Glucosamine-induced alterations of mitochondrial function in pancreatic β-cells: possible role of protein glycosylation

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Anello, Marcello, Daniela Spampinato, Salvatore Piro, Francesco Purrello, and Agata Maria Rabuazzo. Glucosamine-induced alterations of mitochondrial function in pancreatic β-cells: possible role of protein glycosylation. Am J Physiol Endocrinol Metab 287: E602–E608, 2004. First published May 18, 2004; 10.1152/ajpendo.00320.2003.—Glucosamine (GlcN) increased F1-F0-ATP-synthase protein levels but decreased ATP levels and ATP/ADP ratio compared with control groups. To further evaluate mitochondrial function and ATP metabolism, we then studied uncoupling protein-2 (UCP2), F1-F0-ATP-synthase, and mitochondrial membrane potential, a marker of F1-F0-ATP-synthase activity. UCP2 protein levels were unchanged after chronic exposure to GlcN on both pancreatic islets and INS-1 β-cells. Due to the high number of cells required to measure mitochondrial F1-F0-ATP-synthase protein levels and mitochondrial membrane potential, we used INS-1 cells, and we found that chronic culture with GlcN increased F1-F0-ATP-synthase protein levels but decreased glucose-stimulated changes of mitochondrial membrane potential. Moreover, F1-F0-ATP-synthase was highly glycosylated, as demonstrated by experiments with N-glycosidase F and glycoprotein staining. Tunicamycin (an inhibitor of protein N-glycosylation), when added with GlcN in the culture medium, was able to partially prevent all these negative effects on insulin secretion, adenine nucleotide content, mitochondrial membrane potential, and protein glycosylation. Thus we suggest that GlcN-induced pancreatic β-cell toxicity might be mediated by reduced cell energy production. An excessive protein N-glycosylation of mitochondrial F1-F0-ATP-synthase might lead to cell damage and secretory alterations in pancreatic β-cells.

In a normal β-cell, glucose regulates insulin secretion through its metabolism. Mitochondria represent the site where important metabolites that regulate cellular process are generated (17). In the past decade, several studies have focused attention on the adenine nucleotides as regulators of insulin secretion in the pancreatic β-cell. In particular, the increase of the ATP-to-ADP (ATP/ADP) ratio tightly associates to glucose-induced insulin granules release (1, 6, 7).

In this study, we analyzed the effect of long-term exposure to high GlcN levels on rat pancreatic islets and β-cell line (INS-1) function. In particular, we investigated whether there was an association between alteration of insulin secretion and mitochondrial function. We also tested tunicamycin, an inhibitor of N-linked glycosylation, to prevent GlcN effects.

MATERIALS AND METHODS

Materials. Crude collagenase was obtained from Boehringer Mannheim (Mannheim, Germany). Pancreatic islet culture medium CMRL 1066, heat-inactivated fetal calf serum (FCS), glutamine, and gentamicin were obtained from Gibco (Glasgow, Scotland, UK). INS-1 culture medium RPMI 1640, β-mercaptoethanol, glucose, sodium pyruvate, ATP, ADP, phosphoenolpyruvate, pyruvate kinase, rhodamine-123 (Rh123), carboxyl cyanide p-trifluoromethoxyphenylhydrazide (FCCP), tunicamycin, trichloroacetic acid (TCA), nicotinamide (NAD), and N-glycosidase F (PNGase F) were from Sigma (St. Louis, MO), and the Pro-Q glycoprotein blot stain kit was from Molecular Probes (Eugene, OR). All other chemicals were of analytical grade.

Pancreatic islet isolation and INS-1 cell culture. Pancreatic islets were isolated by the collagenase method from 200- to 250-g fed male Wistar rats injected intraperitoneally with 0.2 ml of a 0.2% pilocarpine solution 2 h before being killed by decapitation. Purified islets were cultured overnight at 5.5 mM glucose in CMRL 1066 medium containing 10% FCS, 2 mM L-glutamine, and gentamicin at 37°C in a 95% air-5% CO2 atmosphere and then for 48 h at 5.5 mM glucose with or without 10 mM GlcN. In some experiments, pancreatic islets were cultured in the presence of GlcN with or without 2 μg/ml tunicamycin, an agent that inhibits NH2-terminal glycosylation in rough endoplasmic reticulum (20, 24), or 5 mM NAD, a well-known radical scavenger (13).

INS-1 cells were cultured as previously described (4), in RPMI 1640 medium containing 11 mM glucose, 10 mM HEPES, 10% heat-inactivated FCS, 2 mM glutamine, 1 mM sodium pyruvate, 50 mM β-mercaptoethanol, 100 IU/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere (5% CO2-95% air). Cells were cultured for 72 h in RPMI 1640 medium with or without 5 mM GlcN, and, where indicated, 1 ng/ml tunicamycin was added.

Insulin release. At the end of the culture period, islets were carefully washed in Krebs-Ringer-HEPES (KRH) buffer (containing 136 mM NaCl, 5.4 mM KCl, 2.5 mM CaCl2, 0.8 mM MgSO4, 0.3 mM

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GLUCOSAMINE AND PANCREATIC \( \beta \)-CELL FUNCTION

\[ \text{Na}_2\text{HPO}_4, 0.4 \text{ mM KH}_2\text{PO}_4, 10 \text{ mM HEPES, 0.25% BSA, pH 7.35}. \]

Batches of five purified islets were then incubated in 1 ml of buffer with 2.8 or 22.2 mM glucose (30-min incubation at 37°C). Insulin in the medium was then measured by radioimmunoassay (RIA). Results are expressed as insulin released in the medium (pg-islet\(^{-1}\)·30 min\(^{-1}\)). INS-1 cells were plated into 24-well plates (10^3 cells/well) and cultured as described above. At the end of the culture period, cells were washed with phosphate-buffered saline (PBS) and preincubated for 30 min at 37°C in 1 ml of KRH buffer (containing 134 mM NaCl, 3.6 mM KCl, 0.5 mM NaH\(_2\)PO\(_4\), 0.5 mM MgCl\(_2\), 1.5 mM CaCl\(_2\), 10 mM HEPES, 2.8 mM glucose, 0.1% BSA, pH 7.35). Cells were then incubated for 1 h in 1 ml of fresh buffer containing 2.8 or 22.2 mM glucose. The insulin concentration in the medium was measured by RIA using rat insulin as standard. Total protein content was measured with the Lowry method, after SDS (0.03%) extraction. Results are expressed as insulin secreted in the medium (ng·µg protein\(^{-1}\)·h\(^{-1}\)).

Measurement of adenine nucleotide content. Adenine nucleotides were measured as previously reported (3). After the islet or INS-1 cell incubation with 2.8 or 22.2 mM glucose, the experiment was stopped by the addition of 0.125 ml of TCA to a final concentration of 5%. The tubes were then vortexed, left on ice for 5 min, and centrifuged in a microfuge (Beckman). A fraction (0.4 ml) of the supernatant was mixed with 1.5 ml of diethyl ether, and the ether phase containing the TCA was discarded. This procedure was repeated three times to ensure complete elimination of TCA. The extracts were then diluted with 0.4 ml of a buffer containing 20 mM HEPES, 3 mM MgCl\(_2\), and KOH as required to adjust pH to 7.75 (assay buffer). The diluted extract with 300 µl of assay buffer supplemented with 1.5 mM phosphoenolpyruvate and 2.3 U/ml pyruvate kinase, with incubation at room temperature for 15 min. Samples with known concentrations of ADP, without ATP, were run in parallel to check that the transformation was complete. ATP was measured by the addition of 100 µl of an ATP-monitoring reagent containing luciferase and luciferin (Sigma). The emitted light was measured in a luminometer (Turner TD-20/20). To measure only ATP, the same previously described procedure was followed, except that in the first incubation step pyruvate kinase was lacking. ADP levels were then calculated by subtracting ATP from the total ATP plus ADP. Blanks and ATP standards were run through the entire procedure, including the extraction steps.

Mitochondrial membrane potential in INS-1 cells. Mitochondrial membrane potential (\( \Delta \psi_m \)) was measured using Rh123 as an indicator of \( \Delta \psi_m \) changes in a cell suspension under glucose stimulation (22.2 mM) (8). INS-1 cells, after a culture period in RPMI medium, were loaded in KRH buffer containing 2.8 mM glucose and 10 µg/ml Rh123 for 15 min at 37°C. After centrifugation, cells were resuspended in the same buffer without Rh123 and transferred to a fluorometer cuvette, and the fluorescence excited at 490 nm was measured at 530 nm at 37°C with gentle stirring. Results are expressed as the percentage of basal fluorescence (at 2.8 mM glucose).

UCP2 and F\(_1\)-F\(_0\)-ATP-synthase protein levels. The UCP2 protein levels were measured by Western blot analysis as previously described (19). Briefly, at the end of the culture period, groups of 200 islets or INS-1 cells (3 × 10^5) were washed twice in PBS and homogenized by sonication in SDS-PAGE sample buffer. Pancreatic islet and INS-1 cell proteins were denatured by boiling for 3 min, and equivalent amounts of proteins were used in the different experimental groups. Homogenates (25 µg of total cellular protein for pancreatic islets and 50 µg for INS-1 cells) were separated on a 12% SDS-polyacrylamide gel, blotted to nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). Blotting efficiency, as well as the position of protein standards, was assessed by Ponceau staining. Membranes were then washed at room temperature with PBS containing 1% nonfat dried milk and 0.2% Tween-20 (3 changes within 30 min) and blocked with the same buffer containing 10% nonfat dried milk at room temperature for 1 h. After blocking, the membranes were washed twice, as described above, and incubated with a rabbit polyclonal anti-UCP2 antibody (Alpha Diagnostic International, San Antonio, TX) at 1:2,000 dilution in blocking solution at 4°C overnight. Membranes were then washed and blotted with a donkey anti-rabbit IgG peroxidase-linked whole antibody (Pierce, Rockford, IL) diluted 1:2,000 for 1 h at room temperature. Peroxidase activity was detected by using enhanced chemiluminescence (Amer sham Pharmacia Biotech). As verified in each experiment by Ponceau red staining, the total amount of protein load was similar in each lane for the different culture conditions.

To measure F\(_1\)-F\(_0\)-ATP-synthase protein levels from mitochondrial fraction at the end of culture, INS-1 cells were homogenized in a buffer containing 80 mM Tris·HCl (pH 6.8), 250 mM sucrose, 5 mM MgCl\(_2\), 5 mM EDTA, 1 µl/ml protease inhibitor cocktail (Sigma cat. no. P83840). After a low-speed centrifugation (800 g for 3 min), the supernatant was centrifuged for 10 min at 10,000 g. The pellet of this second centrifugation was used as the mitochondrial fraction and sonicated in a lysing solution containing 5% SDS. Proteins (50 µg) from each sample were then treated with PNGase F to achieve protein deglycosylation. Both treated and not treated samples were resolved in 12% SDS-PAGE. Membranes were incubated overnight at 4°C with a goat polyclonal anti-F\(_1\)-ATP-synthase antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:1,000 dilution. Membranes were then blotted with a monoclonal anti-goat IgG peroxidase conjugate (Sigma) diluted 1:10,000.

Detection of glycosylated F\(_1\)-F\(_0\)-ATP-synthase. To further investigate the glycosylation of F\(_1\)-F\(_0\)-ATP-synthase, we used a different method, which specifically detects glycosylated proteins, the Pro-Q glycoprotein blot stain kit. This method uses wheat germ agglutinin, a lectin that binds to N-acetylgalactosamine and N-acetylenuraminic acid residues in glycoproteins. INS-1 cells, at the end of culture, were homogenized by sonication in SDS-PAGE sample buffer. One milligram of total proteins was then immunoprecipitated with an anti-F\(_1\) ATP-synthase antibody coated with protein A-Sepharose for 16 h at 4°C. After centrifugation at 12,000 g for 5 min, the pellet was washed three times with a buffer containing 0.5 M NaCl, 10 mM sodium phosphate (pH 7.4), 0.5% Nonidet P-40, 2 mM EDTA, 0.1% SDS, and 0.04% BSA and boiled in sample buffer. Samples were then centrifuged at 12,000 g for 5 min, and the supernatant was separated on 12% SDS-polyacrylamide gel and electrophoretically transferred onto nitrocellulose membrane. Blotting efficiency was assessed by SYPRO ruby staining. The membrane was then processed with the Pro-Q glycoprotein blot stain kit with wheat germ agglutinin to detect glycoprotein, as described in the kit instructions.

Statistical analysis. The statistical significance of differences between means was assessed by an analysis of variance (ANOVA) followed by a Newman-Keuls test for comparison of more then two groups of data.

RESULTS

Studies in rat pancreatic islets. A time course of GlcN exposure indicated that 24-h culture with 10 mM GlcN slightly affected insulin secretion in response to 22.2 mM glucose (764.5 ± 95 vs. 1,040.7 ± 102 pg·islet\(^{-1}\)·30 min\(^{-1}\), mean ± SE; \( n = 5, P < 0.05; \) Fig. 1A). The decrement of glucose-stimulated insulin release was more pronounced after 48 h of exposure (621.6 ± 80.2 vs. 1,015.3 ± 72.8 pg·islet\(^{-1}\)·30 min\(^{-1}\); \( n = 5, P < 0.001; \) Fig. 1B). GlcN at 5 mM did not significantly affect insulin release either after 24 h (922.8 ± 133 vs. 1,040.7 ± 102 pg·islet\(^{-1}\)·30 min\(^{-1}\); \( n = 5, \) Fig. 1A) or 48 h (868.7 ± 49.6...
prevent the negative effects of GlcN. After 48 h of culture an agent that inhibits protein N-glycosylation, was able to prevented (863 vs. 1,015.3 pg·islet⁻¹·30 min⁻¹, n = 5; Fig. 1B). Islet insulin content, after 48-h culture, was not statistically different between control islets and islets cultured with 5 or 10 mM GlcN (41.7 ± 7.2, 34.5 ± 4.7, and 34.0 ± 4.7 ng/islet; n = 4). To elucidate the mechanism by which GlcN impairs β-cell secretion, we studied whether tunicamycin, an agent that inhibits protein N-glycosylation, was able to prevent the negative effects of GlcN. After 48 h of culture with 10 mM GlcN and 2 μg/ml tunicamycin, the inhibitory effect of GlcN on glucose-induced insulin release was prevented (863 ± 99 vs. 477.4 ± 54.2 pg·islet⁻¹·30 min⁻¹ in islets cultured with or without tunicamycin, respectively; n = 4, P < 0.001), and secretion was not significantly different from control islets (863 ± 99 vs. 1,013.2 ± 80 pg·islet⁻¹·30 min⁻¹; n = 4). Tunicamycin had no effect in control islets (Fig. 2A).

Because it has been reported that GlcN may induce oxidative stress on pancreatic β-cell (15), we also tested the effect of nicotinamide. Pancreatic islets were cultured for 48 h with 10 mM GlcN with or without 5 mM NAD. Islets exposed to GlcN plus NAD secreted normally in response to 22.2 mM glucose (806.3 ± 113.2 pg·islet⁻¹·30 min⁻¹) compared with islets exposed to GlcN alone (370.1 ± 76.6 vs. 1,157.9 ± 73.6 pg·islet⁻¹·30 min⁻¹; n = 6, P < 0.001). NAD had no effect on insulin secretion in control islets (Fig. 2B).

The ATP/ADP ratio plays a critical role in glucose-induced β-cell insulin secretion; therefore, we measured adenine nucleotide content in pancreatic islets in the presence of basal (2.8 mM) or stimulating (22.2 mM) glucose concentrations (Table 1). In control islets, in response to high glucose concentration, both ATP levels and, as a consequence, the ATP/ADP ratio clearly increased. In GlcN preexposed islets, basal ATP did not further increase under

![Fig. 1. Effect of 24-h (A) and 48-h (B) culture without (open bars) and with 5 mM (hatched bars) or 10 mM (filled bars) glucosamine (GlcN) on glucose-stimulated insulin release. Islets were cultured in medium containing 5.5 mM glucose (open bars) or 5.5 mM glucose + 10 mM glucosamine (filled bars) with or without 2 μg/ml tunicamycin, (A) and with or without 5 mM NAD (B), as indicated. At the end of culture, batches of 5 islets were incubated for 30 min in the presence of basal (2.8 mM) or stimulated (22.2 mM) glucose concentrations. Results are presented as absolute values. Data represent means ± SE of 5 separate experiments (**P < 0.05; ***P < 0.01).](http://ajpendo.physiology.org/)

![Fig. 2. Preventive effect of tunicamycin (A) or nicotinamide (NAD; B) on GlcN-induced decrement of glucose-stimulated insulin release. Islets were cultured in medium containing 5.5 mM glucose (open bars) or 5.5 mM glucose + 10 mM glucosamine (filled bars) with or without 2 μg/ml tunicamycin, (A) and with or without 5 mM NAD (B), as indicated. At the end of culture, batches of 5 islets were incubated for 30 min in the presence of basal (2.8 mM) or stimulated (22.2 mM) glucose concentrations. Results are presented as absolute values. Data represent means ± SE of 4 separate experiments (***P < 0.001) for A and of 6 separate experiments (***P < 0.001) for B.](http://ajpendo.physiology.org/)

### Table 1. Influence of 48-h culture with 10 mM GlcN on glucose-induced adenine nucleotide content in rat pancreatic islets

<table>
<thead>
<tr>
<th>Adenine Nucleotides, pM/islet</th>
<th>Glucose, 2.8 mM</th>
<th>Glucose, 22.2 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20.7 ± 1.6</td>
<td>40.7 ± 4.3†</td>
</tr>
<tr>
<td>ATP</td>
<td>4.1 ± 0.4</td>
<td>3.45 ± 0.45</td>
</tr>
<tr>
<td>ADP</td>
<td>5.2 ± 0.25</td>
<td>11.8 ± 0.3†</td>
</tr>
<tr>
<td>GlcN, 10 mM</td>
<td>28.9 ± 4.7</td>
<td>27.0 ± 7.4</td>
</tr>
<tr>
<td>ATP</td>
<td>4.46 ± 0.6</td>
<td>4.8 ± 1.3</td>
</tr>
<tr>
<td>ADP</td>
<td>6.43 ± 0.4</td>
<td>5.6 ± 0.15*</td>
</tr>
<tr>
<td>GlcN + tunicamycin</td>
<td>12.7 ± 2.3</td>
<td>35.2 ± 2.0†</td>
</tr>
<tr>
<td>ATP</td>
<td>4.1 ± 0.7</td>
<td>3.1 ± 0.45</td>
</tr>
<tr>
<td>ADP</td>
<td>3.1 ± 0.5</td>
<td>11.3 ± 0.25†</td>
</tr>
</tbody>
</table>

Values are means ± SE from 6 separate experiments. Islets were cultured for 48 h in medium containing 5.5 mM glucose with or without 10 mM glucosamine (GlcN) and with or without 2 μg/ml tunicamycin. Batches of 5 islets were incubated in 1 ml of medium containing indicated glucose concentrations. At the end of the incubation, islets were processed for measuring of adenine nucleotides. *P < 0.05 or less vs. control islets. †P < 0.05 or less vs. 2.8 mM glucose.
GLUCOSAMINE AND PANCREATIC β-CELL FUNCTION

Studies in INS-1 cells. To elucidate the mechanism by which GlcN reduced ATP levels, we studied mitochondrial function, as these organelles are the site where most cellular ATP is synthesized. In particular, we measured mitochondria membrane potential, a marker of the activity of F₁–F₀-ATP-synthase, an enzyme that catalyzes ATP synthesis. Because a large number of cells are needed to measure mitochondrial membrane potential in cell suspension, we used INS-1 insulinoma cells (16).

We first investigated the culture conditions that reproduce in INS-1 cells the effects of GlcN and tunicamycin that we had previously observed in rat pancreatic islets. A dose response for tunicamycin has been tested for INS-1 cells, since the same concentration used for islets experiments was toxic for INS-1 cell viability. Differently from pancreatic islets, in the experiments with INS-1 cells, a longer exposure to GlcN and tunicamycin and a lower concentration were needed to reproduce similar results. An increased sensitivity of tumor cells to inhibitors of glycosylation has been already reported (5, 23).

Culture with 5 mM GlcN for 72 h decreased insulin release compared with control cells (4.22 ± 0.5 vs. 7.79 ± 0.28, ng·µg protein⁻¹·h⁻¹, n = 6, P < 0.001). The simultaneous presence of tunicamycin (1 ng/ml) at the beginning of the culture period was able to prevent the inhibitory effect of GlcN on glucose-induced insulin secretion in INS-compared with control cells (9.70 ± 1.6 vs. 7.79 ± 0.28 ng·µg protein⁻¹·h⁻¹ in cells cultured in the presence or in the absence of tunicamycin; n = 6). Tunicamycin had no effect in control INS-1 cells (Fig. 3).

As in pancreatic islets, also in INS-1 cells the ATP/ADP ratio increased under glucose stimulation (22.2 mM). In GlcN-preexposed cells, this increase was blunted, but the presence of tunicamycin in the culture medium was able to prevent this defect (Table 2). Tunicamycin itself had no effect on the ability of INS-1 cells to increase the ATP/ADP ratio under glucose stimulation (Table 2).

Table 2. Influence of 72-h culture with 5 mM GlcN on glucose-induced adenine nucleotide content in INS-1 β-cell line

<table>
<thead>
<tr>
<th>Adenine Nucleotides, pmol/10⁶ cells</th>
<th>Glucose, 2.8 mM</th>
<th>Glucose, 22.2 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.84 ± 0.58</td>
<td>10.92 ± 0.7†</td>
</tr>
<tr>
<td>ATP</td>
<td>1.74 ± 0.19</td>
<td>0.72 ± 0.08†</td>
</tr>
<tr>
<td>ADP</td>
<td>5.6 ± 0.5</td>
<td>15.2 ± 1.5†</td>
</tr>
<tr>
<td>ATP/ADP</td>
<td>1.12 ± 1.1</td>
<td>10.7 ± 1.2</td>
</tr>
<tr>
<td>ATP</td>
<td>1.08 ± 0.18</td>
<td>1.13 ± 0.14</td>
</tr>
<tr>
<td>ADP</td>
<td>12.3 ± 1.3</td>
<td>9.7 ± 0.6</td>
</tr>
<tr>
<td>Control + tunicamycin</td>
<td>7.3 ± 0.2</td>
<td>9.8 ± 0.1†</td>
</tr>
<tr>
<td>ATP</td>
<td>1.57 ± 0.2</td>
<td>0.75 ± 0.19†</td>
</tr>
<tr>
<td>ADP</td>
<td>5.15 ± 0.9</td>
<td>13.3 ± 2.5†</td>
</tr>
<tr>
<td>ATP + tunicamycin</td>
<td>7.0 ± 0.1</td>
<td>10.4 ± 0.28†</td>
</tr>
<tr>
<td>ADP</td>
<td>1.71 ± 0.31</td>
<td>1.02 ± 0.09†</td>
</tr>
<tr>
<td>ATP/ADP</td>
<td>5.3 ± 0.66</td>
<td>10.2 ± 0.05†</td>
</tr>
</tbody>
</table>

Values are means ± SE from 4 separate experiments. INS-1 cells were cultured for 72 h in RPMI medium with or without 5 mM GlcN and with or without 1 ng/ml tunicamycin. Batches of 10⁶ cells were incubated in medium containing indicated glucose concentrations. At the end of the incubation, cells were processed for measuring of adenine nucleotides. *P < 0.05 or less vs. 2.8 mM glucose.

We then measured under these experimental conditions the glucose-induced changes in ΔΨₘ, a marker of F₁–F₀-ATP-synthase activity. This protein is located in the mitochondrial inner membrane and is responsible for the ATP synthesis (17). The Rh123 fluorescence was recorded at the end of a 72-h exposure to 5 mM GlcN or to GlcN plus 1 ng/ml tunicamycin, as indicated in graph section. In control INS-1 cells, when glucose concentration was increased to 22.2 mM, fluorescence decreased (−9.54 ± 0.4%; n = 5), indicating the glucose-induced hyperpolarization of ΔΨₘ (Fig. 4, A–D). The addition of the uncoupler FCCP (1 µM) readily depolarized ΔΨₘ. Cells cultured with 5 mM GlcN showed a decreased hyperpolarization of ΔΨₘ when glucose was raised to 22.2 mM (−5.76 ± 0.59%; n = 5, P < 0.01; Fig. 4, B–D). Tunicamycin (1 ng/ml) prevented the effect of GlcN on glucose-induced hyperpolarization of ΔΨₘ (−8.49 ± 0.89%; n = 5; Fig. 4, C–D).

To better understand whether alterations of F₁–F₀-ATP-synthase activity were associated with its N-glycosylation, we extracted, at the end of each culture condition, a purified mitochondrial fraction and then measured F₁–F₀-ATP-synthase protein levels by Western blot. N-glycosidase F (an agent that deglycosylates proteins) was then used to see whether GlcN exposure led to excessive glycosylation of F₁–F₀-ATP-synthase. The enzyme protein levels were increased in cells exposed to GlcN and distinctly reduced by N-glycosidase F, thus suggesting an important role of protein glycosylation (Fig. 5). Tunicamycin was able to partially prevent this increase (Fig. 5).

Glycosylation of F₁–F₀-ATP-synthase was further confirmed with a specific staining for glycoproteins, the Pro-Q Glycoprotein blot stain kit. Figure 6 shows that glycosylation of F₁–F₀-ATP-synthase was clearly increased in GlcN-exposed INS-1 cells and that this increase was prevented by the presence of tunicamycin.

Fig. 3. Effect of GlcN and tunicamycin on INS-1 β-cell insulin secretion. Cells were cultured for 72 h without (open bars) or with 5 mM GlcN (filled bars) in the absence or presence of 1 ng/ml tunicamycin, as indicated. Thereafter, INS-1 cells were incubated for 1 h in the presence of basal (2.8 mM) or stimulating (22.2 mM) glucose concentrations. Results are presented as absolute values. Data represent means ± SE of 6 separate experiments (**P < 0.001).
UCP2 protein expression. Western blot analysis on both pancreatic islets (Fig. 7A) and INS-1 β-cells (Fig. 7B) did not show, after GlcN exposure, any change in UCP2 expression.

DISCUSSION

Our data indicate that chronic exposure of rat pancreatic islets to 10 mM GlcN reduced insulin release in response to glucose. The impaired insulin release was associated with a reduced ability of glucose to increase ATP synthesis and ATP/ADP ratio. In addition, in INS-1 β-cells, chronic culture with GlcN also affected glucose-stimulated changes of N-glycosylation of F1-F0-ATP-synthase, possibly leading to alteration of its function. In contrast, UCP2 levels were unaffected by GlcN exposure, both in pancreatic islets and INS-1 β-cells.
excess of glucose occurs, does not enter glucose oxidative metabolism and, therefore, does not generate ATP. Accordingly, we did not observe in these islets any change in UCP2 expression.

The role of mitochondria in GlcN-induced β-cell damage has been confirmed in a recent study, where an increase in GlcN production is associated with mitochondrial oxidative stress leading to increased reactive oxygen species production and β-cell death (15). Accordingly, we also found that NAD could prevent GlcN-induced inhibition of insulin secretion, confirming that oxidative stress is, in part, involved in β-cell alterations. In addition, similar results have been observed in adipocytes, where GlcN-induced insulin-resistance was caused by depletion of intracellular ATP (14).

To elucidate the pathway underlying the inhibitory effects of GlcN on pancreatic islet function, we studied the ability of tunicamycin to prevent β-cell impairment in pancreatic islets chronically cultured with GlcN. Tunicamycin is an agent that inhibits NH2-terminal glycosylation in rough endoplasmic reticulum by interfering with the dolichol-phosphate reaction (24). Previous studies reported that, in several tissues, nonenzymatic glycosylation might mediate the toxic effect of hyperglycemia, and therefore, a similar mechanism might be involved in our experimental conditions. In our study, we demonstrated that tunicamycin, both in pancreatic islets and in INS-1 cells, was able to prevent all the inhibitory effects induced by GlcN on β-cell function. In particular, the reduced ability of glucose to increase ATP levels in β-cells exposed to GlcN seems to be the result of impaired F1-Fo ATP-synthase activity induced by GlcN and prevented by tunicamycin, thus suggesting a role of protein glycosylation. To more directly investigate F1-Fo ATP-synthase glycosylation, we used two different reagents (PNGase F and wheat germ agglutinin). By use of both methods, our results indicate that GlcN increases F1-Fo ATP-synthase glycosylation, and tunicamycin could prevent this effect, although we were unable to detect a change in the protein size. Although our data do not establish a direct cause-effect relationship between glycosylation of ATP-synthase and the reduction of ATP levels, pieces of several indirect evidence support this suggestion. Under our experimental conditions, GlcN increases ATP-synthase glycosylation and decreases glucose-stimulated changes of mitochondria membrane potential (a marker of ATP-synthase activity) and ATP levels. Conversely, tunicamycin was able to prevent simultaneously the increase of ATP-synthase glycosylation and the decrease of glucose-stimulated changes of mitochondria membrane potential and ATP levels.

In summary, our data show that chronic exposure to GlcN impairs F1-Fo ATP-synthase activity, ATP synthesis, and insulin secretion. An excessive N-glycosylation of mitochondrial ATP synthase might be responsible for the reduced ATP production. Tunicamycin, an inhibitor of NH2-terminal protein glycosylation, partially prevented the adverse effects of GlcN on the ΔΨm, ATP/ADP ratio increase, and insulin release and also prevented ATP-synthase glycosylation. These data suggest that GlcN may mediate the toxic effect of chronic hyperglycemia on pancreatic β-cells through alterations of normal mitochondrial function due to both an excessive N-glycosylation process and oxidative stress.

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Our findings indicate that, as observed after chronic exposure to high glucose, GlcN also impairs insulin secretion by reducing mitochondrial function and, in particular, ATP synthesis and the ATP/ADP ratio.

In pancreatic β-cells, a critical role is played by the correct balance between new ATP synthesis, produced by F1-Fo ATP-synthase, and proton leak induced by UCP2, a protein that dissipates energy when overproduced (25). Pathological conditions, like hyperglycemia, might bring on cell substrate overload, leading to an abnormal increase of cellular oxidative processes. In this condition, mitochondrial ability to control energy production is impaired. Accordingly, we recently observed that, in pancreatic islets chronically exposed to high glucose, the blunted insulin response to glucose is associated with impaired mitochondrial function and reduced ATP synthesis due, at least in part, to UCP2 overexpression (19). Similarly, the present data obtained in islets chronically exposed to GlcN also indicate that the impaired insulin release is associated with low ATP production. However, different from high glucose, GlcN impairs ATP synthesis (as indicated by the change of mitochondria membrane potential) and does not affect UCP2 expression.

We, then, propose that the impairment of insulin release induced by high glucose or GlcN, although in both cases involving reduced ATP levels, occurs via different mechanisms: a decrease of ATP synthesis after GlcN exposure and an increase of UCP2 expression after high glucose. Why this difference? We speculate that, in islets cultured with high glucose, the excess of glucose greatly increases substrate influx through the metabolic mitochondria pathways, thus overwhelming the oxidative capacity of pancreatic β-cells. As a result, large amounts of high-energy substrates are generated. In this condition, the β-cell protects itself from fuel overload by increasing energy disposal through an increase of UCP2 expression, thus leading to a decrease of ATP content. In contrast, GlcN, although produced when an
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