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The neurosurvival factor Humanin inhibits β-cell apoptosis via signal transducer and activator of transcription 3 activation and delays and ameliorates diabetes in nonobese diabetic mice

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Abstract

Pancreatic β-cell apoptosis is important in the pathogenesis and potential treatment of type 1 diabetes mellitus. We investigated whether Humanin, a recently described survival factor for neurons, could improve the survival of β-cells and delay or treat diabetes in the nonobese diabetic (NOD) model. Humanin reduced apoptosis induced by serum starvation in NIT-1 cells and decreased apoptosis induced by cytokine treatment. Humanin induced signal transducer and activator of transcription 3 and extracellular signal–regulated kinase phosphorylation over a 24-hour time course. Specific inhibition of signal transducer and activator of transcription 3 resulted in nullifying the protective effect of Humanin. Humanin normalized glucose tolerance in NOD mice treated for 6 weeks, and their pancreata revealed decreased lymphocyte infiltration and severity. In addition, Humanin delayed/prevented the onset of diabetes in NOD mice treated for 20 weeks. In summary, Humanin treatment decreases cytokine-induced apoptosis in β-cells in vitro and improved glucose tolerance and onset of diabetes in NOD mice in vivo. This indicates that Humanin may be useful for islet protection and survival in a spectrum of diabetes-related therapeutics.

1. Introduction

Type 1 diabetes mellitus (T1DM) is characterized by the progressive destruction of pancreatic β-cells after lymphocytic infiltration of the islet, resulting in insulin deficiency. Interleukin (IL)-1, tumor necrosis factor (TNF)–α, and interferon (IFN)-γ are released by T cells and macrophages during this autoimmune response and are important mediators of β-cell destruction [1]. Compelling evidence suggests that apoptosis is the principal mode of β-cell death during the development of T1DM [2]. In addition, β-cell loss by apoptosis also occurs after islet graft [3,4].

Humanin is a recently discovered, 24–amino acid, potent cell survival peptide originally thought to be encoded from a region within the mitochondrial 16S ribosomal RNA. A recent report suggests the existence of 13 nuclear loci predicted to maintain the open reading frames of 15 distinct full-length Humanin-like peptides [5], thus inviting the question of the true origin(s) of Humanin peptide. Humanin was identified to be a survival factor for dying neurons by screening a complementary DNA library from brain [6] and antagonizes Bax [7] and insulin-like growth factor binding protein–3 (IGFBP-3) [8]. Additional recent work indicates that Humanin is a wide-spectrum survival factor [9]. Secondary to the prosurvival effect of Humanin observed in neuronal cells, we hypothesized that Humanin could be a survival factor for neuroendocrine β-cells. Our data demonstrate that Humanin is a survival factor for neuroendocrine β-cells and delays the onset of diabetes in nonobese diabetic (NOD) mice.

2. Methods

2.1. Cell lines and reagents

The NOD/Lt mouse–derived pancreatic β-cell line NIT-1 [10] was purchased from ATCC (CRL-2055; Rockville,

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MD) and maintained in F12K medium supplemented with 10% fetal calf serum (Life Technologies, Carlsbad, CA), 100 U of penicillin per milliliter, and 100 U of streptomycin per milliliter in a humidified environment with 5% CO_2. Antibodies against phospho–signal transducer and activator of transcription (STAT) 3 (Y705), total STAT3, phospho–extracellular signal–regulated kinase (ERK) 1/2 (T202/Y204), and total ERK1/2 were purchased from Cell Signaling Technology (Danvers, MA). The Stat3 inhibitor VII was obtained from Calbiochem (San Diego, CA). The NIT-1 cells are characterized by glucose-responsive insulin secretion and ultrastructural features of differentiated mouse β-cells. Briefly, cells (2×10^5 per well) were seeded in 24-well plates and precultured for 48 hours. Cells were changed to serum-free (SF) media overnight and exposed to IL-1β, IFN-γ, and TNF-α (Sigma, St Louis, MO). For Stat3 inhibitor experiments, cells were serum starved for 24 hours before treatment with HNG with or without Stat3 inhibitor VII as indicated. Humanin peptide was synthesized by Genemed Synthesis Biotechnologies (South San Francisco, CA).

2.2. Mice

Nonobese diabetic female mice were purchased from Taconic (Germantown, NY). The mice were housed and fed under specific pathogen-free conditions and were cared for according to an approved protocol from the University of California, Los Angeles, Animal Research Committee. Diabetes onset was diagnosed by the presence of nonfasting blood glucose greater than or equal to 300 mg/dL on 2 consecutive days using a blood glucose meter and test strip (One Touch Ultra; Lifescan, Milpitas, CA).

2.3. Humanin treatments

Nine-week-old female NOD mice underwent a 6-week injection of Humanin (0.7 mg/[kg d] intraperitoneally [IP], once a day) or saline (n = 12 per group), after which they underwent intraperitoneal glucose tolerance test (IPGTT); and pancreata were harvested and analyzed. For the diabetes prevention study, 5-week-old female NOD mice were injected with 2 mg/kg Humanin or equivalent volume saline IP per day (n = 10 per group) until the development of diabetes as described above.

2.4. Intraperitoneal glucose tolerance test

The IPGTT was performed after Humanin treatment (at 15 weeks of age, n = 6 in each group). Glucose (1.5 g/kg) was administered IP to conscious animals after an overnight fast. Blood samples were collected from the tail vein, and the blood glucose level was measured with a glucometer (One Touch Ultra, Lifescan).

2.5. Immunostaining

Pancreata were resected from 15-week-old female NOD mice after 6 weeks of IP Humanin or saline (n = 6, in each group) and fixed in 10% phosphate-buffered neutral formalin. Paraffin-embedded sections were stained with hematoxylin and eosin to observe mononuclear cell infiltration in and around the islet. The grade of insulitis was scored on hematoxylin and eosin–stained sections. For semiquantitative evaluation of infiltration, sections containing 6 or more islets were selected; and at least 50 islets per pancreas were evaluated. The degree of cellular infiltration was scored from 0 to 5 as follows: score G2 = intraislet infiltration less than 50% of the islet, without islet derangement; score G3 = extensive infiltration, greater than 50% of the islet, cell destruction, and prominent cytoarchitectural derangement. The evaluation was carried out by 2 observers.

2.6. Caspase assays

The caspase assay was done using Apo-ONE homogenous caspase -3/-7 assay (Promega, Madison, WI) and performed according to manufacturer's instructions.

2.7. Apoptosis enzyme-linked immunosorbent assay

Photometric cell death detection enzyme-linked immunosorbent assay (ELISA) (Roche Molecular Biochemicals, Indianapolis, IN) was performed to quantitate the apoptotic index by detecting the histone-associated DNA fragments (mono- and oligonucleosomes) generated by the apoptotic cells. The reaction products in each 96-well plate were read using a Bio-Rad microplate reader (model 3550-UV). Averages of the values ± SD from double absorbance measurements of the samples were plotted.

2.8. Immunoblotting

Cell lysates containing 20 μg protein were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). Membranes were blocked in 0.2% I-block (Applied Biosystems, Foster City, CA) in phosphate-buffered saline containing 0.1% Tween-20 for 3 hours at room temperature and then probed with the appropriate primary and secondary antibodies. Antibody-antigen complexes were visualized by Chemilucent enhanced chemiluminescence detection system (Millipore, Billerica, MA) and autoradiography.

2.9. Statistical analysis

Mean, SD, and statistical significance (P value) were calculated using Microsoft (Redmond, WA) Excel or GraphPad (La Jolla, CA) statistical application. Because the same numbers of test and control values were compared, a paired 2-tailed t test was used unless specified. Log-rank analysis was performed to compare diabetes incidence (hyperglycemia) of the test group with that of the control group. Fisher exact test was used for comparing the total number of infiltrated islets in the test groups vs the control group. A P value $\leq .05$ was considered significant.

3. Results

3.1. Dose-dependent protection of β-cells from serum-starvation–induced apoptosis by Humanin

Secondary to the prosurvival effect of Humanin observed in neuronal cells [8] and neuroendocrine PC12 cells [11], we hypothesized that Humanin could be a survival factor for neuroendocrine β-cells. Mouse NIT-1 insulinoma cells were serum starved for 24 hours as control, and compared with cultures coincubated with increasing doses of Humanin ranging from 1 to 10 000 nmol/L. Apoptosis was quantitated by a specific histone-associated DNA ELISA. Humanin potently protected β-cells from serum-starvation–induced apoptosis in a dose-dependent manner (Fig. 1), decreasing apoptosis by 50% in a similar manner to that for serum-starved PC12 cells [11].

3.2. Humanin reduces cytokine-induced apoptosis in β-cells

Inflammatory cell infiltration into islets in T1DM is a T-cell–mediated process. In addition, cytokines including IL-1β, TNF-α, and IFN-γ, released by T cells and activated macrophages, are present and have a role in β-cell destruction. Other groups have described apoptosis and caspase activation in response to these cytokines alone or in combination [12,13]. The NIT-1 cells were exposed to TNF-α (5 ng/mL), IL-1β (1 ng/mL), and IFN-γ (5 ng/mL) for 48 hours; and apoptosis was measured by caspase activation. All cytokines increased caspase-3/7 activation by 70% to 93% over untreated control cells (Fig. 2A). Pretreatment of cells for 2 hours with 1000 nmol/L Humanin before IFN-γ treatment (5 ng/mL) for 48 hours significantly reduced baseline caspase activation in addition to IFN-γ–induced caspase activation (Fig. 2B). Pretreatment with a similar dose

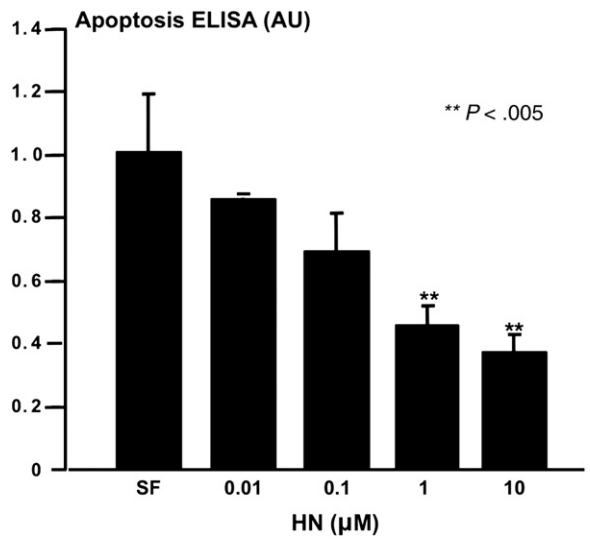

Fig. 1. Humanin protects β-cells from serum-starvation–induced apoptosis. NIT-1 cells were serum starved as control (SF) and compared with cultures coincubated with increasing doses of Humanin. Apoptosis was quantitated by specific histone-associated DNA ELISA. **$P < .05$ compared with SF.

of Humanin for 2 hours additionally significantly reduced baseline apoptosis and TNF-α–induced caspase activation after 48-hour incubation (Fig. 2C). Combination treatment with TNF-α and IFN-γ demonstrated robust caspase activation (Fig. 2D); and pretreatment with Humanin was able to significantly reduce caspase activation in these cells, albeit to a much reduced degree. Pretreatment with Humanin was ineffective in reducing caspase activation by IL-1β as a sole agent (data not shown).

3.3. Humanin activates ERK and STAT3

Previous studies implicate that neuroprotection by Humanin in F11 cells is mediated by the STAT3 transcription factor [14]. An ultrapotent Humanin analogue, HNG, has been shown to be neuroprotective in vivo at least in part by inhibiting ERK activation [15]. To investigate the mechanism of Humanin-induced β-cell survival, we treated NIT-1 cells with 100 nmol/L Humanin in SF media. Cell lysates were harvested, and phosphorylated ERK1/2 and STAT3 were assessed by immunoblotting. Phosphorylation of ERK (activation) was an early event at 15 minutes after adding Humanin in NIT-1 cells and remained elevated throughout the 24-hour time course. In addition, phosphorylation of STAT3 (activation) was a later event at 4 hours after adding Humanin in NIT-1 cells (Fig. 3A). As ERK activation has been described to decline dramatically in the 24 hours post–human, porcine, and canine islet isolation [3], this initial description of ERK activation by Humanin in β-cells is consistent with a potential role in β-cell survival.

We then asked whether a specific inhibitor of STAT3 activation and transcription of STAT3 target genes that exhibits little effect against pathways involved in Akt/mTOR, Erk1/2, or STAT5 activation [16] would have an effect on protection by Humanin from serum-starvation–induced caspase activation. As previously described in Fig. 1, treatment with 1 μmol/L Humanin decreased serum-starvation–induced apoptosis by about 50%. Cotreatment with a specific STAT3 inhibitor abolished the protective effect of Humanin (Fig. 3B), indicating that activation of STAT3 may be important in apoptosis prevention by Humanin.

3.4. Treatment with Humanin in vivo improves glucose tolerance in the NOD mouse

As proof of principle, we examined whether a 6-week course of daily IP injected Humanin (0.7 mg/[kg d]) to euglycemic 9-week-old NOD mice could improve glucose tolerance as compared with saline-injected control mice (n = 12 per group). Daily IP injections were tolerated well. There was no difference in food intake or weight in Humanin-treated vs control mice (data not shown). At the conclusion of the treatment, mice were fasted overnight and divided into groups that were subjected to IPGTT (n = 6 per group). Mice that were treated with Humanin showed a nondiabetic

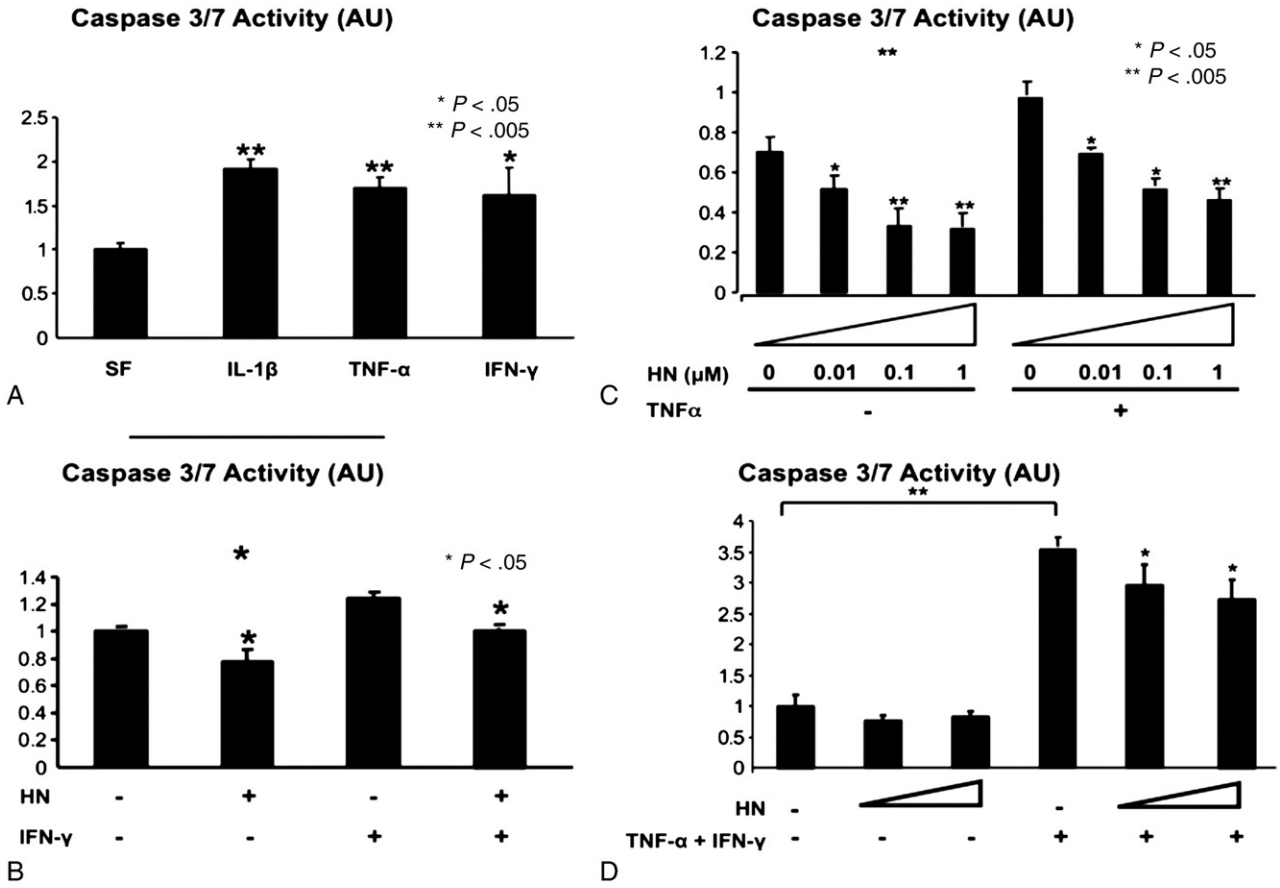

Fig. 2. Reduction of cytokine-induced apoptosis by Humanin. A, NIT-1 cells were exposed to TNF-α (5 ng/mL), IL-1β (1 ng/mL), and IFN-γ (5 ng/mL) for 48 hours; and apoptosis was measured by caspase activation. B, Pretreatment of cells for 2 hours with 1 μmol/L Humanin before IFN-γ treatment (5 ng/mL) for 48 hours. C, Pretreatment with a similar dose of Humanin for 2 hours before TNF-α (5 ng/mL) treatment. D, Humanin pretreatment (1 and 10 μmol/L) before combination treatment with TNF-α (5 ng/mL) and IFN-γ (5 ng/mL) for 48 hours.*$P < .05$, **$P < .005$ when compared with SF.

response to glucose challenge, whereas control mice showed the expected diabetic profile (Fig. 4A). Recipients of Humanin injections showed a significantly higher percentage of intact islets (G0 = 27% treated vs 5% control) with less severe infiltration (G3 = 27% treated vs 60%) compared with saline-treated control mice (Fig. 4B), suggesting that Humanin reduces islet inflammation during the development of T1DM in NOD mice.

3.5. Humanin treatment delays diabetes in NOD mice

To determine whether Humanin administration delays diabetes in NOD mice, female NOD mice (5 weeks old) were injected with 2 mg/kg Humanin or equivalent volume saline IP per day (n = 10 per group) until the development of diabetes (glucose >300 mg/dL on 2 consecutive days). There was a marked delay in the group treated with Humanin. After 20 weeks of therapy, only 40% of the control group was normoglycemic, whereas 70% of the Humanin treatment group was diabetes-free (Fig. 5, $P < .05$ by log rank). Humanin treatment reduced diabetes incidence by 50%, as only 30% of the treatment cohort became diabetic.

4. Discussion

The sustenance of viable, robust neuroendocrine β-cells remains a fundamental question that has implications for the prevention of T1DM, the treatment of T1DM postonset, islet transplantation strategies, and ultimately cure. Humanin is a 24–amino acid peptide that was cloned as a neuroprotective protein that antagonizes apoptosis-related neurotoxicity caused by Alzheimer disease–relevant insults and is being proposed as a potential therapy for Alzheimer [9]. Humanin is thought to bind cell surface receptors (formylpeptide receptor-like–1 [FPRL-1]) [17,18] and to activate kinase signaling and Stat3 [14], and has been shown to physically bind and antagonize the Bcl-2 family members Bax, Bim, and Bid [7,19,20] as well as proapoptotic IGFBP-3 [8]. Stable transfection of Humanin into K562 human leukemia cells did not reveal survival differences, suggesting that this may be a cell type–specific phenomenon [21].

A pertussis toxin-sensitive G protein–coupled receptor, human FPRL-1, has been reported to be activated by Humanin [17]. To our knowledge, this receptor has not been identified on β-cells or islets. It was thought not to be the sole

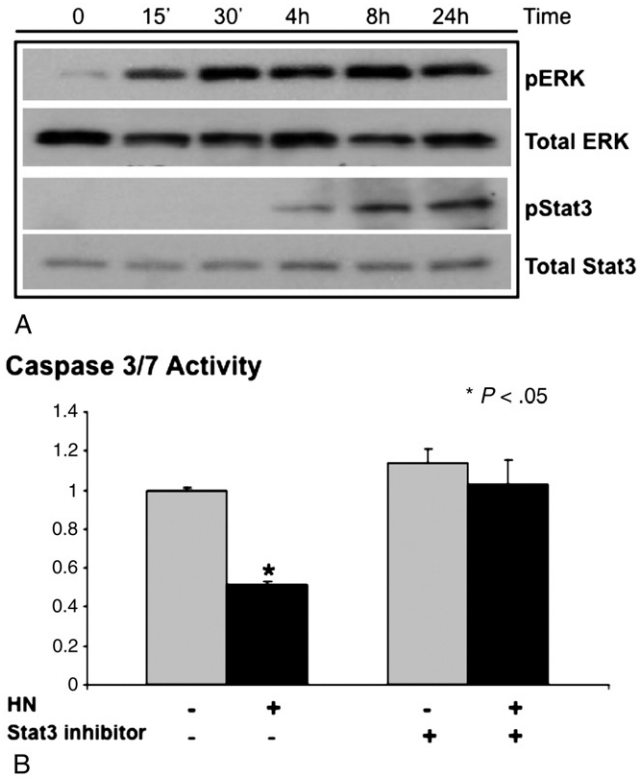

Fig. 3. Activation of ERK and STAT3 by Humanin. A, NIT-1 cells were treated with. 100 nmol/L Humanin in SF media. Cell lysates were harvested, and phosphorylated ERK1/2 and STAT3 were assessed by immunoblotting. B, NIT-1 cells were serum starved for 24 hours and treated with and without Humanin (1 μmol/L) and a specific Stat3 inhibitor (1 μmol/L) for an additional 24 hours.

receptor of Humanin binding, as a specific small interfering RNA to the mouse homologue failed to disrupt the protective effect of Humanin in rodent F11 cells [14]. Importantly, FPRL-1 is known to be an immunoregulatory receptor; and it is possible that some of the observed effects of Humanin in

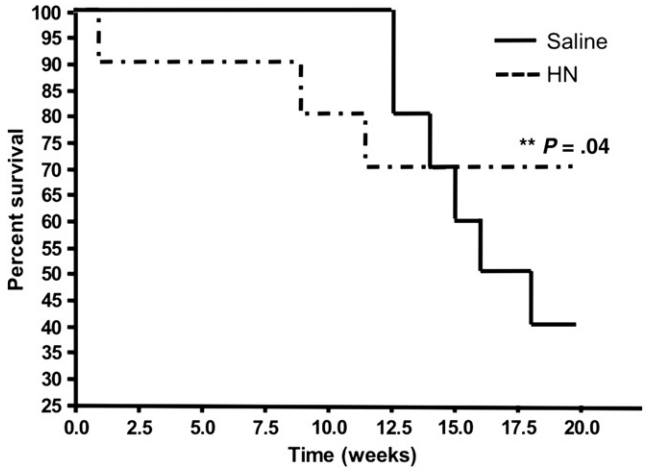

Fig. 5. Prevention of diabetes by Humanin. Female NOD mice (5 weeks old) were injected with 2 mg/kg Humanin or equivalent volume saline IP per day (n = 10 per group) until the development of diabetes (glucose >300 mg/dL on 2 consecutive days).

the 20-week prevention trial were not simply a result of increased β-cell survival, but were possibly due to modulation of the autoimmune process.

Indeed, a recent article reports binding of Humanin to a novel neurocytokine receptor complex of WSX-1, CNTFR, and gp130 [22]. This is particularly intriguing because 2 submodules, among others, of the gp130 transmembrane receptor exist, with one activating Stat3 [23] and the other activating the Ras-ERK pathway [24].

Neither FPRL-1 nor the newly described neurocytokine receptor complex has been described in β-cells as of yet, but they could well be involved in the mechanism of action described here because a Humanin/gp130 interaction is especially appealing given our observations of Stat3 and ERK activation. In our experiments, however, only Stat3 activation is necessary for the protective effect of Humanin against serum starvation.

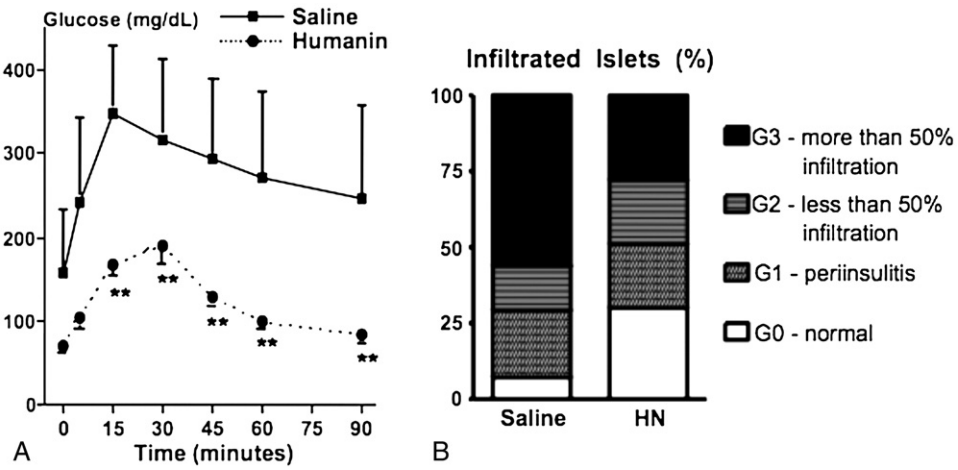

Fig. 4. Improvement in glucose tolerance in Humanin-treated NOD mice. A, Glucose tolerance testing post a 6-week course of daily IP injected Humanin (0.7 mg/[kg d]) to euglycemic 9-week-old NOD mice (n = 12 per group). B, Insulitis scoring of pancreata from Humanin-treated NOD mice.

Stat3 is recognized as an important survival signaling protein in β-cells, mediating the prosurvival effects of various growth factors and cytokines, including hepatocyte growth factor [25,26], β-cellulin [27], leukemia-inhibiting factor [28], and heparin-binding EGF-like growth factor [29]. In addition, rat acinar exocrine cells have been "reprogrammed" into β-cells by a procedure that uses epidermal growth factor and leukemia-inhibiting factor, agonists of the JAK2/STAT3 signaling pathway [28,30]. Enhanced STAT3 signaling protected β-cells from destruction induced by a genotoxic stress (streptozotocin) [31] and is a mechanism by which islet cell autoantigen 512/IA-2, an intrinsic tyrosine phosphatase–like protein of the secretory granules, activates a pathway for β-cell proliferation [32]. In addition, transduction of exogenous constitutively activated Stat3 into dispersed islets induces proliferation of rat pancreatic β-cells [33]. Clearly, Humanin activates an important β-cell survival pathway. As we have described rapid ERK activation by Humanin in β-cells, ERK activation and phosphorylation have been described to play a key role in glucose-mediated pancreatic β-cell survival [34]. In contrast, other data suggest that glucose- and IL-1β–induced β-cell secretory dysfunction and apoptosis are Ca^{+2} influx and ERK dependent in rat islets [35]. Our observed results of ERK activation are contrary to previous reports [15,21], and the role of ERK in Humanin induced cytoprotection remains unclear.

Recent studies have demonstrated that isolated human islets express the proapoptotic protein Bax at a higher level than the antiapoptotic protein Bcl-2 and suggest that the balance between prosurvival and proapoptotic molecules is one of the main mechanisms underlying islet cell death by apoptosis [36]. In addition, a recent publication describes the Bcl-2 family member Bid as being essential for death receptor– and inflammatory cytokine–induced apoptosis in β-cells [37]. This is particularly intriguing given that Humanin has been described to physically bind and inactivate both Bax and Bid [7,20]. We have not observed internalization of Humanin (data not shown), and the possibility of an intracrine mechanism of Humanin has not been explored in this system.

We have previously described the induction of proapoptotic IGFBP-3 by IL-1β in RIN cells. In addition, antisense IGFBP-3 oligonucleotides inhibit cytokine- and serum withdrawal–induced apoptosis in RIN and HIT cultures. Both exogenous IGFBP-3 and endogenous IGFBP-3 induced apoptosis in the insulin-secreting cell lines studied. IGFBP-3 aggregated in the nuclei of both RIN and HIT nuclei upon exposure to cytokines, supporting a predominantly intracellular action, consistent with data from other systems. Observations that antisense IGFBP-3 oligonucleotides inhibit cytokine-induced apoptosis, coupled with confocal data demonstrating cytokine-induced nuclear aggregation, are also consistent with an intracellular (paracrine or autocrine) mechanism for IGFBP-3–induced apoptosis in insulin-secreting cells [38]. Antagonism of IGFBP-3 by Humanin may be an additional mechanism of cell survival. However, IGFBP-3 induction was observed in cell lines and has yet to be reported in primary β-cells.

Reinnervation is another important issue in islet transplantation because transplanted islets are denervated. Studies of the endocrine response to stress in islet autografted dogs revealed differences consistent with loss of neural regulation [39]. It is an attractive hypothesis that Humanin treatment may promote reinnervation in transplanted islet grafts.

In summary, pancreatic β-cell function and survival depend on multiple intrinsic and environmental factors. It has been well documented that Humanin potentiates neuronal survival, and we have begun to study the role of Humanin on β-cell survival. We have demonstrated the utility of Humanin in potentiation of β-cell survival by the inhibition of apoptosis. Humanin is a potential treatment of evolving T1DM in the perinew onset period and delays the onset of diabetes in a genetically prone host. We therefore propose further investigation of the molecular mechanisms by which Humanin promotes β-cell survival as a potential therapeutic in the treatment/prevention of T1DM.

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