

The antioxidants N-acetyl-L-cystein and manganese(III)tetrakis (4-benzoic acid)porphyrin do not prevent β -cell dysfunction in rat islets cultured in high glucose for one week.

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ABSTRACT

We previously showed that the stimulation of heme-oxygenase 1 expression by high glucose and hydrogen peroxide (H_2O_2) in cultured rat islets is prevented by antioxidants and suggested that this effect of high glucose results from an oxidative stress. However, the role of oxidative stress in high glucose-induced β -cell dysfunction is unclear. We therefore compared the preventative effects of N-acetyl-L-cysteine (NAC), a free radical scavenger, and manganese(III)tetrakis (4-benzoic acid)porphyrin (MnTBAP), a superoxide dismutase/catalase mimetic agent, on the alteration of stimulus-secretion coupling induced in rat islets by overnight exposure to hydrogen peroxide (H_2O_2 -treated islets) or one-week culture in 30 vs 10 mmol/l glucose (High-glucose vs Control islets). The features of β -cell dysfunction differed between the two groups : reduced glucose-induced insulin secretion without changes in glucose sensitivity in H_2O_2 -treated islets ; increased sensitivity to glucose with parallel reductions in insulin content and maximal rate of glucose-induced insulin secretion in High-glucose islets. The latter alterations were accompanied by a decrease in preproinsulin without changes in PDX1 mRNA levels. The functional alterations induced by H_2O_2 were significantly prevented by addition of NAC or MnTBAP in the culture medium. In contrast, neither NAC nor MnTBAP affected the functional alterations induced by high glucose. These results suggest that β -cell dysfunction induced by 1 week culture in high glucose does not result from an increase in oxidative stress.

INTRODUCTION

The causal role of chronic hyperglycemia in the onset and progression of diabetic complications is well established (7; 58). At the cellular level, the deleterious effects of hyperglycemia may result from several mechanisms, including increased glucose flux through the polyol and hexosamine pathways, protein glycation, activation of receptors for advanced glycation end products, protein kinase C and mitogen-activated protein kinase activation, and excessive mitochondrial production of reactive oxygen species (ROS)¹ (28; 57; 61). It has been proposed that an increase in oxidative stress, defined as a serious imbalance between enhanced ROS production and reduced antioxidant capacity, may be a common denominator for these various mechanisms of diabetic complications (1; 4; 5; 39; 50; 63).

In insulin-secreting pancreatic β -cells, chronic hyperglycemia or in vitro exposure to supraphysiological glucose concentrations progressively induces reversible and irreversible phenotypic alterations, including loss of glucose-induced insulin secretion within the physiological range of glucose concentrations, reduced expression of specific genes (loss of β -cell differentiation), hypertrophy, reduced survival, and increased expression of genes normally repressed in fully differentiated β -cells (20; 21; 26; 31-33; 41; 42; 56). These global alterations of the β -cell phenotype probably contribute to the progressive worsening of glucose-induced insulin secretion in type 2 diabetic patients (27; 59).

Because of their low antioxidant defense status, β -cells are highly sensitive to the toxic insult of pro-oxidants (55). The alterations of β -cell gene expression, function and survival by chronic hyperglycemia may therefore result from an increase in oxidative stress (11; 12; 14; 45; 46). In support of this hypothesis, it has been shown that 4-hydroxy-2-nonenal-modified proteins and 8-hydroxy-deoxyguanosine, markers of lipid and DNA oxidation respectively, are more abundant in islets from type 2 diabetic patients and rodent models of the disease

than in control islets (13; 17; 48). It has also been reported that high glucose increases ROS production in isolated islets by a mechanism requiring its metabolism (3; 54), but these results have recently been challenged by the observation that glucose acutely represses rather than stimulates ROS production in cultured purified rat β -cells by increasing NAD(P)H and FADH₂/FMNH₂ intracellular concentrations (35).

We previously characterized the effects of a one-week culture in high glucose on selected events of stimulus-secretion coupling and on the expression of heme-oxygenase 1 (HO1), a sensitive marker of oxidative stress, in rat islets (19; 25). We also showed that the stimulation of islet HO1 expression by overnight exposure to high glucose or low hydrogen peroxide (H₂O₂) concentrations is prevented by the antioxidant N-acetyl-L-cysteine (NAC), which suggests a role of oxidative stress in these effects of high glucose (10). However, it remains unclear whether the alterations of β -cell function after one-week culture in high glucose also result from an increase in oxidative stress. We therefore evaluated the ability of NAC, a free radical scavenger, and of manganese(III)tetrakis (4-benzoic acid)porphyrin (MnTBAP), a superoxide dismutase/catalase mimetic agent (6; 36), to prevent the alterations of stimulus-secretion coupling by exposure of rat islets for one week in 30 instead of 10 mmol/l glucose (High-glucose vs Control islets) or for one night to H₂O₂ in the presence of 10 mmol/l glucose (H₂O₂-treated vs Control islets).

MATERIALS AND METHODS

Solutions. All experiments were performed at 37°C with bicarbonate-buffered Krebs solution containing (mmol/l) NaCl (120), KCl (4.8), CaCl₂ (2.5), MgCl₂ (1.2), NaHCO₃ (24), 1g/l bovine serum albumin and various glucose (G) concentrations. This solution was continuously gassed with O₂:CO₂ (94:6) to keep pH at 7.4. When the concentration of KCl was raised to 30 mmol/l, that of NaCl was reduced to 94.8 mmol/l to keep osmolarity of the medium unchanged. Diazoxide, kindly provided by Schering-Plough Avondale (Rathdrum, Ireland), was dissolved to 50 mmol/l in NaOH 0.1N and used at the final concentration of 250 µmol/l.

Islet isolation and culture. Islets from male Wistar rats (Animal facility of the Faculty of Medicine, UCL, Brussels, Belgium) were obtained by collagenase digestion of the pancreas followed by density gradient centrifugation using Histopaque 1077 (Sigma, St. Louis, Mo., USA) as previously described (25). Islets of similar size (≤ 150 µm in diameter) were then cultured for 7-8 days at 37°C in RPMI 1640 medium containing 10 or 30 mmol/l glucose (G10 vs G30) with or without 1 mmol/l N-Acetyl-L-Cysteine (NAC) (Merck, Darmstadt, Germany) or 50 µmol/l manganese(III)tetrakis (4-benzoic acid)porphyrin (MnTBAP) (Alexis Biochemicals, Lausen, Germany). The medium was renewed every other day.

In another series of experiments, the islets were cultured for one week in RPMI medium containing G10 with eventual addition of 50 µmol/l MnTBAP for the last 24h. They were then cultured for 18-24h in the same medium supplemented with 12-58 µmol/l hydrogen peroxide (H₂O₂)(Acros organics, Geel, Belgium) with or without 1 mmol/l NAC or 50 µmol/l MnTBAP.

All animal experimentations were conducted in accord with accepted standards of humane animal care and were approved by the institutional Committee on Animal Experimentation from the Faculty of Medicine of the University of Louvain.

Morphological analysis. At the end of the culture period, the islets were fixed for 4h in Bouin's fluid. Four- μ m-thick sections of paraffin-embedded islets were processed for hematoxylin and eosin staining as described previously (25).

Measurements of insulin secretion and insulin content. After culture, batches of 30-50 islets were perfused (~ 1 ml/min) with a medium containing various glucose concentrations but no H_2O_2 , NAC or MnTBAP. Insulin was measured in 2 min effluent collections by RIA using rat insulin as a standard (16). After perfusion, the islets were disrupted by sonication in TNE buffer (Tris 10 mmol/l, NaCl 0.2 M, EDTA 10 mmol/l) for measurement of their insulin (RIA) and DNA contents (fluorescence assay using bisbenzimidazole)(30).

Measurements of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$). After culture, the islets were loaded for 2 h with 2 μ mol/l furaPE3 acetoxymethylester (Teflabs, Austin, TX, USA) in a medium containing the same glucose concentration as that used during culture but no H_2O_2 or NAC. $[Ca^{2+}]_c$ was then measured in perfused islets by microspectrofluorimetry using Quanticell 700m (alternate excitation at 340/380 nm, emission at 510 nm) (Visitech, Sunderland, UK) as previously described (25).

Measurements of mitochondrial membrane potential. After culture, the islets were loaded for 20 min with 10 μ mol/l rhodamine 123 (Molecular probes, Eugene, OR, USA) in a medium containing the same glucose concentration as that used during culture but no H_2O_2 or NAC. Changes in rhodamine 123 fluorescence were then measured in perfused islets by microspectrofluorimetry (excitation at 490 nm, emission at 530 nm). After background subtraction, fluorescence data from each islet were normalized to the maximal fluorescence measured 2-5 min after addition of 5 mmol/l azide in the presence of G20. Light transmission (phase contrast) and fluorescence microscopy images of furaPE3- and rhodamine 123-loaded islets were acquired with an Olympus IX70 inverted fluorescence microscope (20 $\times$ objective) coupled to a CCD camera (T.I.L.L. photonics, Martinsried, Germany) using

excitation/emission wavelengths of 360/510 nm for furaPE3 and 490/530 nm for rhodamine 123.

Measurements of islet heme-oxygenase 1 and preproinsulin mRNA levels. After culture, islet total RNA was extracted and reverse transcribed into cDNA using random hexamers and 200 units of M-MLV Reverse Transcriptase RNase H⁻ Point Mutant (Promega, Madison, WI, U.S.A.). cDNAs for heme-oxygenase 1 (HO1, 142 bp amplicon), pancreatic and duodenal homeobox gene 1 (PDX1, 169 bp amplicon), preproinsulin (PPI, 148 bp amplicon), TATA-box binding protein (TBP, 157 bp amplicon), and cyclophilin (400 bp amplicon) were amplified with the iCycler iQ Real Time PCR Detection System using iQ Sybr Green Supermix (Bio-Rad, Hercules, CA, U.S.A.) and 300 nmol/l sense and antisense oligonucleotide primers (HO1 sense, 5'-ACA GCA TGT CCC AGG ATT TGT C-3' ; HO1 antisense, 5'- AAG GAG GCC ATC ACC AGT TT-3' ; PDX1 sense, 5'-CGG ACA TCT CCC CAT ACG-3' ; PDX1 antisense, 5'-AAA GGG AGA TGA ACG CGG-3' ; PPI sense, 5'-TCT TCT ACA CAC CCA TGT CCC-3' ; PPI antisense, 5'-GGT GCA GCA CTG ATC CAC-3' ; TBP sense, 5'-ACC CTT CAC CAA TGA CTC CTA TG-3' ; TBP antisense, 5'-ACT TCG TCC CAG AAA TCC TGA-3' ; cyclophilin sense, 5'-AAC CCC ACC GTG TTC TTC-3' ; cyclophilin antisense, 5'-TGC CTT CTT TCA CCT TCC C-3'). The thermal cycle profile was a 3-min denaturing step at 95°C to release DNA polymerase activity followed by 40 cycles of amplification, each composed of a 15-s denaturing step at 95°C, a 60-s annealing step at 62°C for HO1, PDX1 and TBP or at 60°C for PPI and cyclophilin, and a 15-s step at 82°C for HO1, PDX1 and TBP or at 84°C for PPI and cyclophilin. The mRNA levels of HO1, PDX1, PPI, TBP, and cyclophilin were determined by comparing samples to a standard curve prepared by serial 4-fold dilutions of a mixture of all cDNAs tested in that PCR. The ratio of HO1 to TBP, PDX1 to TBP and PPI to cyclophilin mRNA levels were computed and expressed relative to the ratio measured in control islets cultured in G10.

Data Analysis. The results are means $\pm$ S.E.M. for the indicated number of islets from at least 3 different preparations, unless otherwise specified. The statistical significance of differences between means was assessed by one-way ANOVA followed by a test of Newman-Keuls or by two-way ANOVA followed by a test of Bonferroni, as specified. Differences were considered significant if $P \leq 0.05$.

RESULTS

Effects of N-acetyl-L-cysteine and manganese(III)tetrakis (4-benzoic acid)porphyrin on the changes in islet heme-oxygenase 1 (HO1) and preproinsulin (PPI) mRNA expression induced by hydrogen peroxide and high glucose. After one-week preculture in RPMI medium containing 10 mmol/l glucose (G10), overnight culture in the presence of 12-58 $\mu\text{mol/l}$ hydrogen peroxide (H_2O_2) dose-dependently increased the islet HO1/TBP mRNA ratio. The effect of 58 $\mu\text{mol/l}$ H_2O_2 was completely prevented by addition of 1 mmol/l N-acetyl-L-cysteine (NAC) to the culture medium, and was significantly reduced in islets treated with 50 $\mu\text{mol/l}$ manganese(III)tetrakis (4-benzoic acid)porphyrin (MnTBAP) 24h before and during exposure to H_2O_2 (Fig. 1A-B). After one-week culture in G30 instead of G10 (High-glucose vs Control islets), the HO1/TBP mRNA ratio was also significantly increased whereas the PPI/cyclophilin mRNA ratio was decreased (Fig. 1C-D) and the PDX1/TBP mRNA ratio was unaffected (1.17 ± 0.25 in Controls and 1.11 ± 0.07 in High-glucose islets, $n=4$, $P=0.8$ by unpaired t-test). NAC completely prevented the increase in HO1/TBP mRNA ratio by high-glucose (Fig. 1C). In contrast, NAC decreased the PPI/cyclophilin mRNA ratio in both types of islets but did not prevent its reduction by high glucose (Fig. 1D). In comparison, MnTBAP significantly increased HO1 and decreased PPI mRNA levels in Control islets, while high glucose had no further effect in the presence of MnTBAP (Fig. 1C-D).

Effects of NAC on the alterations of islet morphology by H_2O_2 and high glucose.

Control islets (G10, Fig. 2A) displayed only rare morphological signs of β -cell apoptosis and had an apparently normal architecture. After overnight exposure to 23 and 58 $\mu\text{mol/l}$ H_2O_2 , loosen cells with condensed nuclei were consistently observed at the islet periphery, whereas only rare morphological signs of apoptosis were observed in the center of the islets. These effects of H_2O_2 were prevented by NAC (Fig. 2A). In contrast, compared with Control

islets (G10, Fig. 2B), High-glucose islets only displayed a few dispersed apoptotic bodies that were also observed after culture in the presence of NAC (Fig. 2B).

Effects of NAC and MnTBAP on the changes in islet insulin and DNA contents induced by H₂O₂ and high glucose. After one-week preculture in G10, overnight exposure to 23 μ mol/l H₂O₂ decreased the islet DNA content by ~25% without affecting the insulin to DNA content ratio (Table 1). In comparison, one-week culture in G30 instead of G10 did not affect the islet DNA content but reduced the insulin to DNA content ratio by ~50%. NAC prevented the effects of H₂O₂ without affecting those of G30, but tended to reduce the islet insulin to DNA content ratio by ~ 25% under all culture conditions (Table 1). MnTBAP did not prevent the reduction in islet insulin/DNA content ratio by G30 (Table 1).

Effects of NAC and MnTBAP on the alteration of glucose stimulus-secretion coupling events induced by H₂O₂. After one-week culture in G10 and further overnight culture in G10 (Control islets), rhodamine 123 loading was uniform in the whole islet, as shown by comparison of phase contrast and fluorescence microscopy images of the same islets (Fig. 2C, a-b, left panels). In contrast, after overnight exposure to 23-58 μ mol/l H₂O₂, rhodamine 123 loading was almost undetectable in the mantle of loosen peripheral islet cells detected by phase contrast microscopy, but was uniform in the center of the islet (Fig. 2C, a-b, middle panels). In both Control and H₂O₂-treated islets, subsequent stimulation from 0.5 to 6 and 20 mmol/l glucose induced a concentration-dependent decrease in rhodamine 123 fluorescence reflecting mitochondrial membrane hyperpolarization (Fig. 3A). Relative to its maximal level measured in the presence of azide, rhodamine 123 fluorescence was significantly lower at all glucose concentrations after overnight exposure to 58 μ mol/l H₂O₂. However, relative to its level in G0.5, the decrease in rhodamine 123 fluorescence triggered by acute glucose stimulation was unaffected by overnight exposure to H₂O₂ (Table 2). These alterations were all prevented by addition of 1 mmol/l NAC to the culture medium (Fig. 2C, a-b, right panels ; Fig. 3B).

In contrast with rhodamine 123, furaPE3 loading was uniform in both Control and H₂O₂-treated islets (Fig. 2C, c-d, left and middle panels). In Control islets, stepwise glucose stimulation from G0.5 to G6 and G20 induced a transient decrease sometimes followed by a slight increase in [Ca²⁺]_c in G6, and a rapid [Ca²⁺]_c rise with eventual oscillations in G20. Subsequent plasma membrane depolarization with 30 mmol/l extracellular K⁺ (K30) further increased [Ca²⁺]_c to a plateau (Fig. 3C). In other experiments, addition of the ATP-sensitive K⁺ channel opener diazoxide to G20 rapidly decreased islet [Ca²⁺]_c as expected (Fig. 5A). Compared with Control islets, islets exposed overnight to 23-58 μmol/l H₂O₂ displayed a dose-dependent increase in resting [Ca²⁺]_c on top of which glucose- and K30-induced [Ca²⁺]_c rises were still observed (Figs. 3C and 5A ; Table 3). Analysis of [Ca²⁺]_c changes in sub-regions of islets exposed to 58 μmol/l H₂O₂ indicated that [Ca²⁺]_c was higher at the periphery than in the center, whereas glucose-induced [Ca²⁺]_c changes were better preserved in the center (data not shown). These alterations were all prevented by addition of 1 mmol/l NAC to the culture medium and were significantly reduced in islets treated with 50 μmol/l MnTBAP 24h before and during exposure to H₂O₂ (Figs. 3D and 5B ; Table 3).

In Control islets, stimulation from G0.5 to G6 did not affect or only induced a small transient increase in insulin secretion (compare Fig. 3E with Fig. 4E and 5E). Subsequent stimulation with G20 triggered a rapid increase in insulin secretion that was rapidly inhibited by diazoxide. Subsequent plasma membrane depolarization with K30 further increased insulin secretion to a level that was higher than that measured in G20 (Fig. 5E). Overnight exposure to 23 μmol/l H₂O₂ did not significantly affect the rate of secretion in G0.5 or G6, but reduced by ~65% the stimulation of insulin secretion by G20 (Fig. 3E). This reduction, which persisted when insulin secretion was expressed in percent of the islet insulin content ($P < 0.01$), was no longer observed in islets exposed overnight to 58 μmol/l H₂O₂ in the presence of 1 mmol/l NAC (Fig. 3F).

Effects of NAC and MnTBAP on the alteration of glucose stimulus-secretion coupling events induced by high glucose. In contrast with overnight exposure to H₂O₂, one-week culture in G30 instead of G10 (High-glucose vs Control islets) did not affect rhodamine 123 and furaPE3 loading (Fig. 2D). Compared with Control islets, High-glucose islets displayed various functional alterations, including mitochondrial membrane hyperpolarization at low glucose with only small statistically non significant hyperpolarization upon subsequent glucose rise (Fig. 4A ; Table 2), sustained elevation of [Ca²⁺]_c irrespective of the glucose concentration (Figs. 4C and 5C ; Table 3), and increased sensitivity to glucose for the stimulation of insulin secretion with reduced maximal rate of insulin secretion in G20 and G20K30DZ (Figs. 4E and 5E). Addition of 1 mmol/l NAC to the culture medium did not affect the glucose stimulus-secretion coupling events in Control islets nor correct their alteration induced by one-week culture in G30 (Fig. 4 B, D, F). Addition of 50 µmol/l MnTBAP to the culture medium was also unable to prevent the alterations of [Ca²⁺]_c and insulin secretion in High-glucose vs Control islets (Fig. 5 D, F ; Table 3). However, during perfusion with a medium containing G6-G20, [Ca²⁺]_c was significantly higher after culture in the presence of MnTBAP (Table 3).

DISCUSSION

The present study demonstrates that the alterations of rat islets exposed overnight to low concentrations of hydrogen peroxide (H_2O_2 -treated vs Control islets) markedly differ from those observed after one-week culture in 30 instead of 10 mmol/l glucose (High-glucose vs Control islets)(25). Thus, H_2O_2 -treated islets displayed several signs of cell death by apoptosis or necrosis at the periphery but not in the center of the islets (condensed nuclei and reduced rhodamine 123 uptake) and parallel reductions in insulin and DNA contents without changes in insulin to DNA content ratio. In contrast, High-glucose islets displayed few condensed nuclei scattered throughout the islets, lower insulin content without changes in DNA content, and unaffected rhodamine 123 loading. These results indicate that H_2O_2 -treated islets had fewer cells whereas High-glucose islets were degranulated.

The alterations of key events of glucose stimulus-secretion coupling induced by H_2O_2 and high glucose were also different. In H_2O_2 -treated islets, acute stimulation with glucose was still able to decrease rhodamine 123 fluorescence, to increase the already high basal $[\text{Ca}^{2+}]_c$ and to stimulate insulin secretion, although the insulin response was smaller than that in Control islets. Such a reduction in the maximal rate of glucose-induced insulin secretion (reduced glucose responsiveness) with increased β -cell death largely resembles the alterations described in islets exposed *in vitro* to inflammatory cytokines, streptozotocin and other pro-oxidant agents (8; 15; 43). These alterations are, however, different from those observed during or shortly after acute exposure to high H_2O_2 concentrations which almost totally suppress glucose-stimulated mitochondrial membrane hyperpolarization, ATP production and insulin secretion while increasing $[\text{Ca}^{2+}]_c$ by a mechanism that could involve, among others, reduced Ca^{2+} uptake by the endoplasmic reticulum (ER), increased ER Ca^{2+} leak or stimulation of Ca^{2+} influx independent from L-type Ca^{2+} channels (29; 34; 38; 47). In contrast with H_2O_2 -treated islets, High-glucose islets show a mitochondrial membrane potential that is already hyperpolarized in G0.5 and only slightly further increased upon

stimulation with G6 or G20. These changes, which have previously been shown to correlate with an increase in islet ATP/ADP ratio at low glucose concentrations (25), were associated with an increased glucose sensitivity. Thus, insulin secretion by High-glucose islets was maximally stimulated by G6-G10 rather than G20, and the maximal rate of glucose-induced insulin secretion was reduced by ~50%. However, this reduction, in contrast with the reduction in H₂O₂-treated islets, was no longer observed when insulin secretion was expressed in percent of the islet insulin content. To our knowledge, no similar increase in glucose-sensitivity for changes in mitochondrial ATP production and stimulation of insulin secretion has ever been reported in β -cells submitted to various types of oxidative stress. High-glucose islets also displayed an elevation of basal $[Ca^{2+}]_c$ with no detectable $[Ca^{2+}]_c$ rise upon glucose stimulation. Again, this contrasts with H₂O₂-treated islets in which glucose-induced $[Ca^{2+}]_c$ changes were still observed despite an even greater elevation of $[Ca^{2+}]_c$ at low glucose.

A decrease in preproinsulin (PPI) mRNA levels and an increase in the mRNA levels of various antioxidant enzymes have been well documented in β -cells exposed to chronic hyperglycemia in vivo (21; 26; 62). Interestingly, PPI mRNA levels were also significantly decreased, and HO1 mRNA levels increased, in our islets exposed to high glucose in vitro for one week. However, PDX1 mRNA levels did not change under these conditions, in contrast with data obtained in hyperglycemic rats and in clonal insulin-secreting cells cultured several months in high glucose concentrations (20; 21; 49; 64). It therefore appears that one-week culture in the presence of high glucose does not reproduce all effects of prolonged in vivo hyperglycemia on β -cell gene expression. It does, however, reproduce many functional and morphological alterations observed after chronic exposure to hyperglycemia, such as a moderate increase in β -cell apoptosis and reversible loss of glucose-induced insulin secretion within the physiological range of glucose concentrations (5-10 mmol/l) (21; 25).

The many differences between the effects of H_2O_2 and high glucose in this study suggest that the functional alterations in High-glucose islets are independent from an increase in oxidative stress. This conclusion is further supported by the observation that islet alterations induced by H_2O_2 were significantly prevented by the free radical scavenger N-acetyl-L-cysteine (NAC) and, to a lesser extent, by the superoxide dismutase/catalase mimetic agent manganese(III)tetrakis (4-benzoic acid)porphyrin (MnTBAP), whereas those induced by culture in high glucose were not significantly affected by either antioxidant. These observations can be interpreted in three different ways : 1) both H_2O_2 and high glucose induce oxidative stress, but the antioxidants NAC and MnTBAP, which effectively scavenge H_2O_2 in the culture medium, are poorly active at the site of ROS production by high glucose ; 2) NAC and MnTBAP significantly reduce oxidative stress after culture in high glucose, but the functional alterations of β -cells are independent from the increase in ROS production ; 3) in contrast with H_2O_2 , one-week culture in high glucose does not induce oxidative stress in cultured rat islets, in agreement with a recent study showing that high glucose acutely represses rather than increases ROS production in purified rat β -cells (35).

Overnight exposure to H_2O_2 and one-week culture in G30 similarly increased islet HO1 mRNA levels, and this increase was prevented by NAC and significantly reduced by MnTBAP. Of note, the decrease in islet PPI mRNA levels by one week-culture in high glucose was also suppressed by MnTBAP. That NAC is a potent antioxidant in β -cells was recently demonstrated by its ability to prevent the increase in ROS production and decrease in glucose-induced insulin secretion in rat islets exposed to 2 mmol/l D-glyceraldehyde in the presence of G10 (52). The ability of MnTBAP to prevent ROS production was also demonstrated in purified rat β -cells exposed to glucose concentrations below 5 mmol/l (35). Because an increase in HO1 mRNA levels is a sensitive marker of oxidative stress in various cell types including β -cells (9; 13; 19; 31; 40), these results therefore suggest that both antioxidants effectively prevented an increase in oxidative stress during culture in G30.

The dissociation between, on one hand, the beneficial effects of NAC and MnTBAP on the alterations of β -cell HO1 expression by culture in high glucose and, on the other hand, the small effects of NAC and MnTBAP on β -cell dysfunction under these conditions may seem in contradiction with the literature. Thus, similar concentrations of NAC protected insulin-secreting cell lines from the reduction in insulin gene expression during months of culture in high glucose (reviewed in (45)) and significantly delayed the development of hyperglycemia in Zucker Diabetic Fatty rats (46; 53). Furthermore, other antioxidants such as vitamin E, probucol, cysteine, and aminoguanidine have been claimed to attenuate the alterations of both glucose tolerance in in vivo models of type 2 diabetes and glucose-induced insulin secretion in rodent islets exposed in vitro to high glucose concentrations (13; 18; 23; 60). However, the beneficial effects of antioxidant treatment reported in these studies were modest, suggesting that oxidative stress only slightly contributed to the alterations of β -cell function. This conclusion is strongly supported by our own results and by a previous study in which vitamin E failed to improve β -cell function of islets exposed to hyperglycemic conditions in vivo and in vitro (51). Interestingly, it has recently been shown that NAC was also unable to prevent the inhibition of insulin secretion by palmitate (37). In contrast with these models of prolonged exposure to high glucose or free fatty acid concentrations, the loss of glucose-induced insulin secretion observed under conditions causing a strong increase in oxidative stress, namely culture in the presence of ribose, streptozotocin, H_2O_2 , xanthine oxidase/hypoxanthine etc., has been consistently prevented by various antioxidant strategies (2; 15; 22; 24; 44; 53; 54).

In conclusion, although high glucose concentrations may cause an increase in β -cell oxidative stress responsible for the stimulation of islet HO1 expression, this effect is unlikely to be the cause of the reversible functional alterations observed in rat islets cultured for one week in G30 instead of G10. These results clearly demonstrate that the various alterations of the β -cell phenotype induced by supraphysiological glucose concentrations may result from several mechanisms with different sensitivity to antioxidants.

FOOTNOTES

¹Abbreviations used in this paper: $[Ca^{2+}]_c$, cytosolic Ca^{2+} concentration; cDNA, complementary DNA; DZ, diazoxide; GN, N mmol/l glucose; HO1, heme-oxygenase 1; mRNA, messenger ribonucleic acid.; ROS, reactive oxygen species; PDX1, pancreatic and duodenal homeobox gene 1; PPI, preproinsulin; TBP, TATA-box binding protein.

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LEGENDS TO FIGURES

Figure1. Effects of N-Acetyl-L-Cysteine (NAC) and manganese(III)tetrakis (4-benzoic acid)porphyrin (MnTBAP) on the changes in islet heme-oxygenase 1 (HO1) and preproinsulin (PPI) mRNA levels induced by exposure to hydrogen peroxide (H₂O₂) for one night or to high glucose for one week. A, after one-week preculture in RPMI medium containing 10 mmol/l glucose (G10), the islets were cultured 18h in G10 with 0, 12, 23 or 58 μ mol/l H₂O₂ in the absence (open circles) or presence (closed circle) of 1 mmol/l NAC. B, after preculture in G10, the islets were eventually pretreated for 24h with 50 μ mol/l MnTBAP before being exposed for 18h to 58 μ mol/l H₂O₂ in the same medium; C-D, rat islets were cultured for one week in RPMI medium containing G10 (open columns) or G30 (filled columns) in the absence or presence of 1 mmol/l NAC or 50 μ mol/l MnTBAP, as indicated. At the end of culture, islet total RNA was extracted and the HO1/TBP and PPI/cyclophilin mRNA ratios were determined by real-time RT-PCR and expressed in percent of the ratio in islets cultured in G10. Data are means $\pm$ S.E.M. for 4-5 independent experiments. *, $P < 0.05$ or less for the effect of H₂O₂ or G30; #, $P < 0.05$ or less for the effect of NAC or MnTBAP (One-way ANOVA + test of Newman-Keuls (A) or two-way ANOVA + test of Bonferroni (B-D)).

Figure 2. Effects of NAC on islet morphology and rhodamine 123 and furaPE3 loading after overnight treatment with H₂O₂ or one-week culture in high glucose. A, C, rat islets were cultured for one week in G10 and further cultured 18h in G10, G10 + 23-58 μ mol/l H₂O₂ with eventual addition of 1 mmol/l NAC, as indicated on the top of the figures. B, D, rat islets were cultured for one week in G10 or G30 with eventual addition of 1 mmol/l NAC throughout the culture period, as indicated. A-B, at the end of culture, the islets were fixed and processed for microscopical examination as described in "Methods". White stars indicate (at their lower side) intra-islet structures characteristic of apoptotic bodies (condensed chromatin and fragmented nuclei). C-D, after culture, the islets were rinsed with a medium devoid of H₂O₂ and NAC before being loaded with rhodamine 123 (a, b) or furaPE3 (c, d). Light

transmission (phase contrast microscopy)(a, c) and fluorescence images (excitation/emission 490/530 nm and 360/510 nm for rhodamine 123 and furaPE3 respectively)(b, d) were captured consecutively for each group of islets. Rhodamine 123 and furaPE3 fluorescence intensity are shown in green and blue pseudocolors respectively. Red lines delimiting the whole islets or their center and periphery (H_2O_2 -treated islets) were drawn on phase contrast microscopy images and copied on fluorescence images. Data are representative for 2-3 independent experiments.

Figure 3. Effects of NAC on the alteration of glucose stimulus-secretion coupling events induced by overnight exposure to H_2O_2 . After one-week preculture in G10, islets were cultured 18h in G10 with 0, 23 or 58 $\mu\text{mol/l}$ H_2O_2 and eventual addition of 1 mmol/l NAC as indicated. They were then rinsed twice in the same medium devoid of H_2O_2 and NAC before being loaded with rhodamine 123 (A,B) or furaPE3 (C,D). Glucose-induced changes in mitochondrial membrane potential (A, B), cytosolic calcium concentration ($[\text{Ca}^{2+}]_c$) (C, D), and insulin secretion (E, F) were measured in perfused islets in the absence of H_2O_2 and NAC. Data are representative traces (C, D) or means $\pm$ S.E.M. (A, B, E, F) for 3-4 experiments.

Figure 4. Effects of NAC on the alteration of glucose stimulus-secretion coupling events induced by one-week culture in high glucose. Islets were cultured for one week in G30 instead of G10 (High-glucose vs Controls) with eventual addition of 1 mmol/l NAC as indicated. They were then rinsed twice in the same medium devoid of NAC before being loaded with rhodamine 123 (A,B) or furaPE3 (C,D). Glucose-induced changes in mitochondrial membrane potential (A, B), $[\text{Ca}^{2+}]_c$ (C, D), and insulin secretion (E, F) were measured in perfused islets in the absence of NAC. Data are representative traces (C, D) or means $\pm$ S.E.M. (A, B, E, F) for 3-4 experiments.

Figure 5. Effects of MnTBAP on the alterations of glucose stimulus-secretion coupling events induced by exposure to H₂O₂ for one night or to high glucose for one week. A-B, after one-week preculture in G10, the islets were eventually pretreated for 24h with 50 µmol/l MnTBAP before being exposed 18h to 58 µmol/l H₂O₂ (H₂O₂-treated islets) in the same medium. C-F, islets were cultured for one week in G30 instead of G10 (High-glucose vs Controls) with eventual addition of 50 µmol/l MnTBAP as indicated. Glucose-induced changes in [Ca²⁺]_c (A-D) and insulin secretion (E, F) were measured in perfused islets in the absence of H₂O₂ and MnTBAP. Data are representative traces (A-D) or means ± S.E.M. (E, F) for 4-5 experiments.

Table 1 : Effects of NAC and MnTBAP on the changes in islet insulin and DNA contents induced by exposure to H₂O₂ for one night or to high glucose for one week.

Culture conditions	n	DNA content (ng/islet)	Insulin/DNA (ng/ng)
Overnight experiments			
G10 (Control islets)	4	18.7 ± 1.5	2.2 ± 0.3
G10 + 23 µmol/l H ₂ O ₂	4	14.3 ± 0.7 *	2.3 ± 0.4
G10 + NAC	4	21.1 ± 1.5	1.7 ± 0.2
G10 + 58 µmol/l H ₂ O ₂ + NAC	4	20.7 ± 1.7	1.8 ± 0.5
One week experiments			
G10 (Control islets)	8	29.1 ± 4.3	2.2 ± 0.2
G30 (High-glucose islets)	8	28.6 ± 3.1	1.3 ± 0.1 *
G10 + NAC	4	27.4 ± 4.1	1.6 ± 0.4
G30 + NAC	4	29.3 ± 3.4	0.8 ± 0.1 *
G10 + MnTBAP	4	30.8 ± 7.1	2.6 ± 0.4
G30 + MnTBAP	4	28.4 ± 2.6	1.7 ± 0.1 *

Islet insulin and DNA contents were measured at the end of the perfusions shown in Figs. 3E-F, 4E-F and 5E-F. Results are means ± S.E.M. for the indicated number of experiments (n). *, p<0.05 or less for the effect of H₂O₂ or G30 (one-way ANOVA + test of Newman-Keuls).

Table 2. Effects of NAC on the changes in glucose-induced decrease in rhodamine 123 fluorescence induced by exposure to H₂O₂ for one night or to high glucose for one week.

		Change in rhodamine 123 fluorescence	
		(% of G0.5)	
Culture conditions	n	G6	G20
Overnight experiments			
G10 (Control islets)	12	-24.0 ± 1.3	-29.7 ± 1.5 ^a
G10 + 23 µmol/l H ₂ O ₂	12	-24.3 ± 1.2	-32.7 ± 1.3 ^a
G10 + 58 µmol/l H ₂ O ₂	6	-25.1 ± 1.7	-33.3 ± 2.2 ^a
G10 + 58 µmol/l H ₂ O ₂ + NAC	6	-24.4 ± 2.1	-29.7 ± 1.7 ^a
One week experiments			
G10 (Control islets)	4	-21.0 ± 2.1	-28.7 ± 2.8 ^a
G30 (High-glucose islets)	4	-2.7 ± 1.0 [#]	-5.7 ± 1.4 [#]
G10 + NAC	4	-16.7 ± 1.1	-24.2 ± 2.0 ^a
G30 + NAC	4	-3.5 ± 0.4 [#]	-7.2 ± 0.3 [#]

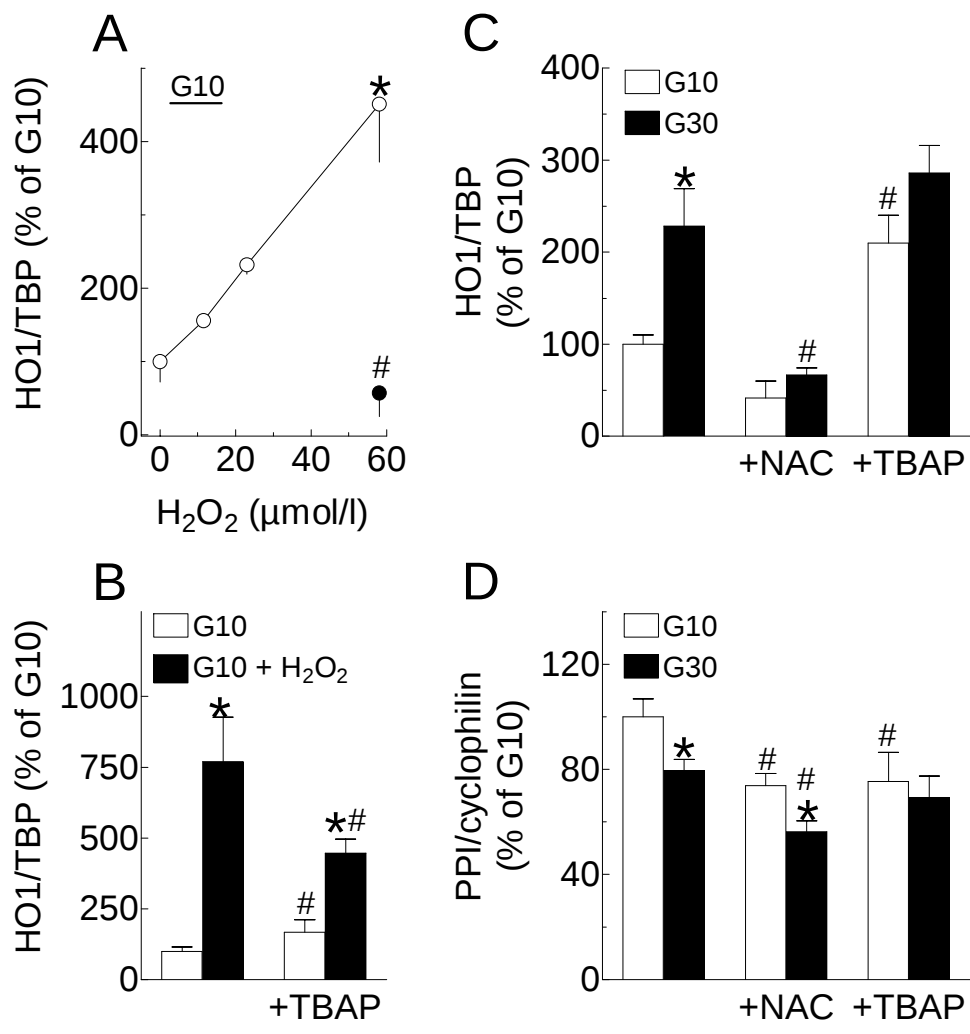
In each experiment illustrated in Figs. 3A-B and 4A-B, the mean absolute rhodamine 123 fluorescence level during the last 5 min of each glucose step was calculated and expressed as a percentage of that in G0.5. Results are means ± S.E.M. for the indicated number of islets (n). ^a, $P < 0.05$ or less for the effect of G20 vs G6; [#], $P < 0.001$ vs Control islets (two-way ANOVA + test of Bonferroni).

Table 3. Effects of NAC and MnTBAP on the changes in islet $[Ca^{2+}]_c$ induced by exposure to H_2O_2 for one night or to high-glucose for one week.

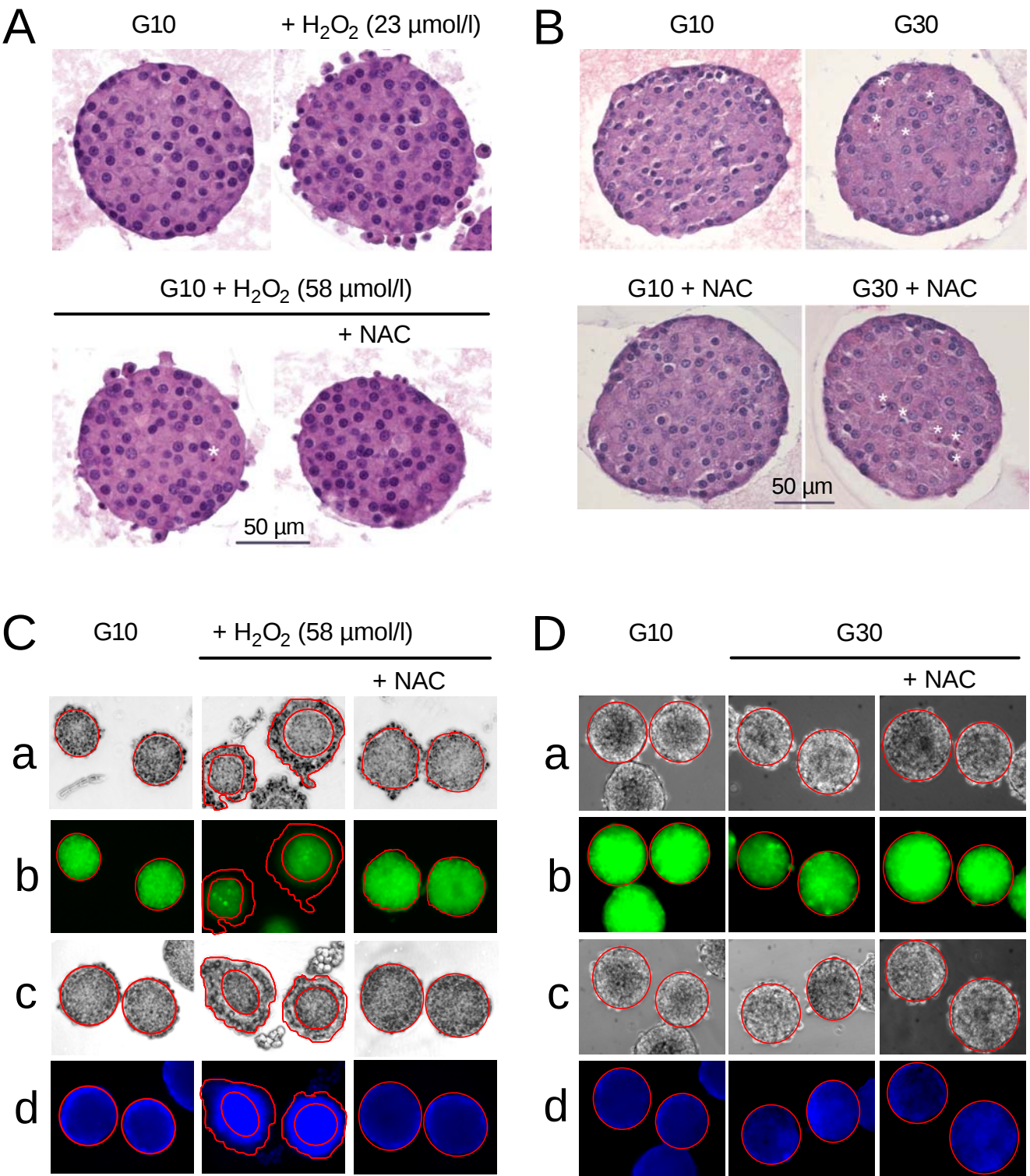
		[Ca ²⁺] _c (nmol/l)			
Culture conditions	n	G0.5	G6	G20	G20K30DZ
Overnight experiments					
G10 (Control islets)	19	152 ± 6	152 ± 7	237 ± 11	319 ± 10
G10 + 23 µmol/l H ₂ O ₂	14	297 ± 18 [#]	298 ± 19 [#]	362 ± 24 [#]	420 ± 23 [#]
G10 + 58 µmol/l H ₂ O ₂	11	536 ± 37 [#]	578 ± 36 [#]	679 ± 43 [#]	747 ± 53 [#]
G10 + 58 µmol/l H ₂ O ₂ + NAC	8	179 ± 5 ^b	173 ± 5 ^b	271 ± 11 ^b	364 ± 9 ^b
G10 + MnTBAP	5	133 ± 8	148 ± 9	219 ± 17	287 ± 20
G10 + 58 µmol/l H ₂ O ₂ + MnTBAP	6	243 ± 31 ^b	282 ± 36 ^{*, b}	375 ± 40 ^{#, b}	437 ± 47 ^{#, b}
One week experiments					
G10 (Control islets)	12	119 ± 3	123 ± 3	203 ± 8	298 ± 12
G30 (High-glucose islets)	11	152 ± 6 [#]	158 ± 6 [#]	169 ± 7 [#]	290 ± 8
G10 + NAC	3	120 ± 6	116 ± 3	214 ± 14	273 ± 24
G30 + NAC	3	175 ± 15 [*]	174 ± 13 [#]	179 ± 11	296 ± 15
G10 + MnTBAP	9	125 ± 5	149 ± 5 ^a	234 ± 3 ^b	304 ± 8
G30 + MnTBAP	9	162 ± 7 [#]	185 ± 7 ^{#, a}	204 ± 6 ^{*, b}	293 ± 9

In each experiment illustrated in Figs. 3C-D, 4C-D and 5A-D, the mean $[Ca^{2+}]_c$ during the last 5 min in G0.5, G6, G20 and G20K30DZ was calculated. Results are means ± S.E.M. for the indicated number of islets (n). *, $P < 0.05$ and [#], $P < 0.01$ or less for the effect of H_2O_2 or G30; ^a, $P < 0.05$ and ^b, $P < 0.01$ or less for the effect of NAC or MnTBAP (two-way ANOVA + test of Bonferroni).

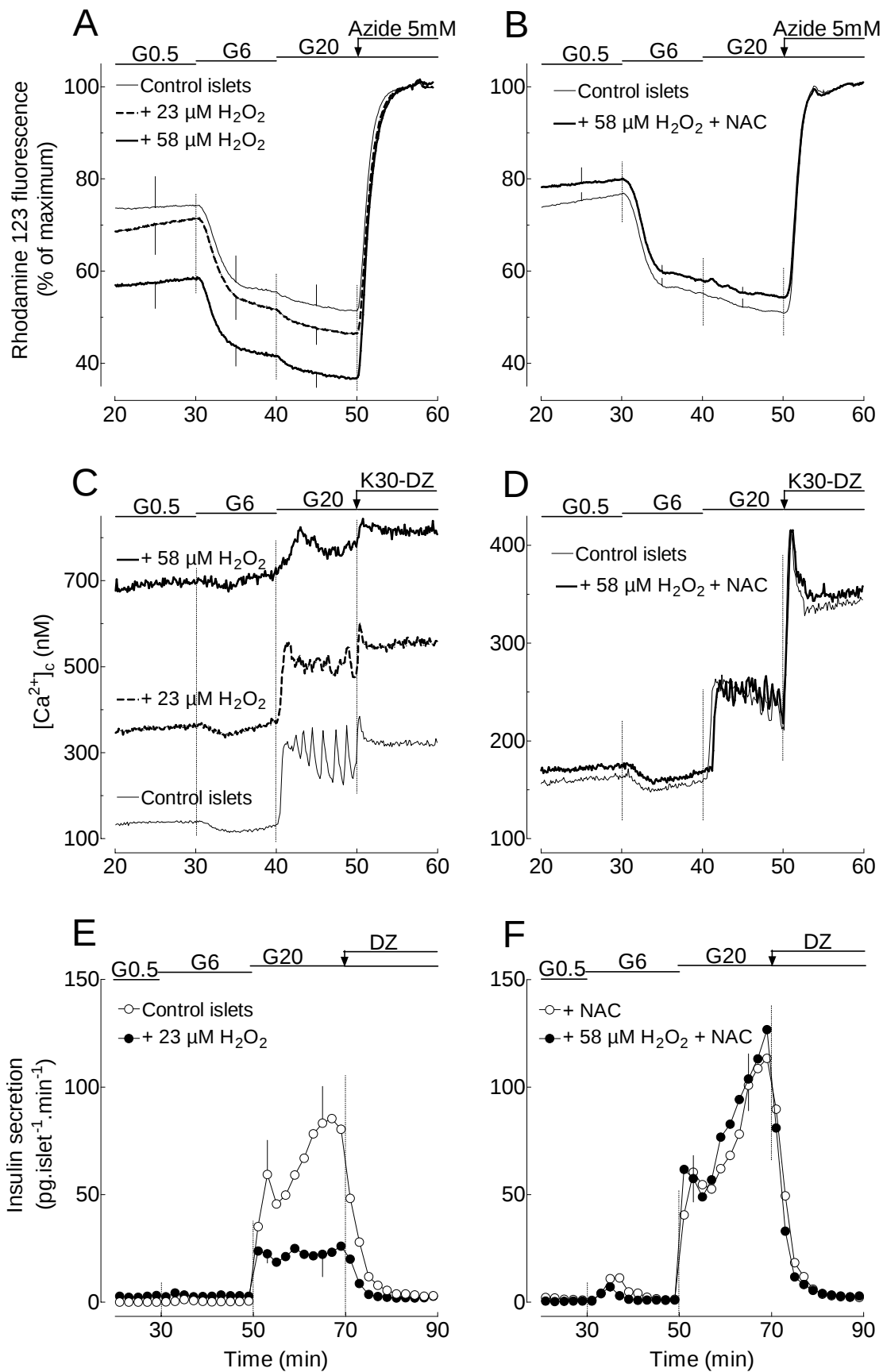
Khaldi et al, Figure 1



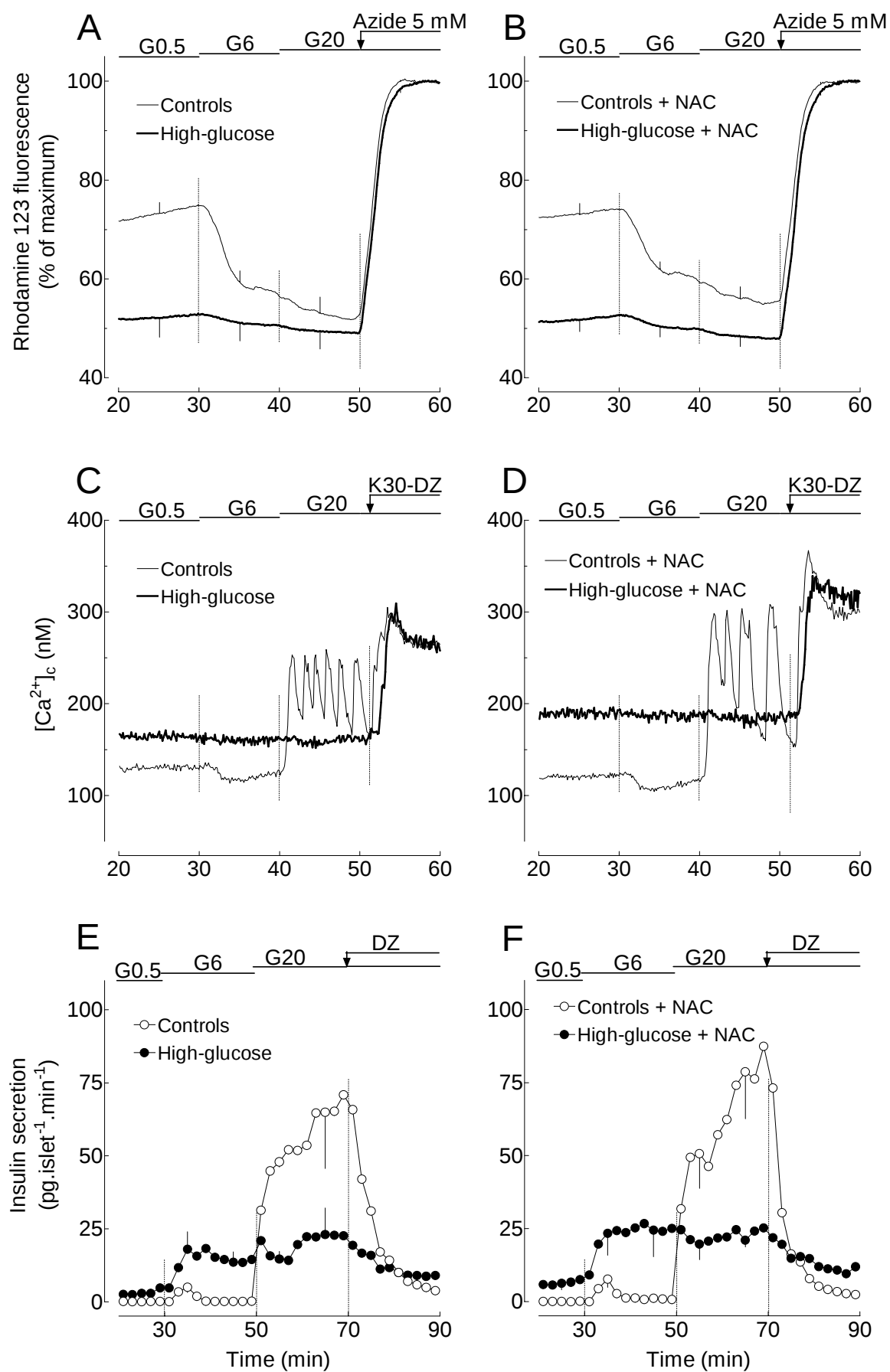
Khaldi et al. Figure 2



Khaldi et al, Figure 3



Khaldi et al, Figure 4



Khaldi et al, Figure 5

