G cells. Interestingly, the S_{0.5} in 11G cells (1.8 μM) was comparable with the S_{0.25} in the 4G cells (1.7 μM), which was also the case in the glucose dose-response curves (Table 1).

Discussion

The work presented here provides compelling evidence to suggest that β-cells cultured in excess nutrients exhibit hypersensitivity to acute stimulation with submaximal glucose concentrations. This hypersensitivity resulted in a left-shifted concentration dependence of GSIS. These data indicated that a shift in the dose-response curve for GSIS may contribute to the hypersecretion of insulin observed in obesity. The shift was not driven by heightened glucose flux into the β-cell or alterations in the consensus pathway by which glucose stimulates insulin secretion. Rather, it appeared that a shift in the sensitivity of exocytosis to Ca^{2+} was at least in part responsible for the left...
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FIGURE 11. 11G cells have a left-shifted sensitivity of exocytosis to Ca²⁺. Cells were permeabilized and exposed to different Ca²⁺ concentrations in the presence of an ATP-regenerating system. Each curve is a mean of four independent experiments. Error bars represent ± S.E. (n = 3–4 wells per condition). The $S_{0.25}$ and $S_{0.5}$ values were derived from the mean of values obtained from each individual experiment ± S.E. A two-tailed Student’s t test was used for comparisons between conditions (*, p < 0.05; #, p < 0.01; †, p < 0.001).

shift in the dose response for GSIS following exposure to excess nutrients.

The effect of fatty acids on β-cell function has been well studied and is clearly a matter of exposure time, concentration, chain length, and saturation level. Acutely, fatty acids potentiate GSIS with LC saturated fatty acids being the most potent (47). Conversely, chronic incubation in fatty acid inhibits GSIS both in vitro and in vivo, especially when combined with elevated glucose (48, 49). Our data highlight the importance of accounting for the altered insulin content when determining and interpreting the functional capacity of the β-cell to secrete insulin in vitro. Under GLT, preservation of or normalization to insulin content resulted in a different interpretation of the secretory capacity of islets. Instead of reduced secretion, the islets actually displayed enhanced secretion at both basal and intermediate glucose concentrations and had unaltered maximal secretion, indicating a left shift in GSIS. Thus, displaying insulin secretion both as an absolute amount and normalizing to insulin content can help to identify alterations in insulin synthesis/stores versus secretion. Under high lipid conditions, there is a clear defect in insulin synthesis/stores that masks the fact that the capacity of the β-cell to secrete insulin is actually enhanced. Loss of insulin content could be a precursor to β-cell failure but when coupled to hypersecretion may result in only mild HI. Further research is needed to clarify this distinction and to determine the relationship between insulin content and secretion in vivo. Interestingly, a previous study showed that DZ administration during sustained glucose infusion preserved insulin content and enhanced GSIS from isolated islets ex vivo (50).

We have developed a model to study this shift through chronic culture of INS-1 cells at physiological and high glucose concentrations. Although it is known that clonal β-cells have a left-shifted dose-response curve for GSIS compared with islets, the underlying cause of this is not known. Given reports that have documented glucose to be a key regulator of lipogenesis in INS-1 cells, we decided to reduce the culture glucose to a more physiological concentration to try to “normalize” these cells to better resemble islets (39). Chronic culture at 4 mM glucose caused a dramatic reduction in intracellular lipid stores compared with cells cultured at 11 mM glucose. This reduction in lipid stores was associated with a right-shifted dose-response curve for GSIS. In addition to its effect on lipid stores, chronic exposure to elevated glucose has also been shown to change the expression of many genes in insulin-secreting cell lines, thus making it difficult to implicate lipid in the initiation of the left shift using this model alone (51). To resolve this, we added oleate overnight to INS-1 cells cultured at physiological glucose. We obtained a similar left shift in the concentration dependence of GSIS when compared with chronic culture at high glucose. This increases the likelihood that lipid was indeed playing a causative role in the left shift induced by chronic incubation in high glucose.

Chronic culture of islets and INS-1 cells in very high glucose (>25 mM) has been shown to induce a similar shift in the concentration dependence of GSIS (41, 52). Studies have implicated an increase in glycolytic flux resulting from enhanced expression of key glycolytic enzymes as the underlying cause (41). This phenomenon has also been observed in vivo in humans following chronic glucose infusion to invoke sustained hyperglycemia (53). Interestingly, this same study showed that fasting induces a right shift in GSIS. Our results build upon these studies by also demonstrating that chronic exposure of β-cells to high glucose alters the dose-response curve for GSIS and implicates lipid accumulation as playing a major role.

Although a clear inverse relationship exists between the $S_{0.25}$ of GSIS and lipid stores in INS-1 cells, it is unlikely that an inert molecule like triglyceride would be driving this shift in GSIS. To more thoroughly investigate the underlying cause of this shift, we examined the known pathways by which glucose stimulates insulin secretion. GK activity was not different between 4G and 11G cells. In response to acute stimulation with 4 mM glucose, both 4G and 11G cells increased NADH, oxygen consumption, and [Ca²⁺]i. We also found no significant differences in the [Ca²⁺]i in response to intermediate glucose. Interestingly, only 11G cells exhibited triggering of insulin secretion in response to this Ca²⁺ influx. 4G cells did not exhibit triggering until the glucose was further raised despite no additional increase in [Ca²⁺]i. We demonstrated that β-cells with high intracellular lipid have an increased sensitivity of exocytosis to Ca²⁺, narrowing the possible sites responsible for the left shift to the exocytotic machinery and the plasma membrane. Modification to the putative exocytotic Ca²⁺ sensor synaptotagmin is a likely target. Interestingly, studies have shown that proteins within the exocytotic machinery, including synaptotagmin and synaptosomal-associated protein 25, are acylated (54, 55). We have shown previously that LC-CoA enhances secretory granule exocytosis in β-cells, an effect that was blocked by cerulenin, which inhibits protein acylation (38). Another likely lipid candidate is monoacylglycerol. We have recently shown that monoacylglycerol stimulates basal insulin secretion, and Zhao et al. have shown that it binds the exocytotic protein Munc13-1 (32, 56). Another possibility is that a change in the lipid composition of the plasma membrane may alter the Ca²⁺ threshold needed for GSIS and thus shift the dose-response curve. Cholesterol and phosphatidylserine are two important membrane
components that have been shown to affect exocytosis in multiple cell types (57, 58).

In summary, we have demonstrated that INS-1 cells and rat islets with high intracellular lipid display a hypersensitivity to acute glucose stimulation. Such conditions lower the S_{0.25} and left-shift the concentration dependence of GSIS. The mechanism underlying this shift appears to emanate from a heightened sensitivity of the Ca^{2+}-induced triggering pathway directly at the exocytotic machinery. The strong relationship between lipid stores and hypersecretion of insulin at submaximal glucose suggests the need for further investigation into the possible role of such lipid species as LC-CoA, cholesterol, and monoacylglycerol among others. Studies on these pathways are currently underway in our laboratory.

Acknowledgments—We thank Dr. Amber Kleckner for helpful comments in the editing of this manuscript. Also, we thank Dr. Michael Kirber and the Boston University Cellular Imaging Core for assistance with imaging.

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