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Biphasic effect of insulin on beta cell apoptosis depending on glucose deprivation

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Abstract **Insulin resistant states are associated with an increase in the beta cell mass and also high levels of circulating insulin. Ultimately the beta cells undergo a failure that leads to diabetes. At this stage, a question arises if those persistent high levels of circulating insulin may contribute to beta cell damage. To address this important issue, we submitted beta cells to a prolonged effect of increasing concentrations of insulin. We observed that a prolonged effect of high levels of insulin on the presence of serum (15–24 h) in glucose-deprived beta cells induced apoptosis. This apoptotic effect was both dose- and cycloheximide-dependent.**

Keywords: Beta cell; Insulin; Glucose deprivation; Apoptosis

1. Introduction

Type 2 diabetes results from a combination of insulin resistance and impaired insulin secretion. While there is some debate about the primary defect in type 2 diabetes, insulin resistance is the most relevant pathophysiological feature in the prediabetic state [1]. Insulin resistant states are associated with an increase in the beta cell mass and also high levels of circulating insulin. In fact, owing to compensatory mechanisms, hyperinsulinemia can persist for years until glucose intolerance becomes apparent. Ultimately, the beta cells undergo a failure in the insulin secretion that leads to uncontrolled diabetes [1]. The molecular mechanisms underlying beta cell failure are not yet fully understood, it has been proposed that beta cells die by different pathways that include both glucotoxic and lipotoxic effects [2–4]. Insulin is a factor that increases cell proliferation and promotes cell survival in beta cells [5–9]. In brown adipocytes, a long-term treatment with insulin induces apoptosis [10]. Furthermore, it has been observed that insulin can induce apoptosis in chick embryo retina in the absence of L-glutamine during the development [11]. In addition, insulin and IGF-1 can induce an apoptosis-like phenomenon, named paraptosis, through IGF-1 receptor in human embryonic kidney 293T cells [12]. Overall, these data suggest that insulin can inhibit or stimulate cell apoptosis depending on the microenvironment. To address this important issue, we have submitted neonatal beta cells to a prolonged effect of increasing concentrations of insulin. Besides its survival effect, we have found that long-term treatment (15–24 h) with insulin induced apoptosis in glucose-deprived cells. Furthermore, this deleterious effect of insulin was dose-dependent, as assessed by cleaved caspase-3 expression, caspase-3 activity, cell cycle and cell survival analysis. This proapoptotic effect of insulin was also dependent on protein synthesis as the addition of cycloheximide, an inhibitor of translational elongation in eukaryotic organisms, blocked both the apoptotic effect of glucose deprivation and insulin treatment on glucose-deprived beta cells.

2. Materials and methods

2.1. Reagents

Insulin, cycloheximide, violet crystal, propidium iodide, fetal calf serum, and beta-actin antibody and glucose were from Sigma Chemical Co. (St. Louis, MO). Phospho-p70 (Thr 389), phospho-Akt (Ser 473), BclxL, and cleaved caspase-3 Asp 175 antibodies were from Cell Signaling (Beverly, MA). BclxS and bax antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Cell culture and viability

Pancreatic beta cells from IR loxP mice were generated as described in [6]. Two different beta cell pool lines were used, IR +/+ and IR −/−, both of them immortalized by transfection with attenuated SV40 large T antigen.

Exponentially growing beta cells were cultured in DMEM medium containing 10% fetal bovine serum. Beta cells were transferred to a glucose-free medium immediately before performing the different treatments. In some experiments, cells were exposed to increasing concentrations of glucose with or without the addition of insulin for 24 h. Then, cells were observed under a microscope. The addition of cycloheximide was 30 min prior to insulin or glucose treatment.

2.3. Caspase-3 activity

Cells were scrapped off, collected by centrifugation at $2500 \times g$ for 5 min, and lysed at 4 °C in 5 mM Tris–HCl, pH 8.0, 20 mM EDTA, and 0.5% Triton X-100. Lysates were clarified by centrifugation at $13000 \times g$ for 10 min. Reaction mixture contained 25 μl of cellular lysates, 325 μl of assay buffer (20 mM HEPES, pH 7.5, 10% glycerol, and 2 mM dithiothreitol) and 20 μM caspase-3 substrate (Ac-DEVD-AMC). After 2-h incubation in the dark, enzymatic activity was measured in a luminescence spectrophotometer (LS-50; PerkinElmer Life and Analytical Sciences, Boston, MA) (λ excitation, 380 nm; λ emission, 440 nm).

2.4. Protein determination

Protein determination was performed by the Bradford dye method, using the Bio-Rad reagent and BSA as the standard.

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2.5. Preparation of cytosolic extracts

At the end of the culture time, cells were scrapped off, collected by centrifugation at 2500 × *g* for 5 min at 4 °C, and resuspended in hypotonic isolation buffer (1 mM EDTA, 10 mM HEPES, and 50 mM sucrose, pH 7.6). Then, cells were incubated at 37 °C for 5 min and homogenized. Samples were centrifuged at 10000 × *g* for 10 min, and the supernatants containing the cytosolic protein fractions were collected.

2.6. Western blotting

Western blot analysis was performed as described in [6].

2.7. Violet crystal assay

Cells were grown in 12-well plates at a density of 30000 cells/cm^2 in DMEM supplemented with FBS 10%. On the next day, cells were depleted of glucose and insulin was added at different doses from 100 pM to 100 nM for 24 h for violet crystal assay. In other experiments, cycloheximide was used for 30 min before the addition of agonists, and was maintained until the end of the experiment. Cells were washed with PBS and stained with violet crystal (0.2% in 2% ethanol) for 10 min. Then, plates were rinsed with water, dried, and 1% sodium dodecyl sulfate was added. Absorbance of each plate was read at 560 nm.

2.8. Cell cycle analysis

After induction of apoptosis, adherent and non-adherent cells were collected by centrifugation and fixed with cold ethanol (70% vol/vol). The cells were then washed, resuspended in PBS, and incubated with RNAse for 30 min at 37 °C. After addition of 0.05% propidium iodide, cells were analyzed by flow cytometry.

2.9. Statistics

Statistically significant differences between mean values were determined using paired Student's *t-test*. Differences were considered statistically significant at $P < 0.05$.

3. Results and discussion

3.1. Insulin induces a biphasic effect on beta cells

Insulin is capable of inducing Akt and p70 phosphorylation in beta cells [4,13]. To assess the effect of long-term treatment with insulin on beta cell survival, we stimulated beta cells with insulin from 2 h to 24 h in the absence of glucose but in the presence of serum. Insulin 10 nM induces at short term Akt

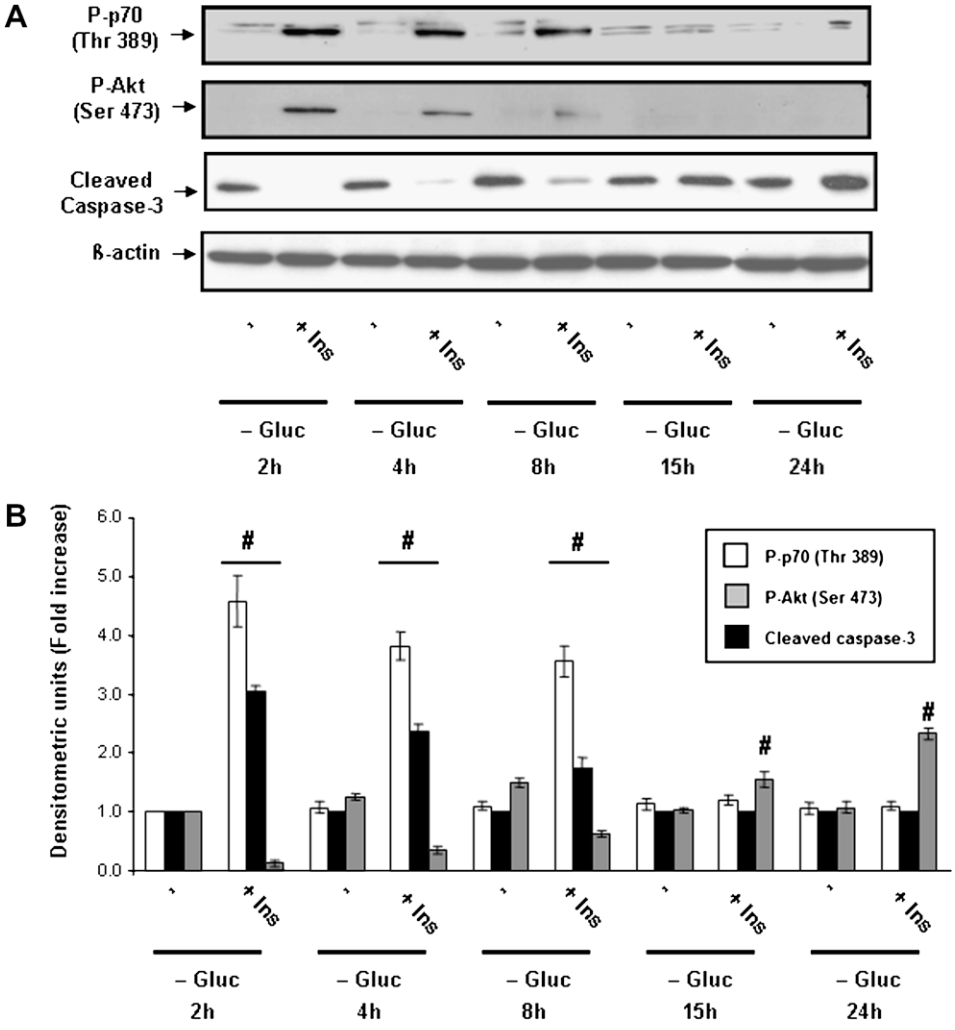

Fig. 1. Insulin induces a biphasic effect on mTOR/p70 pathway and cleaved caspase-3. (A) Beta cells were cultured in DMEM (1 g/l glucose) supplemented with FBS 10%. Beta cells were transferred to a glucose-free medium for different periods of time (from 2 to 24 h) with or without insulin 10 nM for 2–24 h. Total protein extracts were analyzed by immunoblotting with the corresponding antibodies against phospho-p70S_6K (Thr 389), phospho-Akt (Ser 473), cleaved caspase-3 and beta-actin. (B) Densitometric analysis of four independent experiments from the proteins analyzed in (A) is also shown. Results are means ± S.E.M. $^{\#}P < 0.05$ compared with their corresponding non-insulin-stimulated points.