Glucokinase activation is beneficial or toxic to cultured rat pancreatic islets depending on the prevailing glucose concentration.

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Abbreviations: [Ca²⁺]ᵢ, intracellular Ca²⁺ concentration; Dz, diazoxide; Gn, mM glucose; K30, 30 mM extracellular K⁺; roGFP, redox sensitive Green Fluorescent Protein; TUNEL, Terminal deoxynucleotidyl transferase dUTP Nick End Labeling.

Abstract

Background/aim: In rat pancreatic islets, beta-cell gene expression, survival and subsequent acute glucose stimulation of insulin secretion (GSIS) are optimally preserved by prolonged culture at 10 mM glucose (G10) and markedly altered by culture at G5 or G30. Here we tested whether pharmacological glucokinase (GK) activation prevents these alterations during culture or improves GSIS after culture.

Methods: Rat pancreatic islets were cultured 1-7 days at G5, G10 or G30 with or without 3 µM of the GK activator Ro 28-0450 (Ro). After culture, beta-cell apoptosis and islet gene mRNA levels were measured, and the acute glucose-induced increase in NAD(P)H autofluorescence, intracellular calcium concentration and insulin secretion were tested in the absence or presence of Ro 28-0450.

Results: Prolonged culture of rat islets at G5 or G30 instead of G10 triggered beta-cell apoptosis and reduced their glucose responsiveness. Addition of Ro during culture differently affected beta-cell survival and glucose responsiveness depending on the glucose concentration during culture: it was beneficial to beta-cell survival and function at G5, detrimental at G10, and ineffective at G30. In contrast, acute GK activation with Ro increased the glucose sensitivity of islets cultured at G10, but failed at restoring beta-cell glucose responsiveness after culture at G5 or G30.

Conclusions: Pharmacological GK activation prevents the alteration of beta-cell survival and function by long-term culture at G5, but mimics glucotoxicity when added to G10. The complex effects of glucose on the beta-cell phenotype result from changes in glucose metabolism and not from an effect of glucose per se.
Introduction

The glucose stimulation of insulin secretion (GSIS) by endocrine pancreatic beta-cells depends on the acceleration of glucose metabolism through glycolysis and the Krebs cycle, with enhanced production of metabolic coupling factors (25; 39; 41). Besides these rapid effects, glucose exerts complex long-term effects on the beta-cell phenotype (2; 10; 16; 18; 27; 45). During long-term culture of rodent islets, beta-cell gene expression, survival and glucose responsiveness are optimally preserved in the presence of 10 mM glucose (G10), whereas they are markedly altered by culture at either non-stimulating (G5) or very high (G30) glucose concentrations. In other words, culture at G10 vs. G5 is beneficial, whereas culture at G30 vs. G10 is detrimental to beta-cell gene expression, survival and glucose responsiveness. The beneficial effect of culture at G10 vs. G5 is usually attributed to the stimulation of energetic metabolism. In contrast, the deleterious effects of culture at G30 vs. G10, which we later refer to as glucotoxicity, could result from the further increase in metabolism with increased production of reactive oxygen species (ROS) and endoplasmic reticulum stress, or from various mechanisms that do not exclusively depend on glucose metabolism, e.g. through increased glycation of extracellular proteins, activation of the receptor for advanced glycation-end products (RAGE), or a hypothetical osmotic effect of high glucose concentrations (1).

Glucokinase (GK) is a high K_m hexokinase expressed in hepatocytes, pancreatic beta-cells and a few other cell types involved in glucose homeostasis (29; 30). In beta-cells, GK is the glucose sensor that controls the rate of glycolysis, hence insulin secretion, within the physiological range of glucose concentrations (28). Given the pivotal role of GK in glucose homeostasis, small molecule GK activators (GKAs) were developed that augment GSIS and hepatic glucose utilization (17), thereby improving glucose homeostasis in rodent and human type 2 diabetes (T2D) (3; 7; 11; 13; 29; 31; 33; 48). However, although some GKAs may improve beta-cell survival and GSIS under glucotoxic conditions, the loss of GKA effectiveness during long-term treatment of T2D (22; 47) raises questions about their possible toxicity, e.g. through sustained beta-cell stimulation.

In this study, we tested the ability of the GKA Ro 28-0450 (Ro) to prevent or correct the alterations of the beta-cell phenotype by prolonged culture at G5 or G30 vs. G10. We also indirectly tested whether the complex effects of glucose on the beta-cell phenotype in cultured rat islets result from changes in glucose metabolism or from an effect of glucose per se.

Materials and Methods

Materials – Diazoxide and dithiothreitol (DTT) were from Sigma (St-Louis, MI, USA), the glucokinase activator (GKA) Ro 28-0450 (3-Cyclopentyl-2-(4-methansulfonyl-phenyl)-N-thiazol-2-yl-propionamide) was from Axon Medchem (Groningen, The Netherlands), and the GKA Compound A (2-Amino-5-(4-methyl-4H-(1,2,4)-triazole-3-yl-sulfanyl)-N-(4-methyl-thiazole-2-yl)benzamide) was from Calbiochem (Merck, Darmstadt, Germany).

Islet isolation and culture – Male Wistar rat islets were isolated by collagenase digestion of the pancreas, purified by gradient centrifugation using Histopaque 1077, and hand-picked under a stereomicroscope. They were precultured at 37°C and 5% CO_2 in serum-free RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) containing 100 U/ml penicillin, 100 µg/ml streptomycin, 5 g/L BSA and 10 mM glucose (G10). They were then cultured for up to 1 week in the same medium containing G5, G10 or G30 and 3 or 30 µM Ro or vehicle alone (dimethylsulfoxide 1/1000). The islets were transferred to fresh medium every other day. All experiments were approved by the local ethics committee for animal experimentation (project 2013/UCL/MD/016).
Gene mRNA levels - Islet total RNA extraction and reverse transcription were carried out as described (19), except for the use of Tripure (Roche Diagnostics GmbH, Mannheim, Germany), RevertAid™ Reverse Transcriptase and Ribolock™ RNase inhibitor (Thermo Scientific). Real-time PCR were performed with a CFX96 (Bio-Rad) using primers and reaction conditions as in (2; 10).

Cell apoptosis - Histone-associated DNA fragments were measured in islet cell cytosolic extracts using the Cell Death Detection ELISAPLUS kit (Roche Diagnostics), exactly as described in (36). The percentage of apoptotic beta-cells was determined on islet sections by TUNEL using the “In Situ Cell Death Detection Kit” (Roche Diagnostics) followed by insulin immunostaining (9).

Mitochondrial GSH/GSSG redox status - The mitochondrial redox status was assessed by measuring the thiol/disulfide equilibrium with the “redox sensitive Green Fluorescent Protein” targeted to the mitochondria (mt-roGFP1) (14), as described (9; 40). The mt-roGFP1 fluorescence ratio was normalized to the difference between the ratio measured after addition of 10 mM dithiothreitol (set to 0%) and that measured after addition of 1 mM H2O2 (set to 100%).

Total glutathione content - Total glutathione was measured with the DetectX® Glutathione Fluorescent Detection Kit (Arbor Assays, Ann Arbor, MI, USA) and normalized for differences in islet protein content (9; 40).

Intracellular Ca2+ concentration ([Ca2+]i) - After culture, islets were loaded for 2 h with fura2-LR-acetoxymethylester (Teflabs, Austin, TX) in a medium similar to that used during culture. Islets were then perfused (flow rate ~1 ml/min) with a bicarbonate-buffered Krebs solution containing (mM) NaCl (120), KCl (4.8), CaCl2 (2.5), MgCl2 (1.2), NaHCO3 (24), 1 g/l BSA, glucose (0.5 to 20), and various test substances. When the concentration of KCl was raised to 30 mM, that of NaCl was reduced to 94.8 mM to keep the osmolality unchanged. The acute glucose-induced changes in [Ca2+]i were recorded by microspectrofluorimetry as described (21).

NAD(P)H autofluorescence - After culture, islets NAD(P)H autofluorescence was recorded by microspectrofluorimetry in perfused islets as described (21). The data were normalized to the fluorescence level measured 20 min after addition of 10 µM FCCP to G20.

Insulin secretion during culture – After culture, the medium was centrifuged 5 min at 500 rpm (20 x g) and the supernatant was appropriately diluted for determination of insulin concentration by RIA using rat insulin as a standard (16).

Acute insulin secretion – After culture, islets were preincubated for 45 min in a bicarbonate-buffered Krebs solution containing G0.5, then incubated in batches of 5 for 1 h at various glucose concentrations. At the end, the medium was collected for insulin measurement and the islet insulin and DNA contents were measured on each batch of islets (23). Insulin secretion was normalized for variations in islet DNA content.

Statistical analysis - Results are means ± SE for at least 3 preparations. Statistical significance of differences between groups was assessed by 2-way ANOVA followed by a test of Bonferroni, unless specified otherwise. Except in figure 1, these statistical analyses were done separately for the groups G5/G5+Ro/G10 and for the groups G10/G30 +/- Ro. Differences were considered significant at \( P < 0.05 \).

Results

Effects of Ro 28-0450 on the glucose regulation of gene mRNA expression and beta-cell apoptosis in cultured rat islets - We first tested the effects of two chemically-unrelated GKAs, Ro 28-0450 (Ro) and Compound A, during overnight culture at increasing glucose concentrations, on insulin secretion, on the islet mRNA levels of metallothionein 1a
(Mt1a), a sensitive indicator of oxidative stress in rat islets (18), and on the mRNA levels of thioredoxin-interacting protein (Txnip), a sensitive marker of beta-cell glucotoxicity (5). The strong regulation of these genes and of insulin secretion over different parts of the glucose concentration-response curve (2) should provide an optimal system to compare the intensity of the effects of GKAs and glucose in rat islets.

As expected (2), glucose markedly increased insulin secretion in the culture medium between G5 and G30, increased Txnip mRNA expression between G10 and G30, and reduced islet Mt1a mRNA levels between G2 and G10 (Fig. 1). Under these conditions, Ro concentration-dependently shifted to the left the three glucose-response curves without significantly affecting the maximal responses at G30 (Fig. 1A-C). At 30 µM, close to its maximal effective concentration in vitro (13), addition of Ro to G2 tended to reproduce the effects of G10 alone, and its addition to G5 was almost as effective as G30 alone. At 3 µM, close to its semi-maximal effective concentration, addition of Ro to G2 was almost ineffective, its addition to G5 mimicked the effects of G10 alone, and its addition to G10 largely reproduced the effects of G30 alone. The latter concentration of Ro seemed optimal to test whether the complex effects of glucose on the beta-cell phenotype result from the stimulation of glucose metabolism, and we therefore used it in subsequent protocols.

The GKA compound A also shifted to the left the glucose-response curves for changes in insulin secretion and Txnip mRNA expression (Fig. 1D and E). However, compound A induced a ~20-fold increase in Mt1a mRNA levels at G10 (Fig. 1F), suggesting a possible rapid toxic effect of the drug. We therefore stopped testing that compound.

We next tested the effect of 3 µM Ro on insulin secretion, islet gene expression and beta-cell survival during one-week of culture at G5, G10 or G30. The effects of glucose on insulin secretion during culture and on Txnip and Mt1a to Tbp mRNA ratios were similar to those observed after overnight culture (Fig. 2A-C). Compared with prolonged culture at G10 that optimally preserved rat beta-cell function and survival, there were a large decrease in preproinsulin (Ppi) to Tbp mRNA ratio, increase in islet DNA fragmentation and increase in beta-cell apoptosis after culture at G5 (Fig. 2D-F), consistent with the toxic effect of prolonged culture at G5 vs. G10 on beta-cell gene expression and survival. On the other hand, there was a small non-significant decrease in Ppi to Tbp mRNA ratio and a significant ~2-fold increase in the percentage of apoptotic beta-cells after culture at G30 (Fig. 2D and F), confirming the glucotoxic effect of culture at G30 vs. G10 on beta-cell gene expression and survival.

As shown in figure 2A-F, addition of Ro to G5 during one-week culture largely reproduced the effects of culture at G10 alone on all parameters tested, while addition of Ro to G10 reproduced the effects of culture at G30 alone. However, Ro had no significant effect on insulin secretion, islet gene expression or beta-cell apoptosis during culture at G30. These results are easily explained if one considers that i) the long-term effects of culture at G10 vs. G5 result from an increase in glucose metabolism that can be achieved by adding 3 µM Ro to G5, and ii) the long-term effects of culture at G30 vs. G10 result from an increase in glucose metabolism that can be achieved by adding 3 µM Ro to G10.

We have previously shown that the glucose-induced changes in rat beta-cell apoptosis are preceded by parallel changes in the oxidation of mitochondrial redox-sensitive Green Fluorescent Protein (mt-roGFP1) (9; 40), a good indicator of mitochondrial thiol oxidation (32). Similar changes in mt-roGFP1 fluorescence ratio were observed in the present study, with a large increase after culture at G5 vs. G10, and a small significant increase after culture at G30 vs. G10 (Fig. 3A). An increase in islet glutathione content was also observed.
at G5 vs. G10 (Fig. 3B). Again, addition of Ro to G5 prevented the increase in mt-roGFP1 fluorescence ratio and islet glutathione content induced by culture at G5 alone (Fig. 3), in parallel with later changes in beta-cell apoptosis (Fig. 2E-F). However, addition of Ro to G10 did not affect mt-roGFP1 fluorescence ratio as did culture at G30 alone (Fig. 3A), in contrast with the effect of the drug on beta-cell apoptosis (Fig. 2F).

Effects of one week culture at G5, G10 and G30 on islet glucose responsiveness (the data described hereafter correspond to dotted traces and open symbols in all panels from figure 4) - Prolonged culture at a non-stimulating or very high vs. intermediate glucose concentration not only triggers beta-cell apoptosis but also reduces the islet insulin content and induces marked alterations of the beta-cell secretory responses to subsequent acute glucose simulation (9; 21; 40). In agreement with previous studies, after one week culture at G10 alone, rat islets displayed typical changes in NAD(P)H autofluorescence, [Ca\(^{2+}\)], and insulin secretion upon acute stepwise glucose stimulation: a concentration-dependent increase in NAD(P)H autofluorescence (Fig. 4B), a transient decrease in [Ca\(^{2+}\)]i without significant effect on insulin secretion at G6 and a concentration-dependent increase in [Ca\(^{2+}\)]i upon stimulation with G10 and G20 (Fig. 4E), and a concentration-dependent stimulation of insulin secretion above G5 (Fig. 4H). As expected, both [Ca\(^{2+}\)]i and insulin secretion were further stimulated by high K+-induced depolarization in the presence of diazoxide (K30Dz)(Fig. 4E and H).

After one week culture at G5 alone, the islet insulin to DNA content ratio (see legend to fig. 4G-I) and the acute glucose-induced rise in [Ca\(^{2+}\)]i without significant effect on insulin secretion at G6 and a concentration-dependent increase in [Ca\(^{2+}\)]i upon stimulation with G10 and G20 (Fig. 4E), and a concentration-dependent stimulation of insulin secretion above G5 (Fig. 4H). As expected, both [Ca\(^{2+}\)]i and insulin secretion were further stimulated by high K+-induced depolarization in the presence of diazoxide (K30Dz)(Fig. 4E and H).

Effects of long-term GK activation during culture at G5, G10 and G30 on subsequent islet glucose responsiveness - Addition of Ro during one week culture markedly affected the islet insulin to DNA content ratio (see legend to figure 4) and subsequent functional responses to acute glucose and K30Dz stimulation (Fig. 4, thick continuous traces vs. dotted traces, and closed vs. open symbols). Thus, the insulin to DNA content ratio and functional responses of islets cultured at G5 with Ro were similar to those recorded in islets cultured at G10 alone, except for the higher glucose sensitivity of the former (Fig. 4: compare thick traces or closed symbols in panels A, D, G with dotted traces or open symbols in panels B, E, H). Also, the functional responses of islets cultured at G10 with Ro were similar to those recorded in islets cultured at G30 alone (Fig. 4: compare thick traces or closed symbols in panels B, E, H with dotted traces or open symbols in panels C, F, I). Finally, addition of Ro during culture at G30 did not significantly affect the islet insulin to DNA content ratio or the rise in [Ca\(^{2+}\)]i, and insulin secretion upon acute stimulation with glucose and K30Dz (Fig. 4F, I), although their NAD(P)H autofluorescence increased significantly less upon glucose stimulation than in islets cultured at G30 alone (Fig. 4C).
during culture on subsequent islet glucose responsiveness in the absence of the drug. We next tested the acute effects of Ro addition after culture on beta-cell glucose responsiveness during perfusion or 1 h incubations. As shown in figure 5, addition of Ro after one week culture at G10 alone shifted to the left the acute glucose-response curves for changes in NAD(P)H, [Ca\(^{2+}\)], and insulin secretion without affecting their maximal responses to G20 (Fig. 5B, E, H, thick continuous vs. dotted traces, closed vs. open symbols). Addition of Ro during perfusion also shifted to the left the glucose-induced changes in NAD(P)H after 3 days of culture at G5 alone (Fig. 5A) but not after culture at G30 alone (Fig. 5C). However, it did not significantly restore the amplitude of the [Ca\(^{2+}\)], and insulin secretion responses upon acute glucose stimulation, neither in islets cultured at G5 (Fig. 5D,G) nor in islets cultured at G30 (Fig. 5F,I). It also did not improve the lack of effect of K30Dz stimulation on insulin secretion in islets cultured at G5 (Fig. 5G, closed vs. open squares).

**Discussion**

In rodent pancreatic islets cultured for 1-3 weeks at various glucose concentrations, beta-cell gene expression, survival and function are optimally preserved at G10 and markedly altered at G5 and at G30, the latter condition triggering beta-cell glucotoxicity (reviewed in (1; 18; 27)). In this study, acute pharmacological GK activation increased the glucose sensitivity of control islets but failed at restoring beta-cell glucose-responsiveness in rat islets cultured for one week at non-stimulating or very high vs. intermediate glucose concentration. In contrast, chronic GK activation prevented the alteration of beta-cell survival and function by prolonged culture at non-stimulating glucose, was detrimental during culture at G10, and was almost ineffective at G30.

**Impact of GK activation in rat islets cultured at a non-stimulating glucose concentration** – As expected (9; 16; 26), long-term culture of rodent islets at G5 markedly reduced *Ppi* mRNA levels and beta-cell glucose responsiveness while increasing beta-cell apoptosis and markers of oxidative stress (mt-roGFP1 oxidation and *Mt1a* mRNA levels (Fig. 2 and 3). The blunted GSIS could be due to reduced GK expression (24) and adoption of its “wide-open” conformation with low activity and resistance to GKA effect (15; 20). Thus, although Ro 28-0450 significantly increased the glucose sensitivity of islets cultured at G10 (Fig. 5B,E,H), it could not increase the glucose-induced rise in [Ca\(^{2+}\)], nor GSIS after culture at G5 (Fig. 5D,G). However, this lack of effect was not due to a lack of efficacy on glucose metabolism, for Ro significantly shifted to the left the glucose-induced rise in NAD(P)H autofluorescence even if it did not increase its low amplitude (Fig. 5A). Together with our previous observation that the antioxidant drug MnTBAP partially restored the glucose-induced rise in [Ca\(^{2+}\)], without increasing the amplitude of the rise in NAD(P)H autofluorescence or GSIS (40), and with the observation that K30-induced insulin secretion was abrogated despite the preservation of K30-induced rise in [Ca\(^{2+}\)], our results suggest that the defect in GSIS after culture at non-stimulating glucose does not only result from the reduction in glucose metabolism but also from defective coupling between the rise in [Ca\(^{2+}\)] and exocytosis (9; 40).

In contrast with its poor efficacy in the acute setting, moderate GK activation during long-term culture fully prevented all deleterious effects of G5 vs. G10 on beta-cell gene expression, survival and function. These results, which are in good agreement with an earlier study on the effect of GKA on the islet transcriptome after culture at G5 (12), confirm that the deleterious effects of prolonged culture at non-stimulating glucose mainly result from the sustained decrease in glucose metabolism (26; 42).

**GK activation and beta-cell glucotoxicity** – As expected, prolonged culture of rat islets at G30 vs. G10 tended to reduce *Ppi* mRNA levels, increased beta-cell apoptosis and
markers of oxidative stress (Fig. 2 and 3), and induced typical alterations of beta-cell stimulus-secretion coupling events culminating in a strong reduction of GSIS with only slight reduction in K30Dz-induced secretion (Fig. 4C,F,I and 5C,F,I).

Despite its efficacy in islets cultured at G10, Ro did not acutely increase the average glucose-induced rise in \([\text{Ca}^{2+}]\), nor GSIS after culture at G30. Such lack of effect contrasts with recent reports showing that two GKAs (GKA50 and YH-GKA) that are chemically different from Ro acutely reversed the glucotoxic alterations of beta-cell function (35; 46). Although we only tested the effect of Ro in this protocol, other GKAs were found ineffective under similar conditions, indicating that the effect of GKA50 and YH-GKA unlikely resulted from GK activation (46).

When Ro was added during culture at G10, it triggered all glucotoxic alterations of beta-cell gene expression, survival and function recorded after culture at G30 alone, except for the lack of increase in mt-roGFP1 fluorescence ratio. The latter effect aside, these results therefore unequivocally exclude the role of an effect of glucose \(\text{per se}\) in beta-cell glucotoxicity, in agreement with earlier studies in which mice with one-allele GK inactivation did not develop beta-cell glucotoxicity despite stable hyperglycemia (38; 43). These results do not tell us, however, whether these alterations are due to the activation of glucose oxidation, glycogen synthesis, or the pentose phosphate pathway.

The intriguing dissociation between the proapoptotic effect of Ro and its lack of effect on mt-roGFP1 fluorescence ratio at G10 suggests that beta-cell apoptosis induced by Ro and maybe G30 is independent of mitochondrial thiol oxidation. It also sheds light on the mechanism of ROS production during culture at high glucose, suggesting this event is independent of glucose metabolism and acceleration of mitochondrial electron transport chain and might therefore involve other pathways, e.g., AGE’s formation and activation of RAGE (1).

**Implications for the treatment of T2D patients with GKAs** - If GKAs undoubtedly trigger liver steatosis (4; 6; 34), their potential toxicity to beta-cells remains unclear (4; 35; 38; 43; 44; 46). Thus, although some GKAs proved beneficial to beta-cell survival and GSIS under glucotoxic conditions and in islets from T2D patients (8; 35; 46), their secondary failure during long-term treatment (22; 31; 47) reopened the question of their toxicity to beta-cells. This possible caveats of GKAs has been previously considered non-relevant on the ground that beta-cell stimulation is reduced following the GKA-mediated reduction in glycemia (12). However, given the metabolic heterogeneity of beta-cells in rodent islets (37), the present study suggests that GKAs may be beneficial in poorly-responsive beta-cells while being simultaneously toxic to middle- and highly-responsive beta-cells through a variety of mechanisms proposed to contribute to glucotoxicity (1). If such “glucotoxic-like” effect of long-term GKAs treatment at intermediate glucose concentration were confirmed in human islets, it could contribute to the secondary failure of GKAs in T2D (22; 31; 47).

In conclusion, the GKA Ro 28-0450 prevents the toxic effect of long-term culture at non-stimulating glucose while mimicking the glucotoxic alterations of the beta-cell phenotype when added during culture at G10. These results prove that in \(\text{vitro}\) beta-cell glucotoxicity fully depends on the stimulation of glucose metabolism and not on an effect of high glucose \(\text{per se}\). They also provide a plausible explanation for the lack of long-term efficacy of GKAs in T2D.

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Contribution statement

Conceived and designed the experiments: JCJ
Performed the experiments: JD, LPR, JCJ
Analyzed the data: JCJ
Wrote the paper: LPR, JCJ
Corrections/suggestions to the paper: JD

Duality of interest

The authors have no duality of interest associated with this manuscript.

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Legends to the figures

Figure 1. Overnight effects of glucose and two GKAs on insulin secretion, Txnip and Mt1a mRNA levels in cultured rat islets - After one week preculture in serum-free RPMI medium containing 10 mM glucose (G10) and 5 g/l BSA, islets were cultured 18 h at G2, G5, G10 or G30 with 3 µM GKA (closed circles), 30 µM GKA (open squares) or DMSO 1/1000 (open circles). The GKA was Ro 28-0450 (A-C) or compound A (D-F). A, D, insulin secreted during culture. B-C, E-F, islet Txnip and Mt1a to Tbp mRNA ratios normalized to the ratio in G10-cultured islets. Data are means ± SE for 3-4 experiments. *, P<0.05 for the effect of glucose vs. G2; #, P<0.05 for the effect of GKA vs. DMSO.

Figure 2. Long-term effects of glucose and Ro on insulin secretion during culture, islet gene mRNA levels and beta-cell apoptosis - After overnight preculture in serum-free RPMI medium...
containing G10 and 5 g/l BSA, rat islets were cultured one week at G5, G10 or G30 with 3 µM Ro (closed circles) or vehicle alone (open circles). A, average rate of insulin secretion over one week of culture (measured every two days). B-D, islet Txpip, Mt1a and preproinsulin (Ppi) to Tbp mRNA ratios normalized to the ratio in G10-cultured islets. E, cytosolic histone-associated oligonucleosomes. F, percentage of apoptotic beta-cells (TUNEL-positive / DAPI-positive nuclei in insulin-positive cells). Results are means ± SE for 3 experiments (panels A-E) or for 5-7 experiments with 6933-8378 counted cells from 44-71 islets (panel F). *, P<0.05 for the effect of culture at G5 or G30 vs. culture at G10; #, P<0.05 for the effect of Ro during culture at the same glucose concentration. §, P<0.05 for the effect of Ro by Student’s t-test. F, due to the non-Gaussian distribution of the data, the statistical significance of differences between groups was assessed by a test of Kruskal-Wallis with a Dunn’s multiple comparisons post-test.

Figure 3. Effects of glucose and Ro on mt-roGFP1 fluorescence ratio and total glutathione - A, rat islet cell clusters expressing mt-roGFP1 were cultured overnight at G5, G10 or G30 with 3 µM Ro (closed circles) or vehicle alone (open circles). The mt-roGFP1 fluorescence ratio was measured and normalized as described under “Methods”. B, after one week preculture, islets were cultured 18 h at G5, G10 or G30 with Ro (closed circles) or vehicle alone (open circles). Their total glutathione content was measured and normalized to their protein content. Results are means ± SE for 3 experiments (for a total of 12-25 islet cell clusters in panel A). *, P<0.05 for the effect of culture at G5 or G30 vs. culture at G10; #, P<0.05 for the effect of Ro during culture at the same glucose concentration.

Figure 4. Long-term effects of Ro addition during culture at G5, G10 or G30 on subsequent acute glucose stimulus-secretion coupling events in rat islets – The islets were cultured for 1 week at G5 (A, D, G), G10 (B, E, H) or G30 (C, F, I) in the presence of 3 µM Ro (thick traces, closed symbols) or vehicle alone (thin dotted traces, open symbols). They were then perifused or incubated for 1 h at increasing glucose concentrations (Gn) in the absence of Ro. A-C, acute glucose-induced changes in NAD(P)H autofluorescence, expressed as percentage of the fluorescence measured at the end of 10 µM FCCP application. D-I, acute glucose-induced changes in [Ca2+]. The experiments ended by depolarization with 30 mM extracellular K+ in the presence of G20 and 250 µM diazoxide (G20K30Dz). G-I, acute glucose stimulation of insulin secretion in normal Krebs solution (circles) or in the presence of K30Dz (squares). The results were normalized for differences in islet DNA content. The insulin to DNA content ratios (ng insulin per ng DNA, mean ± SE, n=4) were 0.092 ± 0.008 and 0.273 ± 0.004 in islets cultured at G5 with DMSO or with Ro, 0.245 ± 0.011 and 0.173 ± 0.016 in islets cultured at G10 with DMSO or with Ro, and 0.157 ± 0.004 and 0.164 ± 0.007 in islets cultured at G30 with DMSO or with Ro. Results are means ± SE for 7-14 islets from 2-3 isolations (A-C), 20-38 islets from 3-4 experiments (D-F) or 4 experiments (G-I). The statistical significance of differences between groups was computed using the increase in NAD(P)H autofluorescence, [Ca2+]i or insulin secretion above the level measured in G0.5 or before depolarization with K30. * P<0.05 for the effect of culture at G5 or G30 vs. culture at G10; #, P<0.05 for the effect of Ro addition during culture at the same glucose concentration. The significance of the acute glucose effect in each group is not shown.

Figure 5. Acute effects of Ro on glucose stimulus-secretion coupling events in rat islets after one week culture at G5, G10 or G30 – The islets were cultured for a total of 1 week in a medium containing G10 then G5 during the last 3 days (A, D, G), G10 (B, E, H), or G30 (C, F, I). They
were then perfused or incubated for 1 h at increasing glucose concentrations (Gn) in the absence
(thin dotted traces, open symbols) or presence of 3 µM Ro (thick traces, closed symbols). A-F,
acute glucose-induced changes in NAD(P)H autofluorescence and [Ca$^{2+}$]_{i} (see legend to figure 4).
G-I, acute glucose stimulation of insulin secretion in normal Krebs solution (circles) or in the
presence of K30Dz (squares). The insulin to DNA content ratios (ng insulin per ng DNA, mean ±
SE, n=4) were 0.191 ± 0.011 and 0.181 ± 0.003 in islets cultured at G5 and incubated 1 h without or
with Ro, 0.353 ± 0.014 and 0.367 ± 0.021 in islets cultured at G10 and incubated 1 h without or
with Ro, and 0.241 ± 0.016 and 0.287 ± 0.007 in islets cultured at G30 and incubated without or
with Ro. Shown are means ± SE for 18–33 islets from 3-4 isolations (A-C), 22–41 islets from 3-4
experiments (D-F), or 4 experiments (G-I). The statistical significance of differences between
groups were computed as in Fig. 4. * P<0.05 for the effect of culture at G5 or G30 vs. culture at
G10; #, P<0.05 for the effect of Ro addition during the perfusion or incubation in islets cultured at
the same glucose concentration. The significance of the acute glucose effect in each group is not
shown.
Figure 1

(A) Insulin secretion (ng islet⁻¹ h⁻¹) for Ro 28-0450 and Compound A at different glucose concentrations (2, 5, 10, and 30 mM).

(B) Tpxrp1/Pp mRNA (relative to G10) for Ro 28-0450 and Compound A at different glucose concentrations.

(C) Mftr1/Pp mRNA (relative to G10) for Ro 28-0450 and Compound A at different glucose concentrations.
After culture in G5 +/- Ro

Figure 4
Figure 5

After culture in G5

After culture in G10

After culture in G30

A

B

C

D

E

F

G

H

I

NAD(P)H autofluorescence (% of FCCP)

Time (min)

$\text{Ca}^{2+}$ (nM)

Time (min)

Insulin secretion (ng/h per mg of insulin DNA)

Glucose (mM)