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Glucose promotes pancreatic islet β-cell survival through a PI 3-kinase/Akt-signaling pathway

SHANTHI SRINIVASAN,[1] ERNESTO BERNAL-MIZRACHI,[2]
MITSURU OHSUGI,[2] AND MARSHALL ALAN PERMUTT[2]
Divisions of [1]Gastroenterology and [2]Endocrinology, Diabetes and Metabolism, Department of Internal Medicine, Washington University School of Medicine, St. Louis, Missouri 63110

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Srinivasan, Shanthi, Ernesto Bernal-Mizrachi, Mitsuru Ohsugi, and Marshall Alan Permutt. Glucose promotes pancreatic islet β-cell survival through a PI 3-kinase/Akt-signaling pathway. *Am J Physiol Endocrinol Metab* 283: E784–E793, 2002. First published June 25, 2002; 10.1152/ajpendo.00177.2002.—The concentration of glucose in plasma is an important determinant of pancreatic β-cell mass, whereas the relative contributions of hypertrophy, proliferation, and cell survival to this process are unclear. Glucose results in depolarization and subsequent calcium influx into islet β-cells. Because depolarization and calcium (Ca^{2+}) influx promote survival of neuronal cells, we hypothesized that glucose might alter survival of islet β-cells through a similar mechanism. In the present studies, cultured mouse islet β-cells showed a threefold decrease in apoptosis under conditions of 15 mM glucose compared with 2 mM glucose ($P < 0.05$). MIN6 insulinoma cells incubated in 25 mM glucose for 24 h showed a threefold decrease in apoptosis compared with cells in 5 mM glucose (1.7 ± 0.2 vs. 6.3 ± 1%, respectively, $P < 0.001$). High glucose (25 mM) enhanced survival-required depolarization and Ca^{2+} influx and was blocked by phosphatidylinositol (PI) 3-kinase inhibitors. Glucose activation of the protein kinase Akt was demonstrated in both insulinoma cells and cultured mouse islets by means of an antibody specific for Ser^{473} phospho-Akt and by an in vitro Akt kinase assay. Akt phosphorylation was dependent on PI 3-kinase but not on MAPK. Transfection of insulinoma cells with an Akt kinase-dead plasmid (Akt-K179M) resulted in loss of glucose-mediated protection, whereas transfection with a constitutively active Akt enhanced survival in glucose-deprived insulinoma cells. The results of these studies defined a novel pathway for glucose-mediated activation of a PI 3-kinase/Akt survival-signaling pathway in islet β-cells. This pathway may provide important targets for therapeutic intervention.

phosphatidylinositol 3-kinase; apoptosis; islet β-cell mass; depolarization

THE MASS OF INSULIN-PRODUCING islet β-cells is determined by the combined rates of proliferation of existing cells, by neogenesis, and by cell death (5). For example, animals that are insulin resistant develop compensatory hypertrophy and hyperplasia. Under such conditions, growth factors and glucose are thought to be important determinants of islet β-cell mass and function (15). The importance of glucose in these responses has been suggested by observing that animals subjected to infusions of glucose over several days developed increased β-cell mass (51). Similarly, glucose treatment of insulinoma cells in culture resulted in β-cell hyperplasia (24). Pancreatic islet β-cells are unique in their responses to physiological changes in glucose, converting metabolic energy into electrical activity (26). Glucose results in closure of the ATP-activated potassium (K_{ATP}) channels, leading to depolarization of β-cells and an influx of cytosolic calcium. The importance of islet β-cell depolarization in proper β-cell generation and proliferation was recently demonstrated by disruption of the L-type calcium channel α_{1D} subunit in mice (44). Knockout of this calcium channel subunit resulted in a decrease in the number and size of the islets due to a decrease in β-cell generation. These results highlighted the significance of depolarization/Ca^{2+} signaling for normal β-cell development.

Sustained Ca^{2+} overload in neurons is associated with enhanced apoptosis, yet neurons deprived of depolarization and Ca^{2+} also do not survive (53). This has been demonstrated by the fact that promotion of survival of several types of neurons by depolarization can be prevented by dihydropyridine calcium channel antagonists (10, 17, 18, 34). Neurons and islet β-cells share many biochemical and molecular mechanisms. We hypothesized that glucose might affect islet β-cell mass through similar mechanisms. Like neurons, chronic depolarization and Ca^{2+} overload could result in apoptosis (42, 49); yet previous studies suggested that glucose may enhance islet β-cell viability. Isolated rat islet β-cells cultured in 10 mM glucose for 1 wk exhibited marked enhancement of survival relative to those in 3 mM glucose (23). Another study reported increasing apoptosis when islet β-cells were incubated in >12 mM glucose for 40 h; yet interestingly, this study also showed a >80% reduction in apoptosis when β-cells were incubated with glucose increasing from 2 to 12 mM (14). These studies provide preliminary evidence that glucose in the 10–12 mM range, perhaps

Address for reprint requests and other correspondence: S. Srinivasan, Washington Univ. School of Medicine, Campus Box 8127, 660 S. Euclid Ave., St. Louis, MO 63110 (E-mail: ssriniva@im.wustl.edu).

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through activation of depolarization, may enhance survival of β-cells by mechanisms similar to those observed in neuronal cells.

The purpose of the present study was to attempt to confirm previous observations on the survival-enhancing effects of glucose on islet β-cells in culture and, if so, evaluate potential mechanisms. Using cultures of mouse islets and rodent insulinoma cell lines previously shown to secrete insulin in response to physiological changes in glucose concentrations (2–25 mM) (26), we determined that there were significant effects of glucose at >15 mM on survival relative to those in glucose at <5 mM during 24- to 96-h incubations. To elucidate the mechanisms of glucose-mediated survival, we examined signal transduction pathways that might be involved. The results suggested that glucose promotes pancreatic islet β-cell survival through depolarization and subsequent Ca^{2+} activation of a phosphatidylinositol (PI) 3-kinase/Akt survival-signaling pathway.

EXPERIMENTAL PROCEDURES

Antibodies

We used the following antibodies: Akt, phospho-Akt, and cleaved caspase-3 antibodies (New England Biolabs); hemagglutinin (HA) antibody (Santa Cruz Biotechnologies); Alexa 488 goat anti-rabbit IgG antibody, and Alexa 594 goat anti-mouse IgG antibody (Molecular Probes); CY3-conjugated Affinipure donkey anti-guinea pig antibody (Jackson Immunoresearch Lab), and anti-insulin antibody (Biogenex).

Cell Culture

MIN6 cells were cultured in Dulbecco's minimal essential medium (DMEM) containing 15% FCS and 25 mM glucose (passage no. 20–40). INS-1 cells were cultured in RPMI containing 10% FCS and 11.2 mM glucose and PC12 cells in DMEM containing 10% FCS, 5% goat serum, and 25 mM glucose. For detection of apoptosis, cells were plated at 1,000,000 per coverslip, and experiments were performed 24 h after plating at ~70–80% confluence. The protocol for glucose experiments involved washing cells with phosphate-buffered saline (PBS), followed by incubation for 24–96 h with 2–40 mM glucose and other reagents such as mannitol, diazoxide (DZ), PD-98059, wortmannin, LY-294002, and nifedipine.

Isolation of mouse islets was accomplished by collagenase digestion and Ficoll centrifugation, followed by hand picking as described (39). Islets were cultured for 24 h in RPMI with 10% serum and 11.2 mM glucose.

Detection of Apoptosis

The TdT-mediated dUTP nick end labeling (TUNEL) technique was used to detect DNA strand breaks formed during apoptosis (19). Cells on coverslips were fixed with 4% paraformaldehyde for 45 min at room temperature and then permeabalized with 1% Triton X-100. After a rinse with PBS, cells were incubated with fluorescein isothiocyanate (FITC)-labeled dUTP in the presence of enzyme TdT for 1 h at 37°C. Coverslips were mounted on glass slides in mounting medium containing the counterstain propidium iodide (2.5 μg/ml) and visualized using a fluorescent microscope. Five hundred cells were scored (in triplicate) in a blinded fashion to determine the percentage of TUNEL-positive cells. In RESULTS, *n* represents the number of independent experiments.

Activated caspase-3 was detected by immunohistochemistry using an antibody (1:100) to the cleaved caspase-3 (17 kDa) fragment as described (16). Alexa 488 goat anti-rabbit IgG antibody (1:200) was used as a secondary antibody. Five hundred cells were scored in a blinded fashion to determine the percentage of cleaved caspase-3-positive cells by use of a fluorescent microscope.

For detection of apoptosis in cultured islet cells, the islets were isolated and cultured for 24 h in different glucose concentrations (2 and 15 mM). After 24 h of incubation, the islets were dispersed into single cells with dispase (46) and were spun down with a Cytospin machine onto a slide. Cells were fixed with 4% paraformaldehyde for 45 min at room temperature and then permeabalized with 1% Triton X-100. After a rinse with PBS, cells were incubated with FITC-labeled dUTP in the presence of enzyme TdT for 1 h at 37°C. After a wash with PBS, cells were incubated with guinea pig anti-insulin antibody (1:200) for 1 h followed by a 30-min wash with PBS; finally, cells were incubated with secondary antibody (CY3-conjugated donkey anti-guinea pig antibody) for 1 h. After a 30-min wash with PBS, cells were covered in mounting medium and visualized with a fluorescent microscope. No propidium iodide counterstain was used in the TUNEL method. Staining for insulin as described identified β-cells. Five hundred β-cells were scored in a blinded fashion to determine the percentage of FITC-positive (TUNEL-positive) β-cells.

Detection of Necrosis

MIN6 cells were treated with 5 and 25 mM glucose for 24 h, and necrosis was assessed by trypan blue exclusion. Five hundred cells were counted in a blinded fashion, and the number of trypan blue-positive cells determined the percentage of necrosis (2).

Western Blot Analysis

MIN6 cells. MIN6 cells were plated in 100-mm dishes at a seeding density of 5,000,000 cells per dish and cultured in DMEM for 4 days. At ~80% confluence, cells were preincubated in the absence of serum and with 5 mM glucose for 14 h, followed by no serum and no glucose for 4 h. Kinase inhibitors were added 1 h before the addition of glucose. After incubation with glucose (5 or 25 mM) for various time intervals, cells were lysed and assayed on an acrylamide gel (10%) for phospho-Akt as described (1). Protein (40 μg) was loaded per lane. Phospho-Akt was assessed by use of an antibody to Akt phosphorylated at Ser^{473}. The blot was stripped and reprobed for total Akt.

Isolated islets. Isolated islets were cultured overnight in complete medium (11.2 mM glucose and 10% FCS). Cells were incubated in Krebs buffer for 1 h followed by exposure to 2 or 5 mM glucose for 45 min. After incubation with glucose (2 and 15 mM) for various time intervals, cells were lysed and assayed on an acrylamide gel (10%) for phospho-Akt and total Akt.

Akt Kinase Activity

Measurement of Akt kinase activity was performed using a kit (Cell Signaling Technology, Beverly, MA). MIN6 cells were stimulated as described and lysed in cell lysis buffer. Cell lysates containing 300 μg of total protein were immunoprecipitated with immobilized Akt monoclonal antibody slurry with gentle rocking for 3 h at 4°C. After two washes

with cell lysis buffer followed by two washes with kinase buffer (25 mM Tris, pH 7.5, 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM Na_3VO_4, 10 mM $MgCl_2$), the immunoprecipitates were resuspended in 40 μl of kinase buffer added with 200 μM ATP and 1 μg of GSK-3 fusion protein. Samples were incubated for 30 min at 30°C. The reaction was terminated with 20 μl of 3× SDS loading buffer, and then samples were analyzed by immunoblotting with the phospho-GSK-3α/β ($Ser^{21/9}$) antibody (38).

Transient Transfection and Detection of Akt by Immunofluorescence

Akt constructs were a gift of Dr. Philip Stahl (Washington University School of Medicine). Plasmids containing three HA-tagged Akt constructs were used: wild-type Akt (Akt-WT), kinase-dead Akt (Akt-K179M), and a constitutively active form of Akt (Akt-CA) (33). Transfection efficiency was 5%, determined by immunohistochemical detection of the HA tags of the Akt constructs with an antibody to HA (1:200). Cells were transfected with 1 μg of DNA per dish with the use of lipofectamine plus reagent under serum-free conditions for 6 h, and then the cells were replenished with complete medium for 24 h. Next, the cells were cultured for 24 h in different concentrations of glucose. The cells were fixed and assayed for transfection and apoptosis. TUNEL staining was performed first, followed by staining for the HA tag of Akt. No propidium iodide counterstain was used in the TUNEL method. The TUNEL-positive cells appeared green and the HA tag appeared red due to the ALEXA 594 secondary antibody. Apoptosis in transfected cells was detected by scoring yellow cells due to overlap of red and green fluorescence.

Statistical Analysis

Statistical analysis was performed using a two-tailed Student's *t*-test. A P value < 0.05 was considered significant.

RESULTS

Effects of Glucose on Survival of Insulinoma Cells and Cultured Islet Cells

To assess whether glucose affected survival of islet β-cells, apoptosis was measured by the TUNEL method in cultures of isolated mouse pancreatic islets and insulinoma cells incubated in various glucose concentrations. The effects of glucose were assessed on survival of primary mouse islet β-cells maintained in culture for 24 h. β-Cells were identified using a staining protocol for insulin in conjunction with the TUNEL method, as described in EXPERIMENTAL PROCEDURES. Mouse islet β-cells cultured in the presence of 15 mM glucose had a threefold reduction in apoptosis compared with those cultured in 2 mM glucose [29 ± 9.2 vs. 75 ± 8.9% apoptosis ($n = 4$ independent experiments), $P = 0.03$]. In Fig. 1*A*, the percentage of apoptosis is shown relative to apoptosis in 15 mM glucose (2 mM glucose: 258 ± 30.68 vs. 15 mM glucose: 100 ± 31.72, $P = 0.03$). MIN6 insulinoma cells cultured in 5 mM glucose over 24 h showed 6.7 ± 1% TUNEL-positive cells at the end of the incubation ($n = 13$). In contrast, cells incubated in 25 mM glucose exhibited a threefold decrease in TUNEL-positive cells [1.7 ± 0.2% ($n = 11$), $P < 0.001$]. In Fig. 1*B*, the percentage of apoptosis is shown relative to apoptosis in 5 mM glucose (5 mM glucose: 370 ± 61.76 vs. 25 mM glucose: 100 ± 17.54, $P < 0.001$). A representative photograph of MIN6 cells is shown to illustrate TUNEL-positive yellow fluorescent cells (Fig. 1*B*). A similar effect of glucose on apoptosis was also observed in another rodent insulinoma cell line, INS-1 [10 mM glucose: 2.69 ± 0.4% ($n = 6$) vs. 2 mM glucose: 6.02 ± 0.4% ($n = 6$), $P < 0.001$].

Measurement of apoptosis by the TUNEL method was confirmed by detecting apoptosis by means of immunohistochemical detection of cleaved caspase-3 (17-kDa fragment). Activation of caspase-3, one of the key executioners of apoptosis, requires its cleavage into two fragments (17 and 12 kDa). As shown in Fig. 1*C*, MIN6 cells cultured for 24 h under conditions of 5 mM glucose showed a 294.87 ± 54.48% increase in apoptosis by cleaved caspase-3 staining compared with those cultured in 25 mM glucose [100 ± 14.86% ($n = 6$), $P < 0.001$].

MIN6 cells are typically cultured in 25 mM glucose (26). To determine whether the effects of lowering glucose were due to differences in osmolarity, experiments were performed with the addition of mannitol. To maintain osmolarity, cells were incubated in 5 mM glucose plus 20 mM mannitol. This treatment did not change the percentage of apoptosis seen under conditions of 5 mM glucose [%apoptosis; 25 mM glucose: 1.7 ± 0.2 ($n = 11$); 5 mM glucose: 6.7 ± 1 ($n = 13$); 5 mM glucose + 20 mM mannitol: 6.3 ± 1.2 ($n = 6$), $P > 0.05$ vs. 5 mM glucose]. To decrease the rates of proliferation to reduce this as a variable while assessing apoptosis, experiments were performed in the presence of low serum (1%). Even in the presence of low serum concentrations, a three- to fourfold decrease in apoptosis was seen in cells cultured in the presence of 25 mM glucose compared with those in 5 mM glucose [2.8 ± 0.29% ($n = 3$) vs. 7.63 ± 2.2% ($n = 3$), $P < 0.05$]. Min6 cells express predominantly GLUT2, a high-K_m glucose transporter (26, 27). To determine whether the protective effect of 25 mM glucose was a property of cells with high-K_m glucose transporters and glucokinase, the same experiments were performed in a neuronal cell line, PC12. After incubation for 24 h in 5 or 25 mM glucose, no difference in apoptosis was observed [5 mM glucose: 0.26 ± 0.11% ($n = 6$), 25 mM glucose: 0.56 ± 0.07% ($n = 6$), $P > 0.05$]. This result suggested that the correlation between physiological levels of glucose and the regulation of apoptosis might be unique to cells with high-K_m glucose transporters and hexokinases like islet β-cells.

Effect of Glucose on Necrosis in Insulinoma Cells

Cell survival can be determined by apoptosis or necrosis. MIN6 insulinoma cells were treated with glucose (5 or 25 mM) for 24 h. There was no difference in necrosis noted in cells cultured in 5 vs. 25 mM glucose [%necrotic cells, 5 mM: 2.1 ± 0.3 ($n = 4$); 25 mM: 2.3 ± 0.5 ($n = 6$), $P = 0.7$].

Dose Dependency and Time Course of Glucose-Regulated Islet β-Cell Survival

In nonislet β-cells, absence of glucose induces apoptosis, whereas the protective effect on survival is maximal at <2 mM (29). In contrast, in islet β-cells, because of the high-K_m, low-affinity glucose transporter and hexokinase activities, the glucose effect on survival would be predicted to have a K_m in the 5–15 mM range (52). After 24 h of incubation, inhibition of apoptosis was present at 15 mM glucose and was not significantly altered by glucose concentrations up to 40 mM [%apoptosis at various glucose concentrations: 0 mM: 4.64 ± 1.1; 5 mM: 3.08 ± 1.1; 10 mM: 3.04 ± 1.1; 15 mM: 2.43 ± 0.07; 25 mM: 2.08 ± 0.6; 40 mM: 2.33 ± 0.55 (n = 4 in each category), $P < 0.05$ between 0 and >15 mM]. These results indicate that glucose regulation of islet β-cell apoptosis occurs over a physiologically relevant glucose concentration range (5–15 mM).

To assess the time course of the glucose effect on apoptosis, cells were examined at 24-h intervals for up to 96 h of incubation. The rate of apoptosis increased with time in both 5 and 25 mM glucose, whereas at each time point cells incubated in 25 mM glucose had a three- to fourfold reduced rate of apoptosis relative to those in 5 mM glucose [%apoptosis at 24 h at 5 mM glucose: 6.3 ± 1 (n = 13) and 25 mM glucose: 1.7 ± 0.3 (n = 11), $P < 0.001$; at 48 h in 5 mM glucose: 4.16 ± 0.3 (n = 8) and 25 mM glucose: 0.7 ± 0.3 (n = 8), $P < 0.001$; at 72 h in 5 mM glucose: 15.99 ± 4 (n = 6) and 25 mM glucose: 0.9 ± 0.3 (n = 4), $P < 0.0001$; and at 96 h at 5 mM glucose: 34.4 ± 2.4 (n = 6) and 25 mM glucose: 13.6 ± 4.3 (n = 6), $P < 0.05$]. The data in Fig. 2 represent the aforementioned results expressed as a percentage of apoptosis at 24 h of incubation in the presence of 25 mM glucose.

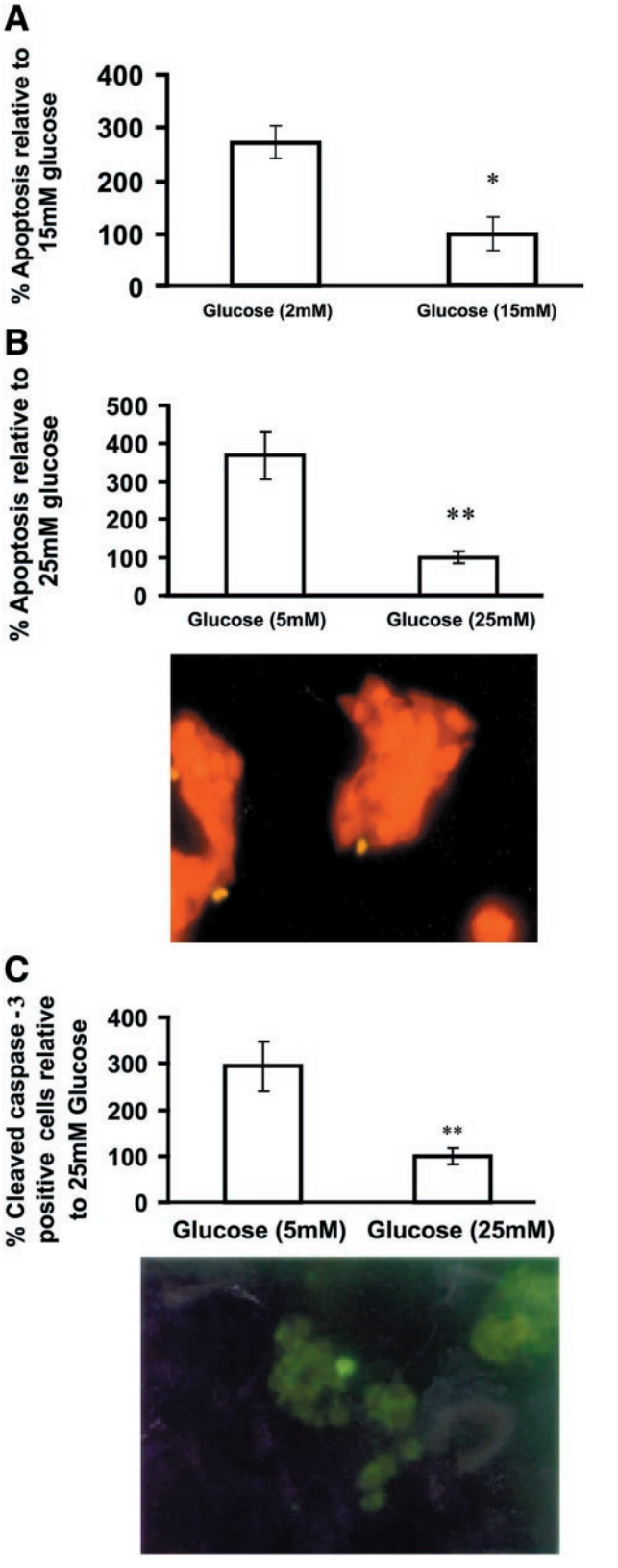

Fig. 1. Effect of glucose on survival in islet β-cells and insulinoma cells. *A*: apoptosis in mouse islets. Isolated mouse islets were treated with 2 or 15 mM glucose for 24 h, and apoptosis was assessed in β-cells by the TdT-mediated dUTP nick end labeling (TUNEL) method as described in EXPERIMENTAL PROCEDURES. Apoptosis was higher in the presence of 2 mM glucose (258.62 ± 30.68%) compared with 15 mM glucose (100 ± 31.72%). Results represent means ± SE; *$P < 0.05$. *B*: MIN6 insulinoma cells were treated with glucose for 24 h at the indicated concentrations and assessed for apoptosis using the TUNEL method as described in EXPERIMENTAL PROCEDURES. Apoptosis was higher in the presence of 5 mM glucose (370 ± 61.76%) compared with 25 mM glucose (100 ± 17.64%). Results represent means ± SE; **$P < 0.001$. A representative photograph to illustrate TUNEL-positive yellow (stained with FITC and propidium iodide) fluorescent cells is shown. *C*: caspase-3 activity assay. MIN6 insulinoma cells were treated with glucose for 24 h at the indicated concentrations and assessed for cleaved caspase-3 staining as described in EXPERIMENTAL PROCEDURES. The cleaved caspase-3-positive cells were higher in the presence of 5 mM glucose (294 ± 54.48%) compared with 25 mM glucose (100 ± 16.66%). Results represent means ± SE; **$P < 0.001$. Representative photograph of immunohistochemical detection of cleaved caspase-3 positive cell is shown. The cleaved caspase-3-positive cells appear green due to Alexa 488-conjugated secondary antibody.

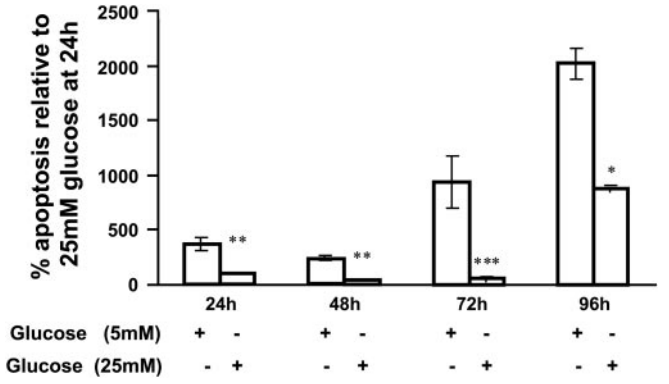

Fig. 2. Time course of glucose-regulated survival in insulinoma cells. MIN6 cells were treated with glucose at the indicated concentrations and time points. Apoptosis was assessed using the TUNEL method as described in EXPERIMENTAL PROCEDURES. Percent apoptosis relative to apoptosis at 24 h in 25 mM glucose; 24 h at 5 mM glucose: 370 ± 61 (n = 13) and 25 mM glucose: 100 ± 17 (n = 11), $P < 0.001$; at 48 h in 5 mM glucose: 244 ± 17 (n = 8) and 25 mM glucose: 41 ± 17 (n = 8), $P < 0.001$; at 72 h in 5 mM glucose: 940 ± 235 (n = 6) and 25 mM glucose: 52 ± 17 (n = 4), $P < 0.0001$; and 96 h at 5 mM glucose: 2,020 ± 141 (n = 6) and 25 mM glucose: 880 ± 31 (n = 6), $P < 0.05$. Results represent means ± SE; *$P < 0.05$, **$P < 0.001$, ***$P <$ 0.0001.

Glucose-Regulated Survival is Dependent on RNA Synthesis, Depolarization, and Ca^{2+} Influx

Having established a model system where reproducible effects of glucose on β-cell survival could be observed, we next sought mechanisms. In neuronal cells, apoptosis induced by growth factor withdrawal is dependent on RNA synthesis (43). Therefore, MIN6 cells were treated for 24 h with actinomycin D (1 μg/ml) to inhibit RNA synthesis in the presence of 5 or 25 mM glucose. This treatment completely attenuated the enhanced apoptosis seen in 5 mM glucose [5 mM glucose: 6.3 ± 1% (n = 13); 5 mM glucose + actinomycin D: 2.06 ± 0.6% (n = 5), P = 0.01]. No change in apoptosis was seen when actinomycin D was added under conditions of 25 mM glucose [25 mM glucose: 1.7 ± 0.2% (n = 11); 25 mM glucose + actinomycin D: 1.11 ± 0.3% (n = 6), $P > 0.05$ vs. 25 mM glucose]. In Fig. 3*A*, these data are represented as percentages relative to apoptosis at 24 h in the presence of 25 mM glucose. These results suggested that the increased rate of apoptosis observed in the presence of 5 mM glucose is dependent

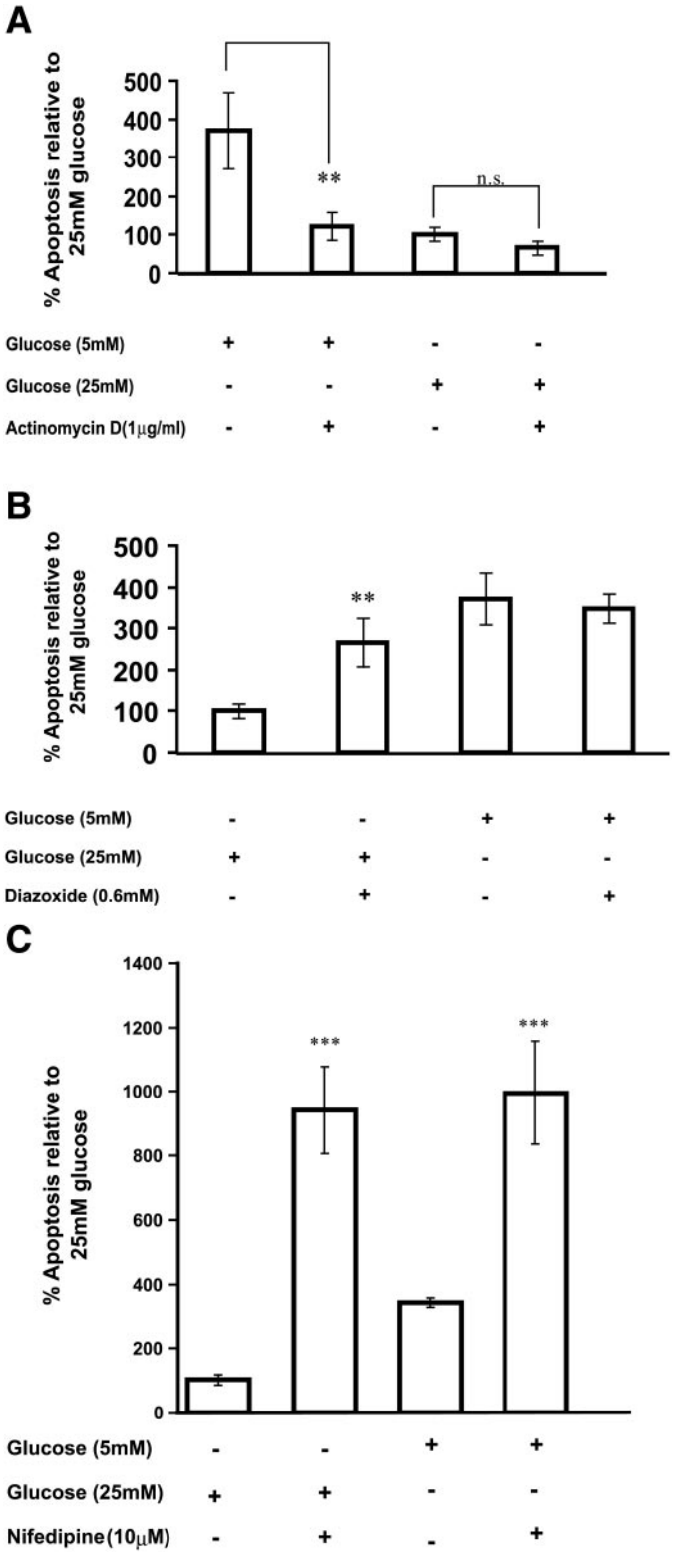

Fig. 3. Glucose-regulated islet β-cell survival is dependent on RNA synthesis, depolarization, and Ca^{2+} influx. *A*: effect of actinomycin D. MIN6 cells were treated for 24 h at the indicated glucose concentrations in the presence or absence of actinomycin D (1 μg/ml) to inhibit RNA synthesis, and apoptosis was assessed by the TUNEL method. Low glucose-induced apoptosis was attenuated in the presence of actinomycin D (%apoptosis relative to 25 mM glucose; 5 mM glucose: 370 ± 61%; 5 mM glucose + actinomycin D: 121 ± 35%; 25 mM glucose: 100 ± 17%; 25 mM glucose + actinomycin D: 65 ± 17%). Results represent means ± SE; **$P < 0.001$ (n.s., not significant, $P >$ 0.05.) *B*: effect of diazoxide. MIN6 cells were treated for 24 h at the indicated glucose concentrations in the presence or absence of diazoxide, and apoptosis was assessed by the TUNEL method. The 25 mM glucose suppression of apoptosis was lost in the presence of diazoxide (%apoptosis relative to 25 mM glucose; 5 mM glucose: 370 ± 61; 5 mM glucose + diazoxide: 345 ± 35; 25 mM glucose: 100 ± 17; 25 mM glucose + diazoxide: 267 ± 58). Results represent means ± SE; **$P < 0.001$. *C*: effect of nifedipine. MIN6 cells were treated for 24 h with glucose at the indicated concentrations in the presence or absence of nifedipine, and apoptosis was assessed by the TUNEL method. Results are all given relative to those in 25 mM glucose and represent means ± SE; ***$P < 0.0001$.

on synthesis of new RNA, whereas the protective effect of 25 mM glucose is not.

To determine whether the glucose effect on survival required membrane depolarization, experiments were performed using DZ, an agent that activates K_{ATP} channels and blocks glucose-mediated depolarization of the β-cell (50). The protective effect conferred by 25 mM glucose was lost in the presence of DZ (0.6 mM), suggesting that depolarization is essential for glucose-mediated protection of the β-cell [25 mM glucose: 1.7 ± 0.3% (n = 11); 25 mM glucose + DZ: 4.54 ± 0.75% (n = 6), P = 0.001 vs. 25 mM glucose; 5 mM glucose 6.3 ± 1% (n = 13), $P < 0.001$ vs. 25 mM glucose; 5 mM glucose + DZ: 5.88 ± 0.6% (n = 6), $P > 0.05$ vs. 5 mM glucose]. In Fig. 3*B*, these data are represented as the percentage of apoptosis relative to that seen at 24 h in the presence of 25 mM glucose.

To determine whether Ca^{2+} influx was required for glucose inhibition of apoptosis, cells were incubated in the presence of the L-type Ca^{2+} channel blocker nifedipine (10 μM). As shown in Fig. 3*C*, the protective effect conferred by 25 mM glucose was lost in the presence of nifedipine, indicating that glucose-dependent Ca^{2+} influx is essential for the glucose-mediated protection of the β-cell at 25 mM glucose [%apoptosis relative to apoptosis in 25 mM glucose; 25 mM glucose: 102 ± 15 (n = 3); 25 mM glucose + nifedipine: 996 ± 161 (n = 6), $P < 0.0001$ vs. 25 mM glucose]. Addition of nifedipine to cells incubated in 5 mM glucose resulted in an almost threefold increase in apoptosis [%apoptosis relative to apoptosis in 25 mM glucose; 5 mM glucose: 343 ± 55 (n = 3), $P < 0.0001$; 5 mM glucose + nifedipine: 942 ± 136 (n = 6), $P < 0.0001$]. Thus it appears that Ca^{2+} influx following glucose-induced depolarization is playing a critical role in survival and that perhaps the further increase in survival in 25 mM is due to the enhanced Ca^{2+} influx known to occur with depolarization.

Signal Transduction Pathways Involved in Glucose-Regulated Survival

Having determined that glucose-induced depolarization and Ca^{2+} influx are important mediators of survival, the signal transduction pathways involved in this process were examined. In islet β-cells, glucose-mediated depolarization and Ca^{2+} influx have previously been shown to result in activation of several serine-threonine kinases, including MAP kinases and PI-3 kinase, and these have been shown to be involved in the survival of other cells (22, 31). MIN6 cells were cultured in either 5 or 25 mM glucose in the presence or absence of the MAPK inhibitor PD-98059 (50 μM) or the PI 3-kinase inhibitors wortmannin (200 nM) or LY-294002 (50 μM). Kinase inhibitors were added 1 h before the change in the glucose concentration of the medium. As shown in Fig. 4, the MAPK inhibitor did not alter the protective effect of 25 mM glucose on apoptosis [%change in apoptosis relative to apoptosis in 25 mM; 25 mM: 101 ± 10 (n = 6); 25 mM + PD-98059: 123 ± 1 (n = 6), P = 0.5; Fig. 4]. However,

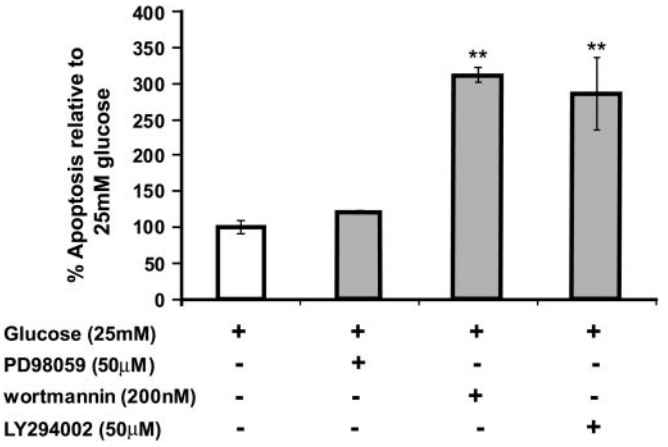

Fig. 4. Signal transduction pathways involved in glucose-regulated islet β-cell survival. MIN6 cells were cultured for 24 h at the indicated glucose concentrations in the presence or absence of the indicated inhibitors, and apoptosis was assessed by the TUNEL method. Results represent means ± SE; $^{**}P < 0.001$.

both PI 3-kinase inhibitors decreased the protective effect of 25 mM glucose, indicating this effect to be PI 3-kinase dependent [%change in apoptosis relative to apoptosis in 25 mM; 25 mM: 101 ± 10 (n = 6); 25 mM + wortmannin: 311 ± 29 (n = 3), P = 0.001; 25 mM + LY-294002: 286 ± 73 (n = 6), P = 0.008; Fig. 4]. No significant change in apoptosis was noted when kinase inhibitors were added to cells cultured in 5 mM glucose compared with apoptosis in 5 mM glucose alone (data not shown).

A major downstream target of activated PI 3-kinase is the serine-threonine kinase Akt, and activation of this enzyme by phosphorylation of Ser^{473} has been associated with inhibition of apoptosis (13, 32). To determine whether glucose-regulated survival observed in MIN6 cells was associated with glucose activation of Akt, cells were treated with glucose as indicated, and protein extracts were immunoblotted with an antibody specific for Ser^{473} phospho-Akt (57 kDa) as a measure of Akt activation (54). At 120 min after addition of 25 mM glucose, there was marked Ser^{473} phosphorylation of Akt (n = 4; Fig. 5*A*), whereas addition of 5 mM glucose showed no phosphorylation at similar time points (data not shown). In separate experiments, it was shown that the activation of Akt by glucose was first observed at 15 min and peaked at 120 min. Under these conditions, there was no change in Akt protein detected by an antibody to total Akt. Addition of kinase inhibitors indicated that glucose activation of Akt was PI 3-kinase dependent and MAPK independent (n = 3; Fig. 5*B*).

To determine whether similar treatment of primary cultures of isolated mouse islets would also result in Ser^{473} phosphorylation of Akt, cells were incubated in 2 or 15 mM glucose for 45 min and immunoblotted with the antibody specific for Ser^{473} phospho-Akt (57 kDa). As shown in Fig. 5*C*, although phospho-Akt was detected in cells incubated in 2 mM glucose, similar treatment with 15 mM glucose resulted in a threefold increase (3.05 ± 0.23, P = 0.01) in phospho-Akt. The

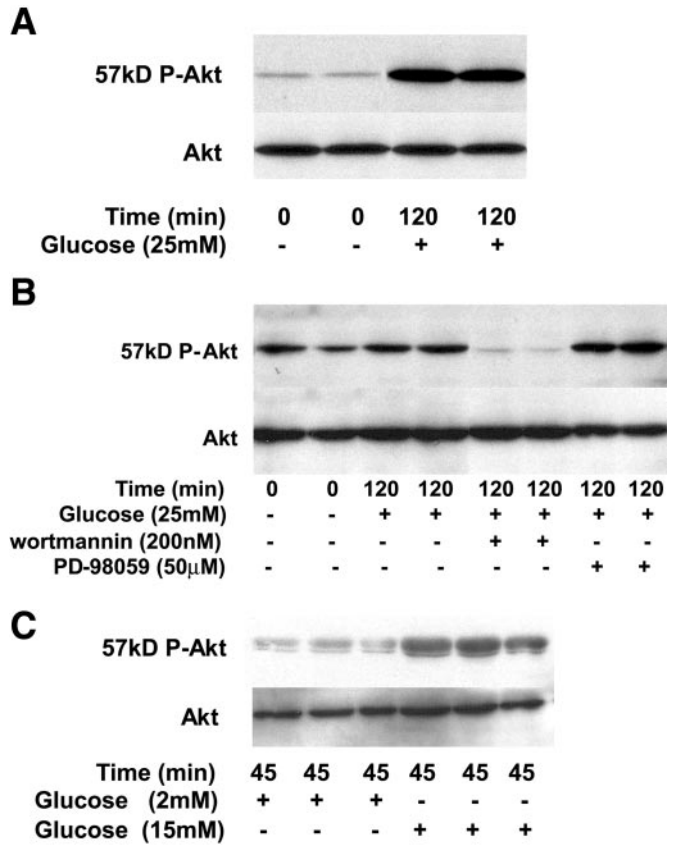

Fig. 5. Glucose activates Akt in insulinoma cells and isolated islets. *A*: assessment of Ser^{473} phospho-Akt (P-Akt). Cells were preincubated in the absence of serum and with 5 mM glucose for 14 h, followed by no serum and no glucose for 4 h and then treatment with 25 mM glucose for the indicated time. Lysates were prepared as described in EXPERIMENTAL PROCEDURES. Protein (40 μg) was loaded in each lane and assayed on an acrylamide gel (10%). Activation of Akt by glucose and total Akt was assessed by use of antibodies specific for Ser^{473} phospho-Akt and Akt, respectively. *B*: Akt activation is phosphatidylinositol (PI) 3-kinase dependent. Cells were treated and assessed as described in *A*. Kinase inhibitors were added 1 h before addition of 25 mM glucose. The effect of glucose on phospho-Akt and total Akt in the presence or absence of MAPK inhibitor PD-98059 (50 μM) or PI-3 kinase inhibitor wortmannin (200 nM) was assessed. *C*: Akt activation in primary mouse islets. Isolated mouse islets were preincubated in Krebs buffer for 1 h and then treated with glucose for the indicated times and concentrations. Lysates were prepared as described in EXPERIMENTAL PROCEDURES. Protein (75 μg) was loaded in each lane and assayed on an acrylamide gel (10%). Activation of Akt by glucose and total Akt was assessed using antibodies specific for Ser^{473} phospho-Akt and Akt.

software Scion Image was used to determine the intensity of bands on the Western blots.

To confirm that Ser^{473} phosphorylation of Akt was associated with Akt kinase activity, we measured the Akt kinase activity under similar conditions. In this assay, immunoprecipitates of Akt are incubated with a substrate GSK-3 fusion protein in the presence of ATP and kinase buffer. Akt phosphorylates GSK-3, which is then detected by Western blot analysis using a phospho-GSK-3α/β antibody. MIN6 cells were incubated in 25 mM glucose overnight and then removed from medium and replaced with Krebs buffer containing glucose or potassium chloride as indicated. After 15 min of treatment, protein was extracted and Akt kinase activity measured with a GSK-3 substrate. As shown in Fig. 6, exposure of cells to 3 mM glucose resulted in a minimal increase in kinase activity, whereas exposure to 25 mM glucose for 15 min resulted in a marked increase in Akt kinase activity similar to that seen with depolarization with 50 mM potassium chloride (Fig. 6).

Akt Activity is Both Necessary and Sufficient for Glucose-Mediated Survival

The previous observations demonstrated that glucose-mediated reduction of apoptosis in insulinoma cells was associated with PI 3-kinase-dependent activation of Akt. To attempt to demonstrate a causal relationship between Akt activation and inhibition of apoptosis, MIN6 cells were transfected with cytomegalovirus promoter-based expression vectors encoding either *1*) wild-type HA-tagged Akt (Akt-WT), *2*) a catalytically inactive mutant (kinase dead) form of HA-tagged Akt (Akt-K179M) with dominant-inhibitory activity toward wild-type Akt kinase activity, or *3*) a constitutively active HA-tagged Akt mutant (Akt-CA) that lacks amino acids 4–129 and that has a 14-amino acid *src* myristoylation signal on the amino terminus of Akt that targets Akt to the membrane (33). Because our rate of transfection was ~5%, as determined by immunohistochemistry using an antibody to HA, we used immunohistochemical staining techniques to identify transfected cells and study apoptosis in these cells. This method has been used successfully in the neuronal literature (13). The constitutively active Akt targets Akt to the membrane, demonstrated by an antibody to the HA tag (data not shown). Figure 7 demonstrates the percentage of apoptosis relative to wild-type Akt-transfected cells cultured in 25 mM glucose. The cells transfected with the kinase-dead plasmid Akt-K179M had increased rates of apoptosis [246 ± 57% increase vs. Akt-WT, 25 mM glucose (n = 8), $P < 0.05$; Fig. 7]. Transfections with constitutively active Akt plasmids in 25 mM glucose reduced the rate of apoptosis [31 ± 7.3% vs. Akt-WT in 25 mM glucose (n = 8), $P < 0.05$; Fig. 7]. Transfections with constitutively active Akt plasmids in 5 mM glucose reduced the rate of apoptosis [31 ± 7.3% reduction vs. Akt-WT in 5 mM glucose (n = 8), $P < 0.05$].

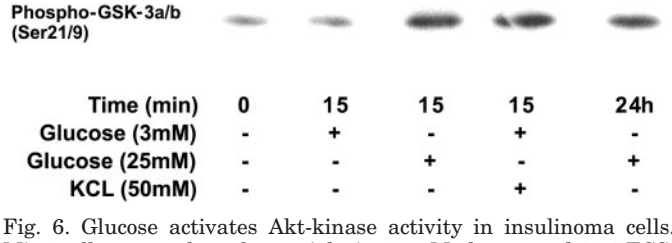

Fig. 6. Glucose activates Akt-kinase activity in insulinoma cells. Min6 cells were cultured overnight in 25 mM glucose and 15% FCS. Cells were then preincubated in Krebs buffer for 1 h (i.e., 0 time point) and then treated with Krebs buffer containing glucose or KCl as indicated. Akt kinase activity was measured as indicated in EXPERIMENTAL PROCEDURES with a GSK-3 substrate at the indicated times.

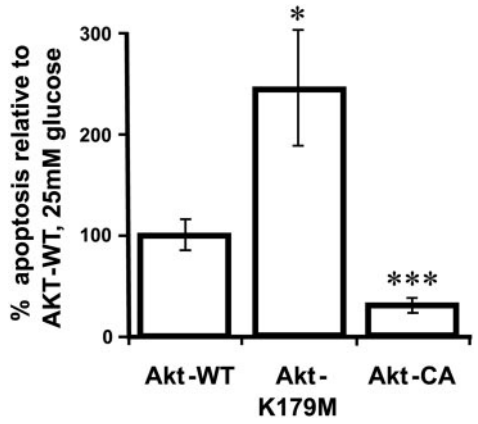

Fig. 7. Akt activity is both necessary and sufficient for glucose-mediated survival. MIN6 cells were transfected with 1 μg of either wild-type Akt (Akt-WT), a catalytically inactive mutant (kinase dead) form of Akt (Akt-K179M), or a constitutively active mutant (Akt-CA) that targets Akt to the membrane and cultured in 25 mM glucose. Apoptosis was assessed by the TUNEL method in transfected cells at 24 h. Results are expressed as percent apoptosis relative to Akt-WT cultured in 25 mM glucose and represent means ± SE; $*P < 0.05$, $***P < 0.0001$.

DISCUSSION

The results of the present study provide evidence for a glucose-mediated PI-3-kinase/Akt-dependent survival pathway in pancreatic islet β-cells. We have demonstrated glucose-mediated activation of Akt in isolated mouse islets and in the MIN6 insulinoma cell line. The results of this study confirm previous observations that glucose activates Akt in insulinoma cells (8). Our findings contribute to the similarities in properties of β-cells and neurons, since activation of Akt in neuronal cells has been shown to enhance survival and to be induced by neurotransmitter-mediated depolarization, Ca^{2+} influx, and PI 3-kinase activation (13). In neurons, survival mediated both by growth factors such as insulin and by depolarization through neurotransmitters seems to be converging on this common survival pathway (56). Certainly, the effects of targeted disruption of the insulin receptor (35) and insulin receptor substrates (36, 57) in islet β-cells, along with the present studies, suggest that the depolarization and growth factor survival pathways may be playing a role in β-cell survival, thus affecting its mass and function.

The novel finding in this study is the nutritional regulation of a survival pathway in islet β-cells through activation of Akt. The importance of the role of glucose and Akt in regulating β-cell mass is seen in the transgenic mice expressing constitutively active Akt in islet β-cells. Expression of constitutively active Akt linked to an insulin gene promoter in transgenic mice resulted in increased islet β-cell mass by altering β-cell size and number (4, 9, 55). These mice are also resistant to streptozotocin-induced diabetes. Although increased β-cell mass was shown to be accounted for, at least in part, by increased proliferation, further experiments will be needed to determine whether these animals have decreased islet β-cell apoptosis as well. Thus Akt activation may be of importance in both islet β-cell proliferation and survival.

Although the present studies demonstrated the effects of glucose and Ca^{2+} influx on the short-term survival of β-cells, they do not address the question of reduced β-cell survival that may be associated with chronic hyperglycemia and excessive Ca^{2+} influx (28, 45). As in neurons, the effects of prolonged sustained hyperglycemia on islet β-cells may result in Ca^{2+} excess and subsequent apoptosis. Some studies have reported increased apoptosis in rodent islets incubated in glucose at >20 mM for periods as short as 2–4 days. However, we did not observe increased apoptosis in Min6 cells cultured in 40 mM glucose for 3 days. The effects of prolonged hyperglycemia on β-cells need to be further addressed.

In the present studies, the increased rate of apoptosis at 5 mM glucose was inhibited by addition of actinomycin D, suggesting the requirement for transcription of proapoptotic proteins. This is similar to the growth factor withdrawal-induced apoptosis in neurons that is also dependent on transcription of new RNA (41). The effects of high glucose on survival in MIN6 cells, however, appeared to be independent of RNA synthesis. This suggests that glucose-induced depolarization and Ca^{2+} influx are rapidly activating a signal transduction pathway mediating survival, perhaps through activation of a serine-threonine kinase. In fact, the results of the present studies in which glucose rapidly activates Akt are consistent with these data. Certain possible mechanisms for this could be through the well known effect of Akt activation on inhibition of proapoptotic transcription factors of the forkhead family (40) or inactivation of BAD (12). The downstream targets of activated Akt and their possible effects on β-cell survival can now be assessed.

The results of the present studies do not rule out the possibility that the glucose/depolarization/Ca^{2+}-mediated survival pathway noted in these experiments is dependent on insulin secretion, perhaps acting in an autocrine/paracrine fashion. Insulin has been shown to inhibit apoptosis in a number of cells (3, 20), and treatment of MIN6 cells with $>10^{-7}$ M insulin did result in increased survival (S. Srivivasan, unpublished observations). Recent studies have suggested that glucose regulation of insulin and L-pyruvate kinase gene transcription are mediated through glucose-stimulated insulin secretion in an autocrine/paracrine fashion (11). Our results are consistent with this hypothesis, and this important question needs to be examined by further studies.

What could the possible significance of glucose-mediated survival be to the normal physiology of islet β-cells? Glucose regulation of islet β-cell survival may play a critical role in the neonatal and postnatal period, a time during which the rates of β-cell apoptosis are high and thus influence the ultimate islet β-cell mass in the adult (21, 48). Epidemiological studies correlate low birth weight and fetal malnutrition with the onset of non-insulin-dependent diabetes later in life (47). In animal models, maternal protein restric-

tion imposed during fetal life and suckling resulted in lower serum glucose levels and a reduction in β-cell mass in the offspring (6, 37). Examples of animal models where hypoglycemia is associated with apoptosis have been reported (7, 42). Studies in rats transplanted with insulinoma cells have demonstrated increased apoptosis in the endogenous islet β-cells in the animals transplanted with insulinomas. These animals have hyperinsulinemia and hypoglycemia. There was a 67 ± 13% reduction in islet cell volume in insulinoma-transplanted animals, with a reduction in β-cell size (7). In the β-cell-specific Kir6.2-dominant-negative transgenic mouse, the neonatal mice develop hypoglycemia (42). These mice have enhanced β-cell apoptosis at 2 wk of age. Subsequent to this, these mice develop hyperglycemia, believed to be secondary to a reduction in β-cell mass. These results suggest that glucose may be important not only in the regulation of insulin synthesis and secretion but also in the survival of the islet β-cell. Increased β-cell apoptosis has been noted in patients with hyperinsulinism-induced hypoglycemia compared with age-matched controls (30). The results of the present study define a molecular mechanism for glucose regulation of an islet β-cell survival pathway that could have important consequences for understanding the etiology of diabetes and that may suggest new means of therapeutic intervention as well.

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