

Glucose Suppresses Superoxide Generation in Metabolically Responsive Pancreatic β Cells*[§]

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High rates of glucose metabolism and mitochondrial electron transport have been associated with increased mitochondrial production of reactive oxygen species (ROS). This mechanism was also proposed as a possible cause for dysfunction and death of pancreatic β cells exposed to high glucose levels. We examined whether high rates of glucose metabolism increase ROS production in purified rat β cells. Glucose up to 20 mM did not stimulate H_2O_2 or superoxide production, whereas it dose-dependently increased cellular NAD(P)H and $FADH_2$ levels with an EC_{50} around 8 mM. On the contrary, glucose concentration-dependently suppressed H_2O_2 and superoxide formation, with a major effect between 0 and 5 mM, parallel to an increase in cellular NAD(P)H levels. This suppressive effect was more marked in β cells with higher NAD(P)H responsiveness to glucose; it was not observed in glucagon-containing α cells, which lacked a glucose-induced increase in NAD(P)H. Suppression was also induced by the mitochondrial substrates leucine and succinate. Experiments with electron transport chain inhibitors indicate a role of respiratory complex I in ROS production at low mitochondrial activity and low NADH levels. Superoxide production at low glucose is potentially cytotoxic, because scavenging by the superoxide dismutase mimetic agent manganese(III)tetrakis(4-benzoic acid)porphyrin was found to reduce the rate of β cell apoptosis. Analysis of islets cultured at 20 mM glucose confirmed that this condition does not induce ROS production in β cells as a result of their increased rates of glucose metabolism. Our study indicates the need of β cells for basal nutrients maintaining mitochondrial NADH production at levels that suppress ROS accumulation from an inadequate respiratory complex I activity and thus inhibit a potential apoptotic pathway.

Chronically elevated glucose levels cause major lesions in retina, kidney, neurons, and vascular tissue (1). The toxic effects of hyperglycemia can be explained by mechanisms that

involve increased glucose metabolism (2, 3). A primary role is often attributed to an excessive formation of reactive oxygen species (ROS)¹ at the mitochondrial electron transport chain (ETC) of cells in an early or late stage of glucose-induced dysfunction (4–6). In endothelial cell lines, glucose-induced ROS production appeared a critical upstream event in the process of hyperglycemic damage (4). Re-oxidation of glucose-generated NADH and $FADH_2$ by the ETC results in a progressively increasing proton gradient across the mitochondrial membrane. In isolated mitochondria such a condition increases superoxide production once the gradient reaches a critical threshold (3, 7). Elevated glucose levels are also suspected of causing dysfunction and/or death of insulin-producing β cells through ROS formation (8, 9). It is unknown whether their toxicity in β cells also involves glucose-induced elevations in cellular NADH and $FADH_2$ levels up to the point that mitochondrial energetic coupling is disrupted and followed by ROS formation. This question needs particular attention, because glucose-responsive β cells are specialized to achieve high rates of glucose catabolism at elevated levels of this nutrient (10). It also bears pathological relevance because β cells appear particularly susceptible to oxidative stress in view of their reportedly low expression of ROS scavenging enzymes (11, 12).

The notion that elevated glucose concentrations induce ROS formation by β cells comes in part from indirect measurements in islets and β cell lines, using up-regulation of ROS scavenging enzymes or a cytoprotective effect of antioxidants as markers for radical formation (13–15). Direct evidence is based on real-time microscopic monitoring of superoxide formation in isolated intact and dissociated rat islets (16, 17). It is however necessary to compare ROS formation with the actual rates of glucose metabolism and/or the corresponding metabolic redox state of the ETC. The present study is conducted on purified rat β cells and islets following exposure to a broad glucose concentration range. It examines whether high glucose increases cellular ROS and superoxide formation, and how ROS formation is correlated with the cellular metabolic redox state; it also investigates whether any of these processes are associated with a higher rate of β cell death.

EXPERIMENTAL PROCEDURES

Materials—MnTBAP (manganese(III)tetrakis(4-benzoic acid)porphyrin) was obtained from Alexis Biochemicals (Lausen, Germany). Hydroethidine (HE) and 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF -DA) were purchased from Molecular Probes (Invitrogen). Atpenin a5 was a kind gift of Dr. Satoshi Omura (The Kitasato Institute,

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[§] The on-line version of this article (available at <http://www.jbc.org>) contains Fig. S1.

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¹ The abbreviations used are: ROS, reactive oxygen species; ETC, electron transport chain; FACS, fluorescence-activated cell sorting; PI, propidium iodide; MFI, mean fluorescence intensity; DCF, dichlorofluorescein; H_2DCF -DA, 2',7'-dichlorodihydrofluorescein diacetate; HE, hydroethidine; MnTBAP, manganese(III)tetrakis(4-benzoic acid)porphyrin; GK, glucokinase.

TABLE I
Oligonucleotide primers and cycling conditions used for reverse transcription-PCR detection of rat islet gene cDNA

| Gene | Sense/antisense | Primer sequence | Annealing ($^{\circ}$ C) | Product size (bp) |
|--------------------------|-----------------|------------------------|---------------------------|-------------------|
| Catalase | S | TCTGCAGATACCTGTGAACTG | 60 | 357 |
| | A | TAGTCAGGGTGGACGTCAGTG | | |
| Glutathione peroxidase 1 | S | CTCGGTTTCCCGTGCAATCAG | 65 | 431 |
| | A | GTGCAGCCAGTAATCACCAG | | |
| TATA-box-binding protein | S | ACCCTTACCAATGACTCCTATG | 62 | 190 |
| | A | ATGATGACTGCAGCAATTCGC | | |

Tokyo, Japan). All other substrates or inhibitors were obtained from Sigma-Aldrich.

Purification of Islet α and β Cells and Analysis of Their Metabolic Redox State—Rat islet cells were prepared from male Wistar rats (250–300 g, Janvier, Le Genest Saint-Isle, France) and separated into a β cell (>90% purity) and an endocrine non- β cell population that is enriched in glucagon-containing alpha-cells (18, 19). In one series of experiments, β cells were further sorted according to their metabolic responsiveness to 7.5 mM glucose using cellular NAD(P)H intensity as parameter to discriminate metabolically responsive from non-responsive β cells (20, 21). Unless otherwise stated, primary rat islet cells were studied in Ham's F-10 nutrient mixture (Invitrogen) supplemented with 0.5% bovine serum albumin (Cohn Analog, Sigma), 2 mM glutamine, penicillin (100 units/ml), and streptomycin (0.1 mg/ml) at the indicated glucose concentration.

The metabolic redox state of the isolated cell populations was analyzed after 30-min incubation in humidified air (20% O_2 /5% CO_2) at 37 $^{\circ}$ C. It was determined by flow cytometry measuring cellular fluorescence of NAD(P)H (argon laser 351–363 nm/400–470 nm) and mitochondrial riboflavin (FAD and FMN, argon laser 488/530 nm) using a dual laser FACStar+ \oplus system (BD Biosciences) (19). Mean absolute fluorescence intensities (MFI) \pm S.E. are shown for 10,000 propidium iodide (PI, 5 μ g/ml) negative islet cells.

Measurement of H_2O_2 and Superoxide in Single Islet Cells—Cellular ROS levels were measured in single cells (50×10^3 cells in 400 μ l of medium) after 60 min of incubation in humidified air (20% O_2 /5% CO_2) at 37 $^{\circ}$ C. For H_2O_2 detection, cells were pre-loaded for 20 min with 25 μ M H_2 DCF-DA in Ham's F-10 medium (containing 5 μ g/ml PI, 10 mM glucose and a final concentration of 0.05% Me_2SO). Cells were washed twice to remove excess dye and incubated for 60 min prior to simultaneous detection of dichlorofluorescein (DCF, 530 nm bandpass filter) and NAD(P)H fluorescence intensity in PI-negative cells. Presence of H_2 DCF-DA itself did not interfere with glucose-induced NAD(P)H generation (data not shown). Endogenous riboflavin fluorescence was two orders of magnitude lower than baseline DCF fluorescence and did not contribute to the DCF signal.

Superoxide was detected by FACS measurement of cellular HE oxidation, as described by Kroemer *et al.* (22). Following 60-min incubations, 2.5 μ M HE was added (final Me_2SO 0.05%), and cells were incubated at 37 $^{\circ}$ C for an additional 10 min prior to analysis of HE-derived red fluorescence (630 nm bandpass filter). Because this analysis was conducted in the absence of PI, a population of damaged cells (typically 2–3% of total) was excluded based on lower forward scatter and distinctively higher fluorescence in the FL3 channel, the latter effect presumably caused by fluorescence enhancement of DNA-bound oxidized HE (23). Endogenous red fluorescence in unstained cells was not influenced by the glucose concentration. Monitoring the rate of HE oxidation for 15 min after probe addition allowed measurement superoxide generation rate by linear curve fitting (Microsoft Excel, $r^2 > 0.95$).

Glucose effects on metabolic redox state and superoxide generation were also measured in cells that were dissociated from islets that had been cultured at low and high glucose. Freshly isolated islets were first cultured overnight at 10 mM glucose and then for an additional 72 h at 6 or 20 mM glucose (in Ham's F-10 nutrient mixture supplemented as described above) before dissociation (24) and FACS analysis. Dot plots of the dispersed islet cells showed two populations, corresponding to β and endocrine non- β cells (18); mean fluorescence intensities (MFIs) were measured in the β cell population that is characterized by higher forward scatter and higher FAD fluorescence (18).

Assays for β Cell Apoptosis—An earlier described fluorescence microscopic method, using PI and Hoechst 33342 (25), was used to determine the degree of apoptosis in single β cell preparations. Effects on apoptosis were examined by calculating the apoptosis index, which expresses the degree of apoptosis for an experimental condition X relative to that in the control condition (control = 10 mM glucose); this index is calculated as follows: (% apoptotic cells in X - % apoptotic cells

in control)/% living cells in control. In whole islets, the degree of apoptosis was determined by FACS analysis of nuclear fragmentation, as described previously (26).

Semi-quantitative Detection of Catalase and Glutathione Peroxidase 1 mRNA in Islets—Islets were pre-cultured for 5 days in 10 mM glucose prior to exposure for 18 h to the indicated glucose concentration with or without MnTBAP, as described (27). Polyadenylated RNA was extracted with oligo(deoxythymidine)-coated magnetic microbeads (Dyna-beads, Dynal, Oslo, Norway) and RNA reverse transcribed with the GeneAmp RNA PCR kit using random hexamer primers and Moloney murine leukemia virus reverse transcriptase (PerkinElmer Life Sciences) prior to PCR. The primers and the lengths of PCR-generated fragments are listed in Table I. The thermal cycle profile was initiated during a 4-min denaturing step at 94 $^{\circ}$ C to release DNA polymerase activity. It was followed by 23–28 cycles of amplification (1-min denaturation at 94 $^{\circ}$ C, 1-min annealing at 60–62 $^{\circ}$ C, then 1-min extension at 72 $^{\circ}$ C) and a final extension step of 10 min at 72 $^{\circ}$ C. PCR products were analyzed on ethidium bromide-stained agarose gels and photographed under UV transillumination using a Kodak Digital Science DC40 camera (Eastman Kodak Co., Rochester, NY). Data were expressed relative to reverse transcription-PCR signals for the house-keeping TATA-box-binding protein gene.

Statistical Analysis—Data are presented as means \pm S.E. of n independent experiments. H_2 DCF-DA- and HE-derived fluorescence was expressed as a percentage of the value measured in 10 mM glucose exposed cells (relative fluorescence). Statistical analysis was performed using analysis of variance or Student's t test where appropriate. Differences were considered significant when $p < 0.05$.

RESULTS

Metabolic Rate in Islet β Cells Is Inversely Related to Cellular Formation of H_2O_2 and Superoxide—Glucose is a well known regulator of the metabolic redox state in islet β cells. It concentration-dependently increases the cellular levels of the reduced electron carriers NAD(P)H and $FADH_2/FMNH_2$ (Fig. 1A), the latter being reflected by a decrease in mitochondrial riboflavin fluorescence (28). The concentration-response curve for NAD(P)H is sigmoidal with an effect from 2.5 mM onwards and with an EC_{50} of ~ 8 mM; the effect on $FADH_2/FMNH_2$ was less pronounced and became only detectable above 5 mM glucose (Fig. 1A). Consequently, the cells exhibited an increase in the NAD(P)H over FAD/FMN ratio when glucose was increased from 0 to 5 mM glucose.

Glucose was found to suppress ROS production as measured by the oxidation of H_2 DCF-DA that was preloaded in β cells (Fig. 1B). ROS levels were highest in absence of glucose and concentration-dependently decreased, predominantly between 0 and 5 mM, reaching the lowest values at 20 mM glucose (Fig. 1B). Cellular ROS levels in glucose-free media were 40% lower than those following exposure to a toxic concentration of H_2O_2 (10 min, 100 μ M, 2.65- \pm 1.0-fold increase in DCF as compared with 10 mM glucose control, $p < 0.001$, $n = 5$). Cellular ROS production was also suppressed by two other mitochondrial nutrients, leucine (10 mM) and succinic acid (methyl ester, 20 mM) (Fig. 1B). Addition of the NG-nitro-L-arginine methyl ester (1 mM), a competitive inhibitor of nitric-oxide (NO) synthase, or removal of arginine from the culture medium, did not influence low glucose-induced DCF accumulation (data not shown), suggesting that the latter effect was not secondary to an increase in NO production.

β Cells also exhibited a superoxide production that was sup-

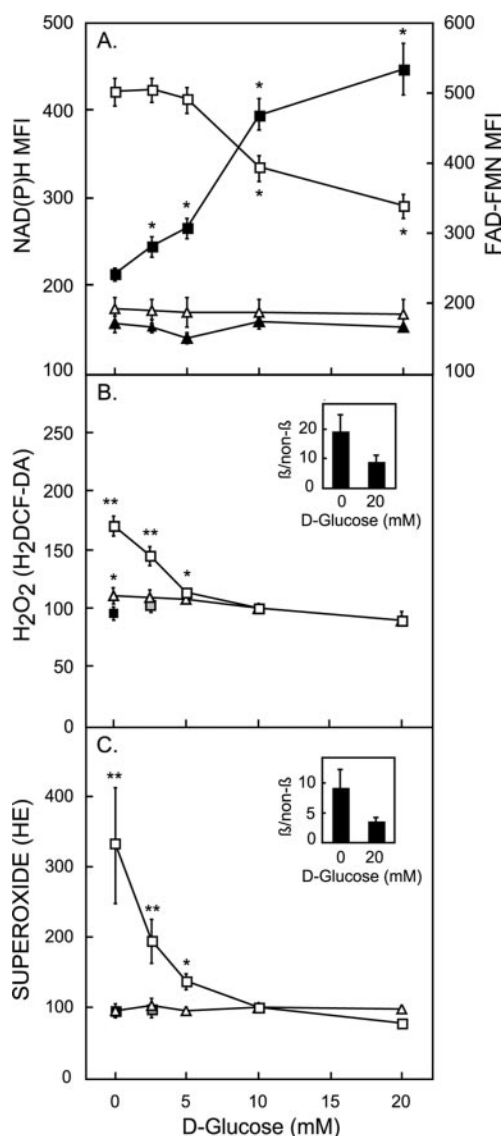


FIG. 1. Effect of glucose on cellular fluorescence intensities expressing variations in NAD(P)H, flavin fluorescence, and ROS levels in rat β and islet non- β cells. *A*, cellular NAD(P)H (filled symbols, left y-axis) and FAD-FMN (open symbols, right y-axis) fluorescence were simultaneously measured in PI negative rat β (squares) and islet non- β cells (triangles) that had been incubated for 30 min at the indicated glucose concentration. Data represent average mean fluorescence intensity (MFI, arbitrary units) \pm S.E. from seven independent experiments. (*, $p < 0.05$ versus the level measured at the preceding glucose concentration.) *B* and *C*, oxidation of pre-loaded H_2 DCF-DA, a marker for cellular H_2O_2 (*B*) and oxidation of hydroethidine (HE), a marker for superoxide generation (*C*) in β cells (squares) and islet non- β cells (triangles) following 1-h exposure to the indicated nutrient concentration. β Cells were also tested in the presence of leucine (10 mM, filled black square) or succinic acid (methyl ester 20 mM, filled gray square) at the indicated glucose concentration. Fluorescence (mean \pm S.E., $n = 7$) is expressed as the percentage of the value measured in the same cell population at 10 mM glucose (relative MFI). (*, $p < 0.05$; **, $p < 0.001$ as compared with the 10 mM glucose; reference value measured in the same cell type.) Insets in *B* and *C* compare absolute levels of DCF- and HE-derived fluorescence, respectively, in islet β and non- β cells. Bars indicate the average ratio of absolute MFI in islet β over non- β cells at 0 and 20 mM glucose ($n = 7$).

pressed by glucose, as indicated by the lower oxidation of HE at increasing glucose concentrations (Fig. 1C). Cellular superoxide levels were 3-fold higher in glucose-free medium than at 10 mM glucose. Increasing the glucose concentration above 10 mM further lowered superoxide levels, not statistically significant at 20 mM (Fig. 1C, $n = 7$) but at 30 mM glucose ($85 \pm 3\%$ of 10

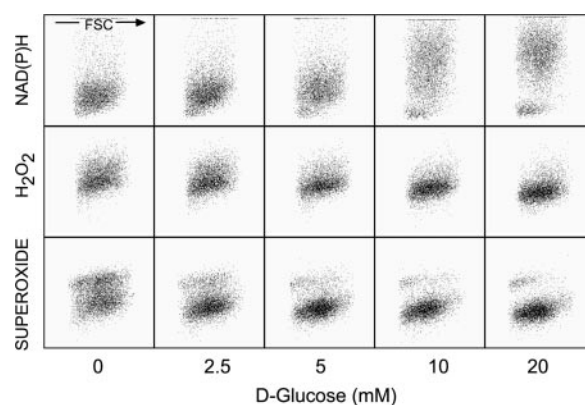


FIG. 2. Intercellular heterogeneity in metabolic responsiveness to glucose (as measured by increase in NAD(P)H) is associated with intercellular differences in H_2O_2 and superoxide generation. Representative FACS dot plots of 1) NAD(P)H fluorescence (upper row, linear scale); 2) H_2 DCF-DA oxidation (middle row, log scale); and 3) cellular HE oxidation (lower row, log scale) as a function of forward scatter (FSC, x-axis) in the same batch of β cells, exposed for 60 min to the indicated glucose concentration.

mM glucose control; $p < 0.05$, $n = 6$). The rate of superoxide generation at low glucose was determined by analyzing the time course of HE oxidation. HE oxidation had linear kinetics ($r^2 > 0.95$) between 5 and 15 min after probe addition. Rates (mean slope \pm S.D.) were calculated by linear curve fitting; the rate of superoxide generation at 0 and 2.5 mM glucose was thus found to be, respectively, 2.2 ± 0.3 - and 1.9 ± 0.4 -fold higher than at 10 mM glucose ($p < 0.01$ versus 10 mM glucose, $n = 5$). As for peroxide-like ROS, superoxide production at low glucose was completely suppressed by leucine and succinate (Fig. 1C).

Islet non- β cells did not exhibit glucose-induced changes in their metabolic redox state as measured by the cellular NAD(P)H and FAD/FMN levels (Fig. 1A). Their H_2O_2 and superoxide production did not vary with the glucose concentration (Fig. 1, *B* and *C*). It was markedly lower than that in β cells, as illustrated by their 10- and 5-fold lower H_2 DCF- and HE-derived fluorescence intensities (insets in Fig. 1, *B* and *C*).

β Cells with Lower Metabolic Responsiveness to Glucose Generate More Superoxide—A comparative analysis of individual β cells indicates a wide variation in their HE- and H_2 DCF-DA-derived fluorescence intensities and in their NAD(P)H response to glucose (representative dot-plots are shown in Fig. 2). At 10 mM glucose, a small subset (around 10%) of β cells can be distinguished with 10-fold higher HE-derived mean fluorescence intensities (Fig. 2, lower row). With decreasing glucose concentrations, the fraction of the cells recovered in this high superoxide-generating subset gradually increased. These data indicate that glucose reduces the number of β cells with elevated superoxide generation but does not succeed in suppressing it in all cells. Previous work has shown intercellular differences in metabolic responsiveness to glucose (20, 21). We therefore examined whether β cells with higher metabolic responsiveness to glucose corresponded to those with a more marked glucose-induced suppression of superoxide generation. Highly responsive β cells were separated from those with lower metabolic responsiveness based on their increased NAD(P)H fluorescence intensity at 7.5 mM glucose (21) and then compared for glucose metabolic rate and superoxide formation. Highly responsive β cells generated more NAD(P)H and $FADH_2/FMNH_2$ (Fig. 3) and did so at lower glucose concentrations. Whereas both subsets presented similar superoxide formation in absence of glucose, the high responsive cells exhibited a much stronger glucose-mediated suppression at all glucose concentrations tested (Fig. 3, lower panel). Addition of 10 or 20 mM glucose only partially suppressed superoxide pro-

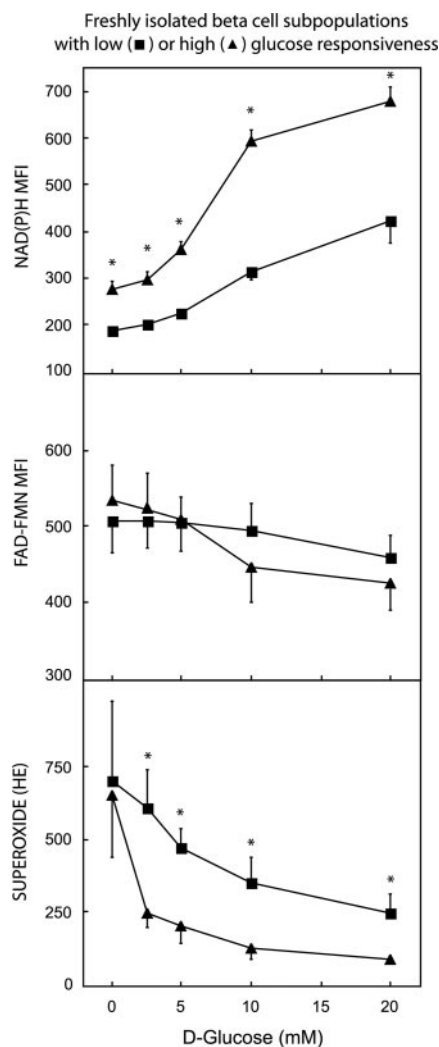


FIG. 3. Lower rates of superoxide generation in β cells with higher metabolic responsiveness to glucose. Freshly isolated β cells were first sorted in two non-overlapping populations with lower (filled squares) or higher (filled triangles) metabolic NAD(P)H response to 7.5 mM glucose. Following 60-min incubation at 37 °C, NAD(P)H levels (upper panel), riboflavin (FAD-FMN) fluorescence (middle panel) and superoxide generation (lower panel) were measured in these β cell subsets. To allow a direct comparison of redox state and superoxide levels, absolute rather than normalized fluorescence is shown. Data represent average MFI \pm S.E. (*, $p < 0.05$ between subpopulations at the indicated glucose concentration, $n = 4$).

duction in the low responsive subpopulation, which is consistent with the heterogeneity in individual levels that was observed in Fig. 2.

Lower Rates of Superoxide Formation in β Cells That Are Metabolically Activated by Sustained Exposure to High Glucose—Short term glucose effects on cellular redox state and ROS formation do not necessarily reflect the effects of chronic hyperglycemia. Therefore, both parameters were measured in β cells following culture of islets at high glucose and subsequent dissociation. As reported previously for purified rat β cells (29), sustained exposure of intact islets to high glucose (20 mM) recruited their β cells to a redox state that exhibits a left and upward shift of the NAD(P)H response curve to glucose when compared with β cells from 6 mM glucose-cultured islets (Fig. 4, upper panel). Their higher sensitivity to glucose is also reflected by comparison of the concentration-dependent decrease in cellular FAD fluorescence (Fig. 4, middle): at 2.5 mM, glucose exerted already $48 \pm 4\%$ of its reducing effect on cellular NAD(P)H and FADH₂ levels. At this concentration, glu-

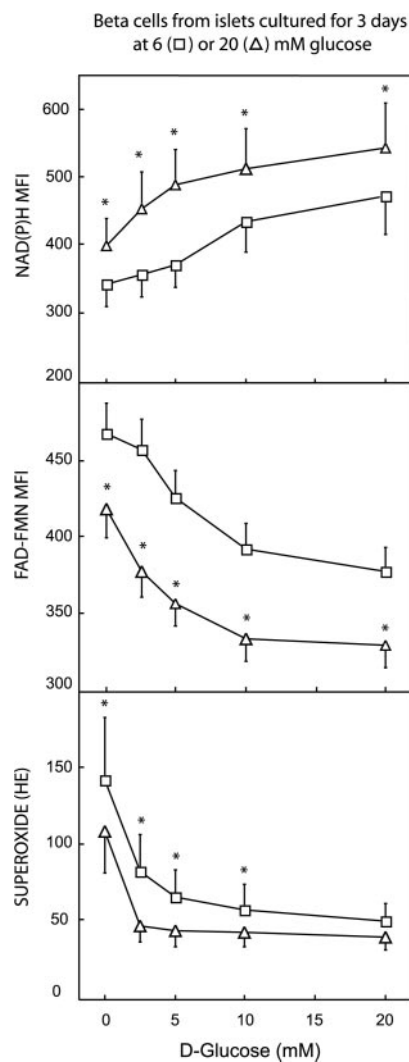


FIG. 4. Lower rates of superoxide generation in β cells metabolically activated by sustained exposure to high glucose. Rat islets were cultured for 72 h in basal (6 mM, open squares) or high (20 mM, open triangles) glucose, dissociated and FACS-analyzed for NAD(P)H (upper panel), riboflavin fluorescence (middle panel), and superoxide generation (lower panel). To allow a direct comparison of redox state and superoxide levels, absolute rather than normalized fluorescence is shown. Data represent average MFI \pm S.E. (*, $p < 0.05$ between 6 and 20 mM exposed cells at the indicated glucose concentration, $n = 4$).

cose completely suppressed superoxide formation in 20 mM cultured β cells, whereas more than 5 mM was needed in 6 mM cultured β cells (Fig. 4). Between 0 and 10 mM glucose, superoxide accumulation was lower in 20 mM cultured β cells than in 6 mM cultured cells (Fig. 4, lower panel).

Role of Respiratory Complex Activity in Superoxide Formation by β Cells—Our observations indicate that glucose-induced suppression of superoxide formation are correlated with an increase in cellular NAD(P)H levels. In freshly isolated β cells, these effects are only partially achieved by 2.5 mM glucose (Fig. 1) but can be amplified by inhibiting NADH consumption through the electron transport chain (ETC). In presence of rotenone (100 nM), an inhibitor of ETC complex I, superoxide formation is completely suppressed (Fig. 5, lower panel), whereby the rise in NAD(P)H is attributed to the rotenone-induced block in H⁺ transfer from NADH (Fig. 5); the associated rise in reduced flavin is compatible with the inhibitor binding downstream of the FMN site in ETC complex I (28, 30). Similar effects were achieved by the complex III inhibitor an-

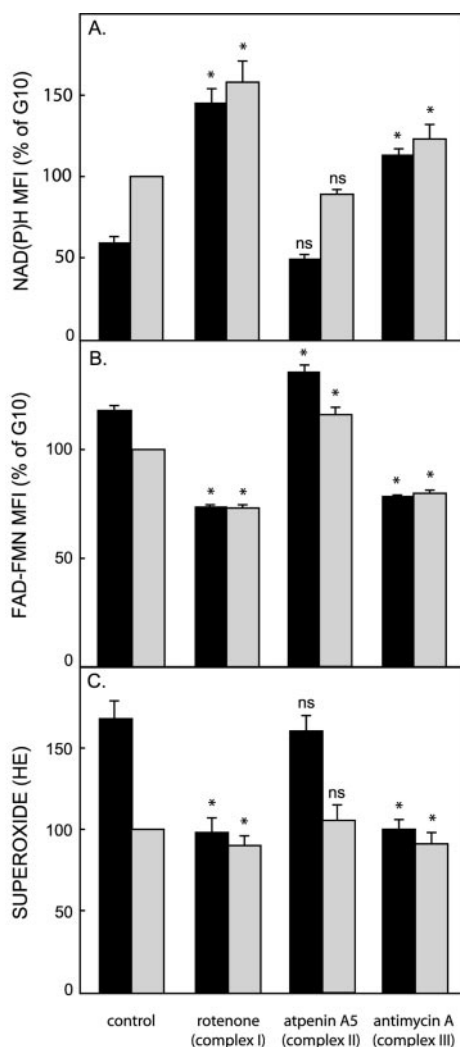


FIG. 5. Effect of respiratory complex inhibitors on glucose-induced superoxide suppression. NAD(P)H (upper panel), FAD-FMN fluorescence (middle panel), and superoxide generation (lower panel) were measured in β cells incubated for 60 min at 2.5 (black bars) and 10 mM (gray bars) glucose with or without blockers of ETC complexes I (rotenone, 100 nM), II (atpenin a5, 100 nM), and III (antimycin A, 200 nM), added 20 min prior to analysis. All data are expressed as relative MFI (% of 10 mM glucose control) and represent mean \pm S.E. of 5–8 independent experiments (*, $p < 0.05$ versus untreated control in the same glucose concentration; ns, not statistically significant).

timycin A (200 nM) (Fig. 5), which is expected to lead to a reduced activity of complex I (30). On the contrary, addition of atpenin a5 (100 nM), a specific inhibitor of complex II-succinate dehydrogenase (31), did not increase NADH and reduced-flavin levels and did not reduce superoxide formation (Fig. 5). This inhibitor did not affect the 10 mM glucose-induced rise in NAD(P)H and suppression of superoxide formation (Fig. 5) but prevented succinate from inducing similar effects (Supplementary Fig. S1). In view of the latter effect, the atpenin a5-induced flavin oxidation (Fig. 5) indicates a specific block in fumarate and FADH₂ formation at the level of complex II-succinate dehydrogenase. These results suggest that respiratory complex activity consumes NADH and thus decreases NADH-induced protection against superoxide accumulation in conditions of inadequate substrate supply to the tricarboxylic acid cycle. The latter condition is then characterized by a low NADH over FAD-FMN ratio as measured in freshly isolated β cells at low nutrient levels (Fig. 1).

Superoxide Generation Contributes to Apoptosis of β Cells with Low Metabolic Activity—Glucose concentration-depend-

ently decreases the susceptibility of rat β cells to apoptotic cell death during culture (25). We tested whether this effect is in part mediated by suppressing superoxide generation. Addition of the superoxide dismutase mimetic MnTBAP (50 μ M) significantly ($p < 0.05$, $n = 6$) reduced superoxide levels in β cells exposed to low glucose concentrations (Fig. 6A, representative dot plots shown in panels B–D). Scavenging of superoxide by MnTBAP also decreased the percent apoptotic, single β cells (Fig. 6E) after 72-h exposure to 3 mM glucose. Similar effects were seen after addition of the mitochondrial fuels leucine (10 mM) or succinate (20 mM) (Fig. 6E), which is consistent with their ability to suppress ROS production in β cells.

A similar protective effect was observed when islets were studied instead of isolated β cells. Islets were cultured for 72 h at 3, 10, or 20 mM glucose, with or without the ROS scavenger MnTBAP. The degree of apoptosis was quantified by FACS analysis of cellular DNA, using cycloheximide-treated islets as positive control (25) (Fig. 7B). Glucose as well as MnTBAP suppressed apoptosis and the MnTBAP effect was more marked at lower glucose levels (Fig. 7, B–E).

We used semi-quantitative reverse transcription-PCR to detect the mRNA levels of catalase and glutathione peroxidase 1, which are considered as indirect indices of cellular ROS (35). Both glucose and MnTBAP suppressed mRNA levels of these H₂O₂-inactivating enzymes (Fig. 7A) in islets.

DISCUSSION

The present work investigated the effects of glucose on oxygen radical formation by primary rat β cells. It was conducted on large populations of intact, primary β cells that were analyzed individually by flow cytometry with parallel measurements of cellular responsiveness to glucose. β Cells exhibited the expected metabolic responses to glucose (19, 32) as shown by the glucose-induced increase in NAD(P)H and decrease in FAD-FMN-fluorescence intensities (28). Production of ROS was measured through oxidation of H₂DCF-DA, a marker for peroxide formation (33). In contrast to previous reports (16, 17) a rise in glucose up to 20 mM did not increase ROS production by β cells but rather suppressed it. The glucose-induced suppression of the H₂DCF-DA signal was shown to occur in the absence of NO production indicating that it was not the result of the ability of the probe to also detect nitric oxide (NO) and peroxynitrite (33). Moreover, similar findings were obtained with HE, which, upon oxidation, is a highly specific marker for cellular superoxide accumulation (34).

Superoxide and ROS formation was not only suppressed by glucose but also by other mitochondrial nutrients such as leucine and succinic acid (methyl ester) (35). These suppressive actions are related to the characteristic metabolic effects of these nutrients in β cells. They were not seen in islet endocrine non- β cells in which glucose-induced changes in NAD(P)H or FADH₂ levels were neither detected. In freshly isolated β cells, the glucose-suppressive effect was predominantly achieved in the lower concentration range (between 0 and 5 mM) where glucose primarily increased NAD(P)H levels with virtually no effect on the FAD redox status (Fig. 1). This might reflect NADH formation during transition of glucose 6-phosphate to acetyl-CoA and subsequent effects on the electron transport chain through complex I (Fig. 5). Three potential effects could reduce superoxide and ROS accumulation (Fig. 8). *First*, the increase in NADH might shift (semi)-oxidized FMN to FMNH₂ and thus suppress superoxide formation at the FMN group in complex I as has been reported for neurons (36, 37). A (semi)-oxidized state of FMN appears to form stable semiquinone species that are thermodynamically capable of superoxide formation (38), and this could thus be avoided by NADH-generating nutrients (39) or artificially by rotenone. *Second*, the

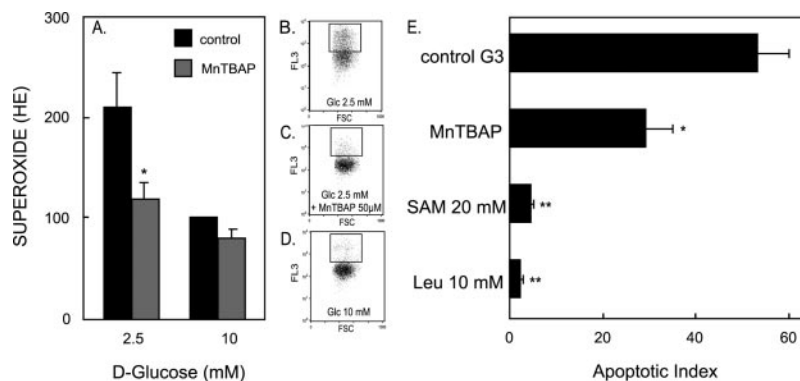


FIG. 6. **Superoxide scavenging reduces apoptotic cell death in low glucose-cultured β cells.** A–D, superoxide dismutase mimetic MnTBAP (50 μ M) decreases superoxide levels in low glucose-exposed β cells (panel A, mean \pm S.E.; *, $p < 0.05$ versus untreated 2.5 mM glucose control, $n = 5$). Panels B–D show representative FACS results illustrating the scavenging effect of MnTBAP. E, apoptosis was detected by fluorescence microscopy in single rat β cells, cultured for 72 h at 3 mM glucose and the indicated substances. Apoptotic rates are expressed as mean apoptotic index \pm S.E. of six duplicate experiments. Indices were calculated using 10 mM glucose-cultured cells as reference (*, $p < 0.05$; **, $p < 0.001$ versus untreated 3 mM glucose control).

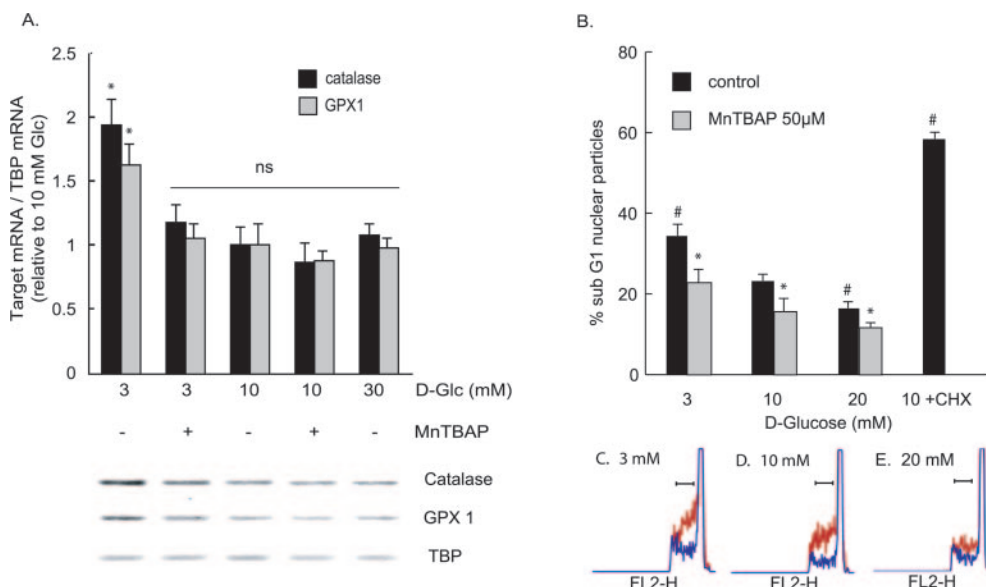


FIG. 7. **Glucose deprivation also induces ROS-mediated apoptosis in cultured pancreatic islets.** A, mRNA levels of catalase (black bars) and glutathione peroxidase (GPX1, gray bars) in islets exposed for 18 h to the indicated glucose concentration with or without MnTBAP (50 μ M) as detected by semi-quantitative reverse transcription-PCR. Bars represent average \pm S.E. ($n = 6$) signal ratio of target over housekeeping TATA-binding protein (TBP) mRNA. Representative bands are shown in the lower part of the panel (*, $p < 0.05$ versus 10 mM glucose control, the vertical line groups conditions with no significant difference, ns). B, apoptosis was measured by FACS analysis of cellular DNA in islets exposed for 4 days to the indicated conditions (CHX, cycloheximide (50 μ g/ml), positive control). Data represent average % sub-G₁ nuclei \pm S.E. ($n = 6$ duplicate experiments; #, $p < 0.05$ versus 10 mM glucose without MnTBAP; *, $p < 0.05$ versus same glucose concentration without MnTBAP). The lower panel shows representative frequency histograms of DNA content of islet cells exposed to 3 mM (C), 10 mM (D), or 20 mM (E) glucose in the presence (blue) or absence (red) of MnTBAP.

NADH-induced activation of complexes I, III, and IV will favor oxygen transition to water instead of radical formation. Third, the generated NAD(P)H equivalents can serve as cofactors for enzymes involved in ROS scavenging, such as glutathione-disulfide reductase (EC 1.8.1.7) and thioredoxin-disulfide reductase (EC 1.8.1.9). The rapid and dose-dependent increase in NAD(P)H levels of glucose-responsive β cells has previously been shown to protect these cells against the cytotoxic effects of *t*-butylhydroperoxide (40). The mitochondrial activity of β cells may thus provide a major protection against oxidative damage, making them less sensitive than is usually inferred from their relatively low expression of antioxidant enzymes (12).

The ROS-suppressive effect of glucose did not occur uniformly in all β cells and was more pronounced in β cell subsets with higher metabolic responsiveness to glucose (20). These β cell subsets have previously been shown to have higher expression and activity of the glucose sensor, glucokinase (GK), enabling them to maintain a markedly higher glucose catabolic

flux than the less responsive β cells (20, 41). These low responsive cells exhibited higher rates of superoxide accumulation probably as a result of their lower GK activity and concomitantly decreased mitochondrial metabolism. Mitochondrial nutrient deprivation also stimulates ROS formation in tumor cells (42). In these cells however, such a metabolic condition occurs only at glucose concentrations below the K_m (≤ 1 mM) of the high affinity hexokinase 1–3 isoenzymes that are expressed in these cells. When β cells were examined after culture of islets at high (20 mM) glucose, they were more sensitive to glucose, both in terms of their metabolic redox regulation as of the suppression of their superoxide formation.

It was recently proposed that glucose-induced accumulation of reduced equivalents, NAD(P)H and FADH₂, predisposed pancreatic β cells to increased ROS formation through a direct effect on the ETC (3, 43). Although such correlation has been directly examined in a number of cell types (4), it has not been demonstrated in β cells. The present work is the first study in

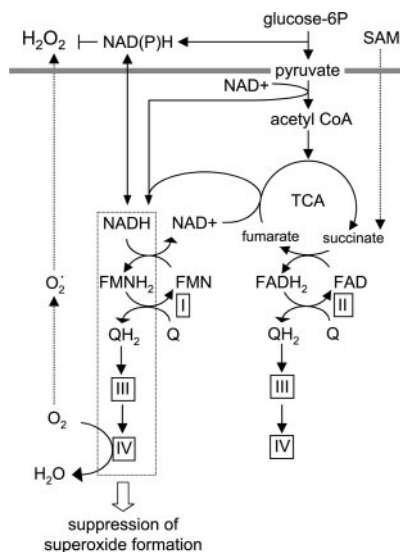


FIG. 8. Schematic representation of the ROS-suppressive action of glucose in pancreatic β cells. Glucose metabolism through glycolysis, oxidative decarboxylation and mitochondrial tricarboxylic acid cycle (TCA) generates NADH and NADPH, increasing the ROS-scavenging potential of the β cell. Mitochondrial NADH and FADH₂ re-oxidation increases electron transport through ETC complexes I–IV (indicated by Roman numbers) and favor oxygen transition to water (complex IV) instead of superoxide. NADH shifts complex I FMN from a (semi-)oxidized to the reduced state FMNH₂, which might lower the known potential of this group (36, 37) for univalent oxygen reduction to superoxide.

which both glucose metabolism and ROS formation are directly measured in primary β cells, both at the level of individual cells and of entire cell (sub)populations. Previous statements on a hyperglycemia-induced oxidative stress in β cells were based on use of indirect ROS markers, such as induction of ROS-scavenging enzymes or of cytoprotection by antioxidants (13, 27, 44). Two recent studies have monitored superoxide accumulation in real time, using fluorescence microscopy of HE-exposed intact rat islets (16) and dispersed islet cells (17) incubated at high glucose, but did not perform simultaneous measurements of glucose metabolism. The importance of the latter is illustrated in a recent report (45) that assessed the effect of glucose on HE and H₂DCF-DA oxidation in a RIN β cell line: high glucose (20 mM) did not induce ROS in normal RIN cells but did so in cells overexpressing GK. Interestingly, the GK overexpressing cells exhibited decreased cellular NADH and ATP levels, which indicates a mitochondrial dysfunction. According to our observations, such impairment can be responsible for an increased ROS production and should thus be considered before attributing it to ROS damage as in the report of Wu *et al.* (45). Non-physiological GK expression could for example disturb mitochondrial glucose metabolism by phosphate trapping (46), thereby decreasing NADH and ATP generation by glucose metabolism and, consequently, stimulate ROS formation. Our findings do not argue against a potential role of oxygen radicals in β cell death or dysfunction as a result of chronic hyperglycemia. They only indicate that such role is unlikely if the β cells have maintained their normal glucose metabolic pathways. However, if they are impaired, or predisposed to become impaired at high glucose, they may, according to our data, lose the glucose suppressive action on ROS accumulation and thus become vulnerable to ROS-mediated dysfunction or injury.

ROS production is not necessarily cytotoxic. Caloric restriction, for example, decreases neuronal susceptibility to death via induction of a mild stress response and up-regulation of protective enzymes (47). Excessive superoxide levels on the

other hand are a well known cause of cellular dysfunction and apoptotic cell death (48). Sustained ROS production during prolonged exposure to low glucose (≤ 6 mM) is also implicated in apoptosis of rat β cells (25) as shown by the protective effect of the superoxide dismutase mimetic agent MnTBAP. Addition of tricarboxylic acid substrates also protected the β cells against ROS-induced apoptosis, suggesting that mitochondrial metabolism, when adequately functioning, is of primary importance for maintaining β cell survival. This may explain the relatively lower abundance of ROS-scavenging enzymes in this cell type.

In conclusion, high rates of glucose metabolism do not increase ROS levels in primary β cells. Instead, they prevent accumulation of superoxide-derived ROS, an effect that is more pronounced in β cells with a higher metabolic responsiveness to glucose. Mitochondrial nutrients reduce superoxide formation and ROS accumulation through stimulating the electron transport chain and increasing ROS scavenging through generation of NADH. These observations prompt for re-evaluating the statement that high glucose levels invariably cause ROS-induced β cell dysfunction and death.

REFERENCES

1. The Diabetes Control and Complications Trial Research Group (1993) *N. Engl. J. Med.* **329**, 977–986
2. Evans, J. L., Goldfine, I. D., Maddux, B. A., and Grodsky, GM (2002) *Endocr. Rev.* **23**, 599–622
3. Brownlee, M. (2001) *Nature* **414**, 813–820
4. Nishikawa, T., Edelstein, D., Du, X. L., Yamagishi, S., Matsumura, T., Kaneda, Y., Yorek, M. A., Beebe, D., Oates, P. J., Hammes, H. P., Giardino, I., and Brownlee, M. (2000) *Nature* **404**, 787–790
5. Vincent, A. M., Olzmann, J. A., Brownlee, M., Sivitz, W. I., and Russell, J. W. (2004) *Diabetes* **53**, 726–734
6. Du, Y., Miller, C. M., and Kern, T. S. (2003) *Free Radic. Biol. Med.* **35**, 1491–1499
7. Korshunov, S. S., Skulachev, V. P., and Starkov, A. A. (1997) *FEBS Lett.* **416**, 15–18
8. Robertson, R. P., Harmon, J., Tran, P. O., and Poutout, V. (2004) *Diabetes* **53**, Suppl. 1, S119–S124
9. Kilpatrick, E. D., and Robertson, R. P. (1998) *Diabetes* **47**, 606–611
10. Schuit, F., De Vos, A., Farfari, S., Moens, K., Pipeleers, D., Brun, T., and Prentki, M. (1997) *J. Biol. Chem.* **272**, 18572–18579
11. Grankvist, K., Marklund, S. L., and Taljedal, I. B. (1981) *Biochem. J.* **199**, 393–398
12. Tiedge, M., Lortz, S., Drinkgern, J., and Lenzen, S. (1997) *Diabetes* **46**, 1733–1742
13. Laybutt, D. R., Kaneto, H., Hasenkamp, W., Grey, S., Jonas, J. C., Sgroi, D. C., Groff, A., Ferran, C., Bonner-Weir, S., Sharma, A., and Weir, G. C. (2002) *Diabetes* **51**, 413–423
14. Tanaka, Y., Gleason, C. E., Tran, P. O., Harmon, J. S., and Robertson, R. P. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 10857–10862
15. Kaneto, H., Kajimoto, Y., Miyagawa, J., Matsuoka, T., Fujitani, Y., Umayahara, Y., Hanafusa, T., Matsuzawa, Y., Yamasaki, Y., and Hori, M. (1999) *Diabetes* **48**, 2398–2406
16. Bindokas, V. P., Kuznetsov, A., Sreenan, S., Polonsky, K. S., Roe, M. W., and Philipson, L. H. (2003) *J. Biol. Chem.* **278**, 9796–9801
17. Krauss, S., Zhang, C. Y., Scorrano, L., Dalgaard, L. T., St-Pierre, J., Grey, S. T., and Lowell, B. B. (2003) *J. Clin. Invest.* **112**, 1831–1842
18. Van de Winkel, M., Maes, E., and Pipeleers, D. (1982) *Biochem. Biophys. Res. Commun.* **107**, 525–532
19. Van De Winkel, M., and Pipeleers, D. (1983) *Biochem. Biophys. Res. Commun.* **114**, 835–842
20. Heimberg, H., De Vos, A., Vandercammen, A., Van Schaftingen, E., Pipeleers, D., and Schuit, F. (1993) *EMBO J.* **12**, 2873–2879
21. Kiekens, R., In 't Veld, P., Mahler, T., Schuit, F., Van De Winkel, M., and Pipeleers, D. (1992) *J. Clin. Invest.* **89**, 117–125
22. Castedo, M., Ferri, K., Roumier, T., Metivier, D., Zamzami, N., and Kroemer, G. (2002) *J. Immunol. Methods* **265**, 39–47
23. Zhao, H., Kalivendi, S., Zhang, H., Joseph, J., Nithipatikom, K., Vasquez-Vivar, J., and Kalyanaraman, B. (2003) *Free Radic. Biol. Med.* **34**, 1359–1368
24. Pipeleers, D. G., In't Veld, P. A., Van de Winkel, M., Maes, E., Schuit, F. C., and Gepts, W. (1985) *Endocrinology* **117**, 806–816
25. Hoorens, A., Van de Casteele, M., Kloppel, G., and Pipeleers, D. (1996) *J. Clin. Invest.* **98**, 1568–1574
26. Van de Casteele, M., Kefas, B. A., Ling, Z., Heimberg, H., and Pipeleers, D. G. (2002) *Endocrinology* **143**, 320–326
27. Jonas, J. C., Guiot, Y., Rahier, J., and Henquin, J. C. (2003) *Diabetologia* **46**, 1234–1244
28. Chance, B., Ernster, L., Garland, P. B., Lee, C. P., Light, P. A., Ohnishi, T., Ragan, C. I., and Wong, D. (1967) *Proc. Natl. Acad. Sci. U. S. A.* **57**, 1498–1505
29. Ling, Z., Kiekens, R., Mahler, T., Schuit, F. C., Pipeleers-Marichal, M., Sener, A., Kloppel, G., Malaisse, W. J., and Pipeleers, D. G. (1996) *Diabetes* **45**, 1774–1782
30. Hatefi, Y. (1968) *Proc. Natl. Acad. Sci. U. S. A.* **60**, 733–740

31. Miyadera, H., Shiomi, K., Ui, H., Yamaguchi, Y., Masuma, R., Tomoda, H., Miyoshi, H., Osanai, A., Kita, K., and Omura, S. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100**, 473–477
32. Bennett, B. D., Jetton, T. L., Ying, G., Magnuson, M. A., and Piston, D. W. (1996) *J. Biol. Chem.* **271**, 3647–3651
33. LeBel, C. P., Ischiropoulos, H., and Bondy, S. C. (1992) *Chem. Res. Toxicol.* **5**, 227–231
34. Bindokas, V. P., Jordan, J., Lee, C. C., and Miller, R. J. (1996) *J. Neurosci.* **16**, 1324–1336
35. Fahien, L. A., and MacDonald, M. J. (2002) *Diabetes* **51**, 2669–2676
36. Liu, Y., Fiskum, G., and Schubert, D. (2002) *J. Neurochem.* **80**, 780–787
37. Kudin, A. P., Bimpong-Buta, N. Y., Vielhaber, S., Elger, C. E., and Kunz, W. S. (2004) *J. Biol. Chem.* **279**, 4127–4135
38. Massey, V. (1994) *J. Biol. Chem.* **269**, 22459–22462
39. Imlay, J. A. (1995) *J. Biol. Chem.* **270**, 19767–19777
40. Pipeleers, D., and Van de Winkel, M. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 5267–5271
41. Schuit, F., Moens, K., Heimberg, H., and Pipeleers, D. (1999) *J. Biol. Chem.* **274**, 32803–32809
42. Lee, Y. J., Galoforo, S. S., Berns, C. M., Chen, J. C., Davis, B. H., Sim, J. E., Corry, P. M., and Spitz, D. R. (1998) *J. Biol. Chem.* **273**, 5294–5299
43. Fridlyand, L. E., and Philipson, L. H. (2004) *Diabetes* **53**, 1942–1948
44. Kubisch, H. M., Wang, J., Bray, T. M., and Phillips, J. P. (1997) *Diabetes* **46**, 1563–1566
45. Wu, L., Nicholson, W., Knobel, S. M., Steffner, R. J., May, J. M., Piston, D. W., and Powers, A. C. (2004) *J. Biol. Chem.* **279**, 12126–12134
46. Wang, H., and Iynedjian, P. B. (1997) *J. Biol. Chem.* **272**, 25731–25736
47. Mattson, M. P., Chan, S. L., and Duan, W. (2002) *Physiol. Rev.* **82**, 637–672
48. Brand, M. D., Affourtit, C., Esteves, T. C., Green, K., Lambert, A. J., Miwa, S., Pakay, J. L., and Parker, N. (2004) *Free Radic. Biol. Med.* **37**, 755–767