Glucose and Tolbutamide Induce Apoptosis in Pancreatic β-Cells

A PROCESS DEPENDENT ON INTRACELLULAR Ca\(^{2+}\) CONCENTRATION∗

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High concentrations of glucose are considered to be toxic for the pancreatic β-cell. However, the mechanisms underlying β-cell dysfunction and resulting cell death are not fully characterized. In the present study we have demonstrated that incubation of pancreatic islets and β-cells from ob/ob mice and Wistar rats with glucose induced a process of apoptotic β-cell death, as shown by DNA laddering, TdT-mediated dUTP-biotin nick end-labeling (TUNEL) technique, and by using DNA-staining dye HOECHST 33342. The obtained results show that the percentage of apoptotic cells was glucose concentration-dependent, because introduction to the culture medium of 50 μM D-600 or 200 μM diazoxide, which blocked glucose- and tolbutamide-induced [Ca\(^{2+}\)]\(_i\), increase, inhibited apoptosis. Thus, this study shows for the first time that high glucose concentrations and tolbutamide induce apoptosis in pancreatic β-cells, and that this process is Ca\(^{2+}\)-dependent.

Glucose is the principal regulator of pancreatic β-cell function. Metabolism of glucose in the pancreatic β-cell leads to generation of ATP, closure of ATP-regulated K\(^+\) channels (K\(_{\text{ATP}}\) channels), plasma membrane depolarization, opening of voltage-dependent Ca\(^{2+}\) channels, increase in cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)), and thereby insulin release (1). Long term exposure to a hyperglycemic environment leads to β-cell dysfunction (2–6), so-called glucose toxicity (7–9), and glucose desensitization (10, 11). The persistent hyperglycemia, which is a feature of diabetes mellitus, may also cause eye, nerve, and kidney microvascular complications.

Despite the convincing evidence for glucose toxicity in pancreatic β-cells, resulting in cell death, the underlying mechanisms have not yet been identified. Cell death could be categorized as either necrotic (accidental) or apoptotic (programmed). Apoptosis is a mechanism of self-destruction, and a variety of stimuli and processes have been shown to induce apoptosis. Among those are DNA damage, growth factor deprivation, environmental toxins, heat shock, viral infections, and immune response (12).

The aim of the present study was to investigate whether glucose and the antidiabetic sulfonylurea drug, tolbutamide, can trigger apoptosis of pancreatic β-cells.

EXPERIMENTAL PROCEDURES

Materials—RPMI 1640 medium and fetal calf serum were obtained from Life Technologies, Inc. IL-1β, tolbutamide, bovine serum albumin, aurintricarboxylic acid (ATA), diazoxide, palmitate, and oleate were purchased from Sigma. The fluorescent dyes fluorescein isothiocyanate (FITC), propidium iodide (PI), HOECHST 33342 (HO 342), and fura-2 acetoxyethyl ester were from Molecular Probes. The kit for TUNEL reaction was received from Boehringer Mannheim. All other reagents were of analytical grade and obtained from MERCK.

Isolation of Pancreatic Islets and β-Cells—10–12-month-old obese (ob/ob) mice, obtained from a local colony, as well as 2–3-month-old Wistar rats were used. Pancreatic islets from ob/ob mice contain approximately 90% β-cells (13). Islets were isolated by collagenase digestion and dispersed into small β-cell clusters in Ca\(^{2+}\)- and Mg\(^{2+}\)-deficient medium, as described previously (14). Cells were cultured on glass coverslips in plastic Petri dishes for 40 h in RPMI 1640 medium containing different concentrations of glucose and other test substances, 10% (v/v) fetal calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin. Fatty acids (sodium salts) were dissolved in 95% ethanol before being added to the culture media, as described previously (15). The final concentration of ethanol in the medium was 1.25% (v/v) at the concentration of 0.25 mM palmitate and 0.125 mM oleate. Control incubations with 1.25% ethanol did not affect the apoptotic process.

Fluorescence Microscopy Analysis of Cell DNA Staining with HOECHST 33342—The percentages of dead cells were assessed in single cell preparations on glass coverslips after a culture period of 40 h. For this purpose, pancreatic β-cells were exposed to the DNA binding dye HO 342 at a concentration of 20 μg/ml for 10 min at room temperature. HO 342 freely passes the plasma membrane of intact cells as well as damaged cells and stains DNA blue. Morphological changes corresponding to dead cells were visualized using an inverted fluorescence microscope with a mercury lamp in combination with excitation filter 340–380 nm and emission filter >430 nm.

DNA Fragmentation Analysis by Gel Electrophoresis—Pancreatic islets were cultured 40 h in a medium containing different concentrations of glucose and other substances. After this period islets were dispersed into a suspension of β-cells as described above. Aliquots from the cell suspensions containing 1 × 10⁶ cells were disrupted by resuspension in ultrapure water containing 10 mg/ml RNase and incubated for 20 min at room temperature. Loading buffer was added to this suspension.
Electrophoresis was run with a constant voltage of 20 V overnight and then 90 V for 1 h on 1.8% agarose gels containing proteinase K. The mixture of pBR 328 DNA-BgI and pBR 328 DNA-HinII was used as a marker. The separated DNA was stained with ethidium bromide, visualized by UV light, and photographed.

**TUNEL Labeling of Pancreatic β-Cells and Double Staining for Confocal Microscopy**—TUNEL technique was used to detect DNA strand breaks in situ. After 40 h of incubation, pancreatic β-cells were washed with phosphate-buffered saline and fixed in 80% methanol at 4 °C. After rinsing with phosphate-buffered saline, cells were covered with 100 μl of reaction mixture for TUNEL enzymatic reaction and were incubated at 37 °C for 1 h. The reaction was stopped by adding a buffer containing 300 mM NaCl and 30 mM sodium citrate. Apoptotic cells were determined using the combination of two fluorescent dyes, FITC and PI. Cells doubled stained with FITC and PI were fixed on glass slides with 50% glycerol in phosphate-buffered saline. Fluorescence was monitored with a Leica TCS NT laser-scanning confocal microscope (Leica Laser-technik GmbH, Heidelberg, Germany), with excitation from the 488-nm line of an argon/krypton laser. Fluorescence emission was detected with a bandpass filter (from Chroma Technology Corp.) centered at 530 nm for FITC and above 590 nm for PI. The pinhole was set to give a confocal section thickness of 1 μm, using a 25 × 0.75 PL Fluoritar oil-immersion objective.

**Measurements of \([\text{Ca}^{2+}]_{\text{i}}\)**—Cells were loaded with 2 μM \([\text{Ca}^{2+}]_{\text{i}}\) fluorescent indicator fura-2 acetoxymethyl ester at 37 °C for 30 min in a basal buffer containing: 125 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl₂, 1.28 mM CaCl₂, 25 mM HEPES, 3.3 mM glucose, and 0.1% bovine serum albumin, pH 7.4. The coverslips formed the bottom of an open chamber and were perfused with the buffer and other additions mentioned in the figure legends at a perfusion rate of 150 μl/min at 37 °C. Measurements of the fluorescence ratio \(F_{\text{340}}/F_{\text{380}}\), reflecting \([\text{Ca}^{2+}]_{\text{i}}\), were made as described previously (16).

**Presentation of Results**—Confocal images were processed using a Corel Photo-Paint and Corel Draw program. Data analysis were performed using the program Sigma Plot for Windows (version 4, Jandel Corp.). Given protocols were tested in pancreatic β-cells from 3–5 preparations from different animals. The statistical significance between means was assessed by Student’s t test for unpaired values.

**RESULTS**

To evaluate the effect of glucose on pancreatic β-cell survival, the cells were incubated for 40 h in RPMI 1640 medium with 0, 3, 5, 8, 11, 14, 17, 20, 23, and 27 mM glucose and then examined for nuclear DNA staining with fluorescent probe HO 342. As shown in Fig. 1, the absence of glucose in the culture medium gave rise to pronounced cell death. β-Cell death was minimal at 8–11 mM glucose and increased at higher concentrations (Fig. 1). Because RPMI 1640 medium with 11 mM glucose was shown to be optimal for culturing of pancreatic β-cells, this condition was chosen as the control.

For evaluation of apoptotic changes after exposure to high glucose concentration, analysis of pancreatic islet DNA fragmentation by gel electrophoresis as well as by TUNEL labeling and double staining of pancreatic β-cells for confocal microscopy were performed.

The extent of DNA laddering is shown in Fig. 2. There was no detectable DNA laddering in gel electrophoresis after incubation of ob/ob mice pancreatic islets in RPMI 1640 medium with 11 mM glucose (standard culture conditions) for 40 h (Fig. 2, first lane). The addition of 100 units/ml IL-1β, a well known inducer of apoptosis in β-cells (17, 18), led to characteristic apoptotic DNA laddering pattern (Fig. 2, second lane) and was used as a positive control for evaluation of apoptosis in our system. The incubation of pancreatic islets in medium containing 17 mM glucose (Fig. 2, third lane) or 27 mM glucose (Fig. 2, fourth lane) during 40 h led to DNA laddering, suggesting that long term exposure to high glucose concentration triggered apoptosis. Interestingly, in this case 17 mM glucose induced more DNA laddering than 27 mM glucose.

It is well known that Ca²⁺ plays an important role in the regulation of the apoptotic process in multiple experimental models (19–22). To investigate whether the increase in \([\text{Ca}^{2+}]_{\text{i}}\), induced by glucose was responsible for the activation of the apoptotic process under our experimental conditions we used diazoxide, an opener of Kₐ₅₆ channels, which hyperpolarizes

![Fig. 1. Percentage of dead cells detected by staining with the DNA binding dye HO 342 using fluorescence microscopy.](http://www.jbc.org/)

**Fig. 1.** Percentage of dead cells detected by staining with the DNA binding dye HO 342 using fluorescence microscopy. The percentages of dead cells were assessed in the single cell preparations on glass coverslips after a culture period of 40 h and subsequent staining with the DNA binding dye HO 342. Dead cells were detected by their fragmented nuclei. In each condition, a minimum of 1000 cells from 3 different isolations was counted. Percentages of dead cells are expressed as mean ± S.E. Statistical significance of differences was calculated by unpaired t test. ***, p < 0.0001 relative to 0 mM glucose; **, p < 0.05 relative to 11 mM glucose; *, p < 0.0001 relative to 0 mM glucose.

![Fig. 2. Analysis of DNA fragmentation by agarose gel electrophoresis and ethidium bromide staining.](http://www.jbc.org/)

**Fig. 2.** Analysis of DNA fragmentation by agarose gel electrophoresis and ethidium bromide staining. Pancreatic islets were cultured for 40 h with 11 mM glucose (Control) or exposed to 100 units/ml IL-1β, 17 mM glucose (3rd and 5th lanes), 27 mM glucose (4th and 6th lanes), 200 μM diazoxide (7th and 6th lanes), and 100 μM tolbutamide (7th lane). DNA size markers (Marker DNA) were included. Fragmented DNA was isolated and run on a 1.8% agarose gel with the method described under “Experimental Procedures.” Results representative of four experiments are shown.
β-cell membrane and thus inhibits glucose-induced [Ca\(^{2+}\)], increase. Fig. 3 shows that the addition of 200 μM diazoxide abolished the increase in [Ca\(^{2+}\)]\(_i\), induced by 17 or 27 mM glucose. The presence of 200 μM diazoxide also decreased DNA laddering induced by 17 mM (Fig. 2, fifth lane) and 27 mM glucose (Fig. 2, sixth lane), suggesting that glucose-induced apoptosis is a Ca\(^{2+}\)-dependent process.

To study whether a [Ca\(^{2+}\)]\(_i\), elevation per se may trigger apoptosis in pancreatic islets, the sulfonylurea tolbutamide was used. Tolbutamide induces Ca\(^{2+}\) influx into β-cells by closure of K\(_{ATP}\) channels and subsequent opening of voltage-dependent Ca\(^{2+}\)-channels (23). Incubation of pancreatic islets with 100 μM tolbutamide for 40 h led to apoptotic changes, as evaluated by DNA laddering (Fig. 2, seventh lane).

To investigate whether the apoptotic changes observed in pancreatic islets also occurred in dispersed β-cells, the TUNEL labeling technique with consecutive FITC/PI double staining and subsequent confocal microscopy analysis were used. The results obtained show that after 40 h of incubation with 11 mM glucose, 7.8 ± 1.4% (n = 8) β-cells underwent apoptosis and showed nuclear condensation and chromatin compaction (Figs. 4 and 5). Exposure of dispersed β-cells to 100 units/ml IL-1β, which served as a positive control in our studies, resulted in an increased formation of apoptotic cells (Figs. 4B and 5B). A decrease in glucose concentration in the incubation medium from 11 to 5.5 mM (Fig. 4, A and H, and 5A) and an elevation from 11 to 17 or 27 mM glucose also gave an increase in the number of apoptotic cells (Fig. 4, A, C, E, and 5A). The addition of 200 μM diazoxide to the culture medium containing 17 or 27 mM glucose diminished the number of apoptotic cells almost to the control level (Fig. 4, D and F, and 5A) but did not affect the number of apoptotic cells when the culture medium contained 5.5 mM glucose (Fig. 5A).

Similar effects were observed when Ca\(^{2+}\) influx into β-cells was inhibited using D-600, a blocker of voltage-gated L-type channels (Fig. 5A). Exposure of pancreatic β-cells to 100 μM tolbutamide raised the number of apoptotic cells (Fig. 4G and 5B), the effect being completely inhibited by 200 μM diazoxide (Fig. 5B).

At a concentration of 100 μM ATA, an inhibitor of endonucleases that prevents apoptotic cell death in many systems (24), almost completely inhibited high glucose-induced apoptosis (Fig. 5A). The percentage of dead cells in the presence of ATA was significantly higher at 27 mM than at 17 mM glucose (Fig. 5C). However, the percentage of ATA-induced decrease in dead cells in the presence of 17 or 27 mM glucose was not different, 20.0 ± 3.7% and 20.1 ± 4.7% at 17 mM and 27 mM glucose, respectively, n = 4.

In a recent publication by Roche et al. (25), it was shown that long term exposure of β-INS cells to elevated glucose concentration increased anaplerosis, lipogenesis, and lipogenic gene expression. To check if an increase in apoptosis of β-cells, caused by lowering of glucose from 11 to 5.5 mM, can be overcome by other metabolic fuels such as fatty acids, experiments involving the addition of 0.25 mM palmitate and 0.125 mM oleate to the culture medium containing 5.5 mM glucose were done. The results obtained show that this mixture of free fatty acids did not significantly affect the number of apoptotic β-cells (Fig. 5A).

As mentioned above, glucose-induced apoptosis of β-cells is a Ca\(^{2+}\)-dependent process. In this context, we were interested in investigating whether there were any differences in the pattern of [Ca\(^{2+}\)]\(_i\), changes at the control glucose concentration of 11 mM and at 17 mM of the sugar (Fig. 6, Table I). The results show that elevation in glucose concentration from 3 mM to 11 and 17 mM did not significantly affect the maximal increase in [Ca\(^{2+}\)]\(_i\) (ΔR\(_{max}\)). However, the average level of [Ca\(^{2+}\)]\(_i\) at 10 min after the addition of glucose (ΔR\(_{10}\)) as well as the area under the curve for 10 min were significantly higher at 17 mM than at 11 mM glucose. Besides that, more than half of the β-cells stimulated by 11 mM glucose had pronounced oscillations in [Ca\(^{2+}\)]\(_i\). At 17 mM glucose, less than 30% β-cells were oscillating, and the oscillatory pattern was less pronounced. At basal glucose concentration (3 mM) there were practically no oscillations at all in [Ca\(^{2+}\)]\(_i\), (Fig. 6). Preincubation of β-cells for 40 h at 17 mM glucose decreased the percentage of cells responding with an increase in [Ca\(^{2+}\)]\(_i\) at 17 mM glucose compared with β-cells preincubated at 11 mM glucose, 72.4 ± 8.8 (n = 5) and 91.7 ± 4.4% (n = 11), respectively (p < 0.05).

All experiments described so far were performed using pancreatic β-cells from ob/ob mice. The data presented in Fig. 7 and 8 show that high glucose concentration and tolbutamide as well as IL-1β also induce apoptosis in pancreatic β-cells from Wistar rats.

**DISCUSSION**

The aim of the present study was to investigate whether insulin secretagogues such as glucose and anti-diabetic sulfonylurea compounds can induce β-cells apoptosis. Our study shows that glucose induces apoptosis of pancreatic β-cells from ob/ob mice as well as normal Wistar rats in vitro. The fact that the endonuclease inhibitor ATA inhibited β-cell toxicity induced by 17 or 27 mM glucose further supports the hypothesis that β-cells were dying by an apoptotic process. An interesting notion is that there was no direct correlation between the β-cell apoptosis and glucose concentration. An increase in glucose concentration from 3 to 11 mM even promoted survival of β-cells. However, further elevation of glucose concentration increased β-cell apoptosis. Hence, RPMI 1640 medium with 11 mM glucose is optimal for culturing of pancreatic β-cells, these results being in agreement with previous studies (26, 27). We have also detected more cells dying by apoptosis after culturing in medium with 17 mM compared with 27 mM glucose. The reason for this is not clear and needs further investigations. One possibility is that at 27 mM glucose there are more cells dying by necrotic cell death, which is not associated with the
typical ladder-like DNA fragmentation. Experiments with ATA (Fig. 5C) favor this suggestion.

As pointed out above, 11 mM glucose protected the \( \beta \)-cell from apoptosis. We were not able to detect apoptotic changes by gel electrophoresis in pancreatic islets incubated in medium with 11 mM glucose. However, we observed a small percentage of apoptotic cells when dispersed \( \beta \)-cells were cultured under the same conditions and apoptotic changes were evaluated by TUNEL technique. There are two explanations for the difference in the results obtained. First, techniques using fluorescent dyes are more sensitive, allowing the detection of apoptotic events before DNA fragments become detectable with DNA laddering technique. Second, islet \( \beta \)-cells are likely to be more resistant than dispersed cells to the induction of apoptotic changes.

Hoorens et al. (28) have reported that an increase in glucose concentration from 3 to 10 mM promoted the survival of human pancreatic islets in a serum-free medium, the results explained by activating synthesis of proteins that suppress a constitutive apoptotic program at elevated glucose. These data are in accordance with our findings. However, in their study there was no significant difference in the rate of cell death when glucose
concentration was increased from 10 to 20 mM. The disagreement between our finding of increased apoptosis at glucose concentrations higher than 11 mM and that of Hoorens et al. (28) may be explained by differences in culture conditions: 40 h in serum-containing medium in our study versus 8 days in serum-free medium in their study and the source of pancreatic islets.

Ca\(^{2+}\) often plays an important role in the regulation of the apoptotic process (19–21). Sustained [Ca\(^{2+}\)] increases can promote endonuclease activation (29) and apoptotic cell death in several cell systems (19–21, 30). On the contrary, in other cell systems Ca\(^{2+}\) appears not to be directly involved in the apoptotic process, and in some systems an increase in [Ca\(^{2+}\)], can even block apoptosis (31). The information on the role of Ca\(^{2+}\) in apoptosis of pancreatic \(\beta\)-cells is rather limited. In a previous study we demonstrated that serum from patients with insulin-dependent diabetes mellitus can activate Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels, resulting in the \(\beta\)-cell destruction by apoptosis (22). In the present study we have shown that high glucose concentration and tolbutamide induced apoptosis in a Ca\(^{2+}\)-dependent manner. Moreover, we have shown that blocking of the voltage-gated Ca\(^{2+}\) channels by diazoxide-induced repolarization and by D-600 inhibited apoptosis induced by both high glucose concentration and tolbutamide.

It was suggested earlier that oscillations in [Ca\(^{2+}\)], may protect against the toxic effects of elevated [Ca\(^{2+}\)], in cells (32, 33). We have compared the pattern of [Ca\(^{2+}\)], handling in pancreatic \(\beta\)-cells at different glucose concentrations with the effect of the sugar on \(\beta\)-cell survival. The obtained data show that the absence of oscillations in [Ca\(^{2+}\)], at 3 mM glucose and the rather low percentage of oscillations at 17 mM glucose correlate with the increased percentage of dead cells at these glucose concentrations. Noteworthy is that the majority of \(\beta\)-cells oscillate at the control glucose concentration of 11 mM. Although these are interesting findings, it is too early to state that oscillations in [Ca\(^{2+}\)], indeed protect the \(\beta\)-cell against glucose-induced apoptosis.

Although our study suggests a role for Ca\(^{2+}\) in apoptosis induced by high glucose concentration and tolbutamide, the reason for the increased number of apoptotic \(\beta\)-cells at low glucose concentration (3 and 5.5 mM) still remains unclear. However, experiments with diazoxide and D-600 show that at these concentrations of glucose the \(\beta\)-cell apoptosis is not dependent on Ca\(^{2+}\) influx into the cell. The increased rate of \(\beta\)-cell apoptosis at 5.5 mM glucose relative to that of 11 mM glucose is

### Table I

Comparison of the kinetic parameters of [Ca\(^{2+}\)], increase after stimulation with 11 and 17 mM glucose

| Parameter | Glucose | \(\Delta R_{\text{max}}\) | \(\Delta R_{10}\) | AUC \(_{10}\) | % of oscillating \(\beta\)-cells |
|-----------|---------|----------------|----------------|----------------|---------------------|
| 11 mM     | 14      | 0.93 ± 0.08    | 0.36 ± 0.04*   | 100 ± 3.5^b     | 57                  |
| 17 mM     | 73      | 1.03 ± 0.08    | 0.63 ± 0.05    | 122 ± 4.6       | 29                  |

\(^* p < 0.05\) vs. 11 mM glucose.  
\(^b p < 0.001\) vs. 17 mM glucose.

**Fig. 5.** Percentage of apoptotic cells detected by confocal microscopy. Several confocal images (15–35) of small clusters of pancreatic \(\beta\)-cells from \(ob/ob\) mice incubated for 40 h with different agents were collected, and the number of apoptotic cells detected by double staining FITC/PI technique (A and B) was counted. In each condition a minimum of 1000 cells from 3–8 different isolations were counted. Control cells (Control) were cultured with 11 mM glucose, A, cells were preincubated with 5.5 mM glucose, 17 mM glucose, and 27 mM glucose; additions of 200 \(\mu\)M diazoxide (DI), 50 \(\mu\)M D-600, 100 \(\mu\)M ATA, and a mixture of free fatty acids (FFA, 0.25 mM palmitate and 0.125 mM oleate) are indicated in the figure. B, incubation of pancreatic \(\beta\)-cells with 100 units/ml IL-1\(\beta\), which served as a positive control for \(\beta\)-cell apoptosis, and 100 \(\mu\)M tolbutamide (Tolb) in the absence and presence of 200 \(\mu\)M diazoxide or 50 \(\mu\)M D-600; C, effect of 100 \(\mu\)M ATA on the percentage of dead cells at 17 and 27 mM glucose, detected by staining with the DNA binding dye HO 342 using fluorescence microscopy. Data are expressed as mean ± S.E. Statistical significance of differences was calculated by unpaired t test. ^# p < 0.05, ^++; p < 0.01 relative to 11 mM glucose; $; p < 0.05, §§§, p < 0.001 relative to 17 mM glucose; xxx, p < 0.001 relative to 27 mM glucose; ¥¥¥, p < 0.001 relative to 100 \(\mu\)M tolbutamide; δδδ, p < 0.05 relative to 17 mM glucose in the presence of 100 \(\mu\)M ATA.

**Fig. 6.** Increases in [Ca\(^{2+}\)] in pancreatic \(\beta\)-cells from \(ob/ob\) mice when glucose (G) concentration was elevated from 5 mM to 17 mM (A) or 11 mM (B). The increase in fluorescence ratio \(F_{340}/F_{380}\) corresponds to increases in [Ca\(^{2+}\)]. The traces are representative of 52 (A) and 8 (B) experiments.
not likely to be accounted for by lack of metabolic fuels, because the addition of free fatty acids to the medium containing 5.5 mM glucose did not overcome apoptosis. These data correspond to what has previously been found by others concerning β-cell survival (26–28). Why is it that the survival of β-cells at 5.5 mM glucose, a concentration that the β-cell should normally encounter in vivo, is less than at 11 mM glucose? The reason for this is unclear but probably reflects the difference in signaling pathways activated in vitro and in vivo. Under in vivo conditions a number of receptor agonists work in synchrony with glucose, giving rise to a complex signaling pattern, maybe not only leading to activation of insulin biosynthesis and secretion but also to the formation of one or several survival factors protecting the β-cell from apoptosis (28).

The majority of the results obtained in this study were obtained using preparations of pancreatic islets and β-cells from ob/ob mice. Nevertheless, experiments using pancreatic β-cells from normal Wistar rats showed the same increase in β-cell apoptosis after elevation of glucose from 11 to 17 mM or addition of tolbutamide, indicating that this is a general phenomenon and not restricted to the ob/ob mouse.

We have shown that both a high glucose concentration and the sulfonylurea tolbutamide trigger apoptosis in pancreatic β-cells. Our data favor the view that glucose- and tolbutamide-induced apoptosis of pancreatic β-cells are Ca²⁺-dependent processes.

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**FIG. 7.** Confocal images of small clusters of pancreatic β-cells from Wistar rats after TUNEL labeling and double staining with FITC/PI. Pancreatic β-cells from Wistar rats were cultured for 40 h in different culture media containing 11 mM glucose (A), 11 mM glucose with 100 units/ml IL-1β (B), 17 mM glucose (C), and 100 μM tolbutamide (D). Apoptotic cells have a yellow color as a result of overlaying green FITC and red PI fluorescence. Results shown are representative of three experiments.

**FIG. 8.** Percentage of apoptotic β-cells from Wistar rats detected by confocal microscopy. Several confocal images (10–17) of small clusters of pancreatic β-cells from Wistar rats incubated for 40 h with different agents were collected, and the number of apoptotic cells detected by double staining FITC/PI technique was counted. In each condition a minimum of 800 cells from 3 different isolations were counted. Control cells (Control) were cultured with 11 mM glucose. Data are expressed as mean ± S.E. Statistical significance of differences was calculated by unpaired Student’s *t* test. ***,* *p < 0.001 relative to 11 mM glucose. Tolb, tolbutamide.
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