Mitochondrial oxidative stress contributes differently to rat pancreatic islet cell apoptosis and insulin secretory defects after prolonged culture in a low non-stimulating glucose concentration

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Abstract

Aims/hypothesis Pancreatic beta cells chronically exposed to low glucose concentrations show signs of oxidative stress, loss of glucose-stimulated insulin secretion (GSIS) and increased apoptosis. Our aim was to confirm the role of mitochondrial oxidative stress in rat islet cell apoptosis under these culture conditions and to evaluate whether its reduction similarly improves survival and GSIS.

Methods Apoptosis, oxidative stress-response gene mRNA expression and glucose-induced stimulation of mitochondrial metabolism, intracellular Ca^{2+} concentration and insulin secretion were measured in male Wistar rat islets cultured for 1 week in RPMI medium containing 5–10 mmol/l glucose with or without manganese(III) tetrakis(4-benzoic acid)porphyrin (MnTBAP) or *N*-acetyl-L-cysteine (NAC). Oxidative stress was measured in islet cell clusters cultured under similar conditions using cytosolic and mitochondrial redox-sensitive green fluorescent protein (roGFP1/mt-roGFP1).

Results Prolonged culture in 5 vs 10 mmol/l glucose increased mt-roGFP1 (but not roGFP1) oxidation followed by beta cell apoptosis and loss of GSIS resulting from

reduced insulin content, mitochondrial metabolism, Ca^{2+} influx and Ca^{2+} -induced secretion. Tolbutamide-induced, but not high K^{+} -induced, Ca^{2+} influx was also suppressed. Under these conditions, MnTBAP, but not NAC, triggered parallel ~50–70% reductions in mt-roGFP1 oxidation and beta cell apoptosis, but failed to protect against the loss of GSIS despite significant improvement in glucose-induced and tolbutamide-induced Ca^{2+} influx.

Conclusions/interpretation Mitochondrial oxidative stress contributes differently to rat pancreatic islet cell apoptosis and insulin secretory defects during culture in a low glucose concentration. Thus, targeting beta cell survival may not be sufficient to restore insulin secretion when beta cells suffer from prolonged mitochondrial oxidative stress, e.g. in the context of reduced glucose metabolism.

Keywords Apoptosis · c-Myc · Catalytic antioxidant · Cytosolic calcium concentration · Haem oxygenase 1 · Insulin secretion · Low glucose concentration · Metallothionein · Mitochondria · MnTBAP · Oxidative stress · Pancreatic beta cell · roGFP

Abbreviations

$[\text{Ca}^{2+}]_i$	Intracellular Ca^{2+} concentration
DTT	Dithiothreitol
GSIS	Glucose-stimulated insulin secretion
MnTBAP	Manganese(III)tetrakis (4-benzoic acid)porphyrin
mt-roGFP	Mitochondrial redox-sensitive green fluorescent protein
NAC	<i>N</i> -Acetyl-L-cysteine
roGFP	Redox-sensitive green fluorescent protein
ROS	Reactive oxygen species

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Introduction

The acute glucose regulation of insulin secretion and proinsulin biosynthesis by pancreatic beta cells plays a critical role in glucose homeostasis. In the long term, however, beta cells also adapt their mass and secretory capacity to prolonged changes in insulin demand, i.e. during prolonged alterations in food supply or under pathophysiological conditions associated with insulin resistance (reviewed by Hinke et al [1] and Karaca et al [2]). For instance, prolonged fasting in rodents reduces islet mRNA and protein expression of genes enriched in beta cells such as preproinsulin, glucose transporter-2, glucokinase and voltage-dependent Ca^{2+} channels, with concomitant reduction in glucose-stimulated insulin secretion (GSIS), and subsequent feeding of the animals corrects these alterations within 1 day [3–5]. Similar alterations in gene expression have been observed in native beta cells of rats implanted with an insulinoma, together with a marked reduction in beta cell mass due to cell atrophy and apoptosis [4, 6].

Also in vitro, rodent beta cell glucose responsiveness and survival are optimally preserved in medium containing 10 mmol/l glucose. Thus, culture of isolated rat islets or purified beta cells in the presence of 2, 3 or 5 mmol/l glucose, i.e. at glucose concentrations lower than the threshold concentration for in vitro stimulation of insulin secretion, markedly altered the expression of genes enriched in beta cells and the ability of beta cells to secrete insulin in response to glucose [7, 8] while increasing their apoptosis [9, 10]. The latter effect has been attributed to (1) decreased mitochondrial ATP production with rise in AMP concentrations and consequent activation of AMP-activated protein kinase [11], (2) decreased protein synthesis and consequent activation of an intrinsic mitochondrial apoptotic programme [12], (3) increased mRNA and protein levels of the pro-apoptotic transcription factor c-MYC [13], and (4) increased oxidative stress with subsequent activation of c-Jun N-terminal kinase [14, 15] (reviewed by Martens et al [16]). The role of oxidative stress is supported by observations that beta cell apoptosis is preceded by increased expression of integrated stress-response and oxidative stress-response genes [8] and that its stimulation by culture in low glucose is significantly reduced by the free radical scavenger, *N*-acetylcysteine (NAC), and by manganese(III)tetrakis(4-benzoic acid)porphyrin (MnTBAP) [14, 15], a superoxide dismutase mimetic, catalase mimetic and peroxynitrite scavenger that also increases the expression of the antioxidant and antiapoptotic enzyme, haem oxygenase 1 [17, 18]. However, the possible link between increased oxidative stress and loss of beta cell glucose responsiveness under these culture conditions has not been thoroughly investigated. Because of the possible relevance of this model to other conditions under which beta cell glucose metabolism is reduced after exposure

to various types of stress [16], we tested the effects of MnTBAP and NAC on alterations in beta cell glucose responsiveness in rat islets cultured for 1 week in a low non-stimulating concentration of glucose.

Methods

Materials Diazoxide, sodium azide, dithiothreitol (DTT) and tolbutamide were from Sigma (St Louis, MO, USA). MnTBAP was from Alexis Biochemicals (Lausen, Germany), and NAC was from Merck (Darmstadt, Germany).

Adenoviruses Adenoviruses encoding redox-sensitive green fluorescent protein (roGFP1) and mitochondria-targeted mt-roGFP1 were generated and amplified using the pAdEasy system (Stratagene, La Jolla, CA, USA), as described (electronic supplementary material [ESM] [Methods](#)) [19].

Islet isolation and culture Islets from ~200 g male Wistar rats were obtained by collagenase digestion of the pancreas followed by density gradient centrifugation [20]. They were cultured for a week at 37°C and 5% CO_2 in serum-free RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) containing 5 g/l BSA, 5 or 10 mmol/l glucose (G5, G10) and various antioxidants. The medium was renewed every other day. All experiments were approved by the local ethics committee for animal experimentation (UCL/MD/2009/009).

Gene mRNA levels Islet total RNA was extracted and reverse transcribed as described previously [21] using RevertAid Reverse Transcriptase and Ribolock RNase inhibitor (Fermentas, Burlington, ON, Canada). Real-time PCR was performed with an iCycler (Bio-Rad, Hercules, CA, USA). For primer sequences and reaction conditions, see ESM Table 1. Gene to *Tbp* (encoding TATA-box binding protein) mRNA ratios were computed as $2^{-(C_{\text{igene}} - C_{\text{Tbp}})}$ and expressed relative to the ratio in G10-cultured islets.

Cell apoptosis Histone-associated DNA fragments (mono- and oligo-nucleosomes) were measured in islet cell cytosolic extracts using the Cell Death Detection ELISA^{PLUS} kit (Roche Diagnostics GmbH, Mannheim, Germany) [22]. The percentage of apoptotic cells on islet sections was determined by TUNEL using the In Situ Cell Death Detection Kit (Roche Diagnostics) [22].

Mitochondrial and cytosolic GSH/GSSG redox status After islet dissociation using trypsin and gentle pipetting in Ca^{2+} -free medium, cell clusters were plated on glass coverslips

and cultured with RPMI medium containing G10 and 10% FBS. After overnight culture, they were infected with roGFP1 or mt-roGFP1 adenovirus ($\sim 1.8 \times 10^6$ infectious units per dish, multiplicity of infection ~ 25 – 50) and further cultured for 24–48 h in G10, then 18–24 h in medium containing G5 or G10 with or without antioxidants. After culture, the coverslip was mounted at the bottom of a 37°C temperature-controlled chamber placed on the stage of an inverted microscope equipped with a $\times 40$ objective. Cell clusters were perfused at a flow rate of ~ 1 ml/min with a bicarbonate-buffered Krebs solution containing (mmol/l) NaCl (120), KCl (4.8), CaCl_2 (2.5), MgCl_2 (1.2), NaHCO_3 (24), 1 g/l BSA (fraction V; Roche, Basel, Switzerland), G5 or G10 with or without antioxidants. This solution was continuously gassed with O_2/CO_2 (94:6) to maintain pH ~ 7.4 .

Long-term changes in cytosolic and mitochondrial redox potential using roGFP1 were measured as described previously [23]. Briefly, cell fluorescence at 535 nm was measured during alternate excitation at 400/480 nm every 30 s. After 20 min of perfusion in a medium similar to that used during the last culture period, cells were perfused with 10 mmol/l DTT to maximally reduce roGFP1, followed by 1 mmol/l H_2O_2 to maximally oxidise it. The 400:480 ratios of fluorescence intensities were expressed as a percentage of the difference between the ratio of the maximally reduced and the maximally oxidised forms of roGFP1.

Total glutathione content Total glutathione was measured with the Glutathione Fluorescent Detection Kit (Arbor Assays, Ann Arbor, MI, USA) and normalised for differences in islet protein content (ESM Methods).

Adenine nucleotides After culture, islets were incubated for 30 min in Krebs medium containing 0.5 mmol/l glucose, and then incubated for 45 min under various conditions. Samples were processed and adenine nucleotides (ATP and ATP+ADP levels) were measured, as described [24], using the ATP Bioluminescence Assay kit CLS II (Roche Diagnostics).

NAD(P)H levels, mitochondrial membrane potential, intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) and insulin secretion After culture, islets were transferred to a 37°C temperature-controlled perfusion chamber either immediately for the measurement of NAD(P)H autofluorescence and insulin secretion, after loading for 20 min with 10 $\mu\text{mol/l}$ rhodamine 123 (Molecular probes, Eugene, OR, USA) to measure the mitochondrial membrane potential, or after loading for 2 h with fura-PE3 acetoxymethyl ester to measure changes in $[\text{Ca}^{2+}]_i$. The loading medium was similar to that used during culture except for the omission of NAC during fura-PE3 loading. Islets were then perfused

(flow rate ~ 1 ml/min) with a bicarbonate-buffered Krebs solution containing 0.5–20 mmol/l glucose and various test substances. When the concentration of KCl was raised to 30 mmol/l, that of NaCl was reduced to 94.8 mmol/l to keep the osmolality unchanged. The acute glucose-induced changes in NAD(P)H levels, mitochondrial membrane potential and $[\text{Ca}^{2+}]_i$ were recorded by microspectrofluorimetry with Quanticell 700 m (Visitech, Sunderland, UK) [20]. GSIS was measured by RIA in 2 min effluent collections from 30–110 perfused islets [25]. At the end of perfusion, the islets were disrupted by sonication in 10 mmol/l Tris, 0.2 mmol/l NaCl, 10 mmol/l EDTA, and their insulin and DNA contents were measured [26].

Statistical analysis Results are mean $\pm$ SEM for at least three islet or cell cluster preparations. Statistical significance of differences between groups was assessed by unpaired Student's *t* test or two-way ANOVA followed by Bonferroni correction. Differences were considered significant if $p < 0.05$.

Results

Effects of MnTBAP on low-glucose-induced oxidative stress and apoptosis in cultured rat islets After 1 week of preculture in G10, further culture of rat islets in G5 vs G10 for 18–72 h significantly increased their total glutathione content (in $\mu\text{g}/\mu\text{g}$ protein) from 0.14 ± 0.01 in G10 to 0.30 ± 0.01 in G5 ($n=3$, $p < 0.001$, unpaired *t* test). After a few days of culture of islet cell clusters in G10, the roGFP1 fluorescence ratio was higher in the mitochondrial matrix (mt-roGFP1) than in the cytosol (roGFP1) ($p < 0.0001$, unpaired *t* test). Overnight culture in G5 vs G10 induced a significant increase in mt-roGFP1 but not in roGFP1 fluorescence ratio (Fig. 1a, c; ESM Fig. 1). Similar changes in mt-roGFP1 oxidation were observed after 3 days of culture (ESM Fig. 2). This increase in mt-roGFP1 oxidation indicates that culture in low glucose increases thiol (probably glutathione) oxidation in the mitochondrial matrix [27]. Accordingly, 1-week culture of rat islets in G5 vs G10 markedly increased the mRNA levels of the oxidative stress-inducible genes, haem oxygenase 1 (*Hmox1*), metallothionein 1a (*Mt1a*) and *c-Myc* (Fig. 1d–f) without affecting the mRNA levels of the antioxidant enzymes Mn-superoxide dismutase (*Sod2*) and catalase (ESM Fig. 3). These changes were associated with a large increase in islet DNA fragmentation and in the percentage of TUNEL-positive islet cells (Fig. 1g, h; ESM Fig. 4), but only slightly reduced the proportion of beta cells (measured by immunohistochemistry on islet sections) from 82% in G10 to 73% in G5 ($n=2$). Except for the increase in *Hmox1* mRNA levels, the addition of 50 $\mu\text{mol/l}$ MnTBAP to the culture medium significantly reduced these alterations by ~ 50 – 70%

Fig. 1 Effects of MnTBAP on the stimulation of mt-roGFP1 oxidation, islet cell apoptosis and expression of oxidative stress-response genes by 1 week of culture in low glucose. **(a–c)** Rat islet cell clusters producing mt-roGFP1 or roGFP1 were cultured overnight in RPMI medium containing 5 mmol/l glucose (G5, thick traces) or 10 mmol/l glucose (G10, thin dotted traces) with 10% FBS in the absence **(a, white columns in c)** or presence **(b, black columns in c)** of 50 μ mol/l MnTBAP. **(a,b)** Ratio of roGFP1 fluorescence intensities (excitation 400/480 nm) was measured during 20 min of perfusion in a medium similar to that used during culture and expressed as a percentage of the difference between the mean ratio measured from 4 to 8 min after addition of 10 mmol/l DTT (set at 0%) and that measured from 14 to 18 min after addition of 1 mmol/l H₂O₂ (set at 100%). Mean recordings in clusters producing cytosolic roGFP1 are shown in ESM Fig. 1. **(d–h)** Rat islets were cultured

for 1 week in medium containing 5 or 10 mmol/l glucose (G5 or G10) and 5 g/l BSA in the absence (white columns) or presence (black columns) of 50 μ mol/l MnTBAP. **(d–f)** Islet *Hmox1*, *Mt1a* and *c-Myc* to *Tbp* mRNA ratios are expressed relative to the levels in islets cultured in G10. Data are means $\pm$ SEM for five experiments. **(g)** Cytosolic histone-associated oligonucleosomes were measured with a commercial ELISA. Data are means $\pm$ SEM for three experiments. **(h)** Percentage of apoptotic islet cells computed as the ratio TUNEL-positive/DAPI-positive nuclei on islet sections. Results are means $\pm$ SEM for 20–86 islets in each group. Representative islet sections are shown in ESM Fig. 4. * p <0.05 for the effect of glucose (G5 vs G10) by two-way ANOVA + Bonferroni correction or by Student's *t* test, respectively. † p <0.05 for the effect of MnTBAP by two-way ANOVA + Bonferroni correction. AU, arbitrary units; Gn, *n* mmol/l glucose

(Fig. 1). MnTBAP also significantly reduced mt-roGFP1 oxidation in cell clusters cultured for 3 days in G10 (ESM Fig. 2) and tended to reduce islet cell apoptosis after 1 week of culture in G10 (Fig. 1g, h), suggesting its slight beneficial effect under control culture conditions associated with minimal apoptosis [8].

Effects of MnTBAP on the alterations of glucose-stimulated $[Ca^{2+}]_i$ rise and insulin secretion by prolonged culture in low glucose To test whether oxidative stress contributes to the alterations in beta cell glucose responsiveness after culture in low glucose, we measured glucose-induced changes in $[Ca^{2+}]_i$ and insulin secretion in islets cultured for 1 week in medium containing G5 or G10 with or without MnTBAP. After culture in G10, acute glucose stimulation with concentrations from 0.5 (G0.5) to 20 (G20) mmol/l induced a transient decrease in $[Ca^{2+}]_i$ followed by rapid

rises in $[Ca^{2+}]_i$ and insulin secretion that were further increased upon islet depolarisation with 30 mmol/l extracellular K⁺ (K30). In comparison, after 1 week of culture in G5, the islet resting $[Ca^{2+}]_i$ was not different, but the glucose-induced $[Ca^{2+}]_i$ rise was greatly reduced by 87%, while the K30-induced $[Ca^{2+}]_i$ rise was unaffected (Fig. 2a; Table 1). These alterations, together with the large reduction in islet insulin to DNA content ratio and preproinsulin mRNA levels (Table 3), led to near-complete suppression (~99% inhibition) of insulin secretion in response to both stimuli (Fig. 2d; Table 2).

Addition of MnTBAP during culture in G10 did not affect the subsequent acute glucose-induced changes in $[Ca^{2+}]_i$ and insulin secretion (Fig. 2b, e; Tables 1 and 2). In contrast, addition of the antioxidant during culture in G5, which tended to increase islet resting $[Ca^{2+}]_i$, significantly increased the high-glucose-induced $[Ca^{2+}]_i$ rise by approximately fourfold, so that it was only 50–60% lower than that in islets cultured in

Fig. 2 Effects of MnTBAP on the alterations in glucose-induced $[Ca^{2+}]_i$ rise and insulin secretion by 1 week of culture in low glucose. Rat islets were cultured for 1 week in the presence of G5 or G10 without (a, d) or with (b, e) 50 μ mol/l MnTBAP. They were then perfused with a medium containing 0.5 mmol/l glucose (G0.5) and acutely stimulated from 0.5 to 20 mmol/l glucose (G0.5 to G20), as indicated on top of the panels (Dz, 250 μ mol/l diazoxide; K30, 30 mmol/l extracellular K^+ concentration). Glucose-induced changes in $[Ca^{2+}]_i$ (a, b) were measured by microspectrofluorimetry and insulin secretion (d, e) by RIA. Thin traces and white symbols, islet cultured in G10; thick traces and closed symbols, islets

cultured in G5. Dotted traces in (a) and (b) are extrapolations of $[Ca^{2+}]_i$ traces obtained in islets perfused in the presence of G0.5 throughout the experiment (c: thin traces, culture in G10; thick traces, culture in G5; continuous traces, culture without MnTBAP; dotted traces, culture with MnTBAP). Insulin secretion data were normalised for differences in islet DNA content. The effect of MnTBAP on insulin secretion after culture in G5 is better shown in (f) where the y-axis scale has been expanded (circles, culture without MnTBAP; triangles, culture with MnTBAP). Results are means $\pm$ SEM for eight independent $[Ca^{2+}]_i$ experiments (corresponding to 12–13 islets) and for four insulin secretion experiments

G10 or G10 with MnTBAP (Fig. 2b, Table 1). However, MnTBAP only tended to increase the islet insulin to DNA content ratio and preproinsulin mRNA levels by ~40% (Table 3). Furthermore, it only slightly increased the insulin secretory response to glucose and K30 stimulation, so that the acute glucose- and K30-stimulated insulin secretion remained 97% lower after a 1-week culture in G5 vs G10 (Fig. 2e, f; Table 2). After normalisation for differences in islet insulin content, however, MnTBAP only tended to improve the insulin secretory response to glucose and no longer improved its stimulation by K30 (Table 2).

Effects of MnTBAP on the alterations in glucose-stimulated mitochondrial metabolism by prolonged culture in low glucose To check whether the beneficial effect of

MnTBAP on beta cell $[Ca^{2+}]_i$ glucose responsiveness after culture in G5 resulted from an increase in glucose metabolism, we next measured the glucose-induced changes in NAD(P)H autofluorescence, rhodamine 123 fluorescence as an indicator of the mitochondrial membrane potential, and adenine nucleotide levels. As expected, acute glucose stimulation from 0.5 to 20 mmol/l rapidly increased NAD(P)H autofluorescence, hyperpolarised the mitochondrial membrane (as shown by the decrease in rhodamine 123 fluorescence), and increased the ATP/(ATP+ADP) ratio in islets cultured in G10. Addition of 5 mmol/l azide, which inhibits electron-transport chain complex IV, further increased NAD(P)H autofluorescence while maximally depolarising the mitochondrial membrane and reducing the ATP/(ATP+ADP) ratio (Fig. 3a, c, e).

Table 1 Effects of MnTBAP on changes in islet $[Ca^{2+}]_i$ (nmol/l) induced by 1 week of culture in low vs intermediate glucose concentrations

Culture condition	n	$[Ca^{2+}]_i$ (nmol/l)		
		G0.5	Δ_1 (G0.5–G20)	Δ_2 (G0.5–G20K30Dz)
G5	12	123±10	9±4*	125±6
G5+MnTBAP	13	148±10	35±4* [†]	94±11* [‡]
G10	12	117±7	71±7	149±10
G10+MnTBAP	13	124±7	85±9	132±11

Results are means±SEM for the indicated number of experiments (n)

In each experiment illustrated in Fig. 2a,b, we calculated the mean $[Ca^{2+}]_i$ during the first 4 min in G0.5, the last 20 min in G20, and the last 5 min in G20K30Dz. Δ_1 and Δ_2 show the mean differences between $[Ca^{2+}]_i$ measured in the presence of G0.5 (dotted lines in Fig. 2) and G20 or G20K30Dz (solid lines in Fig. 2) in different islets during equivalent periods of perfusion

* $p<0.05$ for the effect of glucose (G5 vs G10) and [†] $p<0.05$ for the effect of MnTBAP by two-way ANOVA + Bonferroni correction

[‡] $p<0.05$ by Student's *t* test for the effect of MnTBAP

Dz, diazoxide

In comparison with control islets, the effects of glucose on NAD(P)H autofluorescence were significantly smaller in islets cultured in G5, both in absolute values and when

Table 2 Effects of MnTBAP on the alterations in glucose-stimulated insulin secretion after 1 week of culture in low vs intermediate glucose concentrations

Culture condition	n	G0.5	Δ_1 (G0.5–G20)	Δ_2 (G0.5–G20K30Dz)
Insulin secretion (amol/min per ng islet DNA)				
G5	4	0.53±0.10	0.76±0.02**	5.77±1.05*
G5+MnTBAP	4	0.46±0.06	3.56±0.52* ^{††}	11.97±1.23* ^{††}
G10	4	1.39±0.31	152.1±20.0	413.0±55.5
G10+MnTBAP	4	1.22±0.38	169.1±20.2	429.4±54.7
Insulin secretion (% of insulin content per min)				
G5	4	0.001±0.0001	0.006±0.001**	0.041±0.009**
G5+MnTBAP	4	0.001±0.0003	0.012±0.003**	0.039±0.007**
G10	4	0.004±0.001	0.141±0.011	0.426±0.049
G10+MnTBAP	4	0.001±0.0002	0.156±0.019	0.454±0.105

Results are means±SEM for the indicated number of experiments (n)

In each experiment illustrated in Fig. 2d,e, we computed the average rate of insulin secretion (both in absolute values and relative to the islet insulin content) during the last 8 min in G0.5, the last 30 min in G20, and the last 18 min in G20K30Dz. Δ_1 or Δ_2 show the mean increase in insulin secretion between G0.5 and G20 or G20K30Dz

** $p<0.01$ for the effect of glucose (G5 vs G10) by two-way ANOVA + Bonferroni correction. There was no significant effect of MnTBAP by two-way ANOVA + Bonferroni correction

^{††} $p<0.01$ by Student's *t* test for the effect of MnTBAP

Dz, diazoxide

expressed as a percentage of the difference in fluorescence between G0.5 and G20+ azide (Fig. 3a; Table 4). Regarding rhodamine 123 fluorescence in islets previously cultured in G5 vs G10, it was lower and tended to decrease with time following a one-phase exponential decay curve with a $T_{1/2}$ of approximately 19 min (Fig. 3c; Table 5). When this leakage was taken into account, the amplitude of the mitochondrial responses to high glucose were significantly lower than those in islets cultured in G10, whereas the response to azide was not (Table 5). A possible explanation for this leakage can be found in ESM Table 2.

Concerning the ATP/(ATP+ADP) ratio, it was significantly higher in islets cultured in G5 vs G10 after incubation in G0.5 but not after incubation in G20 (Fig. 3e). However, we have previously shown that a granular pool of adenine nucleotide with low ATP/ADP ratio reduces the ATP/ADP ratio measured in freshly isolated vs cultured mouse islets and its increase by glucose [24]. This granular pool thereby precludes direct comparison of the amplitude of glucose-induced changes in the ATP/(ATP+ADP) ratio between conditions associated with large changes in insulin content, i.e. between islets cultured in G5 vs G10 (Table 3). To circumvent this difficulty, we normalised the ATP/(ATP+ADP) ratios measured in G0.5 and G20 to that measured in G20+azide. On doing so, the glucose-induced increase in normalised ATP/(ATP+ADP) ratio was significantly lower in islets cultured in G5 vs G10 (Fig. 3f). As shown in Fig. 3b, d–f and Tables 4 and 5, the addition of MnTBAP during culture did not increase the amplitude of the acute metabolic response to glucose in islets cultured in G5.

Effects of MnTBAP on the alterations in tolbutamide-induced $[Ca^{2+}]_i$ rise by prolonged culture in low glucose As the beneficial effect of MnTBAP on beta cell $[Ca^{2+}]_i$ glucose responsiveness after culture in G5 did not result from an improvement in glucose metabolism, we next tested the effect of culture in low glucose with or without MnTBAP on the acute $[Ca^{2+}]_i$ response to a maximally effective concentration of tolbutamide, a drug that closes K_{ATP} channels. As shown in Fig. 4 and Table 6, 1 week of culture in G5 vs G10 significantly decreased the $[Ca^{2+}]_i$ response to tolbutamide by ~75% without significantly affecting the response to K30. After culture in the presence of MnTBAP, basal $[Ca^{2+}]_i$ was significantly elevated in islets cultured in G5 and to a lesser extent in islets cultured in G10, and the $[Ca^{2+}]_i$ response to tolbutamide was increased to a larger extent in islets cultured in G5 vs G10, so that the absolute amplitude of this $[Ca^{2+}]_i$ rise was only 55% lower in islets cultured in G5 vs G10. When expressed as a percentage of the difference between basal and K30-stimulated $[Ca^{2+}]_i$, MnTBAP completely prevented the ~65% reduction in the $[Ca^{2+}]_i$ response to tolbutamide induced by culture in G5.

Table 3 Effects of MnTBAP on the alterations in islet insulin and DNA content, insulin to DNA content ratio, and preproinsulin mRNA levels after 1 week of culture in low vs intermediate glucose concentrations

Culture condition	<i>n</i>	Insulin content (pmol/islet)	DNA content (ng/islet)	Insulin/DNA content ratio (fmol/ng)	Preproinsulin/ <i>Tbp</i> mRNA ratio (relative to G10)
G5	4–5	1.61±0.21**	94±12	18.3±3.6**	0.13±0.01**
G5+MnTBAP	4–5	2.29±0.26**	90±6	25.5±1.8	0.20±0.03**
G10	4–5	5.73±1.07	102±12	61.1±15.7	1.00±0.01
G10+MnTBAP	4–5	5.20±0.73	106±21	50.7±3.3	0.76±0.13 [†]

Results are means±SEM for the indicated number of experiments (*n*)

Islet DNA and insulin content were measured at the end of each experiment illustrated in Fig. 2d–f. Insulin/*Tbp* mRNA ratios were measured by real-time RT-PCR and normalised to the mean ratio for conditions G5 plus G10 within each experiment before being expressed relative to the levels in islets cultured in G10

***p*<0.01 for the effect of glucose (G5 vs G10) and [†]*p*<0.05 for the effect of MnTBAP by two-way ANOVA + Bonferroni correction

Effects of NAC alone or in combination with MnTBAP on the alterations in islet cell survival and function after culture in low glucose Because MnTBAP did not fully reduce the stimulation of mt-roGFP1 oxidation, oxidative stress-response gene expression and islet cell apoptosis by culture in G5, we also tested the efficacy of the H₂O₂ scavenger NAC. As shown in ESM Fig. 5a–e, addition of 1 mmol/l NAC during culture markedly decreased the stimulation of *Hmox1* mRNA by culture in G5 vs G10, but was ineffective against the oxidation of mt-roGFP1 and the stimulation of islet cell apoptosis and *c-Myc* and *Mt1a* mRNA expression. Accordingly, 1 mmol/l NAC did not improve the Ca²⁺ response to acute glucose stimulation in islets cultured in G5 (ESM Fig. 5f, g). When used at 10 mmol/l, NAC significantly decreased *Mt1a* mRNA levels, but also increased mt-roGFP1 oxidation and islet cell apoptosis in G5 without improving the Ca²⁺ response to acute glucose stimulation in islets cultured in G5 (ESM Fig. 5h).

MnTBAP may, at least in part, exert its antioxidant action by increasing superoxide dismutation. We therefore tested whether H₂O₂ scavenging with 1 mmol/l NAC, although ineffective alone, could improve the antioxidant efficacy of MnTBAP. However, in comparison with the effect of MnTBAP alone, the combination of the two agents did not further improve beta cell survival and [Ca²⁺]_i glucose responsiveness after 1 week of culture in G5 (ESM Fig. 6).

Discussion

This study shows that prolonged culture of rat islets in a non-stimulating glucose concentration rapidly increases mitochondrial glutathione oxidation, followed by beta cell apoptosis and loss of acute GSIS. Under these conditions, the antioxidant, MnTBAP, triggered a parallel ~50% reduction in both mt-roGFP1 oxidation and beta cell apoptosis, thereby extending previous studies

showing a causal link between oxidative stress and islet cell apoptosis during culture in low glucose [14, 15]. However, despite its antiapoptotic effect, MnTBAP failed to significantly protect against the loss of GSIS, suggesting that the loss of GSIS during prolonged culture in G5 is unrelated to oxidative stress, or that beta cell function is more sensitive than beta cell survival to the level of mitochondrial oxidative stress that persists in the presence of MnTBAP.

Low glucose concentrations increase mitochondrial oxidative stress in rat islet cells The effect of glucose on pancreatic beta cell production of reactive oxygen species (ROS) is controversial, ranging from increased oxidation of ROS-sensitive fluorescent dyes by high glucose concentrations [28] to decreased oxidation of the dyes by glucose stimulation [14, 29–31]. The redox-sensitive fluorescent protein roGFP1 does not directly measure ROS production but rather reflects the balance between ROS-dependent oxidation and NADPH-dependent reduction of thiols (glutathione) by a mechanism involving glutaredoxin [23, 27]. Using this probe, we found that lowering the glucose concentration from 10 to 5 mmol/l for 18 h increases thiol oxidation in the mitochondrial matrix of dispersed rat islet cells. The increase in mt-roGFP1 fluorescence ratio in G5 is unlikely to have resulted from reduced expression of glutaredoxin without changes in thiol oxidation state, as it also occurs upon acute reduction of glucose from 10 to 2 mmol/l [19]. The increase in mt-roGFP1 fluorescence ratio may also have resulted from a decrease in mitochondrial glutathione content without changes in mt-roGFP1 oxidation state, but we consider this hypothesis unlikely because islet total glutathione content increased [27] and the mitochondrial and cytosolic concentrations of GSH are similar in other cell types (reviewed by Lash [32]).

After culture in G10, the roGFP1 fluorescence ratio was greater in the mitochondrial matrix than in the cytosol of rat

Fig. 3 Effects of MnTBAP on the alterations in glucose-induced changes in mitochondrial metabolism by 1 week of culture in low glucose. After 1 week of culture in G5 or G10 without (**a, c**) or with (**b, d**) 50 $\mu\text{mol/l}$ MnTBAP, NAD(P)H autofluorescence (**a, b**), rhodamine 123 fluorescence (**c, d**) and adenine nucleotide levels (**e, f**) were measured in islets perfused or incubated with a medium containing G0.5 (**e, f**, white bars), G20 (**e, f**, hatched bars) and G20+5 mmol/l sodium azide (**e, f**, closed bars), or with medium containing G0.5 throughout the experiment (**c, d**, dotted traces). (**a–d**) Thin lines, culture in G10; thick lines, culture in G5. The rhodamine 123 fluorescence was expressed as a percentage of the fluorescence measured from the 19th to 20th minute of perfusion (last min of perfusion in G0.5 before the stimulation with G20). Data are means $\pm$ SEM for eight independent NADP(H) experiments (corresponding to 14 islets), five independent rhodamine 123 experiments (corresponding to six to ten islets) and 14 batches of ten islets for adenine nucleotide measurements (four independent incubations). Δ_1 and Δ_2 , see Tables 5 and 6. (**f**) Ratio ATP/(ATP+ADP) in (**e**) was normalised to the ratio measured in islets cultured under the same condition and incubated in the presence of G20 + azide. $^{\dagger}p < 0.0001$ for the acute effect of G20 vs G0.5; $^*p < 0.05$ and $^{***}p < 0.001$ for the effect of G5 vs G10 during culture; $^{\dagger\dagger\dagger}p < 0.001$ for the effect of MnTBAP during culture (two-way ANOVA + Bonferroni correction)

Table 4 Effects of MnTBAP on the alterations in glucose-induced changes in NAD(P)H autofluorescence after 1 week of culture in low vs intermediate glucose concentrations

Culture condition	n	Change in NAD(P)H autofluorescence (AU)		
		Δ_1 (G0.5–G20)	Δ_2 (G20–G20 azide)	$\Delta_1/(\Delta_1+\Delta_2)$
G5	14	21.6±2.0**	20.4±1.6**	0.512±0.015**
G5+MnTBAP	14	23.4±1.4**	24.4±1.4	0.489±0.006**
G10	14	39.8±2.8	28.3±1.3	0.579±0.013
G10+MnTBAP	14	37.2±2.0	26.5±1.3	0.583±0.007

Results are means±SEM for the indicated number of experiments (*n*) G0.5 and G20, 0.5 and 20 mmol/l glucose; azide: 5 mmol/l Na⁺-azide. In each experiment illustrated in Fig. 3a, b, the difference in NAD(P)H autofluorescence between G0.5 and G20 (Δ_1) and between G20 and G20 azide (Δ_2) was computed from the average NAD(P)H autofluorescence during the last 5 min in G0.5, the last 20 min in G20, and the last 8 min in G20 azide

***p*<0.01 for the effect of glucose (G5 vs G10) by two-way ANOVA + Bonferroni correction. There was no significant effect of MnTBAP on any of these measurements by two-way ANOVA + Bonferroni correction

AU, arbitrary fluorescence units

islet cells, as reported in cardiomyocytes [33]. The further oxidation of mt-roGFP1 but not roGFP1 after culture in G5 indicates that thiol oxidation at low glucose mainly occurs in the mitochondrial matrix, suggestive of mitochondrial oxidative stress. In agreement, stimulation of dihydroethidine

Table 5 Effects of MnTBAP on the alterations in glucose-induced changes in rhodamine 123 fluorescence after 1 week of culture in low vs intermediate glucose concentrations

Culture condition	n	Change in rhodamine 123 fluorescence (% of basal)	
		Δ_1 (G0.5–G20)	Δ_2 (G20–G20 azide)
G5	10	−8.6±1.6***	21.1±4.0
G5+MnTBAP	10	−8.3±1.4***	17.9±3.2
G10	6	−20.2±1.9	19.6±2.0
G10+MnTBAP	8	−20.3±1.9	22.2±3.0

Results are means±SEM for the indicated number of experiments (*n*) G0.5 and G20, 0.5 and 20 mmol/l glucose; azide: 5 mmol/l Na⁺-azide. In each experiment illustrated in Fig. 3c,d, we computed the mean rhodamine 123 fluorescence from the 23rd to the 60th min, from the 57th to the 60th min, and from the 62nd to the 70th min of perfusion. Δ_1 shows the mean difference between rhodamine 123 fluorescence during the last 37 min of perfusion in the presence of G0.5 (dotted lines in Fig. 3) and G20 (solid lines in Fig. 3). Δ_2 shows the mean difference in rhodamine 123 fluorescence between the last 3 min in G20 and the last 8 min in G20 azide

****p*<0.001 for the effect of glucose (G5 vs G10) by two-way ANOVA+Bonferroni correction. There was no significant effect of MnTBAP on any of these measurements by two-way ANOVA + Bonferroni correction

Fig. 4 Effects of MnTBAP on the alterations of tolbutamide-induced [Ca²⁺]_i rise by 1 week of culture in low glucose. Rat islets were cultured for 1 week in the presence of G5 (a) or G10 (b) in the absence (thin traces) or presence (thick traces) of 50 μmol/l MnTBAP. They were then perfused with a medium containing 0.5 mmol/l glucose (G0.5) and acutely stimulated with 500 μmol/l tolbutamide (Tolbu), as indicated on top of the panels (Dz, 250 μmol/l diazoxide; K30, 30 mmol/l extracellular K⁺ concentration). Shown are traces from individual islets representative of 26–28 islets from three different islet preparations. Mean [Ca²⁺]_i values are shown in Table 6

oxidation in beta cells exposed to low glucose was found to be reduced by rotenone, which inhibits the mitochondrial electron-transport-chain complex I [14, 31]. Nevertheless, because H₂O₂ diffuses through lipid membranes, oxidative stress should also occur in the cytoplasm of beta cells exposed to low glucose, as suggested by the increase in expression of oxidative stress-response genes. It is indeed likely that the almost fully reduced state of cytosolic roGFP1 hampered detection of a moderate increase in thiol oxidation in G5 [27]. In this context, it is also interesting to note that NAC was able to reduce the stimulation of *Hmox1* and *Mt1a* mRNA expression by low glucose but did not reduce mt-roGFP1

Table 6 Effects of MnTBAP on changes in islet [Ca²⁺]_i induced by tolbutamide after 1 week of culture in low vs intermediate glucose concentrations

Culture condition	n	[Ca ²⁺] _i (nmol/l)		
		G0.5	Δ (G0.5+Tolbu)	Δ (G0.5+K30Dz)
G5	26	87±2*	17±3*	121±1*
G5+MnTBAP	28	190±9*†	70±4*†	117±4*
G10	26	79±1	75±5	179±6
G10+MnTBAP	26	105±3†	128±5†	213±8†

Results are means±SEM for the indicated number of experiments (*n*) In each experiment illustrated in Fig. 4, we calculated the mean [Ca²⁺]_i during the first 4 min in G0.5, the last 10 min in G0.5+Tolbu, and the last 5 min in G0.5+K30Dz

**p*<0.05 for the effect of glucose (G5 vs G10) and †*p*<0.05 for the effect of MnTBAP by two-way ANOVA + Bonferroni correction

Dz, diazoxide; Tolbu, tolbutamide

oxidation and beta cell apoptosis. This lack of efficacy of NAC, which has also been observed in INS-1 cells exposed to low glucose [31], may result from its inability to reach the mitochondrial compartment or the nature of the reactive species involved.

Role of oxidative stress in the loss of GSIS after culture in low glucose The almost complete loss of GSIS in islets chronically cultured in low glucose is unlikely to have resulted from the ~10% reduction in beta cell proportion in the islets. It rather resulted from the combination of an ~50% reduction in the glucose stimulation of mitochondrial metabolism, an ~70% decrease in insulin content, an ~90% decrease in glucose-induced $[Ca^{2+}]_i$ rise, and an ~98% reduction in Ca^{2+} -induced insulin secretion. The reduction in the glucose stimulation of mitochondrial metabolism was relatively small, indicating that it better resisted prolonged culture in G5 than other stimulus-secretion coupling events. It may, however, contribute to the loss of GSIS after culture in G5, as it lies upstream of the glucose stimulation of Ca^{2+} influx, Ca^{2+} -induced exocytosis and insulin synthesis [34, 35]. However, the strongly reduced Ca^{2+} response to tolbutamide after culture in G5 indicates that the coupling between mitochondrial metabolism and Ca^{2+} influx was markedly altered in these islets, as was the coupling between K30-induced $[Ca^{2+}]_i$ rise and insulin secretion. The latter two defects thus mainly contribute to the loss of GSIS after prolonged culture in G5.

Interestingly, reducing mitochondrial oxidative stress and apoptosis by 50–70% with MnTBAP did not improve the acute glucose stimulation of NAD(P)H production, mitochondrial membrane hyperpolarisation and ATP production, and only tended to increase insulin mRNA levels, islet insulin content and stimulation of insulin secretion by glucose and K30. It therefore seems that these alterations may have resulted from the lack of glucose per se independently of an increase in mitochondrial oxidative stress. Culture in G5 is indeed associated with decreased expression of many genes that are enriched in beta cells and are involved in the maintenance of their glucose responsiveness [1, 8]. However, one cannot totally exclude the possibility that these functional alterations resulted from their exquisite sensitivity to the lower level of oxidative stress that persists in the presence of MnTBAP, or from the presence of ROS against which MnTBAP is ineffective. Indeed, among the transcription factors that govern beta cell-specific gene expression, MAF-A and PDX-1 have been shown to be highly sensitive to oxidative stress [36, 37]. Mitochondrial metabolism is also highly susceptible to oxidative stress [38], in part because of S-glutathiolation and consequent inhibition of mitochondrial complex I and IV, aconitase and pyruvate dehydrogenase [39]. Finally, several components of the exocytotic machinery, including *N*-ethylmaleimide-sensitive factor and SNAP25, are highly sensitive to oxidative stress,

leading to a marked reduction in SNARE complex formation upon H_2O_2 treatment in other cell types [40, 41].

In contrast, MnTBAP significantly improved the stimulation of Ca^{2+} influx by glucose, indicating that it is oxidative stress that specifically alters the coupling between mitochondrial metabolism and Ca^{2+} influx after culture in G5, as reported in clonal insulin-secreting INS-1 cells exposed to exogenous H_2O_2 [42]. Such uncoupling is unlikely to have resulted from large alterations in voltage-dependent Ca^{2+} channels, as shown by the normal rise in $[Ca^{2+}]_i$ upon membrane depolarisation with K30. It may rather result from subtle alterations in their voltage sensitivity, alterations in ATP-sensitive K^+ channel (K_{ATP} channel) properties [43, 44], or increased expression/activity of a repolarising current, e.g. the large conductance Ca^{2+} -activated K^+ channels [45]. The last two hypotheses (altered K_{ATP} channel properties and increased repolarising current) are supported by the observation that MnTBAP markedly improved the acute $[Ca^{2+}]_i$ rise in response to a maximal concentration of tolbutamide. However, these results do not allow us to distinguish between the two hypotheses.

The pathophysiological relevance of this study is not likely to be restricted to the rare instances when normal beta cells are exposed to sustained hypoglycaemia because of the presence of a focal insulinoma. Indeed, the changes in islet gene expression and beta cell survival observed after culture in low glucose have also been described under conditions associated with a decrease in the glucose sensitivity of beta cells, i.e. after in vitro treatment with cytokines, H_2O_2 and ribose, or in islets isolated from rodent models of type 1 and type 2 diabetes (reviewed by Martens and van de Casteele [16] and Jonas et al [46]). Interestingly, it has recently been shown that increasing glucose metabolism with a glucokinase activator partially protects beta cells from H_2O_2 -induced apoptosis in vitro [47].

In conclusion, MnTBAP efficiently decreases mitochondrial glutathione oxidation and islet cell apoptosis during prolonged culture in low glucose without significantly improving GSIS, indicating that mitochondrial oxidative stress contributes differently to islet cell apoptosis and insulin secretory defects under these conditions. Regarding drug development, targeting beta cell survival may not suffice to restore insulin secretion when beta cells suffer from prolonged mitochondrial oxidative stress, e.g. when glucose metabolism is reduced.

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