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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

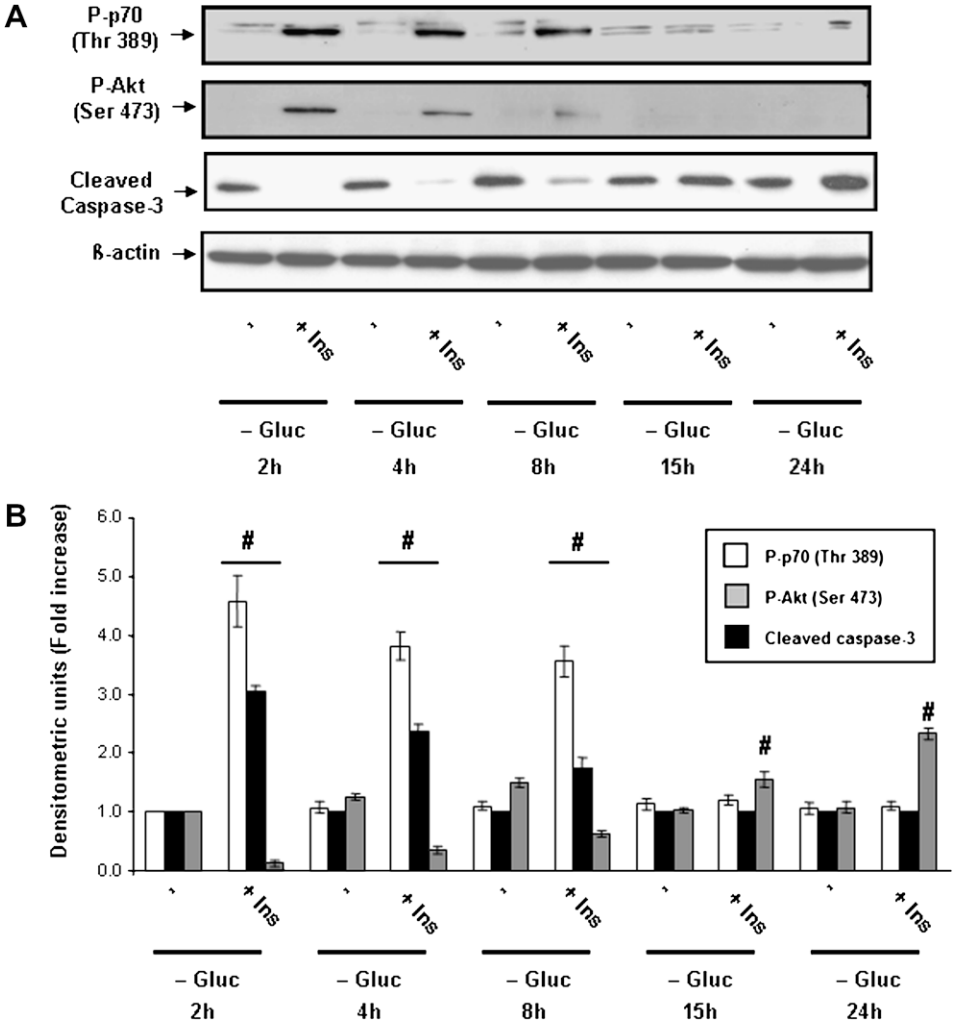

Fig. 1. Insulin induces a biphasic effect on mTOR/p70 pathway and cleaved caspase-3. (A) Beta cells were cultured in DMEM (1 g/l glucose) supplemented with FBS 10%. Beta cells were transferred to a glucose-free medium for different periods of time (from 2 to 24 h) with or without insulin 10 nM for 2–24 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.

and also p70S6K phosphorylation in beta cells, in the absence of glucose (from 2 to 8 h) (Fig. 1). However, insulin's effect on survival signaling is attenuated at 15 h and was totally impaired at 24 h (Fig. 1). In parallel, glucose-deprived beta cells showed enhanced caspase-3 in its active form (cleaved caspase-3). Under the same experimental conditions, insulin inhibited cleaved caspase-3 expression at short term, but increased its expression at long-term (Fig. 1).

3.2. Insulin induces beta cell apoptosis in both dose- and cycloheximide-dependent manner

At 24 h, glucose-deprived beta cells displayed increased caspase-3 activity, increased cleaved caspase-3 levels, an increased number of haplodiploid cells, and the total cell number was decreased (Fig. 2A–D). Under these conditions, there was a shift from inhibitory to stimulatory effects of insulin on caspase-3 activity and also haplodiploid cell number in a dose-dependent manner. Opposite effects were observed regarding beta cell number (Fig. 2B). The stimulatory effect at 24 h of high levels of insulin (from 10 to 100 nM), on cleaved caspase-3 expression, was not observed in beta cells lacking IR stimulated either with insulin at any doses used or stimulated with glucose (5–16–25 mM) (Fig. 2D). In a recent paper, it has been suggested that low nanomolar concentrations of exogenous insulin (0.2–20 nM) were effective in rescuing both human and mouse islets from serum withdrawal-induced apoptosis [14]. However, higher concentrations of insulin (200 nM) did not show additional protective effects. The ability of insulin to modulate apoptosis was assayed in groups of 3–5 islets cultured in serum-free conditions for 5–7 days [14]. This seems to point to a dual effect of insulin in the capacity of beta cell rescue. In our experiments, we are depleting the cells of glucose during a maximum period of 24 h, in contrast to the serum deprivation during 5–7 days in that paper. Furthermore, under glucose deprivation, we clearly observe a dual effect of insulin on beta cell apoptosis, including the loss of insulin's antiapoptotic effectiveness and even the potentiation of cell death. In a recent paper, it has been suggested that in conditions of energy stress, hyperactivation of mTORC1 complex, which can be mediated by growth factors, may induce apoptosis [15]. In haematopoietic cells, including lymphocytes, Glut1/Hexokinase 1 (HK-1)-expressing cells survive to IL-3 withdrawal, and this indicates that glucose and its metabolic derivatives become limiting in growth factor deprivation, and their lack contribute to apoptosis [16–18]. Accordingly, our results point out to the indispensable role of glucose to maintain cell survival in

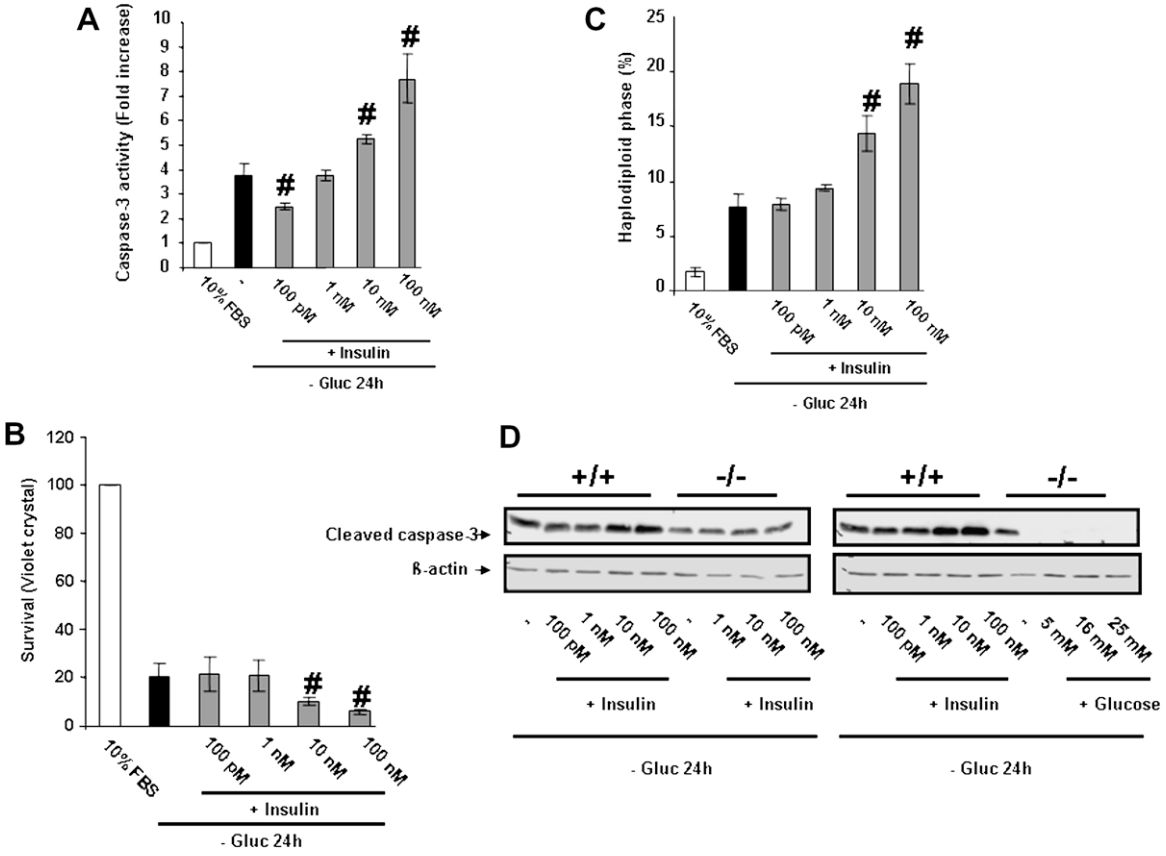

Fig. 2. Insulin stimulates apoptosis in a dose-dependent manner. (A) Beta cells were kept in DMEM in free glucose with or without different insulin doses (100 pM–100 nM) for 24 h. The caspase-3 activity was measured as detailed in materials and methods. (B) Beta cells treated with different doses of insulin from 100 pM to 100 nM. After 24 h violet crystal assay was carried as explained in material and methods. (C) Cells stimulated with different doses of insulin were analyzed by flow cytometry as described in material and methods. (D) Beta cells with insulin receptor (+/+) and without insulin receptor (−/−) were grown in DMEM supplemented with FBS 10% and during the next day, beta cells were kept in glucose-free medium with or without insulin at different doses (100 pM–100 nM) in +/+ and −/− beta cells (left panel) or with insulin in +/+ beta cells or with glucose (5–16–25 mM) in −/− beta cells (right panel) for 24 h. At the end of the experiment, cells were lysed and western-blot was used to determine cleaved-caspase-3 expression. Results are means ± S.E.M. $^{\#}P < 0.05$ compared with glucose-deprived cells.

beta cells. Furthermore, Glut 2/Glucokinase (GK) pathway drives glucose uptake in beta cells, in an insulin-independent manner. In the absence of glucose, a persistent presence of growth factors would be deleterious for the beta cells, and could stimulate apoptosis [19]. In addition, we observed that cell death induced by insulin in glucose-deprived beta cells was both caspase-8 and FAS-FADD association independent, while cytochrome *c* increased within cytosolic compartment (data not shown), indicating the involvement of the intrinsic pathway in the apoptosis of glucose-deprived beta cells.

The presence of cycloheximide (5 μg/ml) abolished caspase-3 activity, cleaved caspase-3 protein and also the number of haplodiploid cells induced by insulin treatment in glucose-deprived beta cells (Fig. 3A, B, and D). Accordingly, cycloheximide treatment increased the number of viable cells (Fig. 3C). All of these data suggest a protection role of cycloheximide in glucose-deprived beta cells either in the presence or in the absence of insulin. This is consistent with the occurrence of a protein or a group of proteins with proapoptotic function that are continuously produced when cells are glucose-deprived. In addition, insulin treatment for 15–24 h enhanced the apoptotic effect induced by glucose deprivation. Under certain conditions, this process can be suppressed by inhibitors of either RNA or protein synthesis, suggesting that its induction occurs after specific protein expression [20]. It has been shown that cycloheximide treatment can both induce or inhibit cell apoptosis depending on cell type and microenvironment [21,22]. Members of the *bcl*-2 protooncogene family encode proteins that function either to promote or to inhibit apoptosis [23,24]. Anti-apoptotic products of this gene family, such as Bcl-2 and BclxL, prevent programmed cell death in response to a wide variety of stimuli [23]. In fact, over expression of BclxL can prevent cell death in beta cells [25]. In contrast, the proapoptotic protein bax has been implicated in beta cell death in response to cytokines [26]. In a recent paper it has been demonstrated that bad and glucokinase reside in a mitochondrial complex and this seems to integrate glycolysis and apoptosis in hepatocytes [27]. Then, we analyzed the role of different proteins of bcl-2 family in the absence of glucose or in the presence of several concentrations of glucose, stimulated or not with insulin. We observed an activation of different proapoptotic proteins (cleaved caspase-3, Bax, and BclxS) and an inhibitory effect on BclxL expression in insulin-stimulated glucose-deprived beta cells (Fig. 4A). In addition, we observed by light microscopy, an increase of cell death in glucose-deprived beta cells stimulated with insulin, which was reversed by increasing glucose concentrations (Fig. 4B). All of these data suggest a proapoptotic effect of insulin in the absence of glucose and an antiapoptotic effect of insulin in the presence of different concentrations of glucose.

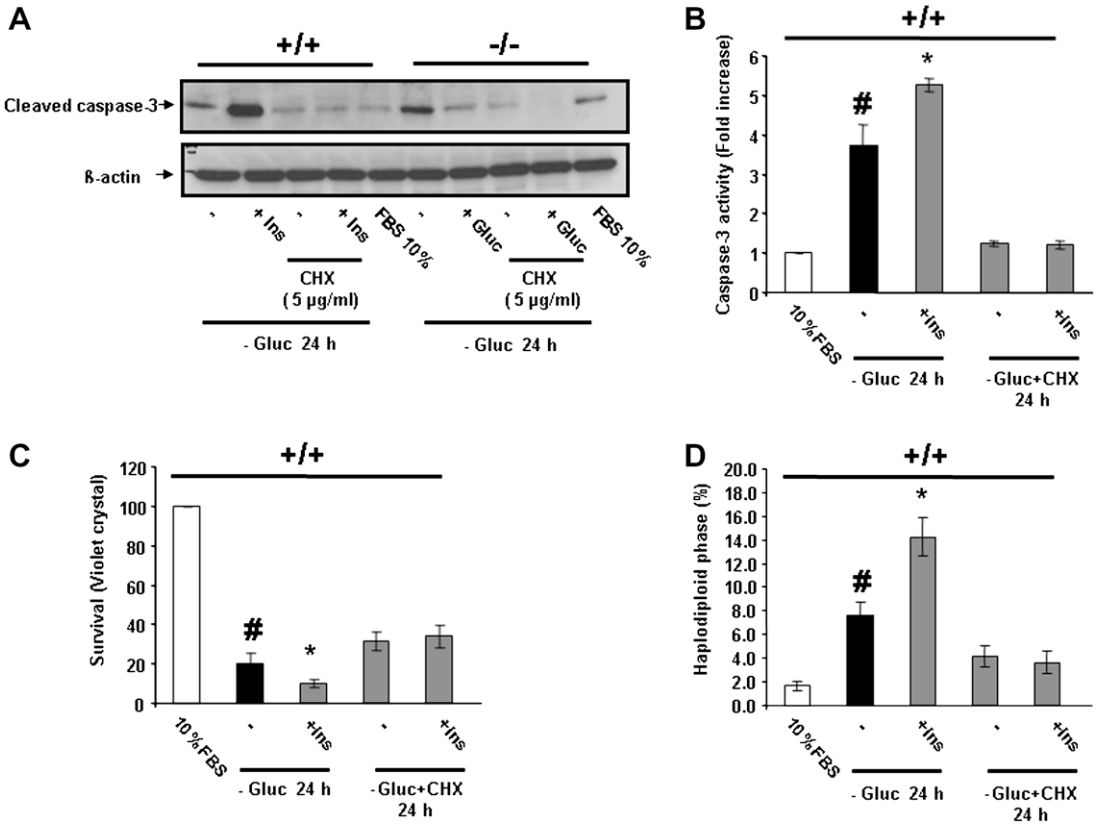

Fig. 3. Beta cell death is cycloheximide-dependent. (A) Cells were kept in DMEM in free glucose with or without cycloheximide (5 μg/ml) for 30 min before the addition of insulin 10 nM or glucose 5 mM for 24 h in +/+ or −/− beta cells, respectively. Total protein was analyzed by immunoblotting with antibodies against cleaved-caspase-3 and beta-actin. (B) Caspase-3 activity was measured as detailed in materials and methods. (C) Beta cells were grown as indicated in material and methods. Then, crystal violet assay was carried to determine the relative number of surviving cells. (D) Beta cells were analyzed by flow cytometry as described in material and methods. Results are means ± S.E.M. $^{\#}P < 0.05$ compared with 10% FBS and $^{*}P < 0.05$ compared with glucose-deprived beta cells.

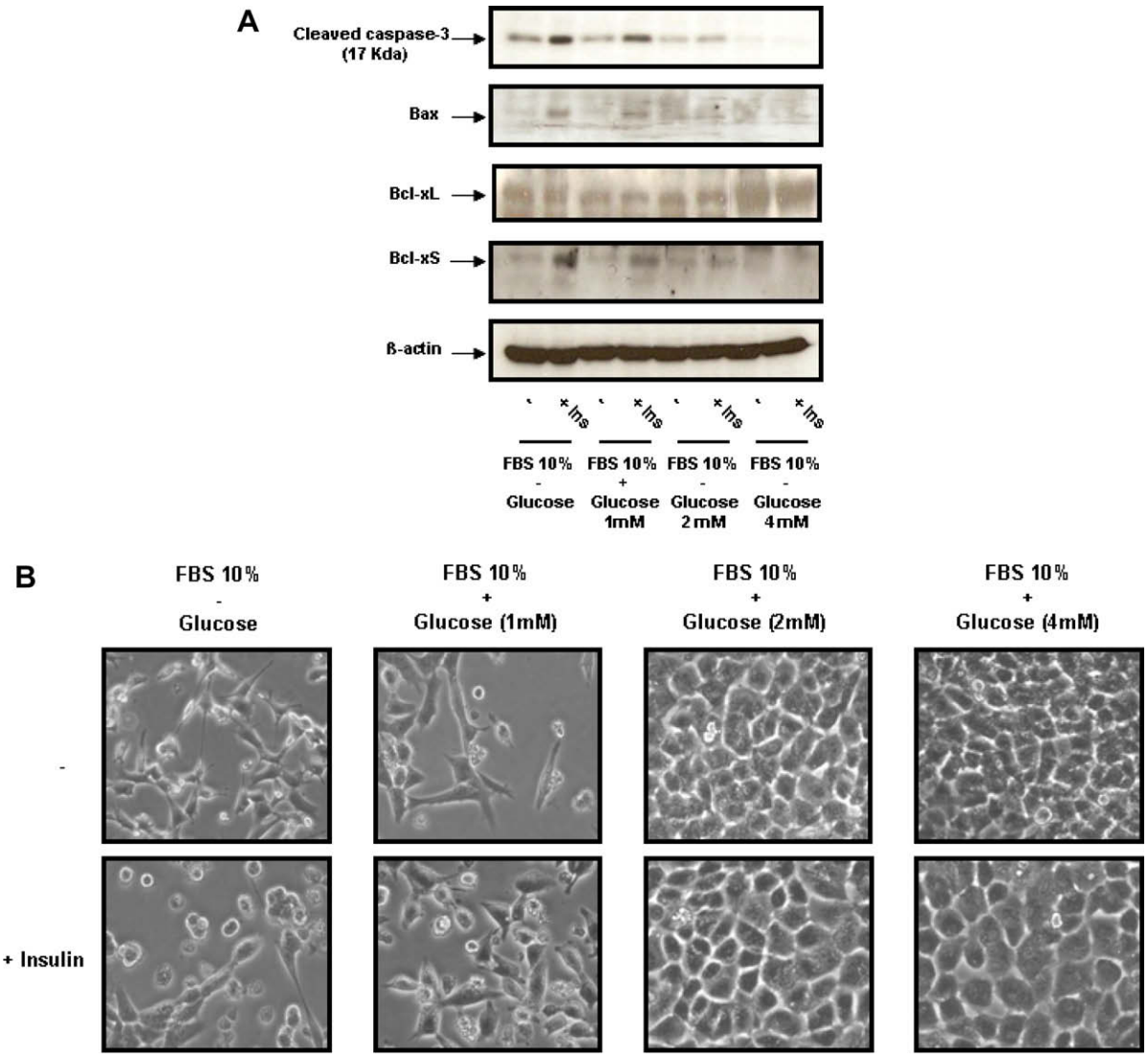

Fig. 4. Role of glucose in the apoptosis induced by insulin. (A) Beta cells were analyzed by immunoblotting with the corresponding antibodies against BclxL, BclxS, cleaved caspase-3, bax and beta-actin. (B) Representative light microscopy view of beta cells with increasing concentrations of glucose with or without insulin.

3.3. Concluding remarks

Insulin inhibits apoptosis and can induce beta cell proliferation [5–9]. In insulin-resistant states, there is a chronic compensatory hyperinsulinemia produced by pancreatic beta cells. Ultimately, there is a failure in the compensatory mechanism as the beta cell mass and function is progressively deteriorated. There is evidence that apoptosis is one of the mechanisms underlying the beta cell mass reduction, but the specific molecular processes are mostly unknown [2,28]. At this stage, we propose that insulin could be, at least in part, a possible mediator of the pancreatic beta cell apoptosis in the progress to type 2 diabetes. We observed that insulin is capable to increase both cleaved caspase-3 protein and activity, increase apoptosis, and diminish cell number. Furthermore, we observed a process that is dependent of protein synthesis. All of these data suggest that, under certain environmental conditions, the prolonged exposure to high levels of insulin may undergo pancreatic beta cell damage.

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