Upregulation of β-cell genes and improved function in rodent islets following chronic glucokinase activation

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Abstract

Glucokinase (GK) plays a critical role in controlling blood glucose; GK activators have been shown to stimulate insulin secretion acutely both in vitro and in vivo. Sustained stimulation of insulin secretion could potentially lead to β-cell exhaustion; this study examines the effect of chronic GK activation on β-cells. Gene expression and insulin secretion were measured in rodent islets treated in vitro with GKA71 for 72 h. Key β-cell gene expression was measured in rat, mouse and global GK heterozygous knockout mouse islets (gk


isoform is an absolute requirement for survival, as gk


Introduction

Glucokinase (GK) exists as two isoforms that have independent tissue-specific promoters. In the pancreatic β-cell, GK acts as a glucose sensor and contributes to the regulation of glucose-stimulated insulin secretion (GSIS). The pancreatic isoform of GK is also found in glucose-sensing cells in the anterior pituitary gland, hypothalamus and entero-endocrine K- and L-cells (Schuit et al. 2001). The liver isoform is specific to hepatocytes, where it is the rate-limiting enzyme for glucose utilisation and glycogen synthesis (Matschinsky 1990, Schuit et al. 2001).

Naturally occurring mutations in man support the importance of GK in the regulation of glucose homeostasis. Loss of both GK alleles results in permanent neonatal diabetes, whereas heterozygous inactivating mutations of GK lead to maturity-onset diabetes of the young (MODY2; Hattersley et al. 1992). Conversely, rare activating mutations of GK result in hyperinsulinaemic hypoglycaemia (Glaser et al. 1998, Christesen et al. 2002, Glynn et al. 2003, Cuesta-Munoz et al. 2004).

Isoform-specific, tissue-specific and global GK knockout (gk


Of potential concern is that sustained stimulation of insulin secretion, without a concomitant increase in insulin biosynthesis, could lead to β-cell exhaustion.

The effect of glucose on the regulation of insulin production has been studied extensively, by direct
measurement of insulin biosynthesis and by measuring gene expression. Prolonged culture of isolated rodent islets in high glucose leads to increased insulin biosynthesis and increased insulin mRNA levels (Brunstedt & Chan 1982, Docherty & Clark 1994, Leibiger et al. 2000, Khaldi et al. 2004). Data generated with GKAs provide support for these molecules acting in a ‘glucose-like’ manner on insulin secretion; we hypothesised that GKAs would also mimic the effect of elevated glucose concentration on insulin biosynthesis. We describe the use of a potent GKA, GKA71, to determine the effect of concentration on insulin biosynthesis. We describe the effect of sustained GK activation on the expression of key β-cell genes, including insulin, in isolated rat and mouse islets.

Materials and methods

Materials

The novel GK activator GKA71 was dissolved in dimethylsulphoxide (DMSO) prior to dilution to the final concentration in the required medium.

Experimental animals and islet isolation

Animals used in these studies were supplied by AstraZeneca Biological Animal Breeding Unit and were kept under standard laboratory conditions with free access to food and water. Male C57Bl/6J mice were 8–10 weeks of age (22–24 g) and male Hannover Wistar rats 8–9 weeks of age (230–260 g). Male global heterozygous GK knockout (gkdel/wt) and wild-type (gkwt/wt) mice were used between 10 and 16 weeks of age; these animals were generated as described previously (Gorman et al. 2008). All procedures were carried out in accordance with the Animals (Scientific Procedures) Act 1986.

Animals were rendered insentient by inhalation of a 5:1 mixture of CO₂:O₂ and killed by cervical dislocation. Animals used in these studies were supplied by AstraZeneca Biological Animal Breeding Unit and were kept under standard laboratory conditions with free access to food and water. Male C57Bl/6J mice were 8–10 weeks of age (22–24 g) and male Hannover Wistar rats 8–9 weeks of age (230–260 g). Male global heterozygous GK knockout (gkdel/wt) and wild-type (gkwt/wt) mice were used between 10 and 16 weeks of age; these animals were generated as described previously (Gorman et al. 2008). All procedures were carried out in accordance with the Animals (Scientific Procedures) Act 1986.

Animals were rendered insentient by inhalation of a 5:1 mixture of CO₂:O₂ and killed by cervical dislocation prior to removal of the pancreas. Islet isolations were carried out as described previously (Johnson et al. 2007) by standard methods using liberase R1 (Roche).

Recombinant GK assays

Human, rat and mouse β-cell GK were cloned from cDNA using PCR. Each was expressed in Escherichia coli with an NH₂-terminal 6His tag and purified using Ni-NTA chromatography. Recombinant proteins were >95% pure as assessed by SDS/PAGE analysis (Brocklehurst et al. 2004). GK was assayed at its S₀5 glucose concentration as described previously (Brocklehurst et al. 2004).

Acute insulin secretion assay

Rat islets were allowed to recover overnight in RPMI medium supplemented with 10% (v/v) FCS, penicillin/streptomycin/glutamine (100 units/ml, 0-1 mg/ml and 2 mmol/l respectively, Invitrogen) and 11 mmol/l glucose. Insulin secretion was assessed according to the method described previously (Johnson et al. 2007); 30 min pre-incubation was followed by 2-h treatment. Insulin concentration was determined by homogeneous time-resolved fluorescence assay (HTRF assay, CisBio, Bognols/Cèze, France) according to the manufacturer’s high-range protocol (0–100 ng/ml).

Chronic treatment of islets

Islets were cultured in RPMI medium supplemented as above. Islets were incubated 20 islets/well in 6-well plates at 37°C in a 5% CO₂ humidified incubator.

Prior to chronic treatment, islets were allowed to recover overnight in medium containing the appropriate glucose concentration. Islets from C57Bl/6J mice and from rats were divided between media containing 5 or 25 mmol/l glucose. For experiments with gkwt/wt and gkdel/wt islets, islets from animals of the same genotype were pooled and then divided between 8 and 25 mmol/l glucose.

Following the overnight incubation, islets were hand-picked into medium at the same glucose concentration containing either 1% (v/v) DMSO (control) or 10 μmol/l GKA71 in 1% DMSO. Islets were treated for 72 h and were hand-picked into fresh treatment medium daily.

RNA isolation and reverse transcription

Following chronic culture, RNA was prepared from pooled samples of 30 (rat) or 40 (mouse) islets (see figure legends for individual experiment replicate numbers). RNA isolation and gene expression analysis were carried out as described previously (Gorman et al. 2008).

Gene expression analysis

Quantitative PCR of single genes was performed by Taqman analysis in 384-well plate assays using ABI Prism 7900HT sequence detection system (Applied Biosystems, Carlsbad, CA, USA). Taqman analysis of mouse samples was carried out using primer and probe sets designed using Primer Express software (TaqMan primer and probe sequences are shown in Supplementary Table 1, see section on supplementary data given at the end of this article). ‘Assay on demand’ primer/probe mixes (Applied Biosystems) were used for analysis of rat samples: insulin (Rn01774648), GLUT2 (Rn00563565), hypoxanthine phosphoribosyltransferase (HPRT) (Rn01527838), cyclophilin (Rn00690933), β-glucuronidase (Rn00566655), IAPP (Rn00561411),...
PDX1 (Rn00755591), GK (Rn00561265), PC1 (Rn00567266) and CEBPβ (Rn00824635). Samples from the C57Bl/6j chronic islet experiment were used to perform Taqman low-density arrays (TLDA), which were carried out as described previously (Gorman et al. 2008).

For both quantitative PCR and TLDA analysis, a number of housekeeper genes were used. For quantitative PCR analysis, the housekeeper genes were HPRT, cyclophilin and β-glucuronidase. For TLDA analysis, the included housekeeper genes were HPRT, 18S and β-tubulin. No qualitative differences were observed when the genes of interest were expressed relative to the different housekeepers; therefore, all data shown are normalised to HPRT expression.

Perifusion studies

Perifusion experiments were performed using a Brandel SF-12 Superfusion apparatus (Brandel, Gaithersburg, MD, USA). Following chronic culture, islets were loaded into perifusion chambers (50 islets/chamber in 200 μl medium), enclosed with polyethylene filters. Islets were perifused in HEPES-balanced Krebs-Ringer phosphate buffer (KRH), prepared as described previously (Johnson et al. 2007), at a flow rate 1 ml/min. During an initial stabilisation period (perifusion 30 min; 3 mmol/l glucose), no samples were collected. Islets were then perifused to obtain a GSIS profile: 30 min with 3 mmol/l glucose; 25 min with 15 mmol/l glucose; 17.5 min with 3 mmol/l glucose and 6 min with 40 mmol/l KCl, during which time samples were collected for analysis of insulin secretion. Insulin was determined using an HTRF kit, prepared according to the normal range protocol (0–10 ng/ml).

Total insulin measurement

Following chronic culture, three islets in 10 μl medium were transferred to individual wells of 96-well plates containing 115 μl KRH. Then, 115 μl 2% (v/v) Triton X-100 were added to each well, and the plates were frozen at −20 °C to completely lyse the islets. For determination of insulin content, samples were thawed, centrifuged (700 g, 2 min) and diluted as appropriate, prior to analysis using the high-range HTRF assay.

Western blotting

Following chronic culture, 500–900 islets from each treatment group were lysed in 50 μl lysis buffer: 20 mmol/l Tris–HCl (pH 7.5), 150 mmol/l NaCl, 2 mmol/l EDTA, 1% (v/v) Triton X-100, with 1 miniprotease inhibitor tablet (Roche)/10 ml. Lyased sample (32.5 μl) was transferred to a tube containing 12.5 μl 4× loading buffer (Invitrogen) and 5 μl 10× denaturing buffer (Invitrogen). Samples were heated at 95 °C for 5 min before storing at −20 °C. Residual lysed sample was used for protein analysis via a DC protein assay (Bio-Rad).

Protein (50 μg/sample) was resolved by electrophoresis on 10% (v/v) Bis–Tris NuPage gels (Invitrogen), using 3-(N-morpholino)propanesulfonic acid buffer. The iBlot system (Invitrogen) was used for transfer onto nitrocellulose. After blocking overnight at 4 °C in 5% (w/v) BSA/0.05% (w/v) polysorbate 20 in Tris-buffered saline, a 2 h room temperature incubation was carried out with rabbit polyclonal antibody raised against full-length human pancreatic GK (used at 1:5000 dilution). Incubation with secondary antibody, HRP-linked donkey anti-rabbit (GE Healthcare, Buckinghamshire, UK), 1:5000 dilution, was for 1 h at room temperature, followed by detection with an enhanced chemiluminescence system (Chemiglow West, Cell Biosciences, Santa Clara, CA, USA). The blot was imaged on a Chemi-imager (Alpha Innotech), and densitometry was performed using software installed on the machine.

Data handling and statistical analysis

Acute insulin secretion induced by GKA71 was compared with the control insulin secretion at each glucose concentration by Student’s t-test. For statistical analysis of the gene expression data, each gene of interest was normalised to the housekeeper gene HPRT and statistical evaluation was carried out on log-transformed data. For each stimulatory phase of the perifusion profile, insulin secretion after treatment with GKA71 or high glucose was compared with 5 mmol/l glucose-treated islets using Student’s t-tests. Two-sided unequal variance Student’s t-tests were used throughout.

Results

GKA71 is a potent activator of human pancreatic GK and acutely stimulates insulin secretion from rat islets

In isolated enzyme assays, GKA71 activated recombinant human pancreatic GK with EC₅₀ 112 nmol/l (n=8, 95% confidence interval (CI) 70–170 nmol/l). GKA71 also activated the rat and mouse pancreatic GK isoforms: EC₅₀ 88 nmol/l (n=3, 95% CI 86–91 nmol/l) and 411 nmol/l (n=3, 95% CI 350–490 nmol/l) respectively.

The acute effect of GKA71 on GSIS from isolated rat islets is shown in Fig. 1. GKA71 increased insulin secretion at 3, 5, 8 and 10 mmol/l glucose; although at 10 mmol/l, the increase was not significantly different from control.

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Chronic activation of GK by GKA71 increases the expression of insulin in mouse islets and changes the expression of other key β-cell genes

Taqman analysis of mouse islets cultured for 72 h revealed that GKA71 led to a 42-fold increase in insulin mRNA levels compared with islets cultured in 5 mmol/l glucose alone (Fig. 2). GK mRNA was also analysed in these samples; however, no significant changes were observed (data not shown). Chronic exposure to 25 mmol/l glucose also significantly increased, ~80-fold, the level of insulin mRNA compared with incubation in 5 mmol/l glucose.

The expression of other β-cell genes was explored in the same samples using TLD analysis. Table 1 summarises the data for those genes in which changes were observed, either greater than twofold or statistically significant (for full gene list, see Gorman et al. (2008)). A marked increase in insulin gene expression was observed, consistent with the individual gene Taqman. IAPP, PDX1, PC1 and GLUT2 also significantly increased, whereas CEBPβ expression decreased by 80%.

To investigate whether sustained GK activation will also maintain islet function where GK activity is catalytically significant (for full gene list, see Gorman et al. (2008)). A marked increase in insulin gene expression was observed, consistent with the individual gene Taqman. IAPP, PDX1, PC1 and GLUT2 also significantly increased, whereas CEBPβ expression decreased by 80%.

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GSIS in rat islets is increased after chronic activation of GK by GKA71

Following chronic culture, rat islets were perifused with sub-stimulatory (3 mmol/l) and stimulatory (15 mmol/l) glucose; the perifusion profile is shown in Fig. 5A. Following the 30-min pre-perifusion phase, all treatment groups had the same basal insulin secretion, indicating that no residual compound remained in the islets. Islets cultured in 5 mmol/l glucose exhibited no measurable GSIS response when stimulated with 15 mmol/l glucose; however, islets cultured in 25 mmol/l glucose exhibited significant GSIS response when stimulated with 15 mmol/l glucose. These changes in insulin mRNA induced by GKA71 were similar in both genotypes to those elicited by 25 mmol/l glucose.

Expression of GK did not significantly change in the presence of GKA71 or 25 mmol/l glucose in islets from gkwt/wt or gkdel/wt mice (Fig. 3B). GK mRNA levels were lower in gkdel/wt islets compared with gkwt/wt islets in basal 8 mmol/l glucose, but in the presence of GKA71 or 25 mmol/l glucose, there was no significant difference in GK mRNA between genotypes.

Figure 2 Chronic treatment of C57Bl/6J mouse islets with GKA71 (10 μmol/l) increases insulin mRNA levels. mRNA levels determined by single-gene Taqman analysis (mean ± S.E.M. of triplicate samples, each of 40 islets). **P < 0.01 versus 5 mmol/l glucose alone.

Figure 1 GKA71 (1 μmol/l) acutely stimulates insulin secretion from rat islets in a glucose-dependent manner. Control islets (black bars) and islets treated with 1 μmol/l GKA71 (striped bars). Values are mean ± s.e.m., n = 6, each of three islets. **P < 0.01, ***P < 0.001 versus the respective control in the absence of GKA71. Data are representative of four separate experiments.
cultured in the presence of GKA71 exhibit clear first- and second-phase insulin secretion and have a profile very similar to that of the islets cultured in 25 mmol/l glucose. The effects of GKA71 on first- and second-phase insulin secretion were quantified by calculating the area under curve (AUC) for each phase. The first-phase (30–39 min) response was 65-fold ($P < 0.001$) greater in islets previously cultured in the presence of GKA71 compared with those cultured in 5 mmol/l glucose alone and not significantly different from islets cultured in 25 mmol/l glucose. AUC for the second-phase response (40–76.5 min) was 585-fold ($P < 0.001$) greater after culture with GKA71 and also similar to islets cultured in 25 mmol/l glucose. These data are shown in Fig. 5B and C.

The response to perifusion with 40 mmol/l KCl was determined from the AUC (76.5–81 min). Insulin release was fourfold ($P < 0.006$) greater in islets treated with GKA71 compared with those cultured in 5 mmol/l glucose alone and similar to that in islets previously cultured in 25 mmol/l glucose (Fig. 5D).

**Table 1 GKA71 influences the expression of key β-cell genes in mouse islets**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Direction of change</th>
<th>Fold change</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IAPP</td>
<td>Increase</td>
<td>4-6</td>
<td>0-005</td>
</tr>
<tr>
<td>Insulin</td>
<td>Increase</td>
<td>15-9</td>
<td>0-001</td>
</tr>
<tr>
<td>PDX1</td>
<td>Increase</td>
<td>4-5</td>
<td>0-012</td>
</tr>
<tr>
<td>PC1</td>
<td>Increase</td>
<td>6-5</td>
<td>0-001</td>
</tr>
<tr>
<td>GLUT2</td>
<td>Increase</td>
<td>29-3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CEBPβ</td>
<td>Decrease</td>
<td>5-0</td>
<td>0-040</td>
</tr>
</tbody>
</table>

**Discussion**

Although many groups have demonstrated the potential of GKAs to drive insulin secretion, no studies, to date, have investigated whether GKAs activate the appropriate pathways to ensure that β-cell exhaustion does not occur in response to prolonged treatment with a GKA. We designed a series of experiments to assess the effect of a high concentration of GKA71 (at least ten times greater than the EC$_{50}$) on isolated islets following a 72-h treatment period. The primary end point for these investigations was the effect of GKA71 on insulin mRNA levels.

We demonstrate that islets chronically treated with a GKA have a greater level of insulin production than islets cultured with a GKA71 resulted in increased GK protein; this change was again consistent with that observed for islets cultured in 25 mmol/l glucose (Fig. 7A and B). Similar findings were observed in islets taken from $gk^{wt/wt}$ and $gk^{del/del}$ mice (Fig. 7C and D).

**Total insulin content in rat islets is increased after chronic activation of GK by GKA71**

We hypothesised that the greater KCl response in islets treated with GKA71 resulted from elevated total insulin content in those islets. Chronically treated islets were lysed and the total insulin content was measured (Fig. 6). The insulin content was 2-4-fold higher in islets cultured in the presence of GKA71 than in those cultured in 5 mmol/l glucose alone. Again, the results for GKA71 were similar to those for islets cultured in 25 mmol/l glucose.

**GK protein is increased in rat and mouse islets with chronic activation of GK by GKA71**

Although GK mRNA levels were not changed by chronic culture with either GKA71 or with 25 mmol/l glucose, GK is known to be regulated at the protein level, rather than at the gene level. Therefore, western blotting was performed to quantify GK protein in chronically treated islet samples. In isolated rat islets, chronic
insulin expression and production, and also on the expression of other key genes, to those induced by high glucose concentration. Therefore, these novel findings again demonstrate the ‘glucose-like’ nature of GKAs, consistent with what is already known about the mechanism of action of these molecules (Leighton et al. 2005).

In preliminary experiments, GSIS of islets from C57Bl/6J mice, and of Wistar rats, was assessed following culture for 72 h. These experiments indicated that while rat islets retained a small GSIS response after 72 h in 5 mmol/l glucose, mouse islets required 8 mmol/l glucose to maintain a similar level of functionality. Therefore, all subsequent mouse islet chronic culture experiments were performed using 8 mmol/l glucose. The insulin mRNA response to GKA71 on C57Bl/6J mouse islets cultured in 5 mmol/l glucose was qualitatively similar to that in gk<sup>wt/wt</sup> islets cultured in 8 mmol/l glucose; however,
Recently, the effect of chronic GKA administration was evaluated in wild-type mice and mice with a β-cell-specific deletion of GK (Nakamura et al. 2009). In both genotypes, β-cell mass was unchanged after 20 weeks’ treatment, but it was less clear whether the potential for GKA to stimulate GSIS was retained. Relative insulin secretion increased in response to feeding, although this could potentially have resulted from improved insulin sensitivity. Although GSIS tended to be greater in islets from GKA-treated mice when evaluated ex vivo, this study did not investigate whether insulin biosynthesis had been maintained. Our own results clearly support the conclusion that chronic exposure to GKA71 results in islets with an increased GSIS response, and indeed provide evidence that insulin content and insulin secretory capacity are significantly improved. It should, however, be noted that in our studies, all end points are compared following 72 h of culture; therefore, our findings do not enable us to differentiate between action of GKA71 to prevent loss of function in low glucose culture and action to increase function during sustained treatment. Study of the kinetics of changes in insulin biosynthesis and GSIS induced by GKA would clarify this.

Although chronic administration of GKA did not change β-cell mass in the above study, sustained exposure of INS1 cells to GKA promoted β-cell replication and, in addition, one compound, GKA50, reduced apoptosis (Nakamura et al. 2009, Wei et al. 2009). Further evidence to support the lack of detrimental effect on β-cell mass comes from demonstration that a short (3 days) treatment in vivo, when glucose concentration was high, increased the rate of β-cell proliferation (Nakamura et al. 2009). Taken together, these two studies, with the current results, suggest that GKAs have the potential to at least prevent and at best reverse the three key elements of glucotoxicity.

Figure 6 Chronic treatment of rat islets with GKA71 (10 μmol/l) increases the total insulin content. Values are the mean ± S.E.M. of six samples, each of three islets. **P<0.01 versus 5 mmol/l glucose alone.

Figure 7 Chronic treatment of rodent islets with GKA71 (10 μmol/l) increases the amount of GK protein. Western blots were performed on cell lysates from rat islets (A: immunoblot lane 1 = 5 mmol/l glucose, lane 2 = 25 mmol/l glucose and lane 3 = 5 mmol/l glucose plus 10 μmol/l GKA71; B: densitometry, 5 and 25 denote glucose concentration in mmol/l with 5 + GKA being 5 mmol/l glucose plus 10 μmol/l GKA71) or gkwt/wt and gkdelp/wt mouse islets (C: immunoblot – lanes 1 and 4 = 8 mmol/l glucose, lanes 2 and 5 = 8 mmol/l glucose plus 10 μmol/l GKA71 and lanes 3 and 6 = 25 mmol/l glucose – lanes 1–3 = gkwt/wt lanes 4–6 = gkdelp/wt; D: densitometry). Data are representative of n=2 experiments with each species.

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impaired proliferation, increased apoptosis and impaired GSIS. Given the effectiveness of GKAs to decrease blood glucose, it may not be physiologically relevant to consider sustained effects at high glucose concentrations.

The mechanism of action of GKAs clearly distinguishes them from sulphonylureas. We have previously demonstrated the difference between GKA50 and tolbutamidine on cytosolic Ca\(^{2+}\) rises in MIN6 cells and isolated rodent islets (Johnston et al. 2007). Although both agents induce insulin secretion, only the GKA does so in a glucose-dependent and 'glucose-like' manner. Administration of sulphonylureas results in eventual loss of insulin secretory capacity (Ball et al. 2004, Urban & Panten 2005), whereas in contrast, the experiments described in this study indicate that GKAs are able to stimulate the cellular mechanisms to increase insulin production, in addition to stimulating insulin secretion.

Type 2 diabetes in man results from failure of insulin secretion to compensate for the increased insulin demand that occurs as insulin resistance develops. Reduced insulin secretory capacity results from declining \(\beta\)-cell mass (Butler et al. 2005) and also reduced secretory capacity of individual \(\beta\)-cells (Ahren 2005). We have demonstrated that chronic GK activation improves or sustains insulin secretory capacity in vitro; studies in human islets and ultimately in diabetic patients are required to support this result. Taken together with the maintenance of \(\beta\)-cell mass, these results reinforce the attractive profile of GKAs as a therapy for type 2 diabetes.

**Supplementary data**

This is linked to the online version of the paper at http://dx.doi.org/10.1530/JME-10-0157.

**Declaration of interest**

All authors of this study were employees of AstraZeneca plc at the time the work was performed.

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**References**


Aramata S, Han SI, Yasuda K & Kataoka K 2005 Synergistic activation of the insulin gene promoter by the beta-cell enriched transcription factors MafA, Beta2, and Pdx1. *Biochimica et Biophysica Acta* 1730 41–46. (doi:10.1016/j.bbadis.2005.05.009)


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