Autocrine Regulation of Single Pancreatic β-Cell Survival

Víctor Navarro-Tableros, M. Carmen Sánchez-Soto, Santiago García, and Marcia Hiriart

Function and survival of cells depend in part on the presence of insulin and nerve growth factor (NGF) on single adult rat pancreatic β-cell survival and hormone secretion. When NGF or insulin signaling were blocked in culture media, cell survival decreased compared with control cells, with apoptosis being the main mechanism of cell death. To further explore the role of glucose in β-cell survival, we cultured the cells for 16 h in 2.6 mmol/l glucose and observed that nearly 17% of the cells died. To further explore the role of glucose, we cultured the cells for 16 h in 2.6 mmol/l glucose and observed that nearly 17% of the cells developed apoptosis; this effect was partially prevented by NGF and almost completely inhibited by insulin treatment. A high K+ concentration had the same effect, suggesting that insulin and NGF secretion by the cells was responsible for the survival effects and not glucose per se. Blocking NGF signaling with an NGF antibody or with K252a reduced insulin biosynthesis and secretion in the cells that survived the treatment. Moreover, the functional β-cell subpopulation with a higher insulin secretion rate is more susceptible to K252a. These results further indicate that NGF and insulin play important autoregulatory roles in pancreatic β-cell survival and function and strongly suggest the need to explore new focuses in diabetes treatment. Diabetes 53: 2018-2023, 2004

Inadequate β-cell mass is a crucial factor in diabetes. Immune destruction of β-cells is the main defect in type 1 diabetes (1), whereas in type 2 diabetes, β-cell mass is decreased to some extent compared with a normal pancreas. In the latter, the remaining cells are not capable of secreting as much insulin as normal β-cells to maintain euglycemic patients (2,3). Apoptosis is the mechanism of pancreatic β-cell death in both types of diabetes (4).

Pancreatic β-cell function and survival depend on a number of intrinsic and environmental factors. Among them, it is widely accepted that glucose promotes survival and prevents apoptosis (5,6); however, this mechanism is not entirely clear.

Glucose also stimulates insulin and nerve growth factor (NGF) secretion (7). Moreover, β-cells express functional receptors for these hormones (8). Insulin and NGF receptors have tyrosine kinase activity that triggers intracellular phosphorylation cascades, including the phosphatidylinositol (PI) 3-kinase/Akt survival-signaling pathway (9–11). It is then possible that glucose regulates an autocrine pathway for β-cell survival by increasing insulin and NGF secretion.

It has been shown that insulin protects different mammalian cells from apoptosis through the activation of insulin receptors and a PI 3-kinase–dependent pathway (12,13). Moreover, it has been recently reported that an insulin analog and, with a lesser potency, exogenous insulin have anti-apoptotic activity in the rat insulinoma cell line INS-1 (14).

Some observations in β-cells also suggest an insulin autocrine regulation. For example, when insulin autoregulation is disrupted in mice homozogous for null alleles of insulin receptor substrate-2 (IRS-2−/−), the animals develop hyperglycemia associated with pancreatic β-cell failure and apoptosis (15).

It is also well accepted that NGF is important for neuronal survival, and recently it was reported that NGF withdrawal induces apoptosis in cultured human β-cells and in the βTC6-F7 cell line (10).

We have previously shown that single rat β-cells cultured at a low density (1,000 cells/cm²) lose their sensitivity to glucose because they secrete the same amount of insulin in different extracellular glucose concentrations, with increasing time in culture (16). This desensitization is not observed in higher-density cultures (10,000 cells/cm²) (17). These observations suggest that autocrine interactions among β-cells, which are increased when cell density is high, are important for the correct function of β-cells.

It is then possible that glucose-stimulated insulin and NGF secretion constitute autocrine/paracrine signals that are required to suppress apoptosis in β-cells and that deprivation of these survival signals results in activation of the apoptosis program.

We investigated the autocrine regulation of single β-cell survival by insulin and NGF. We also explored insulin biosynthesis and secretion by cultured β-cells that survived NGF withdrawal and analyzed β-cell subpopulations that lasted.

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HBSS, Hank’s balanced salt solution; HO 342, Hoechst 33342; LP, large plaque–forming; NGF, nerve growth factor; PI, phosphatidylinositol; TUNEL, Tdt-mediated dUTP nick-end labeling. © 2004 by the American Diabetes Association.

RESEARCH DESIGN AND METHODS

Reagents were obtained from the following sources: collagenase type IV from Worthington (Freehold, NJ); guinea pig insulin antiserum from Biogenesis (Sandown, NH); rabbit anti-mouse NGF 2-5-s antibody, wortmannin, BSA, Hank’s balanced salt solution (HBSS), chromium chloride, staphylococcal protein A, HEPES, pig insulin, 2.5-s NGF, trypsin, triton, sodium citrate, trypsin, tigase, and trypanosoma.
blue, Hoechst 33342 (HO 342), propidium iodide, and poly-L-lysine from Sigma (St. Louis, MO); tissue culture dishes (Corning; K252a from Alomone Labs (Jerusalem, Israel); fetal bovine serum from Equitech-Bio (Ingram, TX); guinea pig complement, RPMI-1640 salts, and penicillin-streptomycin-ampicillin B solution from Life Technologies (Grand Island, NY); and in situ cell death detection kit fluorescein and RNA PCR Core kit from Roche (Mannheim, Germany).

Pancreatic β-cell culture. Animal care was performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH number 89-23, revised 1985). Young adult male Wistar rats (250–280 g) were obtained from the local animal facility, maintained in a 14-h light (0600–2000)/10-h dark cycle, and allowed free access to standard laboratory rat diet and tap water. Animals were anesthetized with sodium pentobarbital (40 mg/kg), and after pancreas dissection, were killed by cervical dislocation.

Pancreatic β-cells were obtained with collagenase digestion, Ficoll gradient centrifugation, and mechanical dissociation in calcium-free solution, as previously described (15). Single cells were cultured in RPMI-1640 (11.6 mmol/l glucose) and supplemented with 200 units/ml penicillin G, 200 mg/ml streptomycin, and 0.5 mg/ml amphotericin B, with 1% of fetal bovine serum, for 16 h to recover from the isolation before starting the experimental procedures.

Cell viability measurement. After the recovery period, islet cells were cultured in the following conditions: 1) 11 days in different densities of 2.1 × 10^5 (low-density cultures), 8.5 × 10^5 (medium-density cultures), 1.6 × 10^6 cells/cm^2 (high-density cultures) in tissue culture dishes, and 2) cells were seeded at a low density for 12, 16, and 48 h on glass coverslips previously treated with poly-L-lysine. Cell viability was measured by trypan blue exclusion by incubating the cells for 10 min with a 0.04% trypan blue in PBS, and incubated during 90 min with the TUNEL reaction mixture at 37°C in a humid chamber protected from light. Positive cells were counted under a Nikon Axiophot inverted microscope connected to a fluorescence lamp.

Reverse hemolytic plaque assay. To identify insulin-secreting cells and measure insulin secretion by single cells, we used the reverse hemolytic plaque assay (18) as described previously (19). Briefly, medium-density cultures were exposed to K252a (200 nmol/l) for 5 days. After this period, cells were challenged for 1 h in HBSS containing 5.6 or 15.6 mmol/l glucose, in the presence of an insulin antiserum (1:20 in HBSS), and further incubated for 30 min with guinea pig complement. Insulin released during the incubation was measured by the presence of hemolytic plaques around secretary cells. The size of the plaques was measured by projecting the image on a monitor attached to a video camera and Nikon Axiovert inverted microscope, with the aid of the JAVA video analysis software (Version 1.40, Jandel Scientific, Corte Madera, CA). The plaque size was expressed as area; cells that formed plaques were counted, and the results were expressed as the percentage of insulin-secreting cells. All experiments were performed by duplicate, and at least 100 cells were counted per experimental condition. The overall secretary activity of β-cells under a given experimental condition was expressed as a secretion index, calculated by multiplying the average plaque area by the percentage of plaque-forming cells (19).

RESULTS

Troponic autocrine effects of insulin and NGF on β-cell survival. We explored the effects of inhibiting autocrine insulin and NGF regulation on β-cell survival and apoptosis in 11.6 mmol/l glucose. As shown in Fig. 1A, single cell viability in control cells was around 67% and tended to decrease with time in culture. When autocrine NGF signaling was disrupted with K252a, the viability of single cells decreased to half of that observed in control cells (Fig. 1B). Interestingly, the percentage of apoptosis did not increase within the first 12 h and nearly 90% of the cells died after 48 h in culture. We investigated if, under these conditions, cell death was apoptotic (Fig. 1B). At 12 h, apoptosis is the main mechanism of death in response to NGF or insulin deprivation in β-cells. We did not find differences between results obtained treating cells with the inhibitor of TrkA phosphorylation, K252a, or a neutralizing NGF antibody (Table 1).

Interestingly, the percentage of apoptosis did not increase within the 48 h in culture in all experimental groups; the additional reduction of viability observed in Fig. 1A corresponds to necrotic death.

We also cultured β-cells with wortmannin to explore whether autocrine modulation of β-cell survival is mediated by the PI 3-kinase pathway. Figure 1 shows that both...
cell viability and apoptosis percentage were similar to those observed with K252a.

We investigated autocrine modulation on in vitro cell survival by calculating cell viability in cells cultured in different densities for 11 days in 11.6 mmol/l glucose. Figure 2 shows that only 20% of cells cultured in a low density survived; this percentage increased by threefold in cells cultured with NGF. This difference was not observed in cells cultured at higher densities, suggesting that autocrine survival modulation by NGF is saturated in a later condition.

**Insulin and NGF prevent low glucose–induced apoptosis.** We explored the possibility that glucose itself would be a survival factor by culturing islet cells for 12 h in RPMI-1640 with 2.6 mmol/l glucose. Figure 3 shows that in this condition, nearly 17% of control cells experimented apoptotic death. When NGF or insulin was added to the culture media, apoptosis decreased by 46 and 80%, respectively, compared with control cells. Interestingly, apoptotic death in cells depolarized with potassium decreased by nearly 90%, indicating that endogenous insulin and NGF secreted in this condition is enough to prevent apoptosis.

**Blocking autocrine NGF signals decreases insulin biosynthesis and secretion.** We analyzed the effect of neutralizing NGF with a monoclonal NGF antibody on insulin biosynthesis in single β-cells cultured for 2 or 5 days. The data are means ± SE for four different experiments.

**TABLE 1**

Percentage of apoptotic cells measured with two different techniques

<table>
<thead>
<tr>
<th>Technique</th>
<th>Control</th>
<th>NGF antibody</th>
<th>K252a</th>
</tr>
</thead>
<tbody>
<tr>
<td>TUNEL</td>
<td>14.8 ± 3</td>
<td>30.2 ± 5</td>
<td>27.9 ± 3</td>
</tr>
<tr>
<td>HO-342</td>
<td>10.9 ± 5</td>
<td>27.1 ± 9</td>
<td>25.9 ± 8</td>
</tr>
</tbody>
</table>

Data are means ± SE.
Treatment of pancreatic β-cells with NGF results in a significant increase in glucose-stimulated insulin secretion, which is due to the activation of the NGF signaling pathway. This is evident from the data presented, where the insulin secretion index, in response to a 1-h challenge in 5.6 and 15.6 mmol/l glucose, decreased by 50 and 76%, respectively, in β-cells cultured with K252a, compared with control cells. This effect resulted from a decrease of both the percentage of insulin secretors and the amount of insulin secreted by single cells cultured with K252a in both glucose concentrations.

It is interesting to note that blocking NGF signaling not only reduced the capability of the cells to respond to glucose but also insulin basal secretion in 5.6 mmol/l glucose.

**Functional β-cell subpopulations.** We have previously described that NGF increases the percentage of LP cells by nearly twofold in 15.6 mmol/l glucose (20). Figure 5 shows the multimodal distribution of plaque areas of control cells that corresponds to insulin secretion of functional subpopulations of pancreatic β-cells in 15.6 mmol/l glucose (19). In contrast, in β-cells that survived K252a treatment, no LP cells can be observed.

**DISCUSSION**

The results of the present study represent, to our knowledge, the first description of an autocrine regulation of normal single rat β-cell survival directly caused by insulin. NGF secreted by β-cells is also an autocrine regulator of survival and preserves insulin biosynthesis and secretion. Moreover, analysis of the functional subpopulations of β-cells that survived NGF withdrawal showed that the subpopulation of high insulin secretors disappears when the NGF pathway is blocked. These observations may contribute toward a better understanding of the pathophysiology of diabetes, where serum insulin and NGF levels are diminished (21,22).

It has been observed that insulin secreted by β-cells can bind to membranal autoreceptors, activating intracellular signaling cascades and promoting insulin gene transcription (23) and secretion (24). Pancreatic β-cells also express the high-affinity NGF receptor TrkA (8,20,25). Among other effects, we have previously observed that exogenous NGF increases glucose-stimulated insulin secretion and content (8,26). Moreover, an increase in Na⁺ and Ca²⁺ current densities is observed in β-cells cultured for 5 days with NGF (17,27). We have demonstrated that adult rat pancreatic β-cells synthesize and secrete NGF in response to increasing extracellular glucose concentrations and to potassium-induced depolarization (7). Endogenous NGF modulates glucose-induced insulin secretion because the acute blockage of NGF signaling with K252a, or with a monoclonal NGF antibody, decreases insulin secretion stimulated by glucose (20).

It has been described that glucose promotes β-cell survival. Among the explanations for this observation are that glucose suppresses a constitutive apoptotic program in β-cells (5) through a PI 3-kinase/Akt signaling pathway.

**TABLE 2**

Insulin secretion by single β-cells treated for 4 days with K252a

<table>
<thead>
<tr>
<th>Glucose (mmol/l)</th>
<th>% of plaque-forming cells</th>
<th>Plaque area (μm²)</th>
<th>Secretion index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 5.6</td>
<td>40 ± 2</td>
<td>2,536 ± 447</td>
<td>1,014 ± 161</td>
</tr>
<tr>
<td>5.6</td>
<td>2,536 ± 447</td>
<td>1,014 ± 161</td>
<td></td>
</tr>
<tr>
<td>15.6</td>
<td>56 ± 1*</td>
<td>4,441 ± 214*</td>
<td>2,487 ± 130*</td>
</tr>
<tr>
<td>K252a 5.6</td>
<td>33 ± 3*</td>
<td>1,528 ± 234*</td>
<td>504 ± 82*</td>
</tr>
<tr>
<td>5.6</td>
<td>36 ± 3*</td>
<td>1,653 ± 238†</td>
<td>595 ± 146†</td>
</tr>
</tbody>
</table>

Data are means ± SE. *P < 0.01 with respect to 5.6 mmol/l glucose; †P < 0.01 with respect to 15.6 mmol/l glucose.
We observed that when insulin is neutralized with a polyclonal antibody or the NGF pathway is disturbed, in the presence of 11.6 mmol/l glucose, the viability of cells decreases with time in culture and that most of the cells die by an apoptotic mechanism. Moreover, when cells are cultured in 2.6 mmol/l glucose, apoptotic death can be partially prevented with NGF and almost completely with insulin. Interestingly, high K⁺ depolarization, which stimulates insulin and NGF secretion, almost completely prevented apoptosis.

Based on these observations, we consider that the effects of glucose on cell survival could be mainly mediated by an autocrine loop of insulin and secondarily by NGF secretion. This consideration is also supported by the observation that NGF increases cell survival in low-density cultures, probably because, in this condition, the amount of NGF secreted by β-cells is not enough to maintain them. In fact, it has been shown that NGF increases β-cell survival through inhibition of apoptosis (10). It is then possible that a critical β-cell mass is required to reach an optimal concentration of insulin and NGF, which exert a positive feedback for β-cell function and survival.

IGF-I is also considered a survival factor that has a widespread antiapoptotic effect on many death signals (28). TrkA, insulin, and IGF receptors are different proteins that have tyrosine kinase activity. When the ligand binds to the receptor, it autophosphorylates on tyrosine residues and activates, initiating cascades of protein phosphorylation. The intracellular signaling cascade of insulin and NGF converge in downstream-located effector proteins, such as PI 3-kinase/Akt, which are associated with the antipoptotic systems in different cell types (9–11). We observed that treatment of cells with the PI 3-kinase blocker wortmannin decreases cell viability and increases the percentage of apoptotic cells, compared with control treated with K252a, which suggests that the trophic effect of NGF on β-cell survival is mediated by the activation of PI 3-kinase; however, we cannot discard the possibility of activation of other survival pathways.

The insulin secretion index in cells that survived NGF withdrawal decreased in both glucose concentrations (5.6 and 15.6 mmol/l). This result can partially be explained because insulin mRNA declined by nearly 40% in cells treated with K252a. It has been shown that there are functional subpopulations of adult rat β-cells (19,29,30). When heterogeneity is studied with the reverse hemolytic plaque assay, we observe that under the same stimulus, one subpopulation of β-cells secretes more insulin (LP cells) than the other one (small plaque cells). It is important to note that LP cells are responsible for nearly 75% of the insulin secreted (18). We have previously observed that LP cells are preferentially modulable by NGF (20). In this study, we demonstrate that the LP subpopulation is more sensitive to NGF withdrawal than the low-rate secreters (small plaque). Moreover, increasing glucose concentrations result in recruitment of β-cells into the secretory pool (19,30). This indicates that the gland has a large reserve of secretory capacity that can be recruited when glucose remains high, for example, in insulin-resistant conditions. It could then be possible that after a prolonged period of hyperglycemia, β-cells with the highest secretion rate become exhausted and type 2 diabetes develops.

The autocrine regulation of β-cell survival has important consequences for understanding β-cell dysfunctions in diabetes and may suggest new means of therapeutic intervention by trying to preserve plasmatic insulin near to normal values in the first stages of type 1 diabetes. Moreover, transplantation of pancreatic islets is a potential treatment for patients with type 1 diabetes; however, a limiting factor for success is insufficient insulin secretion from grafted islets. It has been observed that treatment with NGF and vascular endothelial growth factor increases survival of grafted islets, as well as their reinnervation (31). It is also possible that transplanted islets would exhibit better survival and physiology in an insulin-enriched media.

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