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

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Abbreviations
$[Ca^{2+}]i$, intracellular Ca^{2+} concentration; Dz, diazoxide; MnTBAP, Manganese (III)tetrakis (4-benzoic acid)porphyrin; NAC, N-Acetyl-L-Cysteine; Gn, n mmol/l glucose; K30, 30 mmol/l extracellular K^+; roGFP, redox sensitive Green Fluorescent Protein; TUNEL, Terminal deoxynucleotidyl transferase dUTP Nick End Labeling.

Abstract

Aims/hypothesis: Pancreatic beta-cells chronically exposed to low glucose concentrations present 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 one week in RPMI medium containing 5 to 10 mmol/l glucose ± 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 GFP (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 due to reduced insulin content, mitochondrial metabolism, Ca^{2+} influx and Ca^{2+}-induced secretion. Tolbutamide- but not high potassium-induced Ca^{2+} influx was also suppressed. Under these conditions, MnTBAP, but not NAC, triggered parallel ~50-70% reductions of 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 differently contributes to rat pancreatic islet cell apoptosis and insulin secretory defect 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.

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 in [1,2]). For instance, prolonged fasting in rodents reduces islet mRNA and protein expression of beta-cell enriched genes like preproinsulin, glucose transporter-2, glucokinase and voltage-dependent calcium channels, with concomitant reduction of glucose-stimulated insulin secretion (GSIS), while subsequent feeding of the animals corrects these alterations within one day [3-5]. Similar alterations in gene expression were observed in native beta-cells of rats implanted with an insulinoma, together with a marked reduction of the 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 the *in vitro* stimulation of insulin secretion, markedly altered the expression of beta-cell enriched genes and the beta-cell ability 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 program [12], 3) increased expression 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 in [16]). The role of oxidative stress was supported by the 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-acetyl-L-cysteine (NAC) and by manganese (III)tetrakis (4-benzoic acid)porphyrin (MnTBAP) [14,15], a superoxide dismutase mimetic, catalase mimetic and peroxinitrite scavenger that also increases the expression of the antioxidant and antiapoptotic enzyme heme-oxygenase 1 [17,18]. However, the possible link between increased oxidative stress and the 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 following their exposure to various stress [16], we tested the effects of MnTBAP and NAC on the alterations of beta-cell glucose-responsiveness in rat islets cultured for one week in a low non-stimulating glucose concentration.

Materials and Methods

Materials – Diazoxide, Na^+-azide, dithiothreitol (DTT) and tolbutamide were from Sigma (St-Louis, MI, 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 (ESM methods) [19].

Islet isolation and culture – Islets from ~200g 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 5g/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 reversed transcribed as described [21] using RevertAid^{TM} Reverse Transcriptase and Ribolock^{TM} RNase inhibitor (Fermentas, Burlington, ON, Canada). Real-time PCR were performed with an iCycler (Bio-Rad). For primer sequences and reaction conditions, see ESM Table 1. Gene to *Tbp* mRNA ratios were computed as $2^{-(Ct\textit{Gene}-Ct\textit{Tbp})}$ and expressed relative to the ratio in G10-cultured islets.

Cell apoptosis - Histone-associated DNA fragments (mono- and oligonucleosomes) 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 terminal deoxynucleotidyl transferase-mediated dUTP-nick end-labeling (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 (~$1.8x10^6$ infectious units per dish, MOI~25-50) and further cultured 24-48h in G10, then 18-24h 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 40X objective. Cell clusters were perifused 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 [23]. Briefly, cell fluorescence at 535 nm was measured during alternate excitation at 400/480 nm every 30 s. After 20 min of perifusion in a medium similar to that used during the last culture period, cells were perifused with 10 mmol/l dithiothreitol (DTT) to maximally reduce roGFP1, followed by 1 mmol/l hydrogen peroxide (H_2O_2) to maximally oxidize 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 oxidized forms of roGFP1.

Total glutathione content - Total glutathione was measured with the Glutathione Fluorescent Detection Kit (Arbor Assays, Ann Arbor, MI, USA) and normalized 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, 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 ($[Ca^{2+}]_i$) and insulin secretion - After culture, islets were transferred to a 37°C temperature-controlled perifusion chamber either immediately for the measurement of NAD(P)H autofluorescence and insulin secretion, after loading for 20 min with 10 µmol/l rhodamine 123 (Molecular probes, Eugene, OR, USA) to measure the mitochondrial membrane potential, or after loading for 2 h with furaPE3-acetoxymethylester to measure changes in $[Ca^{2+}]_i$. The loading medium was similar to that used during culture except for the omission of NAC during furaPE3 loading. Islets were then perifused (flow rate ~1 ml/min) with a bicarbonate-buffered Krebs solution containing 0.5 to 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 osmolarity unchanged. The acute glucose-induced changes in NAD(P)H levels, mitochondrial membrane potential and $[Ca^{2+}]_i$ were recorded by microspectrofluorimetry with Quanticell 700m (Visitech, Sunderland, UK) [20]. GSIS was measured by RIA in 2-min effluent collections from 30-110 perifused islets [25]. At the end of perifusion, the islets were disrupted by sonication in TNE (Tris 10 mmol/l, NaCl 0.2 mmol/l, EDTA 10 mmol/l) and their insulin and DNA contents were measured [26].

Statistical analysis - Results are means ± SE for at least 3 islet or cell cluster preparations. Statistical significance of differences between groups was assessed by unpaired Student t-test or by two-way ANOVA followed by a test of Bonferroni. 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 one week preculture in G10, further culture of rat islets in G5 *vs.* G10 for 18-72h significantly increased their total glutathione content (in µg/µg 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 day culture of islet cell clusters in G10, 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 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 (likely glutathione) oxidation in the mitochondrial matrix [27]. Accordingly, one week culture of rat islets in G5 *vs.* G10 markedly increased the mRNA levels of the oxidative stress-inducible genes heme-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, addition of 50 µmol/l MnTBAP to the culture medium significantly reduced these alterations by ~50-70% (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 one week 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 of beta-cell glucose responsiveness after culture in low glucose, we measured the glucose-induced changes in $[Ca^{2+}]_i$ and insulin secretion in islets cultured for one week in a medium containing G5 or G10 with or without MnTBAP. After culture in G10, acute glucose stimulation from 0.5 to 20 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 depolarization with 30 mmol/l extracellular K^+ (K30). In comparison, after one week of culture in G5, the islet resting $[Ca^{2+}]_i$ was not different but the glucose-induced $[Ca^{2+}]_i$ rise was strongly reduced by 87% while K30-induced $[Ca^{2+}]_i$ rise was unaffected (Fig.2a; Table1). These alterations, together with the strong reduction in islet insulin to DNA content ratio and preproinsulin mRNA levels (Table3), lead to a near complete suppression (~99% inhibition) of insulin secretion in response to both stimuli (Fig.2d; Table2).

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; Tables1,2). In contrast, addition of the antioxidant during culture in G5, which tended to increase islet resting $[Ca^{2+}]_i$, significantly improved high glucose-induced $[Ca^{2+}]_i$ rise by approximately 4-fold, so that it was only 50-60% lower than that in islets cultured in G10 or G10 with MnTBAP (Fig.2b, Table1). However, MnTBAP only tended to increase the islet insulin to DNA content ratio and preproinsulin mRNA levels by ~40% (Table3). 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 one-week culture in G5 *vs.* G10 (Fig.2e,f; Table2). After normalization 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 (Table2).

Effects of MnTBAP on the alterations of 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, hyperpolarized 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 depolarizing the mitochondrial membrane and reducing the ATP/(ATP+ADP) ratio (Fig.3a,c,e).

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 expressed as a percentage of the difference in fluorescence between G0.5 and G20 + azide (Fig.3a; Table4). 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 with a $T_{1/2}$ of approximately 19 min (Fig.3c; Table5). 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 (Table5) (a possible explanation for this leakage can be found in ESM-Table2).

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 ATP/(ATP+ADP) ratio between conditions associated with large changes in insulin content, i.e. between islets cultured in G5 *vs.* G10 (Table3). To circumvent this difficulty, we normalized the ATP/(ATP+ADP) ratios measured in G0.5 and G20 to that measured in G20+azide. Doing so, the glucose-induced increase in normalized ATP/(ATP+ADP) ratio was significantly lower in islets cultured in G5 *vs.* G10 (Fig.3f). As shown in figure 3b,d-f and tables 4-5, addition of MnTBAP during culture did not improve the amplitude of the acute metabolic responses to glucose in islets cultured in G5.

Effects of MnTBAP on the alterations of 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 figure 4 and

table 6, one 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 in percentage of the difference between basal and K30-stimulated $[Ca^{2+}]_i$, MnTBAP completely prevented the ~65% reduction of the $[Ca^{2+}]_i$ response to tolbutamide induced by culture in G5.

Effects of NAC alone or in combination with MnTBAP on the alterations of 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 H_2O_2 scavenger NAC. As shown in ESM-figure 4a-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 Ca^{2+}-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 Ca^{2+}-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 H_2O_2 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 both agents did not further improve beta-cell survival and $[Ca^{2+}]_i$ glucose-responsiveness after one week 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 of 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 ROS production 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 that probe, we found that lowering the glucose concentration from 10 to 5 mmol/l for 18h increases thiol oxidation in the mitochondrial matrix of dispersed rat islet cells. The increase in mt-roGFP1 fluorescence ratio in G5 unlikely 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 could also result 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 in [32]).

After culture in G10, roGFP1 fluorescence ratio was greater in the mitochondrial matrix than in the cytosol of rat 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, the stimulation of dihydroethidine oxidation in beta-cells exposed to low glucose was reduced by rotenone, which inhibits mitochondrial electron transport chain complex I [14,31]. Nevertheless, because H_2O_2 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 oxidative stress-response gene expression. 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 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 to 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 unlikely resulted from the ~10% reduction in beta-cell proportion within 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 resist to prolonged culture in G5 than other stimulus-secretion coupling events. It could nevertheless contribute to the loss of GSIS after culture in G5, as it lies upstream 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 hyperpolarization and ATP production, and only tended to increase insulin mRNA levels, the islet insulin content and the stimulation of insulin secretion by glucose and K30. It therefore seems that these alterations could result from the lack of glucose *per se* independently from an increase in mitochondrial oxidative stress. Culture in G5 is indeed associated with decreased expression of many beta-cell enriched genes 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 to the presence of reactive oxygen species 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 unlikely resulted from large alterations of voltage-dependent Ca^{2+}-channels, as shown by the normal rise in $[Ca^{2+}]_i$ upon membrane depolarization with K30. It may rather result from subtle alterations in their voltage-sensitivity, from alterations in ATP-sensitive K^+-channel (K_{ATP} channel) properties [43,44], or from increased expression/activity of a repolarizing current, e.g. the large conductance Ca^{2+}-activated K^+ channels [45]. The latter two hypotheses (altered K_{ATP} channel properties or increased repolarizing 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 distinguishing between both hypotheses.

The pathophysiological relevance of this study is unlikely restricted to the rare instances when normal beta-cells are exposed to sustained hypoglycaemia due to 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 in [16,46]). Interestingly, it has recently been shown that increasing glucose metabolism with a glucokinase activator partially protects beta-cells from hydrogen peroxide-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 differently contributes to islet cell apoptosis and insulin secretory defect 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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Contribution statement
Conceived and designed the experiments: SMP, LPR, JD, JCJ
Performed the experiments: SMP, LPR, JD, JCJ
Analyzed the data: SMP, LPR, JD, JCJ
Wrote the paper: LPR, SMP, JCJ
Corrections/suggestions to the paper: JD

Duality of interest
The authors declare that there is no duality of interest associated with this manuscript.

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Legends to figures

Figure 1. Effects of MnTBAP on the stimulation of mt-roGFP1 oxidation, islet cell apoptosis and oxidative stress-response gene expression by one week of culture in low glucose. a-c, rat islet cell clusters expressing 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 traces) with 10% FBS in the absence (a, open columns in c) or presence of 50 µmol/l MnTBAP (b, solid columns in c). a-b, the ratio of roGFP1 fluorescence intensities (exc 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 H_2O_2 (set at 100 %). Mean recordings in clusters expressing cytosolic roGFP1 are shown in ESM-Fig.1. d-h, rat islets were cultured for one week in a medium containing 5 or 10 mmol/l glucose (G5 or G10) and 5 g/l BSA in the absence (open columns) or presence of 50 µmol/l MnTBAP (solid columns). 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 ± SE for 5 experiments. g, cytosolic histone-associated oligonucleosomes were measured with a commercial ELISA. Data are means ± SE for 3 experiments. h, the percentage of apoptotic islet cells was computed as the ratio TUNEL-positive / DAPI-positive nuclei on islet sections. Results are means ± SE 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 + test of Bonferroni or by Student's t-test respectively. $^{\#}$, $P<0.05$ for the effect of MnTBAP by two-way ANOVA + test of Bonferroni. A.U., arbitrary units.

Figure 2. Effects of MnTBAP on the alterations of glucose-induced $[Ca^{2+}]_i$ rise and insulin secretion by one week of culture in low glucose. Rat islets were cultured for one week in the presence of G5 or G10 without (a, d) or with (b, e) 50 µmol/l MnTBAP. They were then perifused 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 µmol/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 open symbols, islet cultured in G10; Thick traces and closed symbols, islets cultured in G5. Dotted traces in panels a and b are extrapolations of $[Ca^{2+}]_i$ traces obtained in islets perifused in the presence of G0.5 throughout the experiment (panel 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 normalized for differences in islet DNA content. The effect of MnTBAP on insulin secretion after culture in G5 is better shown in panel f where the Y-scale has been expanded (circles, culture without MnTBAP; triangles, culture with MnTBAP. Results are means ± SE for 8 independent $[Ca^{2+}]_i$ experiments (corresponding to 12-13 islets) and for 4 insulin secretion experiments.

Figure 3. Effects of MnTBAP on the alterations of glucose-induced changes in mitochondrial metabolism by one week of culture in low glucose. After one week culture in G5 or G10 without (a, c) or with (b, d) 50 µmol/l MnTBAP, NAD(P)H autofluorescence (a, b), rhodamine 123 fluorescence (c, d) and adenine nucleotide levels (e, f) were measured in islets perifused or incubated with a medium containing G0.5 (e-f, open bars), G20 (e-f, hatched bars) and G20 + 5 mmol/l Na^+-azide (e-f, closed bars), or with a 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 min of perifusion (last min of perifusion in G0.5 before the stimulation with G20). Data are means ± SE for 8 independent NADP(H) experiments (corresponding to 14 islets), 5 independent rhodamine 123 experiments (corresponding to 6-10 islets) and 14 batches of 10 islets for adenine nucleotide measurements (4 independent incubations). Δ_1 and Δ_2, see Tables 5 and 6. f, the ratio

ATP/(ATP+ADP) (e) was normalized to the ratio measured in islets cultured under the same condition and incubated in the presence of G20+azide. §, $P<0.0001$ 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; #, $P<0.001$ for the effect of MnTBAP during culture (Two-way ANOVA + test of Bonferroni).

Figure 4. Effects of MnTBAP on the alterations of tolbutamide-induced $[Ca^{2+}]_i$ rise by one week of culture in low glucose. Rat islets were cultured for one 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 perifused 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 for 26-28 islets from 3 different islet preparations. Mean $[Ca^{2+}]_i$ values are shown in Table 6.

Table 1. Effects of MnTBAP on changes in islet $[Ca^{2+}]_i$ induced by one week culture in low *vs.* intermediate glucose concentrations

		$[Ca^{2+}]_i$ (nmol/l)		
Culture condition	n	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 * [a]
G10	12	117 ± 7	71 ± 7	149 ± 10
G10 + MnTBAP	13	124 ± 7	85 ± 9	132 ± 11

Results are means ± SE for the indicated number of experiments (n). $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; G0.5 and G20, 0.5 and 20 mmol/l glucose, respectively; Dz, diazoxide; K30, 30 mmol/l extracellular K^+. 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 perifusion. *, $P<0.05$ or less for the effect of glucose (G5 *vs.* G10) and §, $P<0.05$ or less for the effect of MnTBAP by two-way ANOVA + test of Bonferroni. [a], $P<0.05$ Student's t-test for the effect of MnTBAP.

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

		Insulin secretion (attomoles/min per ng islet DNA)		
Culture condition	n	G0.5	Δ_1 (G0.5-G20)	Δ_2 (G0.5-G20K30Dz)
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 * [a]	11.97 ± 1.23 * [a]
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 ± SE for the indicated number of experiments (n). G0.5 and G20, 0.5 and 20 mmol/l glucose, respectively; Dz, diazoxide; K30, 30 mmol/l extracellular K^+. 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. Results are means ± SE for the indicated number of experiments (n).*, $P<0.01$ or less for the effect of glucose (G5 *vs.* G10) by two-way ANOVA + test of Bonferroni. There was no significant effect of MnTBAP by two-way ANOVA + test of Bonferroni. [a], $P<0.01$ Student's t-test for the effect of MnTBAP.

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

Culture condition	n	Insulin content (pmol/islet)	DNA content (ng/islet)	Insulin/DNA content ratio (fmol/ng)	*Preproinsulin* / *Tbp* 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 ± SE 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. *Ins* to *Tbp* mRNA ratios were measured by real-time RT-PCR and normalized to the mean ratio for conditions G5 plus G10 within each experiment before being expressed relative to the levels in islets cultured in G10. *, $P<0.01$ or less for the effect of glucose (G5 *vs.* G10) and §, $P<0.05$ or less for the effect of MnTBAP by two-way ANOVA + test of Bonferroni.

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

		Change in NAD(P)H autofluorescence (A.U.)		
Culture condition	n	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 + TBAP	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 + TBAP	14	37.2 ± 2.0	26.5 ± 1.3	0.583 ± 0.007

Results are means ± SE for the indicated number of experiments (n). G0.5 and G20, 0.5 and 20 mmol/l glucose; azide: 5mmol/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 G20and 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. *, $P<0.01$ or less for the effect of glucose (G5 *vs.* G10) by two-way ANOVA + test of Bonferroni. There was no significant effect of MnTBAP on any of these measurements by two-way ANOVA + test of Bonferroni. A.U., arbitrary fluorescence units.

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

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

Results are means ± SE 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 experiments illustrated in Fig.3c-d, we computed the mean rhodamine 123 fluorescence from the 23th to the 60th min, from the 57th to the 60th min, and from the 62nd to the 70th min of perifusion. Δ_1 shows the mean difference between rhodamine 123 fluorescence during the last 37 min of perifusion 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. *, $P<0.001$ or less for the effect of glucose (G5 *vs.* G10) by two-way ANOVA + test of Bonferroni. There was no significant effect of MnTBAP on any of these measurements by two-way ANOVA + test of Bonferroni.

Table 6. Effects of MnTBAP on changes in islet $[Ca^{2+}]_i$ induced by tolbutamide after one week culture in low *vs.* intermediate glucose concentrations

		$[Ca^{2+}]_i$ (nmol/l)		
Culture condition	n	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 ± SE for the indicated number of experiments (n). $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; G0.5, 0.5 mmol/l glucose, respectively; Tolbu, tolbutamide; Dz, diazoxide; K30, 30 mmol/l extracellular K^+. In each experiment illustrated in Fig.4, we calculated the mean $[Ca^{2+}]_i$ 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. *, $P<0.05$ or less for the effect of glucose (G5 *vs.* G10) and §, $P<0.05$ or less for the effect of MnTBAP by two-way ANOVA + test of Bonferroni.

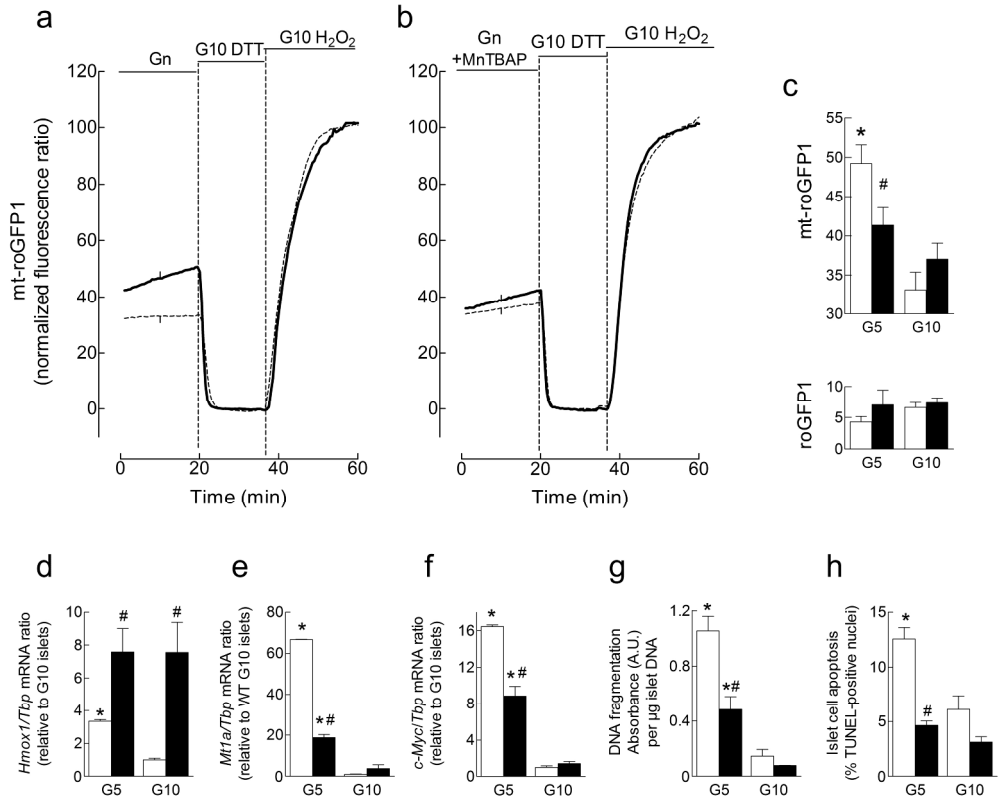

Figure 1. Effects of MnTBAP on the stimulation of mt-roGFP1 oxidation, islet cell apoptosis and oxidative stress-response gene expression by one week of culture in low glucose. a-c, rat islet cell clusters expressing mt-roGFP1 or roGFP1 were cultured overnight in RPMI medium containing 5 mmol/l glucose (G5, thick traces) or 10 mmol/l glucose (G10, thin traces) with 10% FBS in the absence (a, open columns in c) or presence of 50 µmol/l MnTBAP (b, solid columns in c). a-b, the ratio of roGFP1 fluorescence intensities (exc 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 expressing cytosolic roGFP1 are shown in ESM-Fig.1. d-h, rat islets were cultured for one week in a medium containing 5 or 10 mmol/l glucose (G5 or G10) and 5 g/l BSA in the absence (open columns) or presence of 50 µmol/l MnTBAP (solid columns). 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 ± SE for 5 experiments. g, cytosolic histone-associated oligonucleosomes were measured with a commercial ELISA. Data are means ± SE for 3 experiments. h, the percentage of apoptotic islet cells was computed as the ratio TUNEL-positive / DAPI-positive nuclei on islet sections. Results are means ± SE 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 + test of Bonferroni or by Student's t-test respectively. #, P<0.05 for the effect of MnTBAP by two-way ANOVA + test of Bonferroni. A.U., arbitrary units.
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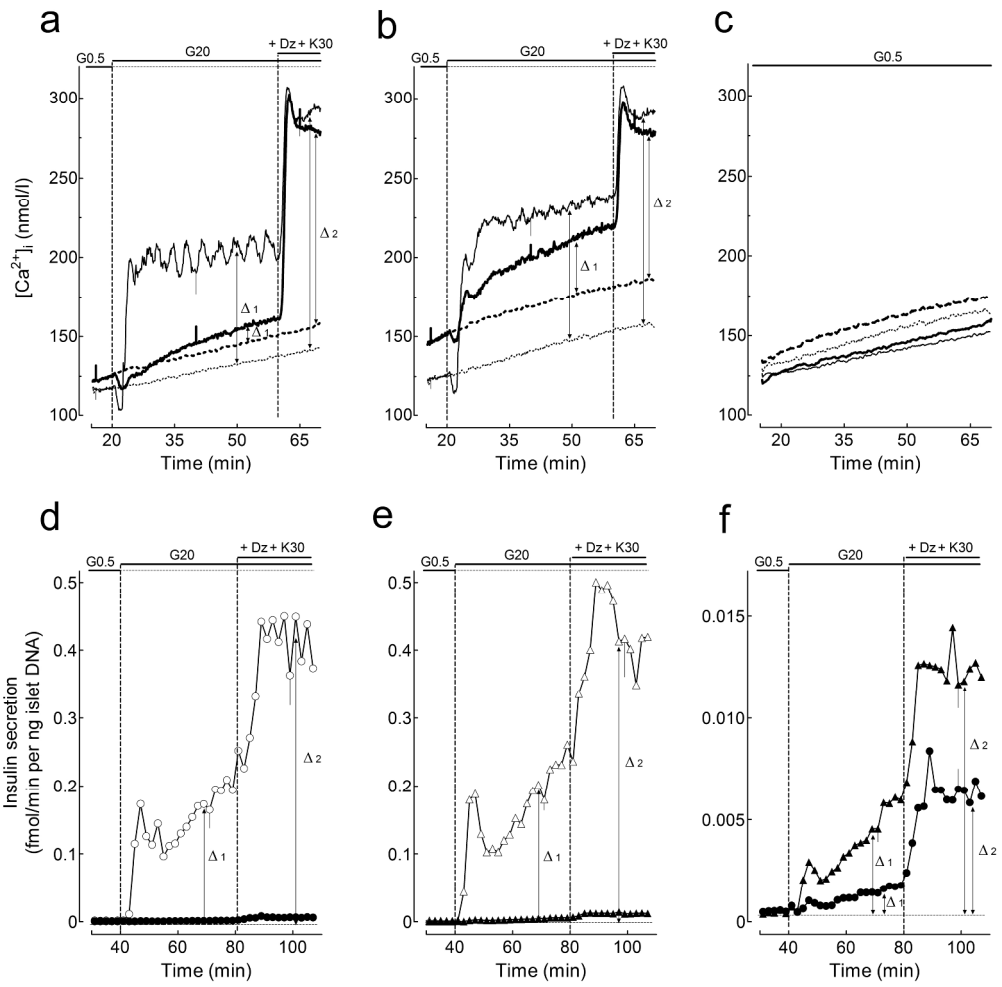

Figure 2. Effects of MnTBAP on the alterations of glucose-induced $[Ca^{2+}]_i$ rise and insulin secretion by one week of culture in low glucose. Rat islets were cultured for one week in the presence of G5 or G10 without (a, d) or with (b, e) 50 µmol/l MnTBAP. They were then perifused with a medium containing 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 µmol/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 open symbols, islet cultured in G10; Thick traces and closed symbols, islets cultured in G5. Dotted traces in panels a and b are extrapolations of $[Ca^{2+}]_i$ traces obtained in islets perifused in the presence of G0.5 throughout the experiment (panel 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 normalized for differences in islet DNA content. The effect of MnTBAP on insulin secretion after culture in G5 is better shown in panel f where the Y-scale has been expanded (circles, culture without MnTBAP; triangles, culture with MnTBAP. Results are means ± SE for 8 independent $[Ca^{2+}]_i$ experiments (corresponding to 12-13 islets) and for 4 insulin secretion experiments.
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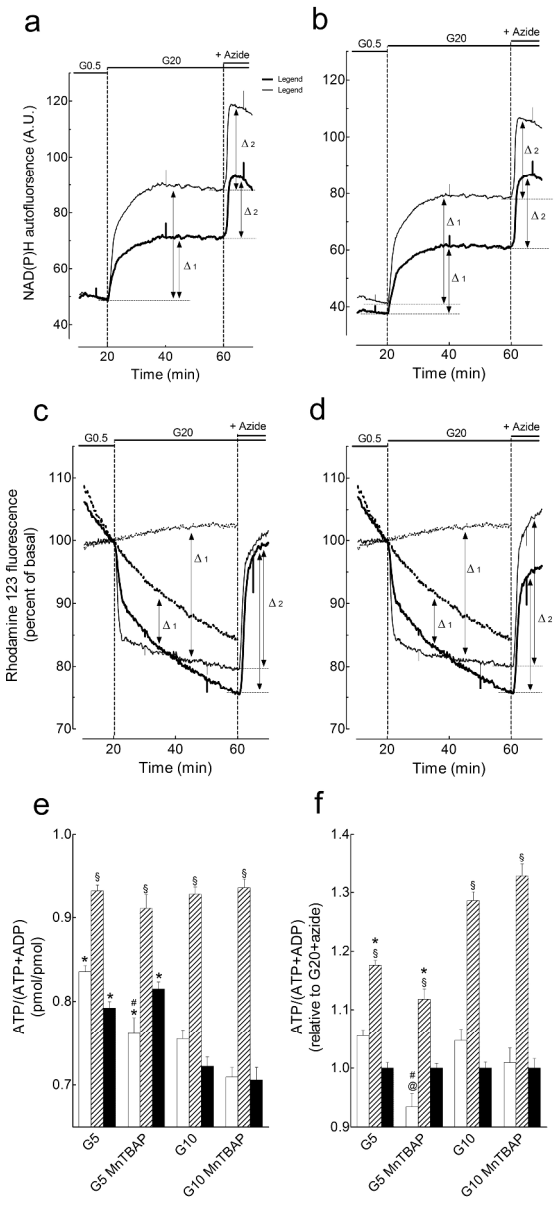

Figure 3. Effects of MnTBAP on the alterations of glucose-induced changes in mitochondrial metabolism by one week of culture in low glucose. After one week culture in G5 or G10 without (a, c) or with (b, d) 50 µmol/l MnTBAP, NAD(P)H autofluorescence (a, b), rhodamine 123 fluorescence (c, d) and adenine nucleotide levels (e, f) were measured in islets perifused or incubated with a medium containing G0.5 (e-f, open bars), G20 (e-f, hatched bars) and G20 + 5 mmol/l Na^+-azide (e-f, closed bars), or with a 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 min of perifusion (last min of perifusion in G0.5 before the stimulation with G20). Data are means ± SE for 8 independent NADP(H) experiments (corresponding to 14 islets), 5 independent rhodamine 123 experiments (corresponding to 6-10 islets) and 14 batches of 10 islets for adenine nucleotide measurements (4 independent incubations). Δ1 and Δ2, see Tables 5 and 6. f, the ratio ATP/(ATP+ADP) (e) was normalized to the ratio measured in islets cultured under the same condition and incubated in the presence of G20+azide. §, 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; #, P<0.001 for the effect of MnTBAP during culture (Two-way ANOVA + test of Bonferroni).
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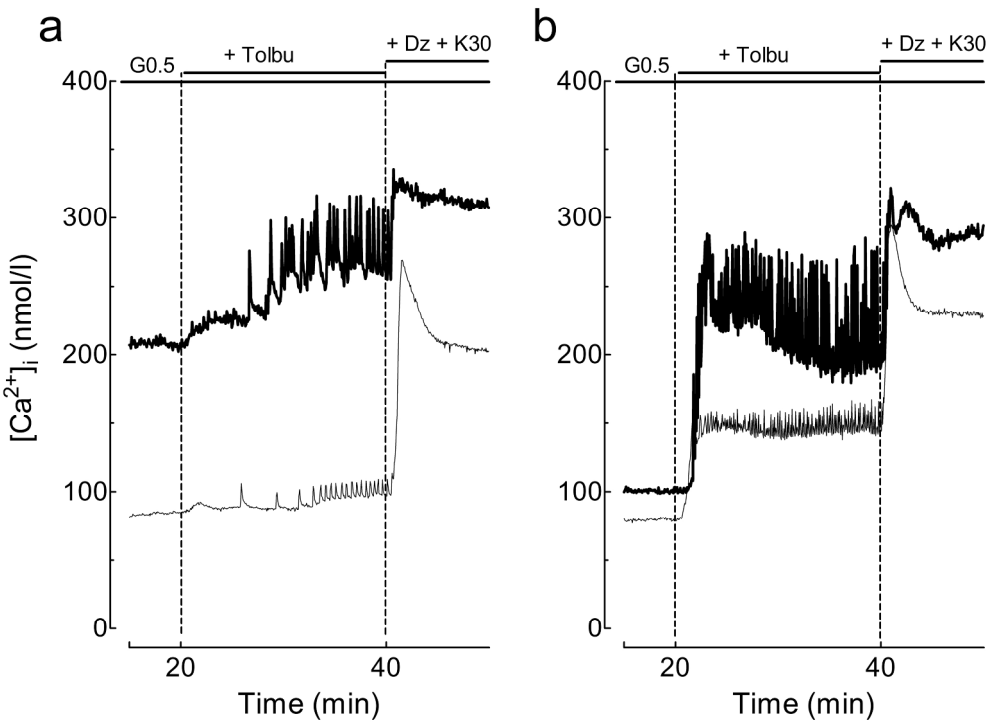

Figure 4. Effects of MnTBAP on the alterations of tolbutamide-induced $[Ca^{2+}]_i$ rise by one week of culture in low glucose. Rat islets were cultured for one week in the presence of G5 (a) or G10 (b) in the absence (thin traces) or presence (thick traces) of 50 µmol/l MnTBAP. They were then perifused 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 for 26-28 islets from 3 different islet preparations. Mean $[Ca^{2+}]_i$ values are shown in Table 6.
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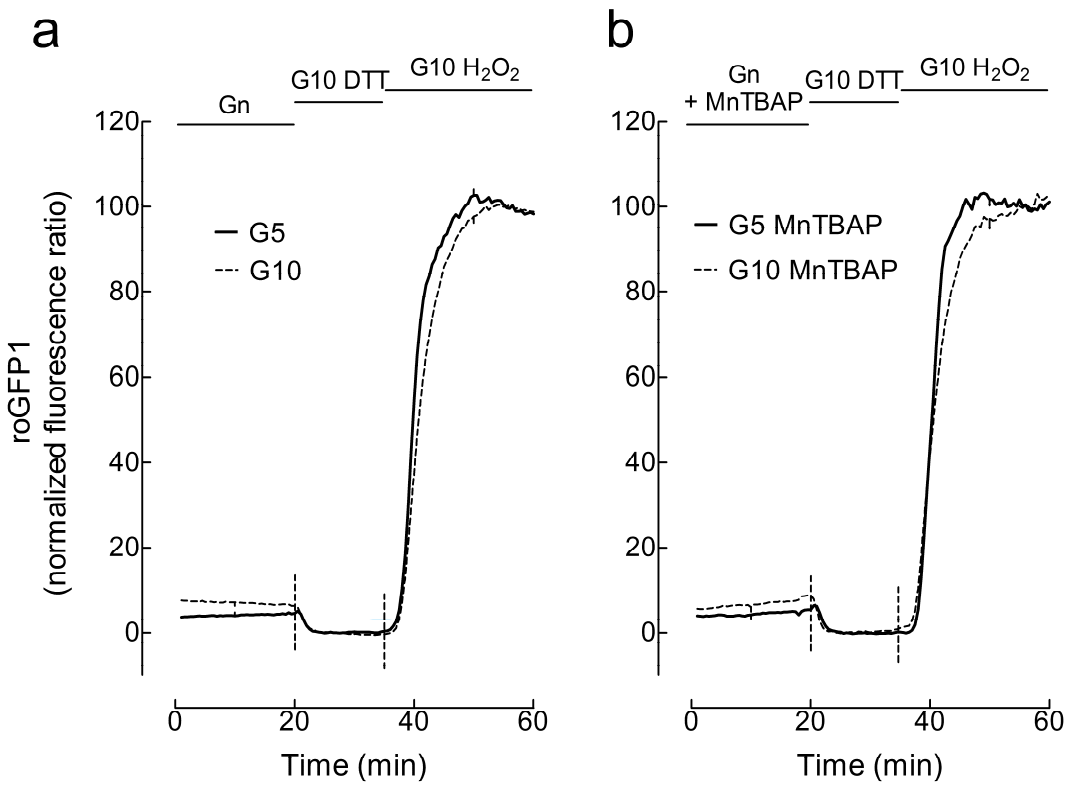
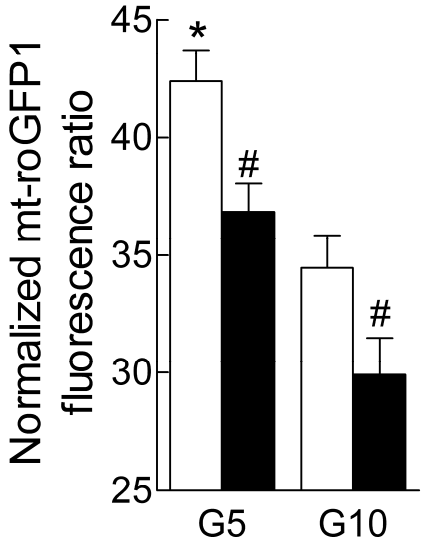
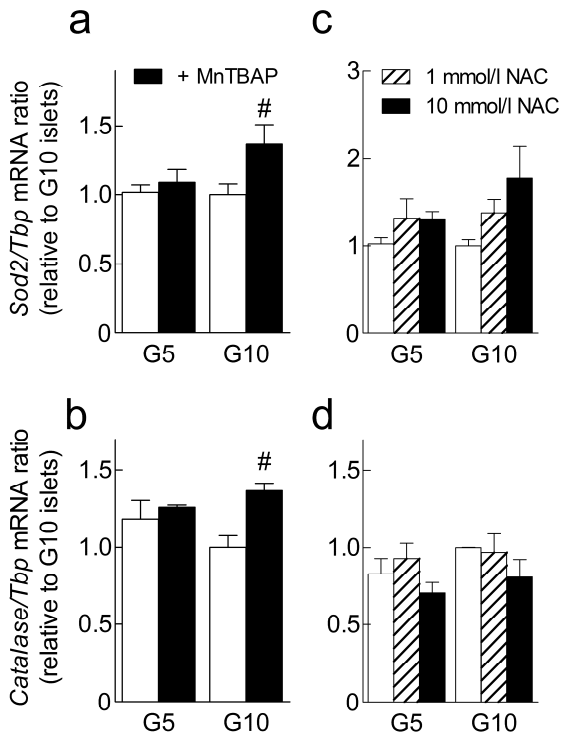


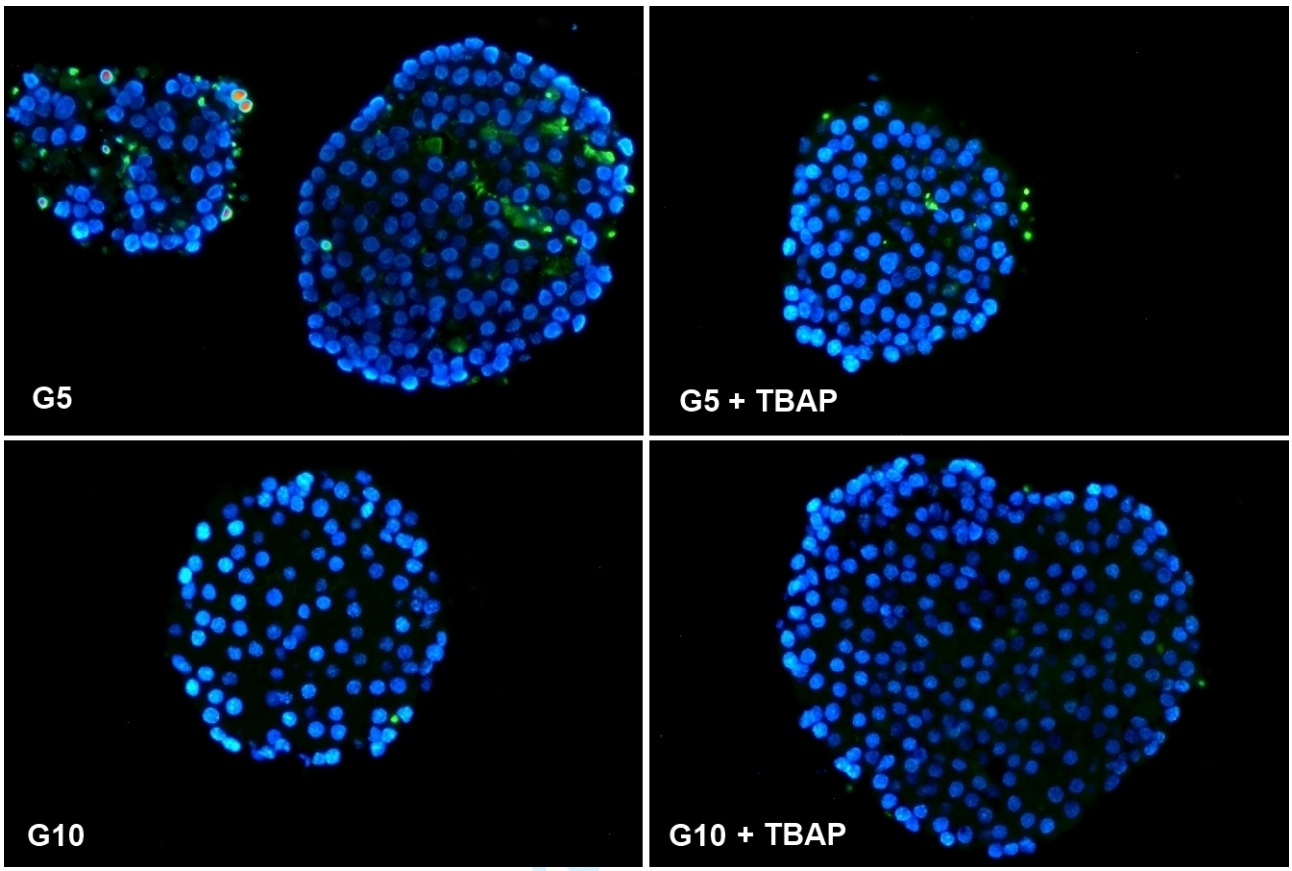
ESM figure 1. Effect of overnight culture on the oxidation of cytosolic roGFP1. Rat islet cell clusters expressing roGFP1 were cultured overnight in RPMI medium containing 5 or 10 mmol/l glucose (G5 or G10) in the absence (a) or the presence of 50 µmol/l MnTBAP (b). The ratio of roGFP1 fluorescence intensities (exc 400/480 nm) was expressed as a percentage of the difference between the mean ratio measured in the presence of 10 mmol/l DTT (set at 0 %) and that measured in the presence of 1 mmol/l H_2O_2 (set at 100 %).



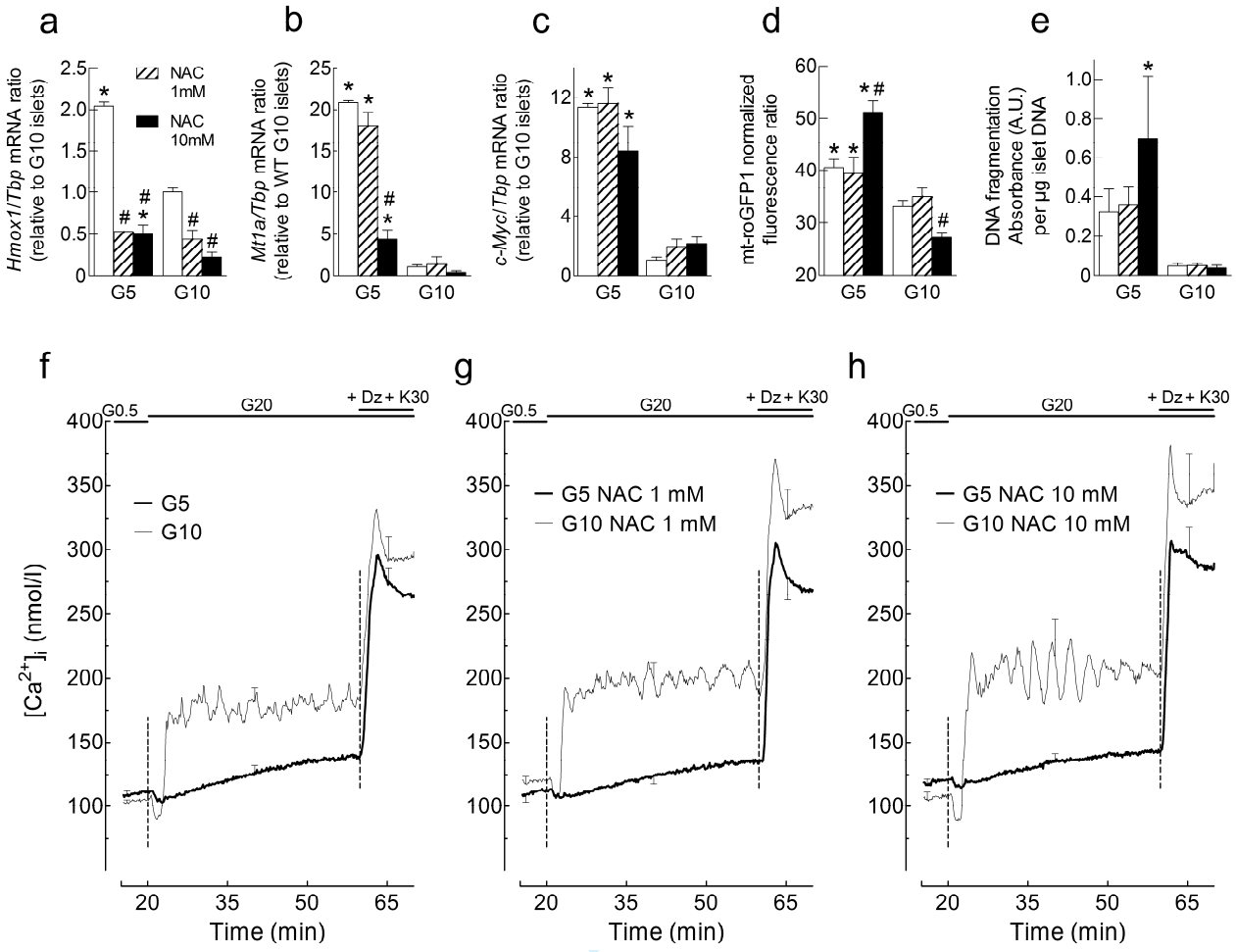
ESM figure 2. Long term effects of low glucose and MnTBAP on the oxidation of mitochondrial roGFP1. Rat islet cell clusters were cultured for 3 days in RPMI medium containing 5 or 10 mmol/l glucose (G5 or G10) in the absence (open bars) or presence (closed bars) of MnTBAP. They were infected with Ad-mt-roGFP1 on the second day of culture. Mt-roGFP1 oxidation was measured and expressed as in figure 1a-c. Results are means ± SE for 14-24 clusters from 3 experiments. *, $P<0.05$ for the effect of glucose (G5 *vs.* G10) and $^{\#}$, $P<0.05$ for the effect of MnTBAP by two-way ANOVA + test of Bonferroni.



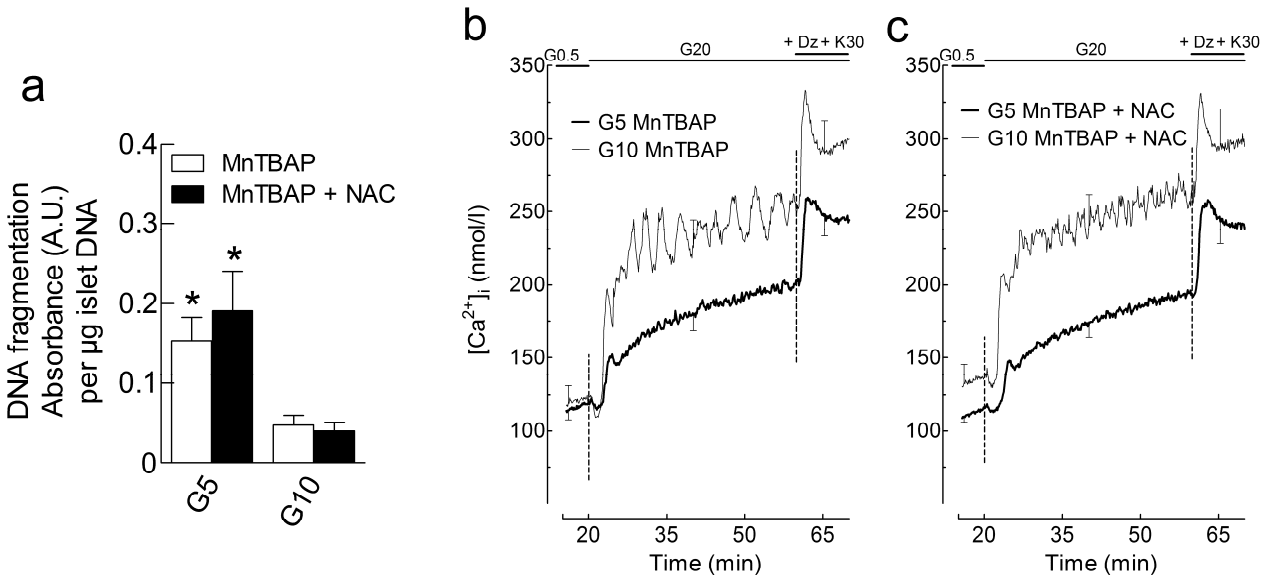
ESM figure 3. Effect of culture on the islet mRNA levels of the antioxidant enzymes Mn-superoxide dismutase (*Sod2*)(a, b) and catalase (c, d). Rat islets were cultured for one week in a medium containing 5 or 10 mmol/l glucose (G5 or G10) in the absence or presence of 50 µmol/l MnTBAP or 1 and 10 mmol/l NAC as indicated. Islet gene mRNA levels were measured and expressed as detailed in legend to figure 1. Results are means ± SE for 5 experiments. $^{\#}$, $P<0.05$ for the effect of MnTBAP by two-way ANOVA + test of Bonferroni.



ESM figure 4. Effect of MnTBAP on the stimulation of islet cell apoptosis by one week culture in low glucose. Rat islets were cultured for one week in a medium containing 5 or 10 mmol/l glucose (G5 or G10) in the absence or presence of 50 µmol/l MnTBAP, as indicated. The percentage of apoptotic islet cells was measured on islet sections by TUNEL using fluorescein-labeled dATP (green). Nuclei were stained with DAPI (blue). Results are representative sections for 20-86 islets in each group.



ESM figure 5. Effects of N-acetyl-L-cysteine on the stimulation of oxidative stress response gene expression and islet cell apoptosis, and on the alteration of glucose-induced $[Ca^{2+}]_i$ rise by one week culture in low glucose. Rat islets were cultured for one week in a medium containing 5 or 10 mmol/l glucose (G5 or G10) in the absence (open columns) or the presence of 1 mmol/l NAC (hatched columns) or 10 mmol/l NAC (solid columns). a-c, islet *Hmox1*, *Mt1a* and *c-Myc* to *Tbp* mRNA ratio were expressed relative to the levels in islets cultured in G10. d, mt-roGFP1 oxidation was measured as depicted in figure 1 a-b in islet cell clusters cultured overnight under the same conditions as in panels a-c. e, the amount of cytosolic histone-associated oligonucleosomes was measured as an indicator of cell apoptosis. Data are means ± SE for 3 experiments (a-c, e) or for 16 clusters from 3 islet cell preparations (d). *, $P<0.05$ for the effect of culture (G5 *vs.* G10) and $^{\#}$, $P<0.05$ for the effect of NAC (two-way ANOVA + test of Bonferroni). A.U., arbitrary units. f-h, after culture, the islets were perifused as described in legend to figure 2. Results are means ± SE for 6-11 islets.



ESM Figure 6. Effects of a combination of NAC and MnTBAP on the stimulation of islet cell apoptosis and the alteration of glucose-induced $[Ca^{2+}]_i$ rise by one week culture in low glucose. Rat islets were cultured for one week in a medium containing 5 or 10 mmol/l glucose (G5 or G10) in the presence of 50 µmol/l MnTBAP alone (a: open columns, b) or in combination with 1 mmol/l NAC (a: solid columns, c). a, the amount of cytosolic histone-associated oligonucleosomes was measured as an indicator of cell apoptosis. Data are means ± SE for 3 experiments. *, $P<0.05$ for the effect of culture (G5 *vs.* G10) (two-way ANOVA + test of Bonferroni). A.U., arbitrary units. b-c, after culture, the islets were perifused as described in legend to figure 2. Results are means ± SE for 4-12 islets.

ESM Table 1. Sequences of oligonucleotide primers and PCR conditions

Gene	5' oligonucleotide	3' oligonucleotide	Input [a]	Annealing (°C-sec)	Extension (°C-sec)	Size (bp)	T_m (°C)
Cat	CTT.CAA.GCT.GGT.TAA.TGC.GAA.TGG	TGG.CGA.TGG.CAT.TGA.AAA.GAT.C	2	60-60	-	155	82
c-Myc	CTG.AGC.CCC.TAG.TGC.TGC.AT	ACC.CTG.ACT.CGG.ACC.TCT.TG	2	62-45	82-15	138	85.5
Hmox1	ACA.GCA.TGT.CCC.AGG.ATT.TGT.C	AAG.GAG.GCC.ATC.ACC.AGC.TT	2	62-45	82-15	142	87.5
Mt1a	GGA.CCC.CAA.CTG.CTC.CCT	CGA.GGC.ACC.TTT.GCA.GAC.AC	2	62-45	82-15	160	89.5
Ppi	TCT.TCT.ACA.CAC.CCA.TGT.CCC	GGT.GCA.GCA.CTG.ATC.CAC	0.5	60-90	84-30	148	91
Sod2	AGA.TTG.CCG.CCT.GCT.CTA.ATC	GGC.TAA.CAT.TCT.CCC.AGT.TGA.TTA.C	2	60-60	-	165	82
Tbp	ACC.CTT.CAC.CAA.TGA.CTC.CTA.TG	ACT.TCG.TGC.CAG.AAA.TGC.TGA	2	60-90	80-30	157	84

The specificity of sense and anti-sense primers was checked by BLAST search. The thermal cycle profile consisted of a 3 min step at 95°C to release DNA polymerase activity followed by 40 cycles of amplification (15 sec denaturation step at 95°C, 60-90 sec annealing step at 60-64°C, and eventual 15-30 sec extension step at 80-84°C). Under these conditions, PCR efficiencies were ~0.95 to 1.0. The melting temperature (T_m) of the amplicons was systematically determined at the end of the PCR to check their specificity. Their size corresponded to that expected from published sequences, as determined by agarose gel electrophoresis. *Cat*, catalase; *Hmox1*, Heme oxygenase1*; Mt1a*, Metallothionein1a*; Ppi*, Preproinsulin I and II*; Sod2*, Mn-superoxide dismutase 2; *Tbp*, TATA-box binding protein. [a], Islet sample cDNA input in 25 µl reactions (ng total RNA equivalent).

ESM Table 2. mRNA levels of glucose affected members of the ATP-binding cassette, sub-family B (MDR/TAP) in cultured rat islets.

Rhodamine 123 leakage in islets cultured in G5 unlikely resulted from the stimulation of islet cell apoptosis, since it was not always observed under conditions associated with apoptosis, i.e. in beta-cells in which c-Myc has been activated for 3 days [1]. It could rather result from an increase in the expression level of multidrug resistance channels (MDR), rhodamine 123 being a well-known substrate of P-glycoprotein [2].

Probe Set ID	MDR/TAP subtype	Gene Symbol	G5	G10
1370464_at	member 1	*Abcb1*	53 ± 2 *	8 ± 3
1370583_s_at	member 1B	*Abcb1 - Abcb1b*	194 ± 22 *	43 ± 7
1388149_at	transporter 1	*Tap1*	277 ± 21 *	87 ± 6

Microarray analysis of glucose-induced changes in rat islet gene expression (18h culture after a one week preculture in G10 [3]) suggests that the mRNA levels of the ATP-binding cassette, sub-family B (MDR/TAP) *Abcb1* and *Tap1* are significantly increased by culture in G5 *vs.* G10. Other members of the MDR/TAP family (*Abcb4-6-8-10* and *Tap2*) were not significantly affected by culture in G5 *vs.* G10 (not shown). Data are means ± SEM of normalized hybridization values from 4 samples. *, $P<0.0008$ by Bonferroni corrected t-test after one-way ANOVA on the 4 glucose conditions.

1. Pascal SM, Guiot Y, Pelengaris S, Khan M, Jonas JC (2008) Effects of c-MYC activation on glucose stimulus-secretion coupling events in mouse pancreatic islets. Am J Physiol Endocrinol Metab 295: E92-E102
2. Chaudhary PM, Roninson IB (1991) Expression and activity of P-glycoprotein, a multidrug efflux pump, in human hematopoietic stem cells. Cell 66: 85-94
3. Bensellam M, Van Lommel L, Overbergh L, Schuit FC, Jonas JC (2009) Cluster analysis of rat pancreatic islet gene mRNA levels after culture in low-, intermediate- and high-glucose concentrations. Diabetologia 52: 463-476