Effects of exposure of human islet beta-cells to normal and high glucose levels with or without gliclazide or glibenclamide

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Abstract

Aim. – To evaluate the effects of exposure to high glucose (HG) levels and sulphonylurea on isolated human islet-cell function, and to investigate some of the mechanisms that might be involved.

Methods. – Islet cells were isolated, using collagenase digestion and gradient purification, from 13 pancreata from non-diabetic multiorgan donors (age: 61.2 ± 11.5 years; gender: 7 men/6 women; body mass index: 25.1 ± 2.8 kg/m²). The cells were then cultured for 5 days with normal glucose (NG) concentrations (5.5 mmol/L), or NG and HG (16.7 mmol/L) levels (alternating every 24 h), with or without the addition of therapeutic concentrations of gliclazide (10 μmol/L) or glibenclamide (1.0 μmol/L). At the end of incubation, functional (glucose-stimulated insulin secretion), morphological (electron microscopy) and molecular (gene and protein expression) studies were performed.

Results. – Insulin secretion differed significantly between study groups, with marked decreases in the presence of HG plus glibenclamide. Compared with NG, insulin expression decreased significantly with HG, and increased similarly with gliclazide as with glibenclamide. However, exposure to gliclazide, but not glibenclamide, significantly induced expression (at both gene and protein levels) of PDX-1, a fundamental beta-cell differentiation transcription factor, and Ki67, a marker of proliferation. However, gliclazide and glibenclamide did not differ in terms of effects on gene expression of the antiapoptotic molecule Bcl2 (increased significantly with both) and the proapoptotic molecule Bax (decreased significantly with both).

Conclusion. – Gliclazide and glibenclamide have different effects on the changes induced by prolonged exposure of human islet cells to high levels of glucose.

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Keywords: Pancreatic islets; Gliclazide; Glibenclamide; PDX-1; Ki67

Résumé

Exposition intermittente de cellules β insulaires humaines à des concentrations élevées en glucose. Effets du gliclazide et du glibenclamide.

Objectif. – Évaluer les interactions entre une exposition intermittente à des concentrations élevées en glucose et des sulfonylurées sur les différentes fonctions d’îlots humains isolés et étudier certains des mécanismes impliqués.

Méthodes. – Treize pancréas de donneurs multi-organes non diabétiques (7 M/6 F ; âge : 61,2 ± 11,5 ans ; IMC : 25,1 ± 2,8 kg/m²) ont été utilisés. L’isolation des îlots a été réalisée par digestion à la collagénase et gradient de purification. Les îlots ont cultivés pendant cinq jours en présence soit de concentrations en glucose stables et normales (NG : 5,5 mmol/L), soit d’une alternance toutes les 24 heures de concentrations en glucose normales et élevées (HG : 16,7 mmol/L), en présence ou non de concentrations thérapeutiques de gliclazide (10 μmol/L) ou de glibenclamide (1,0 μmol/L). En fin d’incubation, ont été réalisées des études fonctionnelles (insulinosécrétion stimulée par le glucose), morphologiques (étude en microscope électronique) et moléculaires (expression des gènes et des protéines).

Résultats. – La sécrétion d’insuline était significativement différente entre les groupes, avec une diminution plus marquée en présence de HG plus glibenclamide. En comparaison avec NG, l’expression de l’insuline a diminué significativement en présence de HG, et a augmenté de manière similaire avec le gliclazide et le glibenclamide. La culture en présence de gliclazide, mais non de glibenclamide, a induit de manière significative l’expression (à la fois du gène et de la protéine) de PDX-1, facteur fondamental de transcription et différenciation des cellules β, et de Ki67, marqueur

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de prolifération. Il n’y avait pas de différence entre gliclazide et glibenclamide concernant l’expression du gène de la molécule anti-apoptotique Bcl2 (augmentation significative avec les deux sulfonylurées) ni de la molécule pro-apoptotique Bax (diminution significative avec les deux).

Conclusion. – Le gliclazide et le glibenclamide ont des effets différents sur les modifications induites par une exposition prolongée de cellules d’îlot humaines à des concentrations élevées en glucose.

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\textbf{Mots clés :} Îlots pancréatiques humains ; Gliclazide ; Glibenclamide ; PDX-1 ; Ki67

\section{1. Introduction}

Type 2 diabetes mellitus is the most common form of diabetes, and its prevalence and associated morbidity continue to increase worldwide \cite{1}. Several algorithms and treatment regimens have been proposed to achieve a reasonable degree of glycemic control and to prevent—or at least delay—the onset of diabetic vascular complications \cite{2–4}. Gliclazide, a second-generation sulphonylurea, is widely used in the treatment of type 2 diabetes \cite{5,6}. The molecule reduces blood glucose levels by increasing insulin secretion from pancreatic beta-cells through interaction with the sulphonylurea receptor (SUR1) of the K\textsubscript{ATP} channel \cite{7}. This interaction is characterized by high affinity and strong selectivity \cite{8,9}. Because of the presence of an aminoazabicyclo-octyl ring in its chemical structure, gliclazide also has antioxidant properties that are independent of its effects on glucose, which might explain the beneficial effects of the drug beyond its antihyperglycaemic activity \cite{10–13}.

Gliclazide appears to have a direct beneficial action on pancreatic beta-cells. It has been reported that gliclazide can protect beta-cells from the deleterious effects of reactive oxygen species \cite{14}. The present authors have shown that the molecule can counteract, at least in part, the damage to isolated human pancreatic islet cells caused by prolonged exposure to high glucose (HG) levels, and that the beneficial action of the drug at the beta-cell morphological and survival levels was associated with reduced oxidative stress \cite{15}. The present study investigated the additional molecular mechanisms involved in gliclazide action, focusing in particular on beta-cell ultrastructural features and genes, as well as on the protein expression of molecules involved in beta-cell insulin secretion and turnover.

\section{2. Materials and methods}

\subsection{2.1. Islet preparation and culture}

Islet cells were isolated by collagenase digestion and density gradient purification, as described elsewhere \cite{15–17}, from the pancreata of 13 human multiorgan donors (age: 61.2 ± 11.5 years; gender: 7 men/6 women; body mass index: 25.1 ± 2.8 kg/m\textsuperscript{2}), according to protocols approved by our local ethics committee. Aliquots containing approximately 100 islet cells were incubated for 5 days under the following conditions:

1. Control M199 medium containing normal (5.5 mmol/L) glucose (NG);
2. Medium containing NG or HG (16.7 mmol/L), alternating every 24 h;
3. Medium as in (1) and (2), but with the addition of 10 \(\mu\)mol/L of gliclazide;
4. Medium as in (1) and (2), but with the addition of 1 \(\mu\)mol/L of glibenclamide.

All sulphonylurea concentrations used were in the therapeutic range \cite{18,19}. The use of intermittent HG was based on the assumption that such a condition would better represent the fluctuating glucose concentrations seen in vivo in diabetic patients, as already discussed and applied elsewhere \cite{20}. At the end of the incubation period, functional, morphological and molecular studies were performed as described below.

\subsection{2.2. Insulin-secretion studies}

In these studies \cite{15–17}, islet cells were initially kept at 37 °C for 45 minutes in Krebs–Ringer bicarbonate (KRB), with 0.5% (vol/vol) albumin, pH 7.4 and containing 3.3 mmol/L of glucose (washout phase). The medium was then replaced with KRB containing 3.3 mmol/L of glucose to assess basal insulin secretion (45 minutes), followed by a further 45 minutes incubation with 16.7 mmol/L of glucose to assess insulin response to the acute challenge. Insulin was quantified using an immunoradiometric assay (Pantec Forniture Biomediche, Turin, Italy).

\subsection{2.3. Electron-microscopy evaluation}

Electron-microscopy studies, as well as morphological and morphometric analyses, were performed as described elsewhere \cite{15–17}. Insulin granules were identified by their dense cores and white haloes \cite{17}. Volume-density (VD) values were derived from evaluations of 3–5 different islet cells from each pancreas, with 12 photomicrographs of each cell at an original magnification of \(\times 7000\). Negatives were printed and enlarged to a final magnification of \(\times 16000\). The cytoplasm was used as the reference area. A graticule (11 × 11 cm) composed of 169 points was superimposed on the photomicrographs to count the number of points that intersected with the insulin granules. The VD of the granules was then calculated according to the formula \(\text{VD} = \text{Pi}/\text{Pt}\), where Pi is the number of points within the subcellular component and Pt is the total number of points, expressed in mL/100 mL of tissue (mL\% \cite{15–17}.

\subsection{2.4. Molecular studies}

Messenger RNA expression of several genes was measured by reverse transcription polymerase chain reaction (RT-PCR), followed by real-time quantitative RT-PCR. The procedure used
by our laboratory has been reported in detail elsewhere [21]. In brief, total RNA was extracted from islet cells using the RNeasy Protect Mini Kit (QIAGEN). Total RNA was quantified by absorbance at \(A_{260}/A_{280}\) (ratio > 1.65) nm by a Perkin–Elmer spectrophotometer, and its integrity assessed after electrophoresis in 1.0% agarose gels by ethidium–bromide staining. For quantitative RT-PCR, the genes of interest were obtained from the US-based GenBank (National Center of Biotechnology Information, NCBI), while the primer and probe sequences for the genes of interest were quantified by a densitometer (GS 690, BioRad Laboratories). Bands of interest were quantified by a densitometer (GS 690, BioRad Laboratories).

### 3. Results

#### 3.1. Insulin-secretion studies

Islet cells cultured for 5 days with NG (5.5 mmol/L) concentrations released 24.3 ± 4.2 and 75.2 ± 16.3 \(\mu\text{U/mL}\) of insulin when acutely challenged with 3.3 and 16.7 mmol/L of glucose, respectively (\(P<0.01\), with a stimulation index (insulin secretion at 16.7 mmol/L of glucose over insulin secretion at 3.3 mmol/L of glucose) of 3.1 ± 0.4. As shown in Table 1, cultures exposed to alternating NG (5.5 mmol/L) and HG (16.7 mmol/L) levels showed significant increases of insulin release in response to 3.3 mmol/L of glucose (\(P<0.01\) by ANOVA), whereas insulin secretion in response to 16.7 mmol/L of glucose was not significantly affected. This led to significant changes in stimulation index values (\(P<0.01\) by ANOVA; Table 1), which resulted, however, in significantly higher values (after Bonferroni correction) in the presence of gliclazide (Table 1).

#### 3.2. Electron-microscopy results

In this part of the study, insulin granules were quantified in the beta-cells following incubation under the 4 different study conditions. The amount of granules differed significantly between groups (\(P<0.05\) by ANOVA):

- 4.9 ± 1.6 mL% in beta-cells with NG concentrations (control cells);
- 2.2 ± 1.0 mL% in cells alternately exposed to NG and HG (\(P<0.05\) vs control cells);
- 3.3 ± 1.4 mL% in beta-cells cultivated with HG plus glibenclamide;
- 3.3 ± 1.5 mL% in those maintained with HG plus glibenclamide (neither significant vs control cells).

#### 3.3. Molecular studies

Insulin gene expression decreased significantly in islet cells maintained with HG and was 34 ± 4% of the value of the control cells (\(P<0.01\) with Bonferroni correction). Incubation with HG plus glibenclamide (244.0 ± 23% of control value; \(P<0.05\)) or glibenclamide (219 ± 23% of control value; \(P<0.05\)) resulted in similar increases in insulin gene expression. As shown in Fig. 1, gene and protein expressions of the differentiation factor PDX-1 tended to be lower in the cells exposed to alternating NG and HG compared with control cells, but did not achieve statistical significance. However, the presence of gliclazide—but not glibenclamide—in the HG medium resulted in a significant increase in PDX-1 expression at both gene and protein levels (Fig. 1).

Similar findings were observed for Ki67, a protein associated with cell regeneration (Fig. 2): its gene and protein expressions tended to decrease after incubation with alternating NG and HG, and were significantly induced by gliclazide, but not glibenclamide. In addition, culture of isolated cells in HG led to a significant decrease (\(P<0.05\)) in gene expression of the antiapoptotic protein Bcl2 (–37 ± 9%) and a significant increase (\(P<0.05\)) in transcription of the proapoptotic protein Bax (+26 ± 9%). The addition of gliclazide and of glibenclamide

<table>
<thead>
<tr>
<th>Control islet cells</th>
<th>HG</th>
<th>HG + glib</th>
<th>HG + glicl</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.3 mmol/L of glucose*</td>
<td>24.3 ± 4.2</td>
<td>37.2 ± 10.4***</td>
<td>41.2 ± 8.8***</td>
</tr>
<tr>
<td>16.7 mmol/L of glucose</td>
<td>75.2 ± 16.3</td>
<td>66.3 ± 21.1</td>
<td>62.3 ± 14.2</td>
</tr>
<tr>
<td>Stimulation index*</td>
<td>3.1 ± 0.4</td>
<td>1.8 ± 0.2***</td>
<td>1.5 ± 0.2***</td>
</tr>
</tbody>
</table>

Stimulation index: ratio of insulin release with 16.7 mmol/L over 3.3 mmol/L of glucose. *: \(P<0.01\) for all values in the row by ANOVA; **: \(P<0.05\) (with Bonferroni correction) versus control cells; ***: \(P<0.05\) (with Bonferroni correction) versus HG + glib; ****: \(P<0.05\) (with Bonferroni correction) versus control cells and HG + glib.
Fig. 1. Gene and protein expressions of PDX-1 in islet cells cultured with normal glucose (NG) or alternating NG and high glucose (HG) concentrations, with or without the presence of gliclazide (glicl) or glibenclamide (glib). *P < 0.05 versus NG, HG and HG + glib; #P < 0.05 versus NG and HG.

Fig. 2. Gene and protein expressions of Ki67 in islet cells cultured with normal glucose (NG) or alternating NG and high glucose (HG) concentrations, with or without the presence of gliclazide (glicl) or glibenclamide (glib). *P < 0.05 versus NG and HG; #P < 0.05 versus NG.

to the HG medium brought about similar significant (P < 0.05) effects, inducing Bcl2 (+102 ± 27% and +82 ± 16% in the HG-exposed cells, respectively) and suppressing Bax (−40 ± 10% and −44 ± 9% in the HG-exposed cells, respectively) mRNA expression.

4. Discussion

The present study shows that alternating exposures to NG and HG induces a condition of lowered responsiveness of human beta-cells to an acute glucose challenge, as reflected by the reduced glucose stimulation index values. This is similar to what happens when islets are constantly exposed to HG, as previously reported in both static and dynamic insulin-release experiments [22,23]. However, while continuous glucose exposure may more profoundly affect insulin release in response to an acute HG challenge [22], alternating exposures can more significantly increase basal insulin release (present study). Clearly, continuous and intermittent HG exposures represent different experimental conditions that may have different effects. In INS-1 cells, more severe toxic effects were induced by intermittent versus sustained HG exposure [24]. In other models, such as human umbilical vein endothelial cells (HUVEC), similar degrees of apoptosis were observed after culture with constant and alternating HG [25].

In the present tests, functional alterations induced by prolonged exposure to HG were associated with fewer insulin granules and decreased expression of the insulin gene. However, proinsulin levels were not measured. Beta-cell degranulation following HG exposure has been reported by some [26], but not all studies [27], but the present study used electron-microscopy evaluation, which is an efficient tool for the assessment of beta-cell ultrastructure.

The deleterious effects of glucotoxicity on islet-cell gene expression have been reported by a number of studies, as recently reviewed [28], which showed reduced transcription of several genes, including that of insulin [28]. Glucotoxicity also has an influence on beta-cell mass. It has been found that the beta-cell apoptosis rate is increased in isolated human islet cells following culture with continuous HG exposure [29], and this was also observed with incubation with intermittent rises in glucose concentration [15]. In the present study, this was associated with increased expression of the proapoptotic molecule Bax and reduced transcription of the antiapoptotic protein Bcl-2. As changes at this level have also been reported after exposure of human islet cells to continuous HG [29], these molecules may be important for regulating death or survival of beta-cells injured by metabolic perturbations.

In addition, it has been reported that the increased beta-cell apoptosis induced by HG exposure is not accompanied by a compensatory increase in beta-cell proliferation or neogenesis [29]. Indeed, under the present experimental conditions, no changes were seen at the level of gene and protein expressions of PDX-1, a master beta-cell differentiation factor [30], nor of Ki67, a molecule associated with cell regeneration [31]. It has been reported that reduction of PDX-1 mRNA levels can occur in beta-cells cultured in the presence of HG, but this can take several weeks of incubation [32].

Furthermore, we assessed the impacts of gliclazide and glibenclamide at therapeutic concentrations. At the beta-cell glucose-stimulated insulin-secretion level, gliclazide’s deleterious effects were less marked, as suggested by a better-maintained stimulation index value. Interestingly, however, both agents induced transcription of insulin and maintained insulin-granule counts, thus counteracting the inhibitory action of HG exposure. A similar effect of glibenclamide on insulin mRNA expression has been described in rodent islets [33], whereas the drug does not appear to affect the transcription of other molecules involved in beta-cell function, such as GLUT2, or the Kir6.2 or SUR-1 subunits of the KATP channel [34,35].
We have previously reported that alternating exposures to NG and HG can lead to increased beta-cell apoptosis and that the addition of glibenclamide can further potentiate beta-cell death [15]. In contrast, gliclazide—probably due to its antioxidant properties—may have a protective action on beta-cell survival [15]. Somewhat surprisingly, however, the present study showed that either agent could increase expression of the antiapoptotic molecule Bcl2 and similarly decrease expression of the proapoptotic protein Bax. Nevertheless, apoptosis is a highly regulated process that is dependent on the balance between several antiapoptotic and proapoptotic mechanisms. It is therefore possible that, under our experimental conditions, beta-cell apoptosis was due, at least in part, to a Bcl2-independent mechanism [36], or that differences might have occurred at the translational or functional level. Clearly, further studies are needed to clarify this issue.

Interestingly, in our study, gliclazide—but not glibenclamide—induced expression (at both mRNA and protein levels) of PDX-1 and Ki67 (molecules involved in beta-cell differentiation and regeneration, respectively), even in islet cells pre-exposed to HG. Although the issue of beta-cell regeneration in humans is a subject of intensive study, most questions still remain unanswered [37,38]. Our data suggest that gliclazide could have beneficial effects on beta-cell differentiation, as demonstrated in adipocytes [39]. In contrast, glibenclamide has been shown to have no or minimal action on beta-cell proliferation in rats [40].

In conclusion, this study confirms that islet cells intermittently exposed to HG levels have altered glucose-stimulated insulin secretion that, in the present study, was further—and differently—affected by the presence of gliclazide and glibenclamide. Both sulphonylureas induced insulin expression and a relative conservation of insulin granules. In addition, gliclazide, but not glibenclamide, induced gene and protein expressions of molecules involved in beta-cell differentiation and regeneration. This observation, together with the previously reported reduced beta-cell apoptosis in the presence of gliclazide [15], suggests that this agent could contribute to beta-cell mass maintenance. If so, this might explain the clinical observation [41] that gliclazide is associated with a lower rate of sulphonylurea treatment failure.

References


