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Oxidative Stress Is a Mediator of Glucose Toxicity in Insulin-secreting Pancreatic Islet Cell Lines*

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Pancreatic β cells secrete insulin in response to changes in the extracellular glucose. However, prolonged exposure to elevated glucose exerts toxic effects on β cells and results in β cell dysfunction and ultimately β cell death (glucose toxicity). To investigate the mechanism of how increased extracellular glucose is toxic to β cells, we used two model systems where glucose metabolism was increased in β cell lines by enhancing glucokinase (GK) activity and exposing cells to physiologically relevant increases in extracellular glucose (3.3–20 mM). Exposure of cells with enhanced GK activity to 20 mM glucose accelerated glycolysis, but reduced cellular NAD(P)H and ATP, caused accumulation of intracellular reactive oxygen species (ROS) and oxidative damage to mitochondria and DNA, and promoted apoptotic cell death. These changes required both enhanced GK activity and exposure to elevated extracellular glucose. A ROS scavenger partially prevented the toxic effects of increased glucose metabolism. These results indicate that increased glucose metabolism in β cells generates oxidative stress and impairs cell function and survival; this may be a mechanism of glucose toxicity in β cells. The level of β cell GK may also be critical in this process.

Pancreatic β cells play a crucial role in maintaining glucose homeostasis through secretion of insulin in response to changes in the extracellular glucose. Glucose-stimulated insulin secretion (GSIS)[1] is regulated by the rate of glucose metabolism within β cells (1, 2), and a key event in this process is the phosphorylation of glucose by glucokinase (GK). GK, also referred to as hexokinase IV, is a member of the mammalian hexokinase (HK) family that catalyzes the initial step in glucose metabolism by most metabolic pathways (*e.g.* glycolysis, pentose phosphate cycle and glycogen synthesis) (3). In pancreatic β cells, GK assumes the rate-limiting role for glucose metabolism (4). GK has a K_m for glucose of ~5 mM, a value that is within the physiological range of blood glucose levels and almost two orders of magnitude higher than any other HK (3–5). Furthermore, the activity of GK is insensitive to feedback inhibition by physiological concentrations of its product, glucose 6-phosphate (Glc-6-P) (3). These characteristics of GK enable β cells to increase glucose metabolism in proportion to elevations in extracellular glucose. Subsequent to glucose phosphorylation, glucose metabolism involves both cytosolic and mitochondrial processes and generates signals leading to insulin secretion (2).

In contrast to the ability of glucose to acutely stimulate insulin secretion, chronic exposure of β cells to increased glucose concentrations results in β cell dysfunction and ultimately β cell death, a phenomenon termed β cell glucose toxicity (glucotoxicity) (6–8). During the progression of type 2 diabetes, glucose toxicity is likely an important factor that contributes to progressive β cell failure and development of overt diabetes. The molecular mechanisms of how chronic exposure to elevated glucose impairs β cell function and survival are incompletely understood. Emerging evidence suggests that oxidative stress contributes to β cell glucose toxicity (8). Cellular oxidative stress results from a persistent imbalance between antioxidant defenses and production of highly reactive molecular species, including reactive oxygen species (ROS) such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2) (9). Since both O_2^- and H_2O_2 are normal byproducts of cellular oxidative metabolism (10), increased glucose metabolism could lead to excessive production of ROS. Pancreatic β cells express low levels of antioxidant enzymes and do not up-regulate these enzymes upon exposure to high concentrations of glucose (11, 12). Thus, increased ROS production in the face of low antioxidant defenses could result in ROS accumulation and oxidative stress in β cells. Elevated ROS affect the function and survival of β cells through direct oxidization of cellular macromolecules such as DNA and lipids (13, 14), and activation of cellular stress-sensitive signaling pathways (15).

To test the hypothesis that accelerated glucose metabolism

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[1] The abbreviations used are: GSIS, glucose-stimulated insulin secretion; GK, glucokinase; HK, hexokinase; ROS, reactive oxygen species; O_2^-, superoxide; MOI, multiplicity of infection; TPEM, two-photon excitation microscopy; HE, hydroethidine; DHR, dihydrorhodamine 123; CM-H_2DCFDA, 5-(and-6)-chloromethyl-2′, 7′-dichlorodihydrofluorescein diacetate; MDA, malonaldehyde; 4HNE, 4-hydroxyalkenals; NIC, nicotinamide; 3AB, 3-aminobenzamide; PARP, poly(ADP-ribose) polymerase; FITC, fluorescein isothiocyanate; PI, propidium iodide; ER, endoplasmic reticulum; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling.

leads to oxidative stress and oxidative damage in β cells, we utilized β cell models in which glucose metabolism was increased by enhancing GK activity and exposing cells to increased concentrations of extracellular glucose. The rationale for this experimental approach is that both protein content and activity of GK in β cells are regulated by extracellular glucose and that elevated glucose concentrations enhance GK activity (16–18). Consistent with these observations, GK activity and glucose metabolism in β cells are increased in several animal models of type 2 diabetes (19–23). Furthermore, the susceptibility to high glucose-induced β cell failure in DBA/2 mice is accompanied by enhanced GK activity and increased glucose metabolism in β cells (24). Our results show that enhanced GK activity accelerates glycolysis when the extracellular glucose is increased and that this is accompanied by accumulation of intracellular ROS, oxidative damage to mitochondria and DNA, and apoptotic cell death. These toxic effects are partially prevented by reducing ROS using a ROS scavenger.

EXPERIMENTAL PROCEDURES

Cell Culture and GK Expression—RIN1046–38 cells were maintained in Dulbecco's modified Eagle's medium containing 25 mM glucose, 10% fetal bovine serum, penicillin, and streptomycin. The recombinant adenovirus carrying the human islet GK cDNA (AdGK) was generated by homologous recombination as previously described (25). The recombinant adenovirus carrying the bacterial β-galactosidase gene (AdLacZ) has been described (25). For adenoviral transduction, multiplicity of infection (MOI) was used to determine the amount of viral stock and was selected by examining GK protein expression and cell survival as a function of increasing MOI. An MOI of 20 for AdGK and AdLacZ was used throughout the study. Cells were transduced with AdGK or AdLacZ for 48 h in medium containing 3.3 mM glucose. Thereafter, cells were exposed to different concentrations of glucose for varying durations as indicated under "Results" and used for subsequent assays.

The parental INS-1 cells expressing the reverse tetracycline-dependent transactivator (INS-1-r9) were kindly provided by Dr. Patrick B. Iynedjian (University of Geneva School of Medicine, Geneva, Switzerland). The plasmid expressing islet GK (PUHD10–3-GKI) was constructed by replacing rat liver GK cDNA with human islet GK cDNA in plasmid PUHD10–3-GK (26) (kindly provided by Dr. Iynedjian). Stable transfection was conducted following the protocol described by Wang and Iynedjian (26). Briefly, INS-1-r9 cells were co-transfected with PUHD10–3-GKI and a hygromycin resistance plasmid using LipofectAMINE reagent (Invitrogen Life Technologies). Individual hygromycin-resistant clones were screened by Southern blotting and maintained as described (26). Two lines, termed INS-1-GK and INS-1-control, were used for this study. GK expression was induced by culturing cells in medium containing doxycycline and 3.3 mM glucose for 48 h. Thereafter, cells were either continuously cultured in 3.3 mM glucose or exposed to 20 mM glucose for different durations and used for subsequent assays.

GK Assay—Protein levels of GK were examined by Western blot analysis and immunocytochemistry followed by flow cytometry (27). Sheep anti-rat/human GK (a gift from Dr. Mark Magnuson at Vanderbilt University Medical Center) was used as primary antibody in these assays. Flow cytometry was carried out on a FACSCalibur flow cytometer using Cellquest v3.1 software (BD Biosiences). Cell debris was excluded, and the fluorescence intensity from GK staining was plotted on a logarithmic scale against cell number. The activity of GK was assayed in cell extracts fluorometrically by a Glc-6-P dehydrogenase-coupled assay (28). Protein content was determined by Bradford assay (Bio-Rad) and used to normalize the results.

Glucose Metabolism and Metabolic Derivatives—The rate of glycolysis was assayed by measuring the production of [^{3}H]water from D-[5-^{3}H]glucose (PerkinElmer Life Science Products). Cells were incubated with D-[5-^{3}H]glucose in the presence of different concentrations of glucose as described (26). [^{3}H]water in the supernatant was separated using borate affinity chromatography on AG 1-X8 anion exchange resin (Bio-Rad). Radioactivity in the water eluate was measured by liquid scintillation counting. Cellular NAD(P)H was assayed by measuring NAD(P)H autofluorescence in live cells using two-photon excitation microscopy (TPEM) (29). Images of NAD(P)H autofluorescence from cell monolayers were acquired in the presence of different concentrations of glucose. Fluorescence intensity of NAD(P)H was analyzed using NIH Image 1.61 (Bethesda, MD). ATP was measured using the Bioluminescent Somatic Cell Assay Kit (Sigma). Cellular glutathione (GSH) was assayed as previously described (30, 31).

Insulin Secretion—INS-1 cells were either continuously cultured in 3.3 mM glucose or exposed to 20 mM glucose for 4 h after GK induction with doxycycline. GSIS was then assayed during a 60-min static incubation as described by Wang and Iynedjian (26). Insulin was measured by radioimmunoassay and normalized for cellular protein (32).

Intracellular ROS—Intracellular O_2^- and H_2O_2 were measured in RIN 1046–38 cells using hydroethidine (HE) and dihydrorhodamine 123 (DHR) (Molecular Probes) as described (33, 34). HE (5 μM) was loaded for 1 h and DHR (5 μM) was loaded for 30 min before harvesting. The cells were then washed and analyzed by flow cytometry. Intracellular peroxides were detected in INS-1 cells using probe 5-(and-6)chloromethyl-2′,7′-dichlorodihydrofluoresceindiacetate(CM-H_2DCFDA) as described (35). CM-H_2DCFDA (5 μM) was loaded for 1 h before harvesting. The cells were then washed and analyzed by flow cytometry.

Oxidative Damage—Lipid peroxidation was examined by measuring malonaldehyde (MDA) and 4-hydroxyalkenals (4HNE) using a colorimetric assay kit (LPO 586, OXIS International, Inc.). The integrity of the mitochondrial membrane was examined using rhodamine 123 (Molecular Probes) as described (33). 5 μM Rhodamine 123 was loaded for 30 min. The cells were harvested and analyzed by flow cytometry. TUNEL assay was used to detect DNA damage. DNA strand breaks were labeled using APO-DIRECT kit (BD Pharmingen) and analyzed by flow cytometry. Poly(ADP-ribose) polymerase (PARP) activity was measured using [2,8-^{3}H]NAD^+ (PerkinElmer Life Science Products) as described (36). DNA content was assayed in parallel as described (37) and used to normalize the results.

Cell Death—Cell death was detected using FITC-labeled annexin V (annexin V-FITC) and propidium iodide (PI) (ApoAlert Annexin V-FITC Apoptosis kit, Clontech). Cells were stained with annexin V-FITC/PI and analyzed by flow cytometry. Cell debris was gated out and the results were displayed as 2-parameter dot plots with the X-axis representing annexin V-FITC and the Y-axis representing PI. This assay separates cells into three populations: viable cells, early apoptotic cells and PI positive cells. Early apoptotic cells and PI-positive cells were pooled together and counted as dead cells. Cell death was examined in the absence or presence of either a ROS scavenger or PARP inhibitors. The ROS scavenger EUK-134 was obtained from Eukarion Inc. (Bedford, MA) (38), dissolved in culture medium. PARP inhibitors Nicotinamide (NIC) and 3-Aminobenzamide (3AB) were purchased from Sigma, and solubilized in culture medium or dimethyl sulfoxide (Me_2SO) respectively.

Statistical Analysis—The data are presented as the mean ± S.E. Statistical analysis was performed using analysis of variance or Student's *t* test where appropriate. Differences were considered significant when $p < 0.05$.

RESULTS

Increased GK Protein and Activity in RIN 1046-38 Cells—Introduction of the islet GK cDNA using AdGK increased both GK protein and activity in RIN 1046-38 cells. Fig. 1*a* shows the results of Western blot analysis of GK protein in AdGK- and AdLacZ-treated cells. To evaluate the percentage of cells with increased GK protein, we performed immunocytochemistry followed by flow cytometry. Fig. 1*b* demonstrates that, compared with AdLacZ-treated cells, the majority of AdGK-treated cells have increased GK protein. To assess the activity of GK in adenovirally transduced cells, we measured the rate of glucose phosphorylation in cell extracts at various glucose concentrations. Increased GK expression enhanced GK activity whereas the activity of HK remained unchanged. The rate of glucose phosphorylation in the presence of 20 mM glucose was ~10-fold higher in AdGK-treated cells as compared with AdLacZ-treated cells (Fig. 1*c*).

Metabolic Response to Glucose in RIN 1046-38 Cells with Increased GK Activity—When RIN 1046-38 cells were transduced with AdGK, as opposed to AdLacZ, they began to undergo cell death in culture medium containing 25 mM glucose (see below). Transduction of cells with the adenoviruses was therefore performed in medium containing 3.3 mM glucose. To investigate the mechanisms underlying the toxic effects of increased GK activity, we examined the following parameters in RIN 1046-38 cells that were exposed to different concentrations

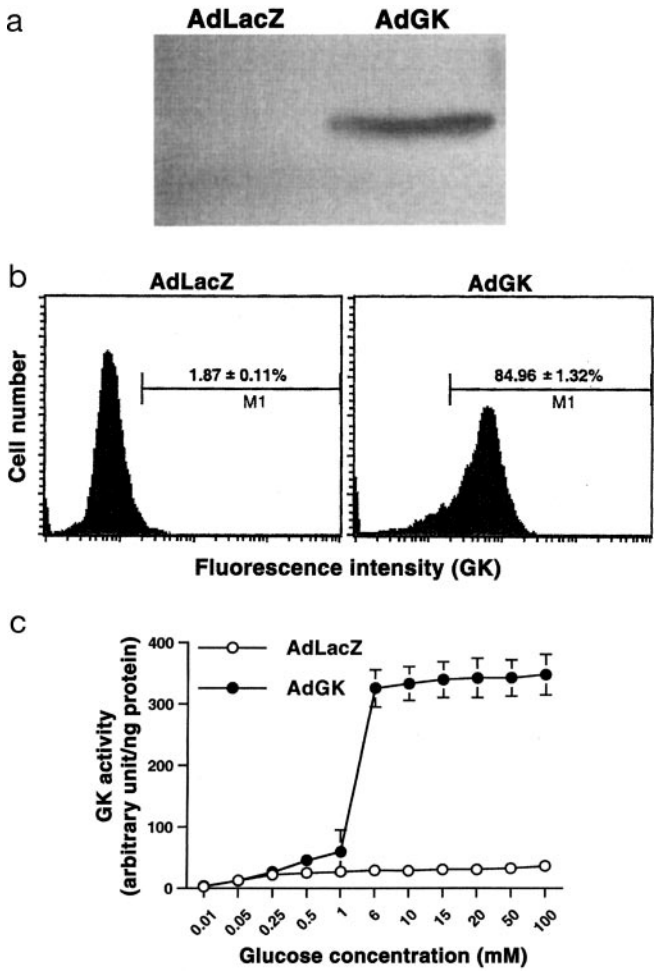

FIG. 1. **Increased GK protein and activity in RIN 1046–38 cells.** Cells were transduced with AdGK or AdLacZ for 48 h in medium containing 3.3 mM glucose. *a*, GK protein was analyzed by Western blot analysis. Ten micrograms of cellular protein were separated on a 10% PAGE gel, and GK was detected using a primary antibody that reacts with both rat and human GK. The experiment was repeated three times, and a representative figure is shown. *b*, immunocytochemistry followed by flow cytometry was used to evaluate the percentage of the cells with increased GK protein. The numbers gated in the M1 areas are mean ± S.E. of three experiments. *c*, GK activity in cell extracts was assayed by measuring the rate of glucose phosphorylation at various glucose concentrations. The experiments were repeated three times, and the mean ± S.E. are shown.

of glucose after adenoviral transduction. We first assayed the rate of glucose metabolism by measuring glycolysis, because β cells respond to elevations in extracellular glucose by facilitating glycolysis and oxidative metabolism. Cells with increased GK activity had an accelerated rate of glycolysis when the extracellular glucose was raised above 5 mM (Fig. 2*a*). The rate of glycolysis in AdGK-treated cells in the presence of 20 mM glucose was ~2-fold higher than AdLacZ-treated cells. In pancreatic β cells, increased glucose metabolism in response to high glucose leads to increases in several metabolic derivatives, such as NAD(P)H and ATP, that are crucial for GSIS. We therefore measured cellular NAD(P)H and ATP. AdGK-treated cells cultured in 3.3 mM glucose had increases in NAD(P)H in response to 10 mM and 20 mM glucose that were comparable to similarly treated AdLacZ-treated cells (data not shown). In sharp contrast and different from the results of glycolysis, exposure of cells with increased GK activity to 20 mM glucose for 2 h significantly decreased the NAD(P)H response to 10 mM and 20 mM glucose (Fig. 2*b*). Similarly, exposure of AdGK-treated cells to 10 and 20 mM glucose significantly reduced cellular ATP (Fig. 2*c*). Since NAD(P)H and ATP are generated during both glycolysis in the cytosol and oxidative metabolism in mitochondria and are utilized during cellular metabolism as an energy source or electron donor, these results suggest increased usage and/or decreased mitochondrial generation of NAD(P)H and ATP.

Accumulation of ROS in RIN 1046-38 Cells with Increased GK Activity after Exposure to High Concentrations of Glucose—One use of NAD(P)H is to provide electrons for the regeneration of different cellular antioxidants. Therefore, decreased cellular NAD(P)H in the face of increased glucose metabolism could result from cellular oxidative stress. To test this, we monitored the levels of intracellular $O_2^-$and H_2O_2 in RIN 1046-38 cells using the intracellular probes HE and DHR, respectively. HE is oxidized specifically by O_2^-, and this process generates a fluorescent product. DHR reacts with H_2O_2 and generates fluorescent rhodamine 123. Cells were either continuously cultured in 3.3 mM glucose, or exposed to 20 mM glucose for different durations. These treatments did not change the levels of intracellular O_2^- and H_2O_2 in AdLacZ-treated cells (Fig. 3, *a* and *b*, *shaded curves*). In contrast, both O_2^- and H_2O_2 accumulated time-dependently in AdGK-treated cells after exposure to 20 mM glucose (Fig. 3, *a* and *b*). The increase in intracellular H_2O_2 became detectable after exposure to 20 mM glucose for 15 min. A further increase was only detected in a subpopulation of AdGK-treated cells at the 4-hour time point (Fig. 3*b*), possibly as a result of diffusion of H_2O_2 out of the cells. Furthermore, a subpopulation of AdGK-treated cells had decreased intracellular H_2O_2 at the 4-hour time point. To ascertain whether this subpopulation represented dead cells, we double labeled the cells with DHR and PI and analyzed by flow cytometry. The results showed that the PI-positive cells (dead cells) had reduced fluorescence intensity of rhodamine 123, reflecting a decreased intracellular H_2O_2 (data not shown).

Oxidative Damage in RIN 1046-38 Cells with Increased GK Activity after Exposure to High Concentrations of Glucose—Elevated ROS can damage β cells by directly oxidizing cellular macromolecules such as lipids and DNA. To investigate if such damage was present in RIN 1046-38 cells with increased GK activity, we exposed cells to 20 mM glucose for 4 h and examined the following parameters. Lipid peroxidation was assayed by measuring MDA and 4HNE, two markers that are commonly used to reflect lipid peroxidation and lipid peroxidation-induced membrane damage (39, 40). Compared with AdLacZ-treated cells, AdGK-treated cells had significantly higher levels of MDA/4HNE after exposure to 20 mM glucose for 4 h, demonstrating the existence of oxidative damage to cellular lipids (Fig. 4*a*). Since ROS are produced primarily in the electron transport chain associated with the mitochondrial membrane, mitochondria may be a major target for oxidative injury. Using rhodamine 123 to assess the mitochondrial membrane, a significantly higher proportion of AdGK-treated cells lost their mitochondrial membrane integrity after exposure to 20 mM glucose for 4 h (reflected by a loss of the ability to retain rhodamine 123 in mitochondria, Fig. 4*b*). We next examined whether DNA damage was present by detecting DNA strand breaks using TUNEL assay. Fig. 4*c* shows that exposure of AdGK-treated cells to 20 mM glucose for 4 h increases the number of DNA strand breaks. Because excessive DNA strand breaks causes overactivation of the DNA-repairing enzyme PARP and depletion of ATP in β cells (41, 42), we then measured PARP activity. AdGK-treated cells had significantly higher levels of PARP activity compared with AdLacZ-treated cells after exposure to 20 mM glucose for 4 h (Fig. 4*d*). Together, these results demonstrate that oxidative stress causes damage to both mitochondria and DNA. When combined with the data

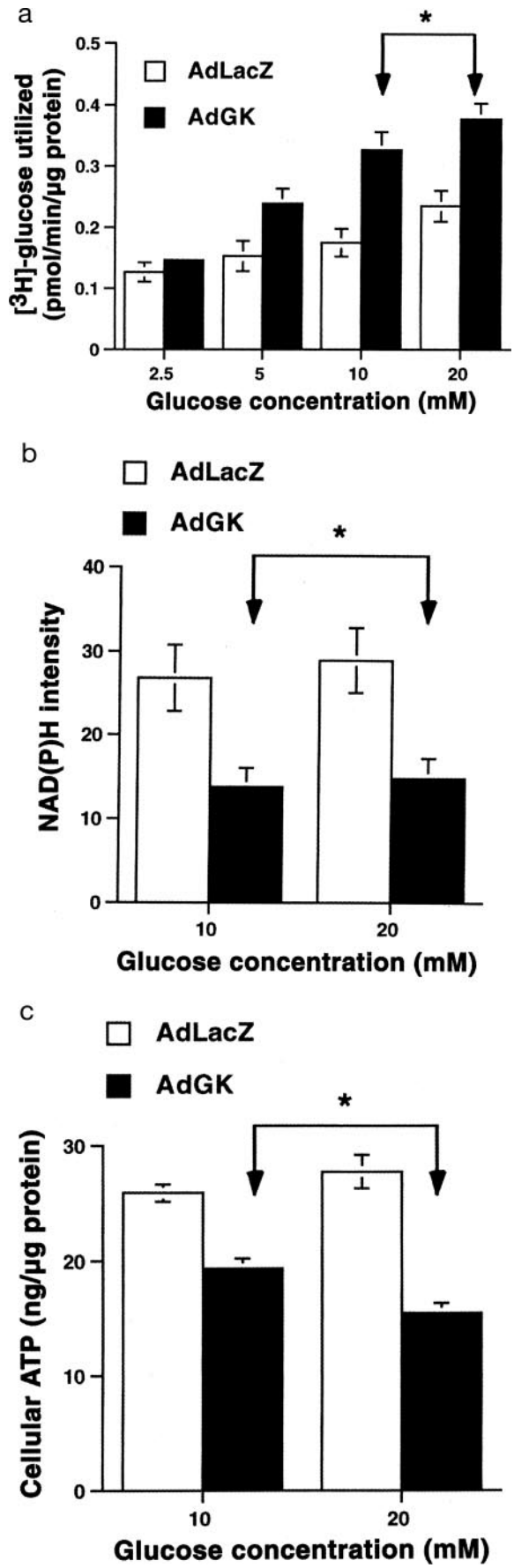

FIG. 2. **Metabolic response to glucose in RIN 1046-38 cells with increased GK activity.** Cells were transduced with AdGK or AdLacZ in culture medium containing 3.3 mM glucose for 48 h. Thereafter, they were either continuously cultured in 3.3 mM glucose or exposed to 20 mM glucose for 2 h before glycolysis and NAD(P)H measurements. *a*, rate of glycolysis was measured using D-[5-^{3}H]glucose with different concentrations of glucose. The mean ± S.E. of three independent experiments from cells exposed to 20 mM glucose are shown. *, significantly different from AdLacZ-treated cells and AdGK-treated cells assayed in 2.5 and 5 mM glucose ($p < 0.05$). *b*, cellular NAD(P)H was measured using TPEM at the indicated glucose concentrations. Results shown are the mean ± S.E. of five separate experiments from cells exposed to 20 mM glucose. *, significantly different from the results of AdLacZ-treated cells ($p < 0.05$). *c*, cells were exposed to 10 and 20 mM glucose for 4 h. The mean ± S.E. of cellular ATP from three independent experiments are shown. *, significantly different from the results of AdLacZ-treated cells ($p < 0.05$).

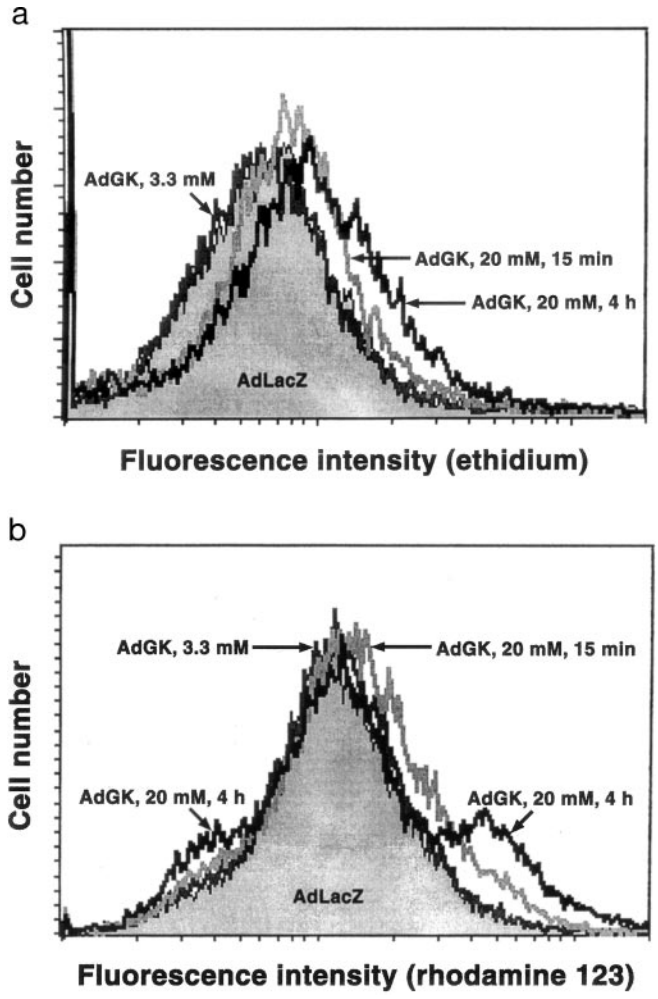

FIG. 3. **Accumulation of ROS in RIN 1046-38 cells with increased GK activity after exposure to high concentration of glucose.** After adenoviral transduction, cells were either continuously cultured in 3.3 mM glucose or exposed to 20 mM glucose for different durations as indicated in the graph. Intracellular O_2^- (*a*) and H_2O_2 (*b*) were labeled using HE and DHR and analyzed by flow cytometry. The results of AdLacZ-treated cells overlap with the curves for AdGK-treated cells cultured in 3.3 mM glucose (*shaded areas*). Representative figures of three independent experiments are shown. The subpopulation of cells with decreased intracellular H_2O_2 was labeled AdGK, 20 mM, 4 h on the *left side* of the graph in *b*.

on cellular NAD(P)H and ATP, these results suggest that mitochondrial damage contributes to reduced cellular NAD(P)H by decreasing the generation of these nucleotides. Likewise, mitochondrial damage may impair ATP generation, in addition to depletion of cellular ATP by PARP overactivation.

Oxidative Stress Causes Apoptosis in RIN 1046-38 Cells with Increased GK Activity—The initial observation during this project was that AdGK-treated RIN 1046-38 cells began to detach from culture plates and eventually died when they were cul-

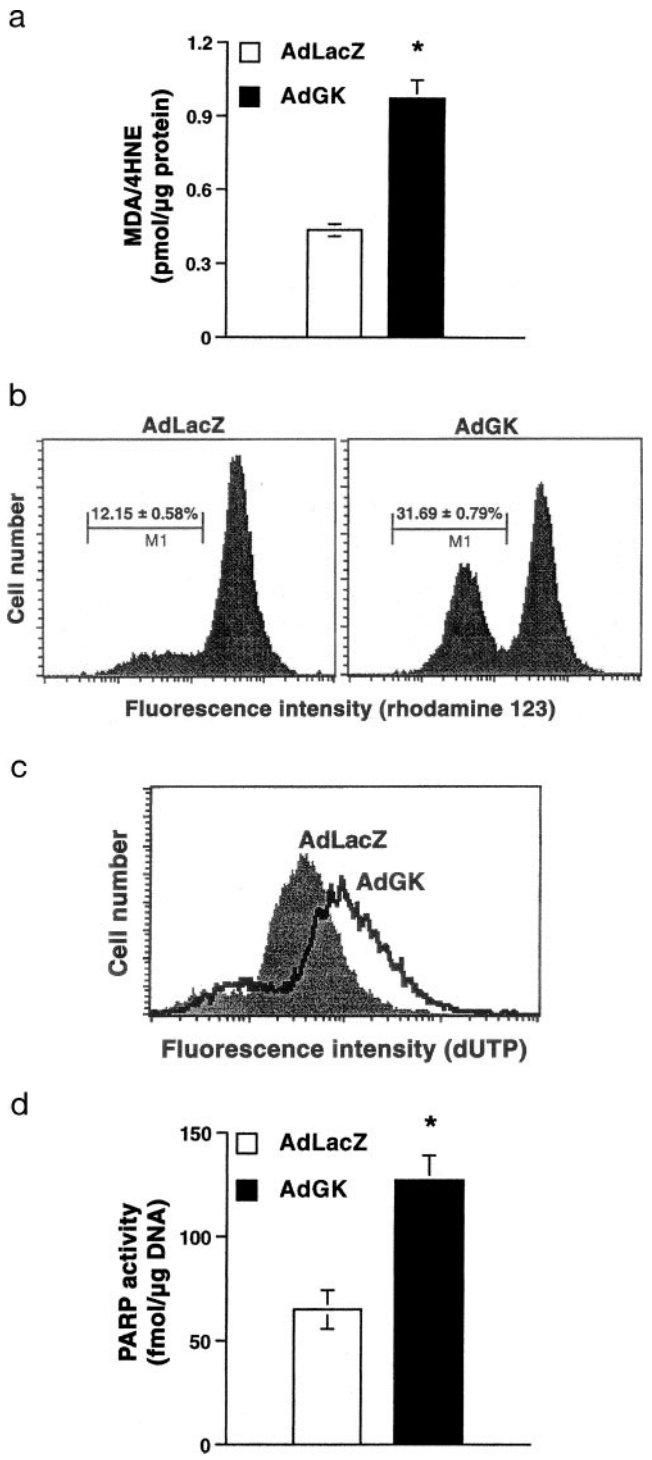

FIG. 4. **Oxidative damage in RIN 1046-38 cells with increased GK activity after exposure to high concentration of glucose.** Cells were exposed to 20 mM glucose for 4 h. *a*, lipid peroxidation was evaluated by measuring MDA and 4HNE. The mean ± S.E. of three experiments are shown. *, significantly different from the results of AdLacZ-treated cells ($p < 0.05$). *b*, a set of representative figures examining mitochondrial membrane integrity is shown. The numbers gated in the M1 regions are mean ± S.E. of three experiments. *c*, DNA strand breaks were detected by TUNEL assay using fluorescence-labeled dUTP. Representative data of three experiments are shown. *d*, mean ± S.E. of PARP activity from three experiments is shown. *, significantly different from the results of AdLacZ-treated cells ($p < 0.05$).

tured in medium containing 25 mM glucose. To characterize the nature of the cell death, we first examined the glucose concentration dependence. Adenovirally transduced RIN 1046-38 cells were exposed to different concentrations of glucose for 4 h, and then analyzed for cell death. High concentrations of glucose did not induce cell death in AdLacZ-treated cells, whereas cells with increased GK activity underwent cell death when they were exposed to 10 and 20 mM glucose (Fig. 5*a*). To determine if cell death requires the presence of metabolizable glucose and to exclude the hyperosmotic effect of high concentrations of glucose, AdGK-treated cells were exposed to 20 mM 3-*O*-methylglucose (3OMG) for up to 16 h. This treatment did not induce cell death (data not shown). Combined with the data on glycolysis, these results indicate that the metabolic consequences of increased glucose metabolism lead to cell death. To characterize the mode of cell death, we stained the cells with annexin V-FITC and PI, followed by flow cytometric analysis. AdGK-treated cells were separated into 3 populations. Viable cells are both annexin V-FITC and PI-negative (Fig. 5*b*; *lower left quadrant of each panel*). Early apoptotic cells are annexin V-FITC-positive but PI-negative (Fig. 5*b*; *lower right quadrant of each panel*). The cells located in the *upper right quadrant* of each panel in Fig. 5*b* are both annexin V-FITC and PI-positive and represent dead cells with disrupted cytoplasmic membrane (referred to as PI-positive cells in this study). After exposure to 20 mM glucose for 4 h, AdGK-treated cells had significantly higher numbers of both early apoptotic cells and PI-positive cells, compared with either AdGK-treated cells cultured only in 3.3 mM glucose or AdLacZ-treated cells (Fig. 5*b*). Together, these results demonstrate that increased glucose metabolism leads to apoptosis.

To investigate if oxidative damage activated apoptosis signaling pathways, we included a ROS scavenger or PARP inhibitors in the cell death assay. EUK-134 is a synthetic superoxide dismutase (SOD)/catalase mimetic that catalytically eliminates both O_2^- and H_2O_2 (38). NIC is a PARP inhibitor and serves as the precursor of NAD^+ (43). 3-Aminobenzamide (3AB) is a more selective PARP inhibitor than NIC (41). Adenovirus-transduced RIN 1046-38 cells were exposed to 20 mM glucose for 4 h, in the absence or presence of EUK-134 (400 μM), NIC (10 mM), or 3AB (10 mM). The number of dead cells was significantly reduced in the presence of these compounds in AdGK-treated cells exposed to 20 mM glucose (Fig. 5*c*). These data demonstrate that oxidative stress and oxidative damage lead to apoptosis.

Increased Glucose Metabolism Leads to Oxidative Stress, Cellular Dysfunction, and Death in INS-1 Cells—To address whether the level of GK expression is an important variable, we performed similar experiments using INS-1 cells with regulated GK expression. Using the doxycycline-inducible gene expression system, both GK protein and activity could be regulated in INS-1-GK but not INS-1-control cells (Fig 6, *a* and *b*). Similar to the results with RIN 1046-38 cells, increased GK protein in INS-1-GK cells enhanced GK activity while the activity of HK remained unchanged. For example, compared with cells cultured in doxycycline-free medium, GK activity measured in the presence of 20 mM glucose was enhanced 1.5- and 2.7-fold in INS-1-GK cells cultured in medium containing 50 ng/ml and 100 ng/ml of doxycycline, respectively (2.16 ± 0.5 and 3.77 ± 0.73 *versus* 1.41 ± 0.23, mean ± S.E. of three experiments). GK activity in INS-1-control cells cultured in different concentrations of doxycycline was similar to INS-1-GK cells cultured in doxycycline-free medium (data not shown). Enhanced GK activity in INS-1 cells leads to accelerated glucose utilization, as reflected by a higher rate of glycolysis in response to increasing extracellular glucose (Fig. 6*c*). Doxycycline at a concentration of 2000 ng/ml induced increases in GK protein and activity comparable to those achieved in AdGK-treated RIN 1046–38 cells (see Fig. 1, *a* and *c*). For

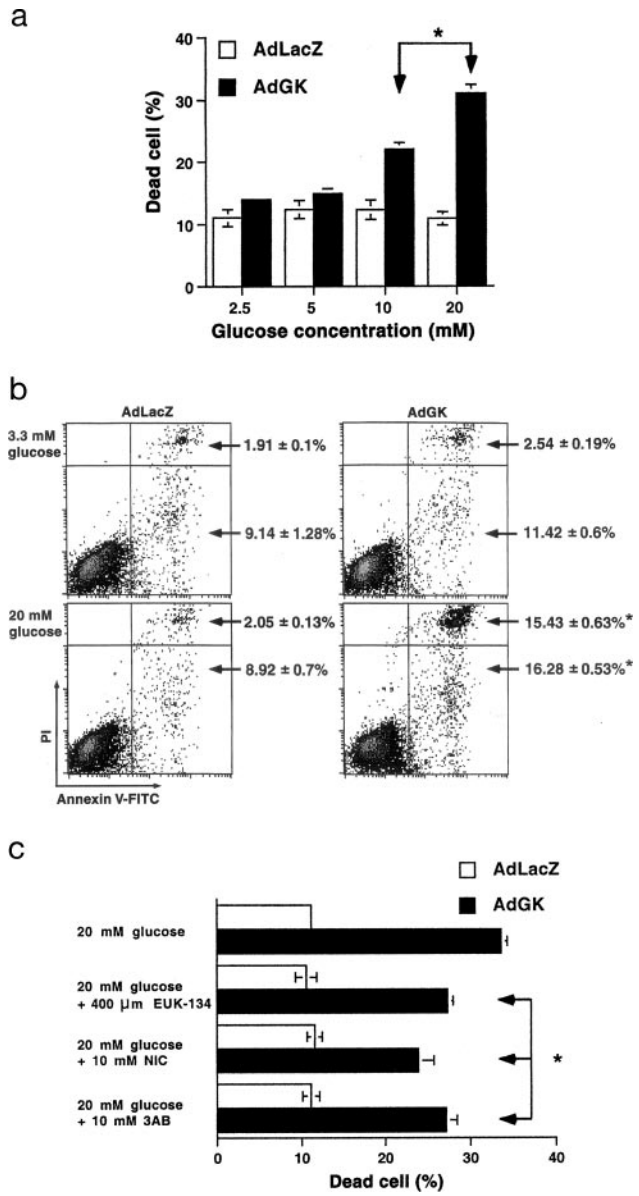

FIG. 5. **Oxidative stress causes apoptosis in RIN 1046-38 cells with increased GK activity.** *a*, cells were exposed to different concentrations of glucose as indicated in the graph for 4 h. Dead cells were detected with annexin V-FITC and PI followed by flow cytometry. The mean ± S.E. of three experiments are shown. *, significantly different from AdLacZ-treated cells and AdGK-treated cells exposed to 2.5 mM and 5 mM glucose ($p < 0.05$). *b*, cells were either continuously cultured in 3.3 mM glucose (*upper 2 panels*) or exposed to 20 mM glucose for 4 h (*lower 2 panels*), then stained with annexin V-FITC and PI, and analyzed by flow cytometry. The experiment was repeated three times, and representative figures are shown. The mean ± S.E. of three experiments are indicated by *arrows*. *, significantly different from AdLacZ-treated cells and AdGK-treated cells cultured in 3.3 mM glucose ($p < 0.05$). *c*, cells were exposed to 20 mM glucose for 4 h, in the absence or presence of EUK-134 (400 μM), NIC (10 mM), or 3AB (10 mM). Cell death was analyzed as described in *a*. Results shown are the mean ± S.E. of three separate experiments. The error bar for AdLacZ-treated cells exposed to 20 mM glucose alone is too small to visualize. *, significantly different from AdGK-treated cells exposed to 20 mM glucose alone ($p < 0.05$).

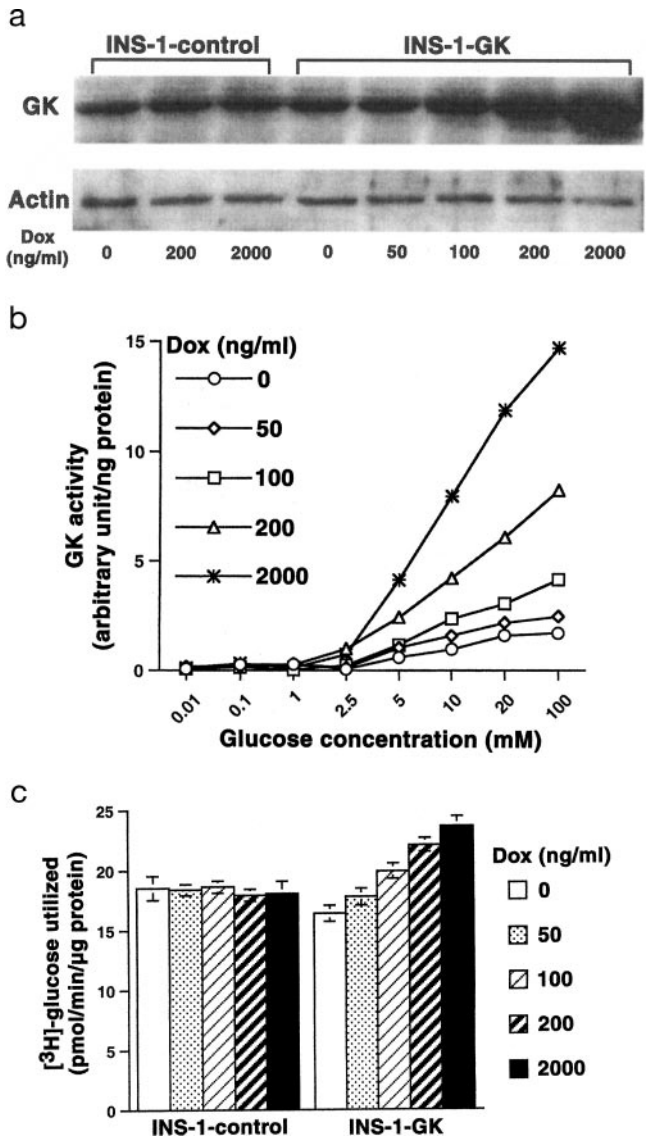

FIG. 6. **Regulated GK expression and glucose metabolism in INS-1 cells.** Cells were cultured in medium containing 3.3 mM glucose and different concentrations of doxycycline for 48 h. *a*, GK protein was detected as described in Fig. 1*a*. Actin was detected using a primary antibody that reacts with β-actin. A representative figure of three independent experiments is shown. *b*, GK activity was assayed as described in Fig. 1*c*. Shown here are the results in INS-1-GK cells from one experiment that was repeated three times. *c*, rate of glycolysis was measured as described in Fig. 2*a*. The mean ± S.E. of three independent experiments are shown.

subsequent experiments, we used doxycycline at concentrations between 0 and 200 ng/ml.

We next examined whether oxidative stress was present in INS-1 cells with increased glucose metabolism. First, we monitored intracellular ROS with the intracellular probe CM-H_2DCFDA, which collectively detected intracellular peroxides (35, 44). When INS-1 cells were exposed to 20 mM glucose, accumulation of intracellular peroxides was detected only in the cells with increased glucose metabolism (Fig. 7*a*). Similar to the results with RIN 1046-38 cells, intracellular peroxides increased in a time-dependent manner after exposure to 20 mM glucose. In addition, varying the level of GK expression altered the levels of intracellular ROS, indicating that generation of ROS was dependent on the rate of glucose metabolism (Fig. 7*a*). Second, we measured intracellular GSH. Similar to cellular NAD(P)H, GSH can be consumed as an electron donor for regeneration of cellular antioxidants and is a marker of oxidative stress. Consistent with our findings of NAD(P)H in RIN 1046-38 cells, increased glucose metabolism in INS-1 cells decreased cellular GSH levels (Fig. 7*b*).

To investigate whether facilitated glucose metabolism leads to impaired cellular function, we next assayed GSIS. In this set of studies, we induced GK expression with 50 ng/ml of doxycycline and exposed the cells to 20 mM glucose for 4 h. As shown

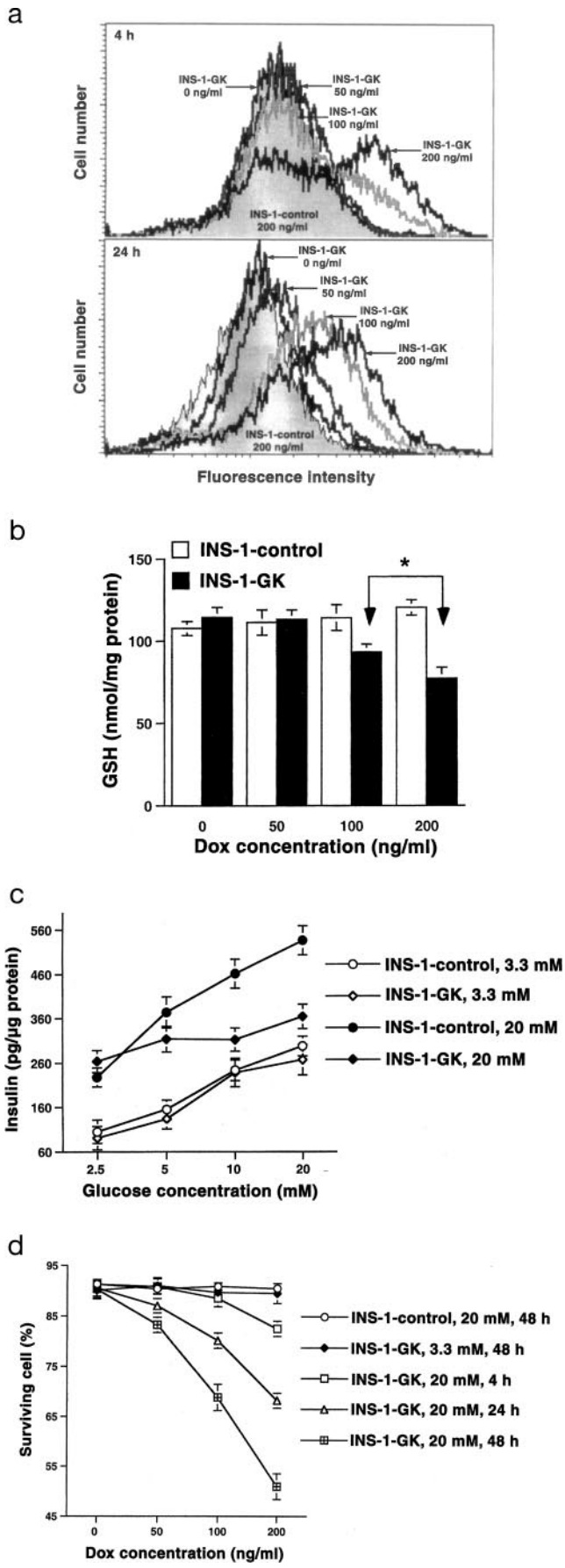

FIG. 7. **Increased glucose metabolism leads to oxidative stress, cellular dysfunction, and death in INS-1 cells.** *a*, cells were cultured in 3.3 mM glucose and different concentrations of doxycycline for 48 h. Thereafter, they were exposed to 20 mM glucose in the presence of doxycycline for 4 or 24 h and used for ROS measurements. The experiment was repeated three times and a set of representative figures is shown. *b*, cells were exposed to 20 mM glucose in the presence of doxycycline for 24 h after GK induction and used for GSH measurements. The mean ± S.E. of three independent experiments are shown. *, significantly different from INS-1-control cells and INS-1-GK cells cultured in 0 and 50 ng/ml of doxycycline ($p < 0.05$). *c*, cells were cultured in 3.3 mM glucose and 50 ng/ml of doxycycline for 48 h. Cells were then either left in 3.3 mM glucose or exposed to 20 mM glucose for 4 h and assayed for GSIS. Shown are the mean ± S.E. of three separate experiments. *d*, cells were cultured in 3.3 mM glucose and different concentrations of doxycycline for 48 h. Thereafter, cells were either left in 3.3 mM glucose or exposed to 20 mM glucose in the presence of doxycycline for the durations indicated in the graph. Cell death was detected as described in Fig. 5*a*. The mean ± S.E. of three independent experiments are shown.

above and further described below, this treatment caused detectable intracellular ROS accumulation in INS-1-GK cells without a significant decrease in cellular GSH or cell survival. However, insulin secretion in response to high glucose (10 and 20 mM) was significantly reduced (Fig. 7*c*). INS-1-GK cells cultured in 50 ng/ml doxycycline and 3.3 mM glucose had a GSIS comparable to similarly treated INS-1-control cells (Fig. 7*c*). This demonstrates that the impaired GSIS is not simply due to level of GK protein, but rather that the metabolic consequences of increased glucose metabolism lead to cellular dysfunction.

Finally, we examined the effect of increased glucose metabolism on cell survival in INS-1 cells. When INS-1 cells were continuously exposed to 20 mM glucose after GK induction, cells that had 1.5-fold increase in GK activity (induced by 50 ng/ml of doxycycline) began to die after 24 h (Fig. 7*d*). When GK activity was increased 6-fold (induced by 200 ng/ml of doxycycline), nearly half of the cells underwent apoptosis after exposure to 20 mM glucose for 48 h (Fig. 7*d*). Again, this death-inducing effect was not due to increased GK protein, as INS-1-GK cells cultured in different concentrations of doxycycline and 3.3 mM glucose maintained their viability (Fig. 7*d*). INS-1-control cells cultured in different concentrations of doxycycline remained viable after exposure to 20 mM glucose (Fig. 7*d*). This excludes a hyperosmotic effect of high glucose and any negative effects of doxycycline on cell survival.

DISCUSSION

The current report investigated the molecular mechanisms that mediate the toxic effects of high glucose on insulin-secreting cells. Our results show that increased glucose metabolism, induced by exposing cells with enhanced GK activity to high concentrations of glucose, leads to oxidative stress and oxidative damage. These changes, in turn, impair cellular function and activate apoptosis signaling. Since GK activity and glucose metabolism are increased in β cells of animal models of type 2 diabetes (19–23), these observations may partially explain how chronic hyperglycemia damages β cells *in vivo* (glucose toxicity). Previous studies by several investigators have observed that enhanced GK activity, combined with high concentrations of glucose, negatively affects the function and survival of β cells. Polonsky and coworkers (17) studied the effect of GK activity on GSIS using isolated islets from wild type ($GK^{+/+}$) and heterozygous GK mutant ($GK^{+/-}$) mice. High glucose (30 mM) culture up-regulated both GK protein and activity in $GK^{+/-}$ islets to levels that were comparable to $GK^{+/+}$ islets cultured in 11.6 mM glucose. This was accompanied by improved GSIS. In contrast, increased GK protein and activity in $GK^{+/+}$ islets cultured in high glucose was accompanied by blunted GSIS (17), suggesting an upper limit of GK activity for normal GSIS. Using INS-1 cells with inducible GK expression,

Wang and Iynedjian showed that a 4-fold increase in GK activity resulted in impaired GSIS in cells cultured in 2.5 mM glucose despite the fact that these cells had accelerated glycolysis. After exposure to a high concentration of glucose (24 mM), cells with a 6-fold increase in GK activity had decreased viability as observed by phase-contrast microscopy (26, 45). Our findings expand on these observations by demonstrating that the toxic effects of enhanced GK activity in the presence of high glucose are, at least partially, mediated by increased glucose metabolism, which leads to accumulation of ROS and oxidative stress.

The major biological process leading to generation of ROS is the electron transport chain associated with the mitochondrial membrane (10). Excessive glucose metabolism through oxidation in mitochondria may be a mechanism that leads to overproduction of ROS. Another possible source of increased ROS is the oxidative metabolism of glucose through the pentose phosphate cycle. Normally, this pathway does not contribute significantly to glucose utilization in β cells (1). Furthermore, the flow of glucose through the pentose cycle does not vary with increases in glucose concentrations within the physiological range (1). However, enhanced GK activity in the presence of high concentrations of glucose can result in elevated Glc-6-P, the substrate for both glycolysis and the pentose cycle, as suggested by Wang and Iynedjian (45). The assay we used to measure glucose utilization in this study, namely the formation of $[^3H]H_2O$ from D-[5-^{3}H]glucose, reflects the combined rates of glucose flux through the glycolytic and pentose cycle pathways. In addition, because several rate-limiting enzymes for the pentose cycle are inhibited by NADPH (46), the decreased cellular NAD(P)H levels could activate this pathway and increase the flux of glucose oxidation through the pentose cycle. Recent studies from Robertson and coworkers (35, 47) and other investigators (48, 49) in both rodents and humans have shown oxidative stress and oxidative damage in β cells of type 2 diabetes, and increased intracellular ROS in islets exposed to high glucose; antioxidant treatment or enhancement of the ROS scavenger enzyme glutathione peroxidase protects β cells against glucose toxicity. Studies from Brownlee (50) have demonstrated that overproduction of ROS by the mitochondrial electron transport chain is a mediator of hyperglycemia-induced vascular damage. In these cells, increased ROS is thought to initiate a cascade of biochemical abnormalities such as increased flux through the polyol and hexosamine pathways, activation of NF-κB, and activation of protein kinase C (50). Combined with these observations, our results suggest a common mechanism for glucose toxicity in β cells and vascular endothelial cells. Because β cells naturally posses low antioxidant capacities (11, 12), they are likely quite vulnerable to the increased generation of ROS.

Oxidative stress activates apoptosis signal transduction pathways in many cell types. In this study, we observed accumulation of intracellular ROS and oxidative damage to lipids and DNA in cells with increased glucose metabolism, and we have further demonstrated that oxidative damage is one of the mechanisms that appears to activate apoptosis. The observation that cell death is only partially prevented by either reducing ROS or inhibiting PARP suggests that the death-inducing effect of increased glucose metabolism may also involve other mechanism(s). An additional mechanism was recently suggested by Oyadomari *et al.* (51) who showed that endoplasmic reticulum (ER) stress caused by ER overload results in β cell death and diabetes. Since increased glucose metabolism facilitates insulin biosynthesis and secretion, it is possible that this could lead to ER overload.

Our results show that the levels of cellular NAD(P)H and ATP paradoxically decrease in cells with increased glucose metabolism. These changes are likely among the factors that lead to the impaired GSIS observed in both ours and Wang and Iynedjian's studies (26, 45). Decreased cellular NAD(P)H is likely the result of increased usage and impaired generation in mitochondria as a consequence of oxidative stress. The reduced levels of cellular ATP in our study is consistent with the observations by Wang and Iynedjian (45), and is most likely caused by decreased generation in damaged mitochondria and increased usage as a result of PARP overactivation. In addition, accumulation of Glc-6-P may contribute to ATP depletion, as observed by Wang and Iynedjian (45). Adenovirus-mediated GK expression was previously evaluated in both INS-1 cells and isolated rat islets (52, 53), but the authors did not observe increased glucose-stimulated glucose usage and insulin secretion in cells with enhanced GK activity. Those results of glucose metabolism differ from the observations in Wang and Iynedjian's studies (26, 45) and our findings. One possible explanation is the different experimental protocols used in those studies (52, 53) in that the isolated islets or INS-1 cells were transduced with GK-carrying adenoviruses in medium containing 11.1 mM glucose. We have recently shown that INS-1 cells with more than a 2-fold increase in GK activity undergo apoptosis, while INS-1 cells with a 1.5-fold increase in GK activity have blunted GSIS when the cells are cultured in medium containing 11.8 mM glucose for 48 h.[2]

In conclusion, our results suggest the existence of an upper limit of both GK activity and glucose metabolism for normal function and survival of pancreatic β cells. Future studies to extend our findings to the *in vivo* arena may provide insights into the molecular mechanisms of β cell glucose toxicity and provide information useful for developing new therapeutic strategies for the treatment of type 2 diabetes.

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