IGF-1 Protects Against Dexamethasone-Induced Cell Death in Insulin Secreting INS-1 Cells Independent of AKT/PKB Phosphorylation

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Abstract
Appropriate insulin secretion depends on β-cell mass that is determined by the balance between cell proliferation and death. IGF-1 stimulates proliferation and protects against apoptosis. In contrast, glucocorticoids promote cell death. In this study we examined molecular interactions of the glucocorticoid dexamethasone (dexta) with IGF-1 signalling pathways in insulin secreting INS-1 cells. IGF-1 (50 ng/ml) increased the growth rate and stimulated BrdU incorporation, while dexta (100 nmol/l) inhibited cell growth, BrdU incorporation and induced apoptosis. Dexta-induced cell death was partially antagonized by IGF-1. This protection was further increased by LY294002 (10 µmol/l), an inhibitor of PI3 kinase. In contrast, MAP kinase inhibitor PD98059 (10 µmol/l) significantly reduced the protective effect of IGF-1. The analysis of signalling pathways by Western blotting revealed that dexta increased IRS-2 protein abundance while the expression of PI3K, PKB and ERK remained unchanged. Despite increased IRS-2 protein, IRS-2 tyrosine phosphorylation stimulated by IGF-1 was inhibited by dexta. Dexta treatment reduced basal PKB phosphorylation. However, IGF-1-mediated stimulation of PKB phosphorylation was not affected by dexta, but ERK phosphorylation was reduced. LY294002 restored IGF-1-induced ERK phosphorylation. These data suggest that dexta induces apoptosis in INS-1 cells by inhibiting phosphorylation of IRS-2, PKB and ERK. IGF-1 counteracts dexta-mediated apoptosis in the presence of reduced PKB but increased ERK phosphorylation.

Introduction
There is increasing evidence that insulin and IGF-1 receptor signalling pathways are involved in the regulation of β-cell mass and that alterations in these pathways increase the incidence of diabetes mellitus [1-6]. The pivotal role of IRS-dependent signalling in β-cells was convincingly demonstrated by targeted gene disruption. The IRS-2 knockout mouse developed lethal hyperglycaemia that was rescued by β-cell specific overexpression of IRS-2 [7]. The IRS-1 knockout directed to insulin
secreting β-cells leads to impaired glucose homeostasis [8, 9]. Thereby, both, impaired glucose-induced insulin secretion and the reduction of β-cell mass contribute to these phenotypes [10].

Insulin and IGF-1 trigger autophosphorylation of their respective receptors and activates several divergent signalling cascades. One pathway involves tyrosine phosphorylation of specific insulin receptor substrates (IRS-1 to IRS-4) and subsequent activation of phosphatidylinositol-3 kinase (PI3K), PDK-1 and protein kinase B (PKB/Akt), that regulates gene expression, glucose transport, glycogen synthesis and has anti-apoptotic effects [11-15]. Another pathway leads to the stimulation of the mitogen-activated protein kinase (MAPK) cascade through Grb/SOS and ras inducing gene expression and cell proliferation [16]. Reduced tyrosine kinase signalling, inappropriately high cytosolic Ca²⁺ activity during sustained hyperglycaemia and oxidative stress have been found to induce apoptotic β-cell death [17-19]. In contrast, PKB stimulation through insulin and IGF-1 receptor activation or overexpression of PKB effectively counteracts cell death [13]. The anti-apoptotic effects of PKB signalling depend on the rate and duration of activation, i.e. phosphorylation [20]. Beside PKB activation, a contribution of MAP kinase pathways, especially ERK activation, to proliferation and protection against cell death has been observed but its significance is not completely understood [20-22].

Glucocorticoids are diabetogenic agents due to the stimulation of hepatic glucose production and induction of peripheral insulin resistance. In healthy subjects, glucocorticoid treatment results in stimulation of insulin secretion [23]. However, long term treatment with immunosuppressive synthetic glucocorticoid dexamethasone induces steroid diabetes mellitus as a result of impaired insulin secretion [24-26]. Glucocorticoids have been described to inhibit insulin secretion by increasing α₂-adrenoceptor signalling [27, 28] and ß-channel activity [29] as well as by impairing insulin biosynthesis and glucose metabolism [30-32]. Previously, we showed that dexamethasone (dexamethasone) induces apoptotic cell death in insulin secreting cells, an effect that may contribute to impaired β-cell function [33]. The dexamethasone-stimulated cell death was accompanied by reduced protein levels of the anti-apoptotic protein Bcl-2 and reduced phosphorylation of BAD, changes that are known to trigger mitochondrial dependent apoptosis [33, 34]. Both proteins of the Bcl-2 family are regulated by PI3K and MAPK signalling pathways [34-37].

In the present study, we examined whether dexamethasone affects β-cell survival via interference with PKB or MAPK, or both signalling pathways. We explored the role of these pathways in the insulin secreting cell line INS-1 by manipulating the expression and activation of endogenously expressed proteins by long term treatment with glucose, the synthetic glucocorticoid dexamethasone and IGF-1 in the presence and absence of PKB and MAP kinase inhibitors.

**Materials and Methods**

Reagents

The antibody for PKB (# 06-558) was purchased from Upstate (Lake Placid, NY, USA), the antibodies for P-PKB (Ser 473, # 9271), P-ERK (Thr202/Tyr204, # 9101) and ERK (# 9102) were from Cell Signaling Technology (Beverly, MA, USA). Antibodies against PY-IRS-2 and ß-tubulin-HRP (# sc-5274HRP) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), peroxidase-linked donkey anti-rabbit IgG was from Amersham Biosciences (Buckinghamshire, UK). The IRS-2 antibody was a kind gift from M. White (Harvard Medical School, Boston, USA). Cell culture media were purchased from Gibco (Invitrogen GmbH, Karlsruhe, Germany), fetal calf serum from Biochrom (Berlin, Germany). The protein assay was purchased from Roche Diagnostic GmbH (Mannheim, Germany). All other chemicals were from Sigma (Deisenhofen, Germany) and of analytical grade unless otherwise stated.

Cell culture and cell counting

INS-1 cells (kindly provided by C. B. Wollheim, University of Geneva, Switzerland) derived from a rat insulinoma were cultured in HEPES-buffered RPMI 1640 supplemented with 10 % foetal calf serum (FCS, Biochrom, Berlin, Germany), 10 mmol/l Hepes, 2 mmol/l L-glutamine, 1 mmol/l Na pyruvate, 10 µmol/l ß-mercaptoethanol (Sigma, München, Germany) and antibiotics as described previously [29]. Unless otherwise stated the culture medium contained 10 % FCS and 11 mmol/l glucose (standard condition). For proliferation analysis, cells were seeded at a concentration of 0.2 x 10⁶ cells/11 cm² culture dish, cultured for 2 d and thereafter test substances were added with fresh medium as indicated. At the respective time points cells were detached by trypsin and counted using a hemocytometer.

Cell proliferation assay

Cells were seeded into 96 well plates in RPMI 1640 medium. They were cultured for 24 h in serum-free medium containing 5 mmol/l glucose. IGF-1 (50 ng/ml) and LY294002 (10 µmol/l) were added 1 d before dexamethasone, a well (100 µmol/l). One day later cells were labelled for 8 h with bromodeoxyuridine (100 µmol/l). Incorporated BrdU was bound by a peroxidase-coupled anti-
BrdU antibody and the immune complexes were detected and quantified in an ELISA reader.

**Cell death detection assays**

INS-1 cells were seeded onto glass cover slips coated with poly-L-ornithine (0.001%) at subconfluent densities and cultured for 2 d. Thereafter, test substances were added with fresh medium as indicated. After incubation for the appropriate time period, cells were fixed with ethanol (100 %) for 5 min at room temperature and then incubated with 4’6-diamidino-2-phenylindole (DAPI, Sigma) at a concentration of 0.1 µg/ml in ethanol for 15 min at 37°C. After washing the cells with cold PBS, the morphology of stained nuclei was analysed under an inverted fluorescence microscope (10 x objective; Nikon, Japan) using an excitation wavelength of 350 nm. Apoptotic nuclei were identified by fractionated and condensed chromatin. More than 200 cells/condition/experiment were counted and the percentage of apoptotic nuclei determined. For TUNEL staining, cells were processed as described by the provider of the kit.

**Western blotting**

Cells were cultured for 3 d, treated with test substances as indicated for each experiment and processed as described previously [33]. Briefly, cells were lysed in buffer containing 125 mmol/l NaCl, 1 % (v/v) Triton X-100, 0.5 % sodiumdeoxycholate, 0.1 % SDS, 10 mmol/l EDTA, 25 mmol/l HEPES pH 7.3, 10 mmol/l NaPP, 10 mmol/l NaF, 1 mmol/l Na-vananate, 10 µg/ml pepstatin A, 10 µg/ml aprotinin and 0.1 mmol/l PMSF. Proteins of a 10000 g supernatant were subjected to a SDS-PAGE (8-12 %) and blotted onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). All antibodies were diluted 1:1000. Quantification of Western blots was performed by measuring the O.D. of the respective protein dot on the film using a Molecular Imager Gel Doc System (Bio-Rad Laboratories).

**Statistical analysis**

Data are provided as means ± S.E., n represents the number of independent experiments. All data were tested for significance using Student’s t-test, ANOVA and Dunnett’s test as post-test. Results with p<0.05 were considered statistically significant.

**Results**

**IGF-1 inhibits while dexamethasone promotes apoptotic cell death of insulin secreting INS-1 cells**

INS-1 cells cultured under standard conditions had a proliferation rate with a doubling time of 2 d (Fig. 1A). IGF-1, 50 ng/ml for 1 d, significantly increased proliferation and BrdU incorporation (Fig. 1A and 1B). Dexamethasone, 100 nmol/l, inhibited cell proliferation by 82 % after 2 - 4 d of treatment. Dexamethasone exerted its effect through the activation of the glucocorticoid receptor as 1 µmol/l RU486, a glucocorticoid receptor inhibitor, antagonized its effect. In the presence of IGF-1, dexamethasone inhibited cell proliferation by 50 % and 78 % after 2 and 4 d, respectively (Fig. 1A). Dexamethasone inhibited IGF-1 mediated BrdU staining (Fig. 1 B). In addition, dexamethasone stimulated cell death 3-fold (Fig. 1C). IGF-1 reduced the amount of condensed apoptotic nuclei by 71 % (Fig. 1C). These observations indicate that IGF-1 and dexamethasone compete for opposing effects on signalling pathways involved in proliferation and cell death.

Fig. 1. Dexamethasone inhibits proliferation and induces apoptotic cell death. (A) INS-1 cell population doubling time was measured as described under Materials and Methods. Cells were incubated with 100 nmol/l dexamethasone, 1 µmol/l RU486 and 50 ng/ml IGF-1 as indicated. (B) BrdU incorporation was measured as described under Material and Methods after 1 d treatment. (C) Cell death is expressed as % of condensed and fractionated nuclei visualized by DAPI staining after 1 d treatment. Shown are means ± S.E. for the given number (n) of independent experiments. * denotes significant effect of dexamethasone to the respective control of untreated cells; § denotes significance to IGF-1 in control cells, # denotes significant different to dexamethasone.
The protection against dexamethasone (DEXA)-induced apoptotic cell death was mediated by IGF-1 and not by LY294002, since LY294002 augmented cell death (Fig. 2A). These results indicate that signalling pathways distinct from PI3K are effective in counteracting dexamethasone-induced cell death when PI3K is inhibited. Indeed, the inhibition of the MAPK pathway by PD98059, 10 µmol/l, reversed the protective effect of IGF-1 on dexamethasone-induced apoptosis, regardless whether LY294002 was present or not (Fig. 2C and 2D). These observations indicate that MAPK pathways are able to exert anti-apoptotic effects in INS-1 cells, especially when the PI3K pathway is concomitantly inhibited.

**Fig. 2.** Dexamethasone (DEXA)-induced apoptosis is counteracted by IGF-1 in the presence of LY294002. INS-1 cells were treated for 4 d with 100 nmol/l dexamethasone (DEXA), 50 ng/ml IGF-1, 10 µmol/l LY294002 (LY) and 10 µmol/l PD98059 (PD) as indicated. (A and C) Cell death is expressed as % of condensed DAPI-stained nuclei. Shown are means ± S.E. for the number (n) of independent experiments (B and D) INS-1 cell apoptosis estimated by TUNEL assay. Shown are representative pictures of INS-1 cells and the correspondent fluorescent stained TUNEL positive cells. * denotes significance to control; # to dexamethasone, & to IGF-1.

Dexa increases IRS-2 protein but inhibits phosphorylation of IRS-2

Next we examined whether long term treatment with dexamethasone changed the expression of components of the IGF-1/insulin signalling cascades. The quantification of cytosolic proteins on Western blots revealed that dexamethasone significantly increased IRS-2 protein by 44 ± 15 (n = 5, Fig. 2A). PI3K, PKB and ERK protein concentrations remained unchanged. To analyse whether dexamethasone affects IRS-2 stimulation, tyrosine phosphorylation of IRS-2 was examined after immunoprecipitation of IRS-2. IGF-1 mediated stimulation of IRS-2 phosphorylation was significantly reduced in dexamethasone-treated INS-1 cells. This observation indicates that dexamethasone blunts the stimulation of the IGF-1 signalling pathways by inhibition of IRS-2 phosphorylation despite increasing IRS-2 protein.

**PI3K inhibition leads to increased ERK phosphorylation in dexamethasone-treated INS-1 cells**

Since IRS-2 stimulates PI3K and the phosphorylation of PKB/Akt further experiments were performed to examine the effects of dexamethasone and kinase inhibitors, LY294002 (10 µmol/l) and PD98059 (10 µmol/l), on PKB and ERK phosphorylation (Fig. 4 and Fig. 5). IGF-1 (50 ng/ml) transiently stimulated PKB and ERK phosphorylation.
Fig. 3. Dexa increased IRS-2 protein and reduced IRS-2 tyrosine phosphorylation stimulated by IGF-1. INS-1 cells were treated with dexa for 1 d and stimulated with IGF-1 for 5 min as indicated. Shown is a representative Western blot for IRS-2, PI3K, PKB and ERK (A) and PY-IRS-2 and reblot for IRS-2 after immunoprecipitation with IRS-2 (B). Data are presented as means ± S.E. The number of independent experiments is given in each column. * denotes significance to control, # to dexa.

Fig. 4. Effect of LY294002 and PD98059 on IGF-1 stimulated PKB phosphorylation. Cells were treated with dexa (100 nmol/l) for 1 d, with kinase inhibitors (each 10 µmol/l) for 1 h before the addition of IGF-1 (50 ng/ml) for 5 min as indicated (A-D). Shown are representative Western blots and means ± S.E. for the indicated (n) number of independent experiments. (A and B) P-PKB of control INS-1 cells; (C and D) P-PKB and PKB of dexa-treated cells; (E and F) P-PKB and PKB after 1 d treatment with dexa, inhibitors and IGF-1 as indicated. * denotes significance to control, # to dexa, § to dexa + IGF-1.

phosphorylations in control cells (Fig. 4A and B, Fig. 5A and B). As expected, PI3K inhibition by LY294002 (10 µmol/l) decreased PKB but not ERK phosphorylation, while MAPK inhibition by PD98059 (10 µmol/l) reduced ERK but not PKB phosphorylation. Dexa-treatment for 1 d reduced PKB phosphorylation, but did not affect the stimulation with IGF-1, 50 ng/ml for 5 min (Fig. 4C and D) and 1 d (Fig. 4E and F). The effect of IGF-1 on PKB phosphorylation in dexa-treated cells was inhibited by LY294002 but not by PD98059. In contrast, ERK phosphorylation was not changed by dexa treatment of the cells for 1 d (Fig. 5C and D). Furthermore, IGF-1, applied for 5 min, was unable to stimulate ERK phosphorylation (Fig. 5C and D). The addition of LY294002 restored stimulation of ERK phosphorylation by IGF-1. This effect was transient and not observed after 1 d treatment with IGF-1 (Fig. 5E and F). These observations support the idea that increased ERK phosphorylation exerts anti-apoptotic effects in dexa-treated cells when PI3K is inhibited.
Discussion

This study demonstrates that dexa-induced apoptotic cell death is effectively inhibited by activation of MAPK pathways in insulin secreting INS-1 cells when PI3K is inhibited. Dexa itself interferes with IGF-1/insulin receptor signalling pathways. Dexa increased IRS-2 protein but inhibited the phosphorylation of IRS-2 and PKB/Akt (Fig. 6). Similar observations have been made in other systems. In adipocytes, dexa also increased IRS-2 protein but decreased IRS-1 [38]. The reduction of IRS-1 and insulin-stimulated phosphorylation of PKB was associated with development of insulin resistance during glucocorticoid excess in adipocytes [38, 39]. Similarly, in rat liver, dexa-treatment in vivo decreased insulin-dependent IRS-1 and IRS-2 phosphorylation, reduced PI3K signalling and PKB/Akt phosphorylation [40]. In insulin secreting cells we were unable to detect an effect of dexa treatment on IRS-1 protein and IRS-1 tyrosine phosphorylation (data not shown). The reduced phosphorylation of PKB may account for the reduced phosphorylation of the pro-apoptotic protein BAD that we observed previously and the activation of apoptotic cell death by the glucocorticoid [33].

Fig. 5. Effect of LY294002 and PD98059 on IGF-1 stimulated ERK phosphorylation. Cells were treated with dexa (100 nmol/l) for 1 d, with kinase inhibitors (each 10 µmol/l) for 1 h before the addition of IGF-1 (50 ng/ml) for 5 min as indicated (A-D). Shown are representative Western blots and means ± S.E. for the indicated number (n) of independent experiments. (A and B) P-ERK of control INS-1 cells; (C and D) P-ERK of dexa-treated cells; (E and F) P-ERK and ERK after 1 d treatment with dexa, inhibitors and IGF-1 as indicated. * denotes significance to control, § to dexa + IGF-1, & to dexa + IGF-1 + LY294002.

Fig. 6. Effects of glucocorticoids on IGF-1 dependent signalling pathways. Glucocorticoids inhibit phosphorylation of IRS-2, PKB, Bad, induce apoptosis and reduce proliferation. IGF-1 counteracts glucocorticoid-dependent apoptosis most efficiently through activation of MAPK pathways.
IGF-1 antagonized dexamethasone-induced inhibition of PKB phosphorylation, an observation that may explain the protective effect of IGF-1 on dexamethasone-induced apoptosis. This conclusion confirms studies from other groups showing that PKB exerts anti-apoptotic effects in insulin secreting cells [13, 15, 41, 42]. Paradoxically, IGF-1 dependent protection against apoptosis was not antagonized but enforced by PI3K inhibition. Thus, although dephosphorylation of PKB/Akt accompanies dexamethasone-mediated apoptosis, increased activation of MAPK pathways by IGF-1 is able to prevent cell death despite PKB inhibition. The anti-apoptotic effect of IGF-1 in the presence of PI3K inhibition suggests that PKB activity is not essential for the survival of insulin secreting cells during glucocorticoid excess.

Interestingly, it has been shown that glucocorticoids inhibit the development of the endocrine pancreas and suppress insulin gene expression through reduced PDX-1 activation [43-47]. Furthermore, dexamethasone increases suppress insulin gene expression through reduced PDX-1 activation [43-47]. This discloses the possibility of in vivo regulation of β-cell mass by glucocorticoids during embryonic development and late pregnancy. Thus, these mechanisms might not only be important for the prevention of steroid diabetes mellitus during treatment with immunosuppressive drugs but also for physiological regulation of insulin secretion in pregnancy and other metabolic situations of increased demand of insulin.

In conclusion, this study demonstrates that phosphorylation of PKB is not essential for the protection against glucocorticoid-induced cell death in insulin secreting cells.

Abbreviations

Ac-DEVD-pNA (p-nitroanilide); PKB/Akt (protein kinase B); dexamethasone; CHAPS (3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate); DAPI (4’6-diamidino-2-phenylindole); DTT (dithiothreitol); EDTA (Ethylene diamine tetraacetic acid); FCS (foetal calf serum); HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid); HRP (horse radish peroxidase); IGF-1 (insulin like growth factor 1); IRS-1, IRS-2 (insulin receptor substrate-1 (-2)); LY294002 (PI3K inhibitor); MAPK (mitogen activated protein kinase); NaPP (sodium pyrophosphate) PD98059 (MAPK inhibitor); PI3K (phosphatidylinositol 3 kinase); PMSF (phenylmethylsulfonylfluoride); SDS (sodium dodecyl sulphate); TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP nick end labelling).

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