Am J Physiol Cell Physiol 310: C558–C567, 2016.
First published January 6, 2016; doi:10.1152/ajpcell.00148.2015.

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Altered expression of uncoupling protein 2 in GLP-1-producing cells after chronic high glucose exposure: implications for the pathogenesis of diabetes mellitus

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Submitted 10 June 2015; accepted in final form 6 January 2016

Urbano F, Filippello A, Di Pino A, Barbagallo D, Di Mauro S, Pappalardo A, Rabuazzo AM, Purrello M, Purrello F, Piro S. Altered expression of uncoupling protein 2 in GLP-1-producing cells after chronic high glucose exposure: implications for the pathogenesis of diabetes mellitus. *Am J Physiol Cell Physiol* 310: C558–C567, 2016. First published January 6, 2016; doi:10.1152/ajpcell.00148.2015.—Glucagon-like peptide-1 (GLP-1) is a gut L-cell hormone that enhances glucose-stimulated insulin secretion. Several approaches that prevent GLP-1 degradation or activate the GLP-1 receptor are being used to treat type 2 diabetes mellitus (T2DM) patients. In T2DM, GLP-1 secretion has been suggested to be impaired, and this defect appears to be a consequence rather than a cause of impaired glucose homeostasis. However, although defective GLP-1 secretion has been correlated with insulin resistance, little is known about the direct effects of chronic high glucose concentrations, which are typical in diabetes patients, on GLP-1-secreting cell function. In the present study, we demonstrate that glucotoxicity directly affects GLP-1 secretion in GLUTag cells chronically exposed to high glucose. Our results indicate that this abnormality is associated with a decrease in ATP production due to the elevated expression of mitochondrial uncoupling protein 2 (UCP2). Furthermore, UCP2 inhibition using small interfering RNA (siRNA) and the application of glibenclamide, an ATP-sensitive potassium (K^+_{ATP}) channel blocker, reverse the GLP-1 secretion defect induced by chronic high-glucose treatment. These results show that glucotoxicity diminishes the secretory responsiveness of GLP-1-secreting cells to acute glucose stimulation. We conclude that the loss of the incretin effect, as observed in T2DM patients, could at least partially depend on hyperglycemia, which is typical in diabetes patients. Such an understanding may not only provide new insight into diabetes complications but also ultimately contribute to the identification of novel molecular targets within intestinal L-cells for controlling and improving endogenous GLP-1 secretion.

intestinal L-cell; glucagon-like peptide-1; UCP2; ATP; gut hormones; incretins; metabolism

GLUCAGON-LIKE PEPTIDE-1 (GLP-1) is an intestinal hormone that is secreted from the enteroendocrine L-cells and is involved in the regulation of glycemia. Once released into the circulation, GLP-1 elicits a potentiation of glucose-stimulated insulin secretion; this phenomenon, referred to as the "incretin effect" (7, 15), accounts for ~70% of postprandial insulin release in healthy individuals (31). The action of gut hormones in the metabolic process has been recognized to be critical, and, as such, incretin-based treatments, including the administration of GLP-1 mimetics or inhibitors of its degradation, represent important therapeutic tools for the treatment of patients with type 2 diabetes mellitus (T2DM).

In these subjects, the incretin response has been suggested to be impaired (33) and decreased GLP-1 levels have been observed (32). However, the pathophysiological mechanism underlying the reduced incretin effect remains incompletely elucidated, although several lines of evidence support the hypothesis that this deficit could constitute an acquired defect that is secondary to impaired glucose homeostasis and insulin resistance rather than a primary pathogenic defect (19, 20, 23, 30, 34). Accordingly, it is likely that environmental factors such as chronically high plasma concentrations of glucose (glucotoxicity) affect L-cell function and nutrient-induced GLP-1 release by chronically stimulating and, consequently, desensitizing these cells.

Considering the link between T2DM, glucotoxicity, and diminished GLP-1 secretory responses, as well as the finding that high levels of glucose induce functional and secretory impairments in different cell types (3, 16, 17, 25, 41), in the present study we investigated whether simulated hyperglycemia affects the physiological responses of intestinal L-cells to acute nutrient stimulation, thus reducing GLP-1 secretory capacity.

Our hypothesis was consistent with the evidence that intestinal L-cells and pancreatic beta cells share many common elements: transporters, channels, granules, glucose metabolism, and ATP activity (39). Because it is universally recognized that glucotoxicity affects pancreatic beta cells (3, 17, 25, 41), we focused our studies on the molecular mechanisms underlying this phenomenon.

GLP-1 is synthesized in intestinal enteroendocrine L-cells expressing the proglucagon gene (11). As in beta cells, increased glucose metabolism and closure of ATP-sensitive po-

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tassium (K^+_{ATP}) channels result in membrane depolarization, Ca^{2+} entry, and GLP-1 release (51); furthermore, both nonmetabolizable and metabolizable sugars can stimulate GLP-1 secretion via the activity of sodium-dependent glucose cotransporters (SGLTs) (18).

Because increased ATP levels play a significant role in glucose-induced GLP-1 release, any defect in the ATP production machinery could be responsible for impaired GLP-1 secretion.

Thus, using an in vitro L-cell model, the GLUTag cell line (6, 13), we evaluated GLP-1 secretion, glucose metabolism, and ATP production in cells that were chronically exposed to high glucose. ATP synthesis can also be regulated by the activity of the mitochondrial uncoupling protein 2 (UCP2) (1, 5, 12, 21, 24, 40, 56), and chronically elevated levels of glucose or free fatty acids were previously shown to induce UCP2 overexpression in different cell types (28, 29, 41, 47, 56, 57). UCP2 can act as a sensor of mitochondrial oxidative stress; by exporting citric acid cycle intermediates from mitochondria, UCP2 negatively controls the oxidation of glucose (53), thus lowering the redox pressure on the mitochondrial respiratory chain, reactive oxygen species (ROS) production, and ultimately ATP synthesis.

Therefore, we hypothesized that glucotoxicity conditions may increase UCP2 activity in L-cells and affect their GLP-1 secretory capacity.

The results of the present study may add information regarding the mechanisms that are responsible for the impairment of the incretin effect that occurs in diabetes patients. Current incretin approaches are effective in controlling blood glucose levels in type 2 diabetes patients. However, an alternative approach could be to increase endogenous GLP-1 secretion via the modulation of the GLP-1 secretory mechanism by improving and restoring L-cell function. Enhancing endogenous GLP-1 release may represent a novel therapeutic strategy that is superior to current incretin therapies and may help to prevent diabetes. However, to modulate endogenous GLP-1 secretion, it is first necessary to fully understand L-cell function and the factors that influence the regulation of GLP-1 secretion. In this study, we demonstrated that glucotoxicity directly affects GLP-1 secretion in GLUTag cells and elucidated the mechanism underlying this phenomenon.

MATERIALS AND METHODS

Chemicals. Fetal bovine serum (FBS) was provided by Invitrogen Laboratories (Carlsbad, CA). The anti-UCP2 antibody was purchased from Alpha Diagnostic International (San Antonio, TX) (3, 36), and the anti-GLUT1, anti-GLUT2, anti-SGLT1, and anti-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cell medium, aprotinin, diprotin A, forskolin, glibenclamide, and all other chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

Cell culture. GLUTag cells, which were kindly provided by Prof. F. M. Gribble (University of Cambridge) with permission from Prof. D. J. Drucker (University of Toronto) (13, 14, 26), were cultured in Dulbecco's modified Eagle's medium (DMEM, 5.6 mmol/l glucose) supplemented with 10% (vol/vol) FBS, penicillin, and streptomycin. The medium was changed once a week, and the cells were trypsinized and reseeded at a 1:4 dilution when 70% confluence was reached (approximately every 5 days). Most of the experiments were conducted with cells at *passages 12–22*; however, the cellular responses were similar for *passages 10–25*.

Chronic glucose exposure. To study the role of systemic hyperglycemia (chronic high glucose exposure), we performed preliminary experiments using different high glucose concentrations (11.2, 16.8, 19.4, and 25.2 mmol/l) for extended periods (12, 24, 48, and 72 h). We selected the dose-time combination of 19.4 mmol/l for 72 h because these conditions caused statistically significant inhibition of acute GLP-1 release. Moreover, to exclude an osmotic effect of the sugar, we performed a set of experiments using mannitol (19.4 and 25.2 mmol/l), but we did not observe any effect on GLP-1 secretion or cell survival.

Twenty-four hours after plating, GLUTag cells were cultured for 72 h at 37°C in complete DMEM (5.6 mmol/l glucose) in the presence or absence of an additional 13.8 mmol/l glucose (to a final concentration of 19.4 mmol/l). The medium containing 5.6 or 19.4 mmol/l glucose was replaced every 24 h. At the end of each experiment, the cells were lysed with RIPA buffer and the lysates were analyzed for total protein content, determined with the BCA assay (Pierce Biotechnology, Rockford, IL). To select the appropriate normalization method, we performed preliminary experiments and measured the cell number and total protein content in wells at the end of the 72-h culture period. No differences in normalized data were observed between the two normalization methods. On the basis of these findings, we chose to normalize all of the measurements to the total protein content per well.

GLP-1 secretion. For the GLP-1 secretion experiments, the cells were plated in six-well plates and cultured for 72 h in medium containing 5.6 or 19.4 mmol/l glucose. On the day of the experiment, the cells were washed twice with 2 ml of glucose-free Krebs-Ringer buffer (KRB) containing (in mmol/l) 120 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 22 $NaHCO_3$, and 0.1 diprotin A, bubbled with 95% O_2-5% CO_2 for 10 min and supplemented with 0.5% (wt/vol) BSA. Experiments were performed by incubating the cells in 2 ml of KRB containing the test reagents for 2 h at 37°C and 5% CO_2. Forskolin was prepared as a stock solution in DMSO, and the final DMSO concentration was adjusted to 0.25%. The experiments were performed in KRB to prevent any interference by other possible nutrient secretagogues, such as fatty acids and amino acids. To investigate the acute stimulatory effects of glucose on GLP-1 secretion, cells were grown in the presence of 5.6 mmol/l (control group) or 19.4 mmol/l glucose (high-glucose group) for 72 h, washed, and acutely exposed to different glucose concentrations (0, 0.5, 5, or 25 mmol/l) for 2 h. In a specific set of experiments, the cells were also acutely exposed to 10 nM glibenclamide as previously described by Gribble et al. (18). At the end of the incubation period, the media were collected and centrifuged to remove any floating cells. The cells were lysed with RIPA buffer, and the lysates were analyzed for total protein content to control for the number of cells. The GLP-1 levels in the supernatants were determined via ELISA for the quantitative determination of bioactive GLP-1 (7–36) (GLP-1 Active ELISA kit; ALPCO Diagnostic). Each sample was analyzed in duplicate. GLP-1 secretion was normalized to the baseline levels in 0 mmol/l glucose, which were measured in parallel on the same day.

Intracellular GLP-1 content quantification. After the GLP-1 secretion experiments, the cells were incubated in a solution containing HCl (10 N) and ethanol (70%) and shaken overnight at 4°C. The samples were then collected in vials and stored frozen at −20°C until analysis. Insoluble material was removed via centrifugation. For determination of the GLP-1 content [total GLP-1 (7–36 and 9–36)], we used a specific ELISA kit (Millipore, Billerica, MA).

Western blot analysis. Protein was extracted from GLUTag cells with RIPA lysis buffer, and Western blottin was performed as previously described (44). All of the immunoblot signals were visualized via autoradiography with the electrochemiluminescence method (Amersham, Little Chalfont, UK) and were subjected to densitometric analyses with ImageJ software version 1.41.

Glucose uptake assay. After being cultured in medium with 5.6 (control group) or 19.4 (high-glucose group) mmol/l glucose for 72 h,

the cells were washed and kept at 37°C for 5 min in glucose-free KRB (glucose deprivation phase).

Cells were then incubated for 30 min in KRB containing 0, 40, or 100 μM 2-deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-D-glucose (2-NBDG) (Cayman Chemical), a fluorescent and nonmetabolizable glucose analog. 2-NBDG enters cells through glucose transporters and is subsequently phosphorylated and trapped inside cells (10, 55). At the end of the incubation period, the cells were washed twice and glucose uptake was determined by measuring fluorescence at a wavelength of 535 nm (excitation wavelength of 485 nm).

Measurement of intracellular ATP content. ATP levels were measured with the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI) according to the manufacturer's protocol. Briefly, equal numbers of cells were grown in the presence of 5.6 (control group) or 19.4 (high-glucose group) mmol/l glucose for 72 h. Cells were then washed and acutely exposed to different glucose concentrations (0, 0.5, 5, or 25 mmol/l). The reaction agent was added to each well, and the plate was incubated at room temperature for 10 min. The luminescent signal was proportional to the amount of ATP. The ATP levels were normalized to the baseline levels in 0 mmol/l glucose, which were measured in parallel on the same day.

Measurement of mitochondrial membrane potential. Glucose-induced changes in mitochondrial membrane potential ($\Delta\Psi_m$) were quantified in GLUTag cells with rhodamine 123 (Rh123) (3). After being grown in the presence of 5.6 (control group) or 19.4 (high-glucose group) mmol/l glucose for 72 h, GLUTag cells were loaded in KRB containing 15 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; glucose was then added to a final concentration of 10 mmol/l, and the fluorescence excited at 490 nm was measured at 530 nm and 37°C with gentle stirring. The uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was added at a concentration of 1 μmol/l to inhibit the respiratory reaction and fully dissipate the $\Delta\Psi_m$.

Lactate measurement. For the measurement of lactate production, GLUTag cells were plated in a 96-well plate in quadruplicate and grown in the presence of 5.6 (control group) or 19.4 (high-glucose group) mmol/l glucose for 72 h. Cells were then washed and acutely incubated in KRB containing different glucose concentrations (0, 0.5, 5, or 25 mmol/l). At the end of the exposure period, lactate production was determined by measuring NADH generated from NAD^+ in the presence of lactate dehydrogenase.

Ucp2 small interfering RNA and transfection of GLUTag cells. *Ucp2* silencing experiments were performed with three specific small interfering RNAs (siRNAs) targeting *Ucp2* (FlexiTube siRNAs Mm_UCP2_2, Mm_UCP2_4 and Mm_UCP2_5; Qiagen, Venlo, The Netherlands) and a nontargeting control (NT) siRNA (AllStars Negative Control siRNA, Qiagen). Twenty-four hours after plating, GLUTag cells were transfected with siRNA at a final concentration of 50 nM with HiPerFect Transfection Reagent (Qiagen) and cultured for 72 h in medium containing 5.6 or 19.4 mmol/l glucose. Seventy-two hours after transfection, we achieved a *Ucp2* knockdown efficiency of ~60% in GLUTag cells under control conditions and 66.7% under high glucose exposure conditions, as determined via real-time RT-PCR and Western blot analysis (see RESULTS).

RNA extraction and quantitative RT-PCR. Total RNA was extracted, quantified, and reverse-transcribed as previously reported (4). *Ucp2* expression and knockdown were evaluated via RT-PCR with Fast SYBR Green Master Mix (Life Technologies) according to the manufacturer's instructions. Hypoxanthine guanine phosphoribosyl transferase (*Hprt*) and β-actin (*Actb*) were used as reference genes to normalize the RT-PCR results.

Statistical analysis. Differences between the means of unpaired samples were analyzed with Student's *t*-test. Comparisons between multiple means were performed via ANOVA followed by post hoc analysis for significance (Bonferroni test). For both tests, the level of significance was set at $P \leq 0.05$. Statistical analyses were performed with GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). Data are expressed as means ± SE.

RESULTS

Chronic high glucose-treatment reduced GLP-1 secretion but not total GLP-1 content. To study the effects of glucotoxicity on GLP-1 release, we investigated acute glucose-stimulated GLP-1 secretion in control cells (5.6 mmol/l glucose) and in cells that had been chronically preexposed to high glucose levels (19.4 mmol/l glucose for 72 h) (Fig. 1*A*). In the control group, 0.5, 5, and 25 mmol/l glucose acutely stimulated GLP-1 secretion in a dose-dependent manner [fold changes of 2.1 ± 0.4 ($P < 0.001$), 2.9 ± 0.2 ($P < 0.001$), and 3.1 ± 0.3 ($P < 0.001$), respectively] compared with its secretion in the absence of acute glucose stimulation. In the same control cells, forsko-

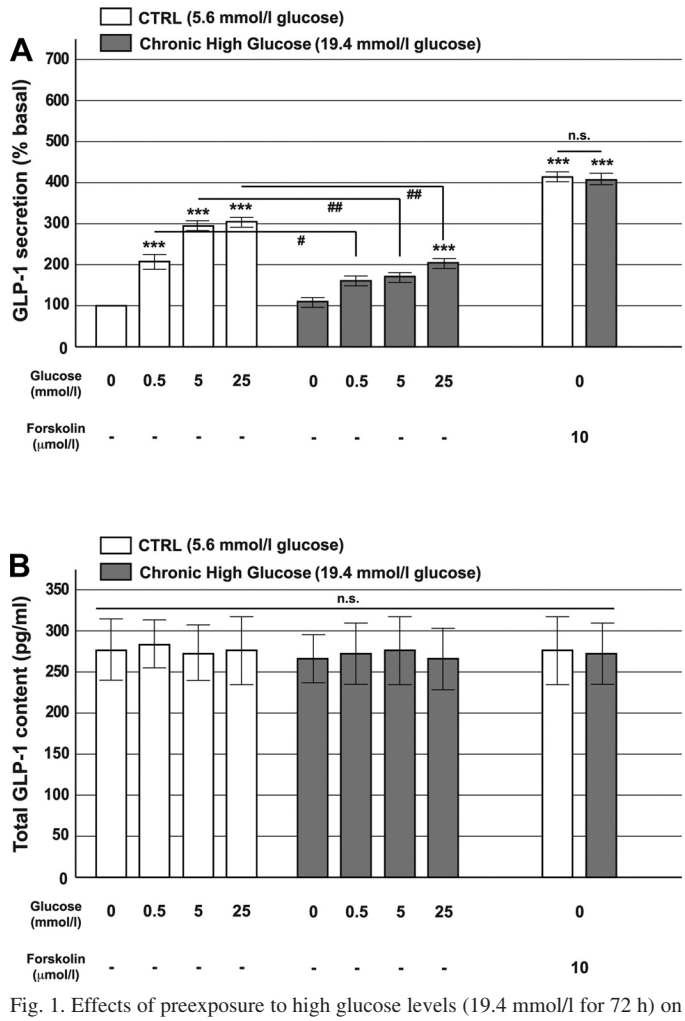
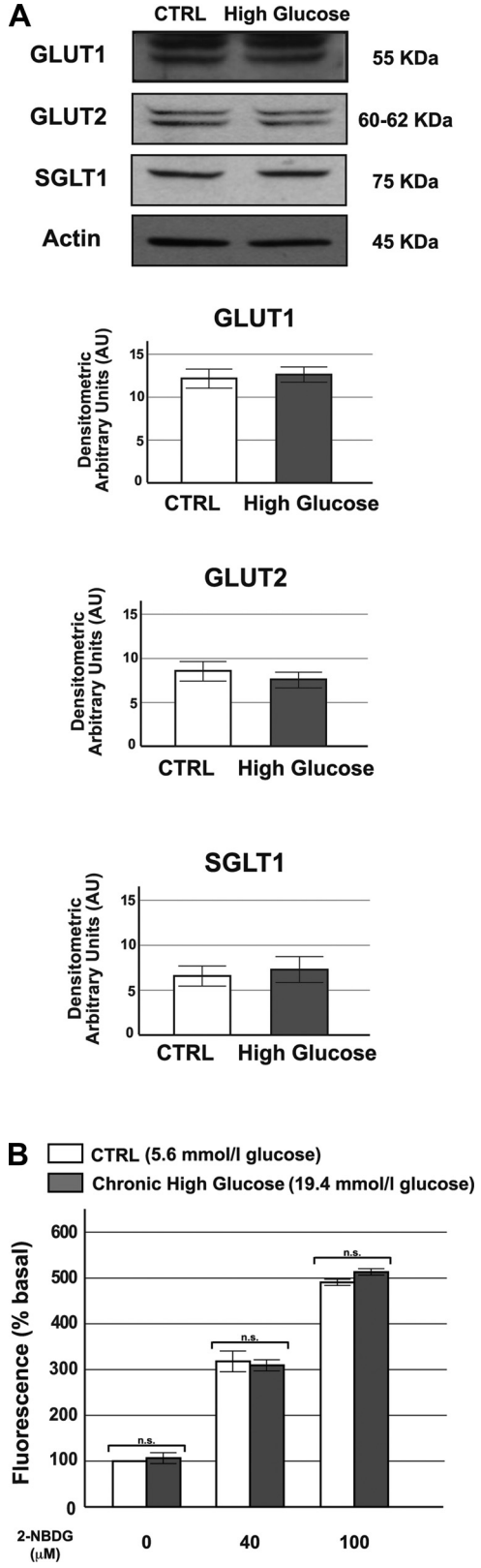


Fig. 1. Effects of preexposure to high glucose levels (19.4 mmol/l for 72 h) on glucagon-like peptide-1 (GLP-1) secretion and content. *A*: after 72 h of incubation in either 5.6 [control (CTRL)] or 19.4 (high glucose) mmol/l glucose, GLUTag cells were washed and cultured in Krebs-Ringer buffer under the indicated conditions; subsequently, the amount of GLP-1 released into the medium was measured (baseline secretory rate in 0 mmol/l glucose: 8.2 ± 0.8 pmol/l in 2 h). *B*: after the GLP-1 secretion experiments, cells were lysed and assessed for total GLP-1 content (basal cell content: 285.7 ± 42.8 pg/ml). $^{***}P < 0.001$ vs. basal level in control group; $^{\#}P < 0.05$ vs. control group; $^{\#\#}P < 0.01$ vs. control group; n.s., not significant (1-way ANOVA followed by Bonferroni test, $n = 4$).

lin (10 μmol/l), which is a direct activator of adenylyl cyclase and a robust cell secretagogue (13), increased the secretion of active GLP-1 compared with the baseline (fold change of 4.6 ± 0.3, $P < 0.001$).



In cells that had been preexposed to 19.4 mmol/l glucose for 72 h, basal GLP-1 secretion was slightly, but not significantly, increased compared with the basal control, and glucose-stimulated GLP-1 release was markedly reduced at all of the tested glucose concentrations.

The effects of forskolin, which served as a positive control, were not altered by chronic exposure to high glucose levels.

In contrast to GLP-1 secretion, total GLP-1 content was similar in cells cultured in the presence or absence of chronically high glucose (Fig. 1*B*).

Chronic high-glucose treatment modified neither glucose transporter expression nor glucose uptake. To evaluate the primary elements involved in intestinal sugar transport in our model, we analyzed the expression of SGLT1 and glucose transporters 1 and 2 (GLUT1 and GLUT2), which are localized to the apical surface and basolateral membrane of L-cells in vivo, respectively (38, 48) (Fig. 2*A*). Western blot demonstrated that both types of transporters were expressed in our cells and that there were no significant differences in the abundance of these transporters between the control and high-glucose-treated cells.

To further investigate the intestinal sugar transport and to exclude this involvement in impaired GLP-1 secretion, we assayed glucose uptake in our model. As shown in Fig. 2*B*, in both groups (control and high glucose) 2-NBDG, a nonmetabolizable glucose analog, was internalized into L-cells in a dose-dependent manner, without any significant difference between the control and high-glucose groups.

Chronic high-glucose treatment reduced ATP production. Because GLP-1 secretion is also triggered by elevations in cytosolic ATP subsequent to metabolic fluxes, we analyzed the ATP concentrations in control cells and in cells that had been preexposed to high glucose levels (19.4 mmol/l for 72 h) (Fig. 3).

In the control group, 0.5, 5, and 25 mmol/l glucose acutely stimulated ATP production in a dose-dependent manner [fold changes of 1.8 ± 0.3 ($P < 0.001$), 2.2 ± 0.3 ($P < 0.001$), and 2.3 ± 0.2 ($P < 0.001$), respectively] compared with the ATP production levels observed in the absence of acute glucose stimulation.

In cells that had been preexposed to 19.4 mmol/l glucose for 72 h, the basal ATP levels were slightly, but not significantly, higher than those in control cells; furthermore, glucose-stimulated ATP production was markedly reduced at all of the tested glucose concentrations. Accordingly, after stimulation with increasing glucose concentrations (0.5, 5, or 25 mmol/l), the ATP levels did not increase further above the baseline level in cells that had been chronically exposed to high glucose levels.

Fig. 2. Effects of preexposure to high glucose levels (19.4 mmol/l for 72 h) on protein expression of glucose transporters and glucose uptake in GLUTag cells. *A*, *top*: representative Western blots for glucose transporters 1 and 2 (GLUT1 and GLUT2), sodium-dependent glucose cotransporter 1 (SGLT1), and actin in control cells (*left*) and in cells that had been exposed to high glucose (*right*). *Bottom*: corresponding densitometric analysis. Data are expressed as means ± SE (paired 2-sample *t*-test, $n = 5$). *B*: quantification of glucose uptake using the nonmetabolizable glucose analog 2-deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-D-glucose (2-NBDG). After 72 h of incubation in either 5.6 or 19.4 mmol/l glucose, GLUTag cells were washed and cultured for 30 min in Krebs-Ringer buffer under the indicated conditions; subsequently, the fluorescence intensity of 2-NBDG was determined (n.s., paired 2-sample *t*-test, $n = 4$).

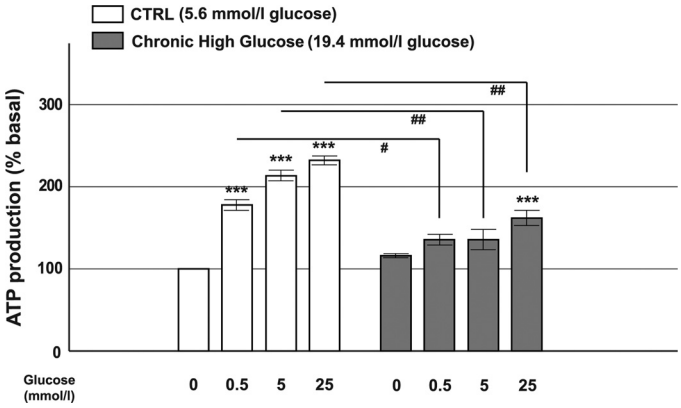


Fig. 3. Effects of preexposure to high glucose levels (19.4 mmol/l for 72 h) on glucose-stimulated ATP production. After 72 h of culture in either 5.6 or 19.4 mmol/l glucose, GLUTag cells were washed and cultured in Krebs-Ringer buffer under the indicated conditions. Subsequently, cellular ATP concentrations were measured (baseline production rate in 0 mmol/l glucose: 13 ± 0.9 μM). ***$P < 0.001$ vs. basal level in control group; #$P < 0.05$ vs. control group; ##$P < 0.01$ vs. control group (1-way ANOVA followed by Bonferroni test, $n = 4$).

Chronic high-glucose treatment increased L-lactate production. Reduced ATP levels can result from a number of events that impact metabolism; we examined the rate of glycolysis in our model to explore the causes of impaired ATP synthesis and to identify the metabolic step affected by high-glucose treatment. Because lactate constitutes the end product of glycolysis, we evaluated L-lactate production in GLUTag cells chronically treated with high levels of glucose (19.4 mmol/l for 72 h). As shown in Fig. 4, in both groups (control and high glucose) lactate was released after stimulation with increasing glucose concentrations in a dose-dependent manner, and in the high-glucose-treated cells the enhancement of lactate production was greater than that in control cells.

Chronic high glucose decreased mitochondrial membrane potential. Because the energy to drive ATP formation is provided by a proton gradient across the inner mitochondrial membrane, we measured glucose-induced changes in $\Delta\Psi_m$. In control cells, when the glucose concentration was increased to 10 mmol/l, fluorescence decreased (−9.6 ± 0.1%, $n = 3$), indicating the glucose-induced hyperpolarization of $\Delta\Psi_m$ (Fig. 5*A*). Cells chronically treated with high levels of glucose (19.4 mmol/l for 72 h) showed decreased hyperpolarization of $\Delta\Psi_m$ when the glucose level was increased to 10 mmol/l (−6.5 ± 0.54%, $n = 3$, $P < 0.001$) (Fig. 5*B*). The addition of the uncoupler FCCP at a concentration of 1 μmol/l readily depolarized $\Delta\Psi_m$ in both experimental conditions.

Glibenclamide abolished high glucose-induced GLP-1 secretion defect. To definitively confirm that mitochondria, ATP, and, consequently, K^+_{ATP} channels represent key components of

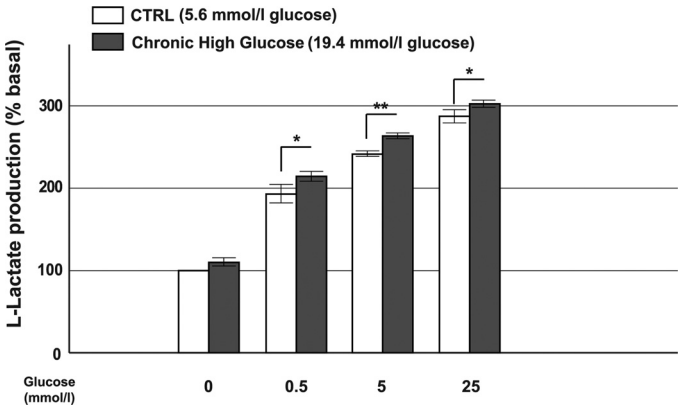


Fig. 4. Effects of preexposure to high glucose levels (19.4 mmol/l for 72 h) on glucose-stimulated lactate production. After 72 h of culture in either 5.6 or 19.4 mmol/l glucose, GLUTag cells were washed and cultured in Krebs-Ringer buffer under the indicated conditions. Subsequently, L-lactate production was measured. *$P < 0.05$, **$P < 0.01$ (1-way ANOVA followed by Bonferroni test, $n = 3$).

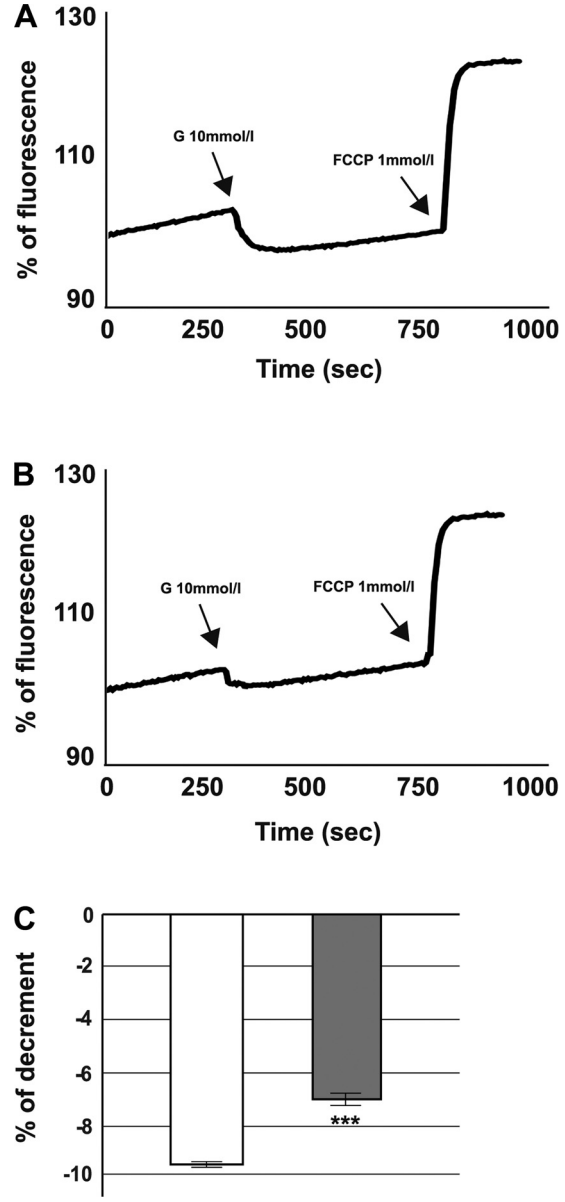


Fig. 5. Glucose-induced mitochondrial membrane potential changes ($\Delta\Psi_m$) in cells preexposed to high glucose levels (19.4 mmol/l for 72 h). *A* and *B*: glucose (G; 10 mmol/l) induced hyperpolarization in GLUTag cells cultured in either 5.6 (*A*) or 19.4 (*B*) mmol/l glucose. Results are expressed as % of basal fluorescence. *C*: summary of $\Delta\Psi_m$ over the basal level in glucose-induced hyperpolarization of mitochondrial membrane potential in control (open bar) and high-glucose-treated (gray bar) cells. Results are % decrease under basal fluorescence. ***$P < 0.001$ (paired 2-sample *t*-test, $n = 3$).

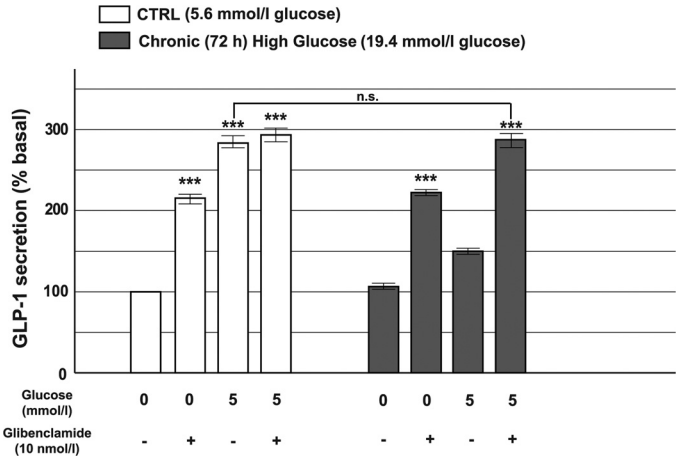


Fig. 6. Effects of glibenclamide on glucose-stimulated GLP-1 secretion in cells preexposed to high glucose levels (19.4 mmol/l for 72 h). After 72 h of culture in either 5.6 or 19.4 mmol/l glucose, GLUTag cells were washed and cultured in Krebs-Ringer buffer under the indicated conditions. Subsequently, the amount of GLP-1 released into the medium was measured (baseline secretory rate in 0 mmol/l glucose: 8.2 ± 0.8 pmol/l in 2 h). ***$P < 0.001$ vs. basal level in control group (1-way ANOVA followed by Bonferroni test, $n = 3$).

the impaired GLP-1 secretory pathway, we examined the effect of acute stimulation with the K^+_{ATP} channel inhibitor glibenclamide on control cells and on cells that had been preexposed to high glucose levels (19.4 mmol/l for 72 h) (Fig. 6). We measured the acute effect of this sulfonylurea at two glucose concentrations (0 and 5 mmol/l). In contrast to acute glucose-induced GLP-1 secretion, glibenclamide-induced secretion was maintained in cells that were cultured in high-glucose conditions.

Chronic high-glucose treatment increased UCP2 mRNA and protein expression. To investigate the pathways involved in mitochondrial energy metabolism, we analyzed the UCP2 transcript and protein levels in control cells and in cells that had been chronically exposed to high glucose (19.4 mmol/l for 72 h) (Fig. 7).

Our data showed a significant ($n = 3$, $P < 0.01$) 1.5-fold increase in *Ucp2* mRNA expression in high-glucose-treated cells compared with matched control cells, as indicated by the increase in $2^{-\Delta\Delta C_t}$ (where C_t is threshold cycle) based on real-time RT-PCR (Fig. 7*A*). UCP2 protein expression was also significantly increased in cells that were exposed to high glucose compared with control cells ($n = 4$, $P < 0.001$), as evidenced by Western blot (Fig. 7*B*).

Ucp2 knockdown reversed GLP-1 secretion and ATP production defects induced by chronic high glucose. To determine whether the impairments induced by chronic high glucose resulted from UCP2 overexpression, we evaluated the effect of UCP2 inhibition (Fig. 8); we analyzed GLP-1 secretion and ATP production in cells treated with *Ucp2* siRNA and cultured in the presence or absence of chronically high glucose levels. The UCP2 mRNA and protein levels after *Ucp2* siRNA transfection were analyzed via qRT-PCR and Western blot analysis, respectively (Fig. 8).

Ucp2 silencing reversed the impairments induced by chronic high glucose exposure; in these cells, glucose-stimulated GLP-1 release and ATP production were increased compared with cells that were cultured in high glucose alone and trans-

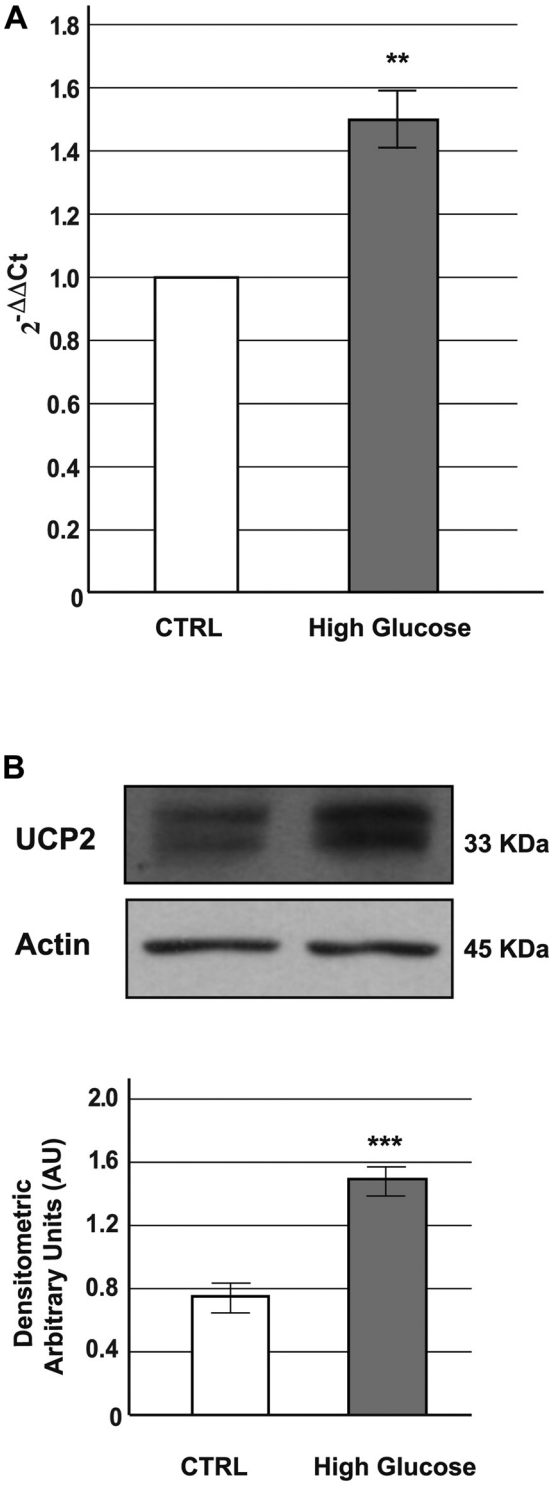


Fig. 7. Effects of preexposure to high glucose levels (19.4 mmol/l for 72 h) on uncoupling protein 2 (UCP2) mRNA and protein expression in GLUTag cells. *A*: after 72 h of culture in either 5.6 or 19.4 mmol/l glucose, *Ucp2* mRNA expression was analyzed via real-time RT-PCR. Data are presented as $2^{-\Delta\Delta C_t}$, where C_t is threshold cycle; *Ucp2* gene expression in GLUTag control cells was normalized to 1. Hypoxanthine guanine phosphoribosyl transferase (*Hprt*) was used as the reference gene. **$P < 0.01$ (paired 2-sample *t*-test, $n = 3$). *B*: after 72 h of culture in either 5.6 or 19.4 mmol/l glucose, UCP2 protein expression was measured via Western blotting. *Top*: representative Western blot for UCP2 and actin in control cells (*left*) and in cells that were exposed to high glucose (*right*). *Bottom*: densitometric analyses. Data are presented as means ± SE. ***$P < 0.001$ vs. control group (paired 2-sample *t*-test, $n = 4$).

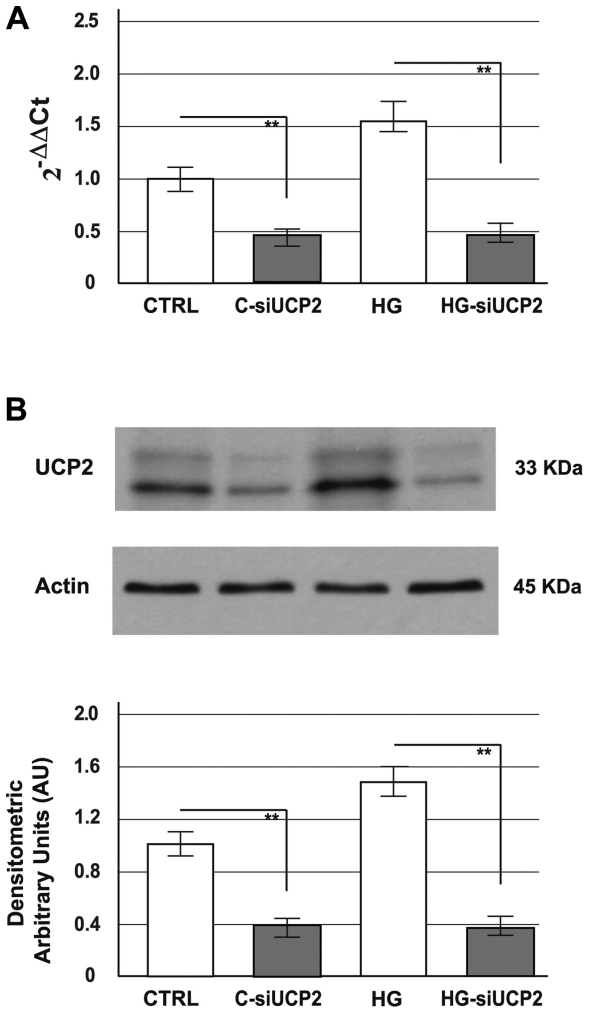


Fig. 8. UCP2 mRNA and protein expression in GLUTag cells treated with *Ucp2* small interfering (siRNA) and cultured in the presence or absence of chronically high glucose levels (19.4 mmol/l for 72 h). GLUTag cells transfected with siRNA at a final concentration of 50 nM displayed a *Ucp2* knockdown efficiency of ~60% in GLUTag cells under control conditions (C) and 66.7% under conditions of high glucose exposure (HG) as determined by real-time RT-PCR (*A*; $^{**}P < 0.01$, paired 2-sample *t*-test, $n = 3$) and Western blot analysis (*B*; $^{**}P < 0.01$, paired 2-sample *t*-test, $n = 3$).

fected with a scrambled siRNA sequence at all examined glucose concentrations (Fig. 9, *A* and *B*, respectively).

DISCUSSION

In this study, we provide evidence that chronic exposure to high glucose significantly impairs the acute glucose-induced secretion of GLP-1.

In addition, we demonstrate for the first time that simulated hyperglycemia induces UCP2 overexpression, which causes a decrease in ATP synthesis, resulting in the impairment of GLP-1 secretion in GLUTag cells.

Hyperglycemia in T2DM patients is toxic to many tissues and cell types, including insulin-secreting beta cells, and causes decreased secretory capacity. Specifically, acute glucose exposure physiologically stimulates insulin secretion from pancreatic beta cells; alternatively, long-term glucose exposure has been reported to impair beta cell function and survival. This condition, referred to as "glucotoxicity," has been clearly associated with a dramatic reduction in glucose-stimulated insulin secretion both in vitro and in vivo (2, 43, 54). Notably, this dysfunctional insulin secretion can be reversed by treatments that reduce glucose levels.

Because plasma GLP-1 levels have been reported to be decreased in T2DM patients (50), glucotoxicity likely contributes to defective GLP-1 secretion in T2DM and to a GLP-1 secretory response that progressively diminishes with increasing diabetes severity. Although it has been widely observed that nutrients and hormones are necessary and sufficient to regulate GLP-1 secretion, whether and through what mechanism chronic exposure to these factors affects L-cell function remains unclear. Existing data are lacking and also somewhat controversial (8, 27, 45, 52). Furthermore, whether glucotoxicity affects the responsiveness of L-cells to acute glucose stimuli remains unclear.

The GLUTag cell line, used as a model of enteroendocrine L-cells in this study, is a stable, immortalized relatively differentiated murine enteroendocrine cell line that expresses the proglucagon gene and that secretes glucagon-like peptides in a regulated manner (6, 26). GLUTag cells appear to be highly differentiated and recapitulate the responsiveness of primary nontransformed cultured rat intestinal cells to physiological and pharmacological secretagogues (6, 13). The GLUTag cell line is one of the best models for studying L-cells because native L-cells are very scarce and dispersed as individual cells along the gastrointestinal tract and cannot be isolated to provide homogenous L-cell cultures (22).

Our results demonstrate that glucotoxicity induces a massive decrease in GLP-1 secretion, suggesting that hyperglycemia may underlie the reduced GLP-1 plasma levels that we observed in T2DM. Furthermore, we report that these effects were associated with impaired ATP synthesis, which resulted from the inability of L-cells to increase ATP production in response to acute glucose stimulation. To investigate the molecular mechanisms underlying glucotoxicity induced by simulated hyperglycemia, we first studied UCP2 expression in the presence or absence of chronic high-glucose treatment, as increased UCP2 activity in response to hyperglycemia or hyperlipidemia has been reported to mediate impaired ATP synthesis and insulin release in insulin-producing beta cells. In addition to observing an increase in UCP2 expression, we effectively reduced these effects of glucotoxicity using specific siRNAs targeting *Ucp2*. Our results appear to indicate that in our model UCP2 plays a role in ATP production and modulates GLP-1 secretion; moreover, UCP2 activity has previously been shown to negatively regulate glucose-induced GLP-1 secretion in L-cells (9, 59) and to modulate GLP-1 release in the mouse gastrointestinal tract (58).

To further characterize the defect in GLP-1 secretion and, in particular, to identify the step of the metabolic pathway at which high-glucose treatment affected ATP production, we analyzed L-lactate production in our model. As previously observed in pancreatic islets harboring defects in mitochondrial oxidative phosphorylation, in which increased glycolytic lactate synthesis has been observed (35, 37), in our model high-glucose treatment caused enhanced lactate synthesis. The observed increase in glycolytic metabolism could represent an attempt to partially compensate for the reduction in ATP production; however, the amount of ATP production originating from the glycolytic process was extremely insufficient to

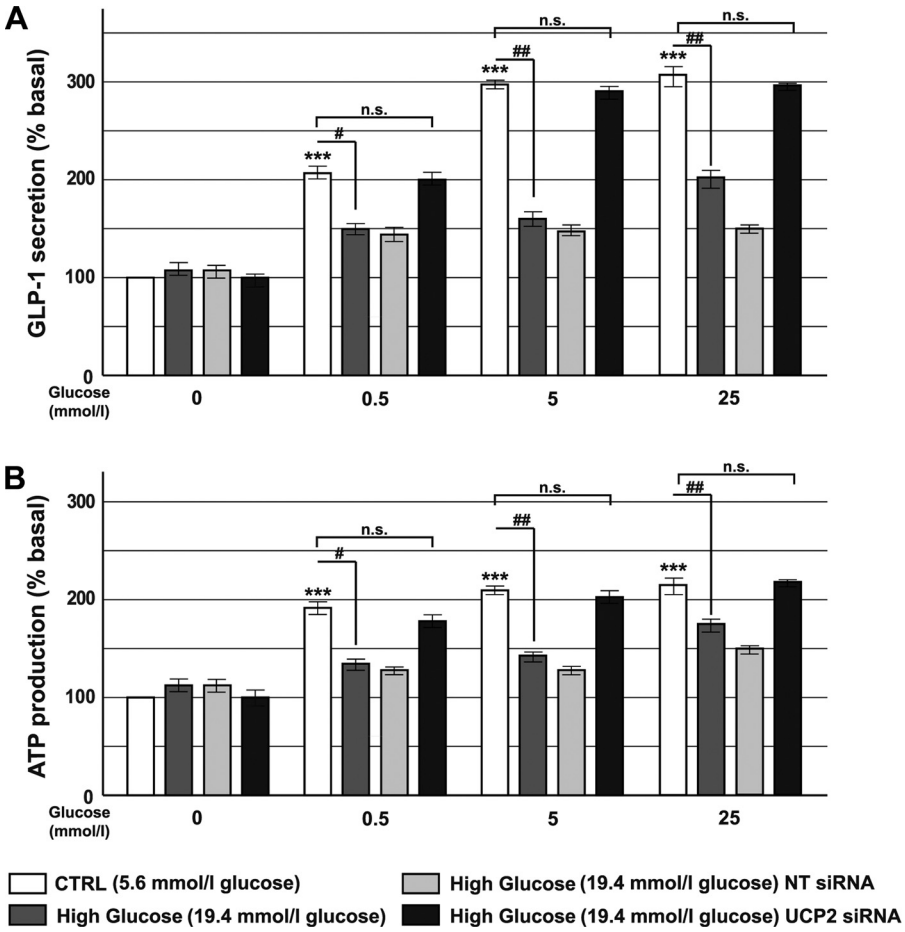


Fig. 9. Effects of *Ucp2* silencing on glucose-stimulated GLP-1 secretion and ATP production in cells preexposed to high glucose levels (19.4 mmol/l for 72 h). Cells were treated with *Ucp2* siRNA and cultured in the presence or absence of chronically high glucose levels for 72 h. Transfected cells (NT, nontargeting control siRNA) were cultured in Krebs-Ringer buffer under the indicated conditions. Subsequently, GLP-1 release (baseline secretory rate in 0 mmol/l glucose: 8.0 ± 0.9 pmol/l in 2 h, $n = 3$) (*A*) and ATP concentrations (baseline production rate in 0 mmol/l glucose: 11.5 ± 0.8 μM) (*B*) were measured ($n = 3$). $^{***}P < 0.001$ vs. basal level in control group; $^{\#}P < 0.05$ vs. control group; $^{\#\#}P < 0.01$ vs. control group (1-way ANOVA followed by Bonferroni test).

compensate for the mitochondrial defect. Moreover, this result ultimately indicates that reduced ATP levels did not result from reduced rates of glycolysis but rather from other events that impact metabolism downstream of glycolytic flux. According to these data, glucose-induced hyperpolarization of the mitochondrial membrane was reduced in cells treated with high levels of glucose. Therefore, by lowering the redox pressure on the mitochondrial respiratory chain, UCP2 negatively controls ATP synthesis and GLP-1 release from intestinal L-cells.

Although it has been observed previously that electrogenic glucose uptake via apically localized SGLT1 is the major glucose-sensing mechanism in L-cells (46), our results indicate that rather than glucose detection or transport, intracellular glucose metabolism and consequent ATP production play a significant role in GLP-1 secretion. In fact, our data indicate that glucotoxicity did not affect the expression of transporters involved in sugar-induced GLP-1 secretion or glucose uptake but rather caused evident impairments in ATP production.

Previous studies have confirmed that the K^+_{ATP} channel subunits $K_{ir}6.2$ and SUR1, in addition to glucokinase, are highly expressed in purified mouse L-cells and that these proteins are also expressed in human cells as evidenced by immunostaining (38, 49). Electrophysiological and secretion studies have demonstrated that K^+_{ATP} channels are functional in murine L-cells and that sulfonylureas can stimulate GLP-1 secretion from primary colonic cultures (49). Accordingly, we found that applying the specific K^+_{ATP} channel blocker glibenclamide abolished the effect of glucotoxicity on GLP-1 secretion, demonstrating the importance of K^+_{ATP} channels and the requirement for appropriate levels of ATP production in this model. These observations strengthen the evidence supporting a relevant role of glucose metabolism in responsiveness to acute glucose stimulation and unequivocally indicate that, as in beta cells (42), the ATP levels and K^+_{ATP} channels are central components of the GLP-1 secretory pathway in L-cells.

Taken together, our results indicate that the loss of the incretin effect observed in diabetes patients may be, at least in part, secondary to glucotoxicity and to the related metabolic machinery defect. In particular, the present study provides novel information regarding the molecular mechanism underlying glucotoxicity and demonstrates that glucotoxicity can be counteracted by UCP2 inhibition and by directly acting on K^+_{ATP} channels in GLP-1-secreting cells. Moreover, our data show many similarities between pancreatic beta cells and intestinal L-cells and reveal interesting new areas of research.

Because GLP-1 is currently used for the treatment of T2DM, it has become crucial to focus on L-cells and on the possible stimulation or control of endogenous GLP-1 release. Enhancing endogenous GLP-1 secretion may serve as a novel and more physiological strategy for incretin-based diabetes therapy. This approach requires an understanding of the mechanisms underlying normal GLP-1 release from L-cells and of all of the factors that could be responsible for modifying this perfectly equilibrated system. In addition to the development

of GLP-1 analogs and DPP-4 inhibitors, the identification of novel molecular targets for improving and enhancing endogenous GLP-1 secretion may represent a potential third approach to treat T2DM via incretin activity.

ACKNOWLEDGMENTS

F. Purrello and S. Piro are the guarantors of this work; as such, they have full access to all data from this study and take responsibility for the integrity of the data and the accuracy of the data analysis.

GRANTS

This work was supported by a grant from Ministero Università e Ricerca Scientifica (2011), Società Italiana di Diabetologia (SID), and Diabete e Ricerca FO.DI.RI.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: F.U. and S.P. conception and design of research; F.U., A.F., D.B., S.D.M., and A.P. performed experiments; F.U., A.F., A.D.P., and D.B. analyzed data; F.U., A.F., and S.P. interpreted results of experiments; F.U. prepared figures; F.U. and A.D.P. drafted manuscript; A.M.R., M.P., F.P., and S.P. edited and revised manuscript; F.U., A.F., A.D.P., D.B., S.D.M., A.P., A.M.R., M.P., F.P., and S.P. approved final version of manuscript.

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