Diabetologia (2008) 51:298–308
DOI 10.1007/s00125-007-0889-4

ARTICLE

Inhibition of c-jun N terminal kinase (JNK) improves functional beta cell mass in human islets and leads to AKT and glycogen synthase kinase-3 (GSK-3) phosphorylation

A. Fornoni · A. Pileggi · R. D. Molano · N. Y. Sanabria · T. Tejada · J. Gonzalez-Quintana · H. Ichii · L. Inverardi · C. Ricordi · R. L. Pastori

Received: 12 July 2007 / Accepted: 26 October 2007 / Published online: 8 December 2007

Abstract
Aims/hypothesis Activation of c-jun N-terminal kinase (JNK) has been described in islet isolation and engraftment, making JNK a key target in islet transplantation. The objective of this study was to investigate if JNK inhibition with a cell-permeable TAT peptide inhibitor (L-JNKI) protects functional beta cell mass in human islets and affects AKT and its substrates in islet cells.
Methods The effect of L-JNKI (10 μmol/l) on islet count, mitochondrial membrane potential, glucose-stimulated insulin release and phosphorylation of both AKT and its substrates, as well as on reversal of diabetes in immunodeficient diabetic *Nu/Nu* mice was studied.
Results In vitro, L-JNKI reduced the islet loss in culture and protected from cell death caused by acute cytokine exposure. In vivo, treatment of freshly isolated human islets and diabetic *Nu/Nu* mice recipients of such islets resulted in improved functional beta cell mass. We showed that L-JNKI activates AKT and downregulates glycogen synthase kinase-3 beta (GSK-3B) in human islets exposed to cytokines, while other AKT substrates were unaffected, suggesting that a specific AKT/GSK-3B regulation by L-JNKI may represent one of its mechanisms of cytoprotection.
Conclusions/interpretation In conclusion, we have demonstrated that targeting JNK in human pancreatic islets results in improved functional beta cell mass and in the regulation of AKT/GSK3B activity.

Keywords AKT · Beta cell viability · c-jun N terminal kinase · Inflammation · Insulin production · Islet transplantation · Mitogen activated protein kinase · Pancreatic islets

Electronic supplementary material The online version of this article (doi:10.1007/s00125-007-0889-4) contains supplementary material, which is available to authorised users.

A. Fornoni (✉) · A. Pileggi · R. D. Molano · N. Y. Sanabria · T. Tejada · J. Gonzalez-Quintana · H. Ichii · L. Inverardi · C. Ricordi · R. L. Pastori (✉)
Diabetes Research Institute,
University of Miami Miller School of Medicine,
1450 NW 10th Avenue,
Miami, FL 33136, USA
e-mail: afornoni@med.miami.edu
e-mail: rpastori@med.miami.edu

A. Fornoni · T. Tejada
Division of Nephrology and Hypertension,
Department of Internal Medicine,
University of Miami Miller School of Medicine,
Miami, FL, USA

Abbreviations

7AAD	7-aminoactinomycin-D
D-JNKI	a protease-resistant form of TAT-derived PTD inhibitor of JNK
GLP-1	glucagon-like peptide-1
GSIR	glucose-stimulated insulin release
GSK-3B	glycogen synthase kinase-3 beta
IEQ	islet equivalents
IR	insulin receptor
JNK	c-jun N-terminal kinase
L-JNKI	cell-permeable TAT peptide inhibitor of JNK
LSC	laser scanning cytometry
NG	Newport Green
PP	pancreatic polypeptide
PRAS40	proline-rich AKT substrate

PTD	protein transduction domain
70S6K	70-kDa-S6 kinase
TMRE	tetramethylrhodamine ethyl ester

Introduction

In the specific area of islet transplantation, pancreas preservation, islet cell processing and implantation are stress conditions associated with the release of pro-inflammatory cytokines that can impair cell viability and function [1–4]. Although the pathways of islet loss during isolation and following cytokine-mediated injury may be different, a role for c-jun N-terminal kinase (JNK) activation has been described in both [1]. In addition, JNK plays a major role in early graft failure in experimental models of islet transplantation [5]. JNK is markedly activated in several tissues from diabetic mice [5]. Moreover, JNK inhibition markedly improved rat islet viability [6] and islet engraftment in a syngeneic animal model [6]. Thus, targeting JNK may improve functional beta cell mass via several mechanisms.

In human pancreatic islets, JNK inhibition via an ATP-competitive agent (SP600125) has been shown to preserve whole-islet mass [4]. In addition, ATP-non-competitive agents, such as small permeable TAT peptide inhibitors of JNK (L-JNKI), have been successfully used by us and others in insulinoma cell line and both murine and porcine islets [7–9]. We have previously shown that protein transduction domain (PTD) technology can be successfully applied to human islet cell biology. We found that anti-oxidative stress proteins such as haeme oxygenase-1 [10] and anti-apoptotic proteins such as B cell lymphoma associated X protein (Bcl-XL) [11] can be effectively delivered to human islets when coupled to a TAT-derived PTD. Similarly, it has recently been shown that a protease-resistant form of TAT-derived PTD inhibitor of JNK (D-JNKI) can effectively transduce rat and human islets [6]. However, PTDs have a length- and dose-dependent toxicity [12] particularly when used in the D-isoform [6, 8]. Although D-JNKI appears safer in human than in rodent islets [6], further investigations are warranted prior to their introduction to clinical islet transplantation.

In human pancreatic islets, JNK inhibition has been shown to reduce caspase-3 activity [4], but the mechanism of islet cell protection by JNK inhibitors remains unclear. Recent data suggest that JNK activation in peripheral tissues affects IRS-1 and/or AKT [13–15]. A similar regulation of AKT in islet cells would be very important, since AKT has been shown to have a strong influence on pancreatic beta cell growth, function and survival [16–18].

In the present study, we investigated whether inhibition of JNK in human islets with a TAT-JNK inhibitory peptide results in improved functional beta cell mass. We demonstrated that L-JNKI protects human islet mass in vitro and partially in vivo. In addition we showed that, under stress generated by exposure to cytokines, L-JNKI activates AKT and down-regulates glycogen synthase kinase-3 beta (GSK-3B), while other AKT substrates such as proline-rich AKT substrate (PRAS40) and 70-kDa-S6 kinase (70S6K) were unaffected.

Methods

Islet isolation and assessment of islet recovery and insulin content We obtained 15 human pancreases from deceased, multi-organ donors. Islets were isolated at the Human Cell Processing facility of our Institute using a modification of the automated method [19, 20]. Donor characteristics are shown in Electronic supplementary material (ESM; ESM Table1). Each experiment was performed on five to 11 different pancreases based on the amount of available islets. Research consents were obtained by the Organ Procurement Organization (Life Alliance Organ Recovery Agency, University of Miami, Miami, FL, USA) from the donor family or next of kin. Freshly isolated islets were treated with either TAT peptide alone or with L-JNKI (10 μmol/l; BioSynthesis, Lewisville, TX, USA). After overnight culture, islet counts were obtained and expressed as islet equivalents (IEQ) after diphenylthiocarbazone staining [21, 22]. Data were expressed as percentage of islet loss as compared with 100% placed in culture at day 0. For the assessment of insulin content, cell lysates were collected and insulin content evaluated by ELISA (Mercodia, Winston Salem, NC, USA).

Assessment of beta cell viability and mitochondrial membrane potential For the analysis of viability in the first overnight after isolation, L-JNKI (10 μmol/l) was added within 2 h of isolation and viability assessment performed 12 h after in five different preparations. For the assessment of islet viability in culture, islets were pretreated for 48 h with JNKI or with TAT peptide (10 μmol/l) based on our prior experiments in rodent islets and insulinoma cells [8]. A cocktail of cytokines (IL-1β 50 U/ml, TNF-α 1,000 U/ml and INF-γ 1,000 U/ml) was added 18 h prior to islet dissociation and analysis. Measurement of viable beta cells was performed as described [23]. Briefly, the zinc-binding dye Newport green (NG) allows identification and quantification of islet beta cells on dissociated islets, based on the abundant zinc content in beta cell secretory granules [24]. The membrane exclusion dye 7-aminoactinomycin-D (7AAD; Invitrogen, Carlsbad, CA, USA) was used as marker of cell death. Tetramethylrhodamine ethyl ester (TMRE; Molecular Probes, Eugene, OR, USA) was used to assess mitochondrial membrane potential. Single-cell suspensions from nine different preparations were incubated for 30 min at 37°C with 110 μmol/l NG and 100 ng/ml TMRE, and stained with 7AAD prior to

analysis. Data were expressed as $TMRE^+$ cells on gated NG^+7AAD^- cells (viable beta cells). Analysis of cellular composition was performed by immunofluorescence in the first five preparations and quantified by laser scanning cytometry (LSC) as previously described [23]. Briefly, dispersed cells were incubated for 1 h with the following antibodies: mouse monoclonal antibody to insulin and rabbit polyclonal antibody to somatostatin (1:100; both: Neo Markers, Fremont, CA, USA); mouse monoclonal antibody to glucagon (1:500; Sigma, St Louis, MO, USA); and undiluted rabbit polyclonal antibody to pancreatic polypeptide (PP; Bio-Genex, San Ramon, CA, USA). After washing, samples were incubated with either goat anti-mouse (Alexa Fluor 488 goat antimouse IgG) or goat anti-rabbit (Alexa Fluor 488 goat antirabbit IgG) antibodies (both from Molecular Probes). Omission of the primary antibody served as negative control.

Assessment of AKT and JNK signalling pathway activation Islets (~250 IEQ) from 11 different preparations were collected in cell lysis buffer (Bio-Rad, Hercules, CA, USA). After sonication, protein concentration was determined and endogenous AKT (S473), insulin receptor (IR) (Y1162/1163), IRS-1 (S312), PRAS40 (T246), GSK3 (S9) and 70S6K (TS421/424) phosphorylated and total protein were quantitatively determined with the Bioplex platform as per manufacturer's recommendations (Invitrogen). Data are expressed as units/μg protein for phosphorylated protein and ng/μg protein for total protein. Total and phosphorylated JNK were assessed by a semiquantitative method with Bioplex platform and GST-pull down assay (Cell Signaling, Danvers, MA, USA) and data expressed as ratio of phosphorylated to total protein. Experiments were performed in the presence or absence of two different JNK inhibitors: L-JNKI (10 μmol/l) in ten experiments or the ATP-competitive JNK inhibitor SP600125 (Sigma-Aldrich, St Louis, MO, USA), in six experiments.

Glucose-stimulated insulin release Dynamic glucose-stimulated insulin release (GSIR) was tested on day 2 after isolation in five different preparations as previously described [25]. Briefly, 100 IEQ per condition were loaded on microcolumns connected to an inflow and an outflow port of a customised perifusion system. Media of defined composition were circulated at a rate of 100 μl/min. Fractions of 100 μl were collected every minute. After 1 h pre-incubation in HEPES buffer, sequential circulation of media containing 3 mmol/l glucose (5 min), 11 mmol/l glucose (10 min), 3 mmol/l glucose (15 min), 25 mmol/l KCl (5 min) and 3 mmol/l glucose (5 min) was performed and insulin content determined by ELISA (Mercodia).

Induction of diabetes in Nu/Nu mice All animal manipulations were conducted under protocols approved by the local Institutional Animal Care and Use Committee. Recipient immunodeficient *Nu/Nu* mice (Harlan, Indianapolis, IN, USA) were rendered diabetic by a single intravenous injection of streptozotocin (200 mg/kg). Glycaemia was monitored using portable glucometers (OneTouch Ultra; Lifescan, Milpitas, CA, USA) on whole-blood samples obtained from the tail vein. Diabetes occurrence was defined as non-fasting glycaemic values >19.5 mmol/l in three consecutive readings.

Islet transplantation in Nu/Nu mice Human islets were treated with 10 μmol/l L-JNKI or with TAT-alone immediately after isolation and on days 1 and 2 of culture, prior to transplantation. Islets (either 2,000, 1,000 or 500 IEQ per mouse) were transplanted in the kidney subcapsular space of diabetic *Nu/Nu* mice under general anaesthesia [26, 27]. Subsequent experiments, where both islets and recipients were treated with L-JNKI (2 h before transplantation and daily for the following 15 days), were performed with an islet mass of 1,000 IEQ. Normoglycaemia was defined as non-fasting blood glucose values ≤11 mmol/l. Assessment of graft performance during IVGTT was performed in conscious animals at 16, 60, 120 and 127 days after transplantation. Briefly, after overnight fasting, an intravenous glucose bolus (0.5 g/kg) was administered to mice that had achieved normoglycaemia. Glycaemia was measured at baseline and over a 20 min period and AUC (in [mmol × min] dl^{-1}) during the first 10 min calculated [20]. Animals with an established graft function (120 days after transplant) received 10 mg/kg L-JNKI intraperitoneally daily for 7 days in order to assess the independent effects of L-JNKI on human islet function in vivo. Preliminary experiments were performed to study peptide transduction efficiency 2 and 24 h after transplantation of islets treated with TAT and/or TAT-FITC intraperitoneal peptide administration (10 mg/kg). Immunofluorescence stainings of graft-bearing kidneys for DAPI (Vector Lab, Burlingame, CA, USA) and insulin (see above) were performed.

Data analysis Results represent the mean of five to eleven independent experiments from different human pancreases performed in duplicate or triplicate. Results are expressed as scattered dot plots. When one-way ANOVA showed statistical significance, results were compared using *t* test after Tukey's correction for multiple comparisons (Graph Pad Prism software; San Diego, CA, USA). Statistical significance was set at $p<0.05$.

Results

L-JNKI preserves islet mass in culture We examined the loss of islet mass in the first overnight culture after iso-

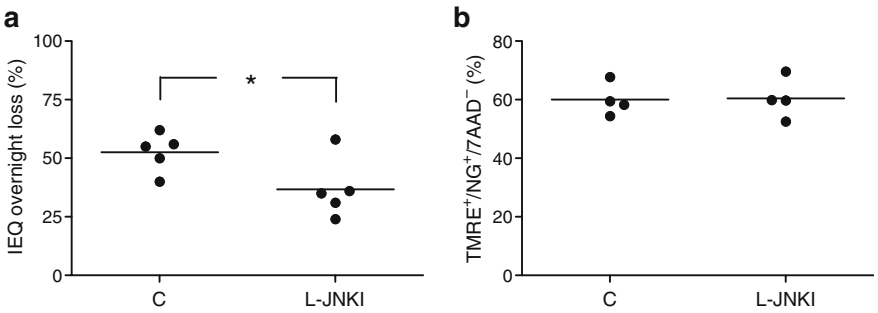

Fig. 1 Effect of L-JNKI on islet equivalents and on islet viability in the first 12 h after isolation. Islets treated immediately after isolation with L-JNKI (10 μmol/l) or TAT peptide alone (C) were compared. **a** Islet equivalent count was performed at isolation and after 18 h of culture and expressed as 100 (count at 18 h/count at 0 h), i.e. percentage of islet loss. **b** Assessment of mitochondrial membrane potential in beta cells (% of $TMRE^+$, NG^+ and $7AAD^-$ cells) was performed 12 h after treatment as in (**a**). *$p<0.05$

lation, when a strong activation of JNK and cell death pathways occurs [1, 3]. Islets were treated at isolation with 10 μmol/l L-JNKI or TAT alone. The percentage of IEQ loss after the first overnight culture was reduced from 52.6±8.2 (control group) to 36.8±12.8% (L-JNKI treated group) (Fig. 1a). Interestingly, there was no difference in the percentage of $TMRE^+$ NG^+ and $7AAD^-$ cells between control and treated islets 12 h after isolation and treatment (Fig. 1b). Furthermore, the insulin: protein ratio was not different between treated and untreated islets (3671.9± 1430.7 vs 3619.0±253.8 nmol/mg protein in L-JNKI treated islets vs control, NS).

L-JNKI protects beta cells from cytokine-induced cell death In order to understand whether L-JNKI may further protect human islet cells that survive the initial overnight culture, and given the fact that JNK activation is transiently low during islet culture at baseline [28], islets pre-incubated with L-JNKI or with TAT alone were subsequently treated for 18 h with a cocktail of inflammatory cytokines, which are able to activate JNK in selected cultured human islets (Fig. 2a,b). We first investigated the relative contribution of different cytokines to JNK phosphorylation in those selected cultures, in which we detected at least a twofold increase in JNK phosphorylation after cytokine exposure and found that IL-1β was mainly responsible for JNK phosphorylation (Fig. 2c). We found that islet pretreatment with a L-JNKI almost completely prevented cytokine-induced beta cell death (Fig. 3a). Notably, this effect appears beta-cell-specific, since no protective effect was observed in NG^- cells (Fig. 3b). Assessment of cellular composition by LSC in the overall population of dissociated islet cells (Table 1) suggested

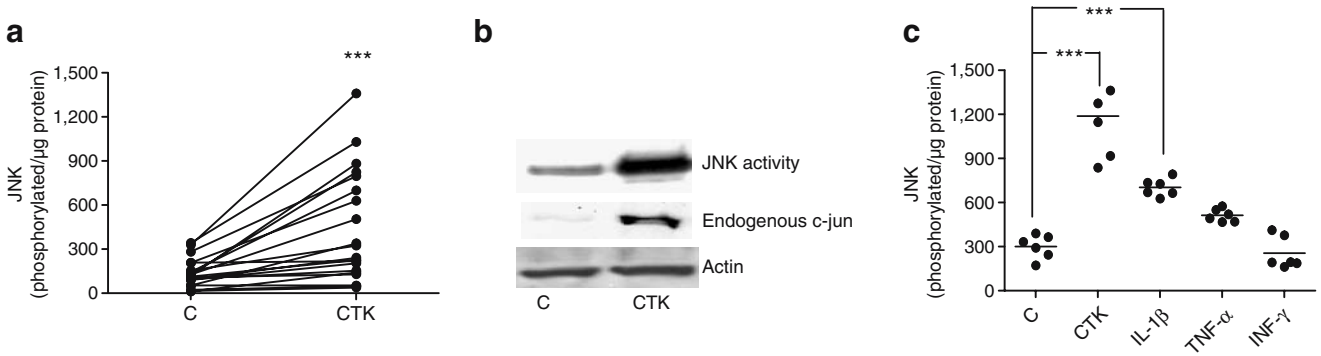

Fig. 2 Effect of cytokines on JNK activity in whole islets. **a** Human islets were cultured in the presence or absence of a cytokine cocktail (CTK; IL-1β 50 U/ml, TNF-α 1000 U/ml and INF-γ 1000 U/ml) for 18 h. Although there was a high variability from preparation to preparation, cytokine-treatment led to a strong activation of phosphorylated JNK. **b** Data obtained with Bioplex were confirmed with GST pull-down assay (JNK activity) and with western blot for endogenous c-jun normalized to actin. **c** To understand the contribution of the different cytokines to JNK activation, human islets with at least a twofold induction of JNK phosphorylation on exposure to the cytokine cocktail were treated either with the cytokine cocktail or with each cytokine alone at the same dose used for the cocktail. IL-1β was the cytokine most responsible for JNK activation in human islets. ***$p<0.001$

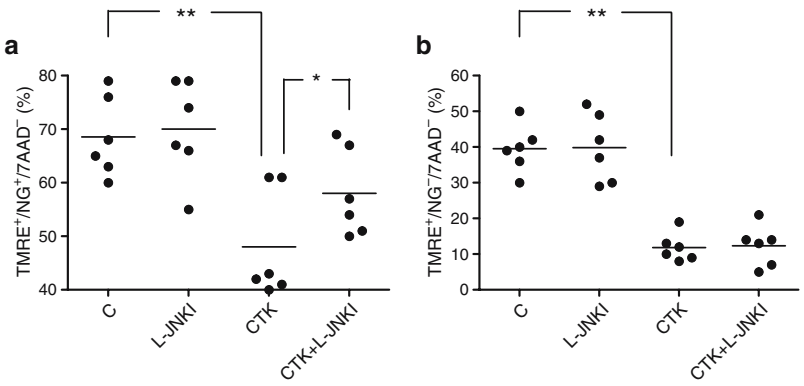

Fig. 3 Effect of cytokines and L-JNKI on mitochondrial membrane potential. Untreated human islets were compared with islets treated immediately after isolation with L-JNKI (10 μmol/l) or with TAT-peptide alone as control (C). A cytokines cocktail (CTK; IL-1β 50 U/ml, TNF-α 1,000 U/ml and INF-γ 1000 U/ml) was added to islets in culture 18 h prior to collection for assessment of mitochondrial membrane potential in beta cells. **a** Beta cell viability in % of $TMRE^+$, NG^+ and $7AAD^-$ cells; **b** non beta cell viability in % of $TMRE^+$, NG^- and $7AAD^-$ cells. Data from six independent experiments conducted in duplicate are shown. *$p<0.05$, **$p<0.01$

that: (1) cytokines predominantly affect beta cell content and this effect is partially prevented by L-JNKI; (2) the reduction of mitochondrial membrane potential observed with cytokines in NG^- cells is accounted for by exocrine tissue; and (3) L-JNKI does not prevent exocrine tissue death. Focusing on islet cells at baseline (i.e. islets untreated with cytokines), we saw that L-JNKI did not affect islet composition or mitochondrial membrane potential either in the beta cell or in the non-beta cell component of dissociated islets cells.

L-JNKI modulates phosphorylation of AKT and GSK-3B AKT has been shown to be an important survival signal for islets [16, 17]; moreover, in several peripheral tissues activation of JNK resulted in downregulation of AKT phosphorylation [15]. We hypothesised that one of the mechanisms of protection of human islets by L-JNKI may occur through maintenance of an intact autocrine insulin action through AKT, as recently suggested [29]. We found that L-JNKI upregulated AKT phosphorylation (S473) in human islets exposed to inflammatory cytokines (Fig. 4a). Among several substrates downstream of AKT, we looked at GSK-3B (S9), PRAS40 (T246) and p70S6K (TS421/424). JNK inhibition resulted in downregulation of GSK-3B activity (resulting from increased GSK-3B phosphorylation) (Fig. 4b), while both PRAS40 and P70S6K were unaffected (Fig. 4c,d). Interestingly, cytokines stimulated P70S6K irrespectively of whether L-JNKI was present or not. JNK inhibitor affected neither IR (Y1162/1163) nor IRS-1 (S312) (data not shown).

JNKI improves functional beta cell mass in vivo We transplanted human islets treated with L-JNKI or TAT-alone immediately after isolation and then, in order to correct for the observed preservation of islet mass in each culture condition, recounted to allow an equal amount of IEQ to be transplanted in control and experimental mice.

Table 1 Laser scanning cytometry analysis of dissociated human islets

Treatment	Ins (%)	Glu (%)	Som (%)	PP (%)
C	65.3±10.2	28.3±16.4	4.2±2.1	5.5±0.7
LJNKI	58.1±11.9	34.8±14.2	4.7±1.6	5.4±1.2
Cytokines	49.6±7.1[b]	37.5±12.6	7.6±2.7	9.6±1.9
Cytokines+LJNKI	62.0±10.7[a]	28.1±10.5	5.3±3.6	4.5±1.8

Values are mean±SD of the percentage of cells positive for insulin (Ins), glucagon (Glu), somatostatin (Som) and PP among endocrine cells. Percentages were obtained as the ratio of total number of cells over purity

Cells were dissociated and stained as described in the Methods section. Five different experiments with five independent preparations were performed

C Control receiving TAT peptide alone, *LJNKI* L-TAT-JNKI-treated islets, *Cytokines* IL-1β 50 U/ml, TNF-α 1,000 U/ml and INF-γ 1000 U/ml treated islets

[a] $p<0.05$ for cytokines+LJNKI vs cytokines

[b] $p<0.01$ for cytokines vs C

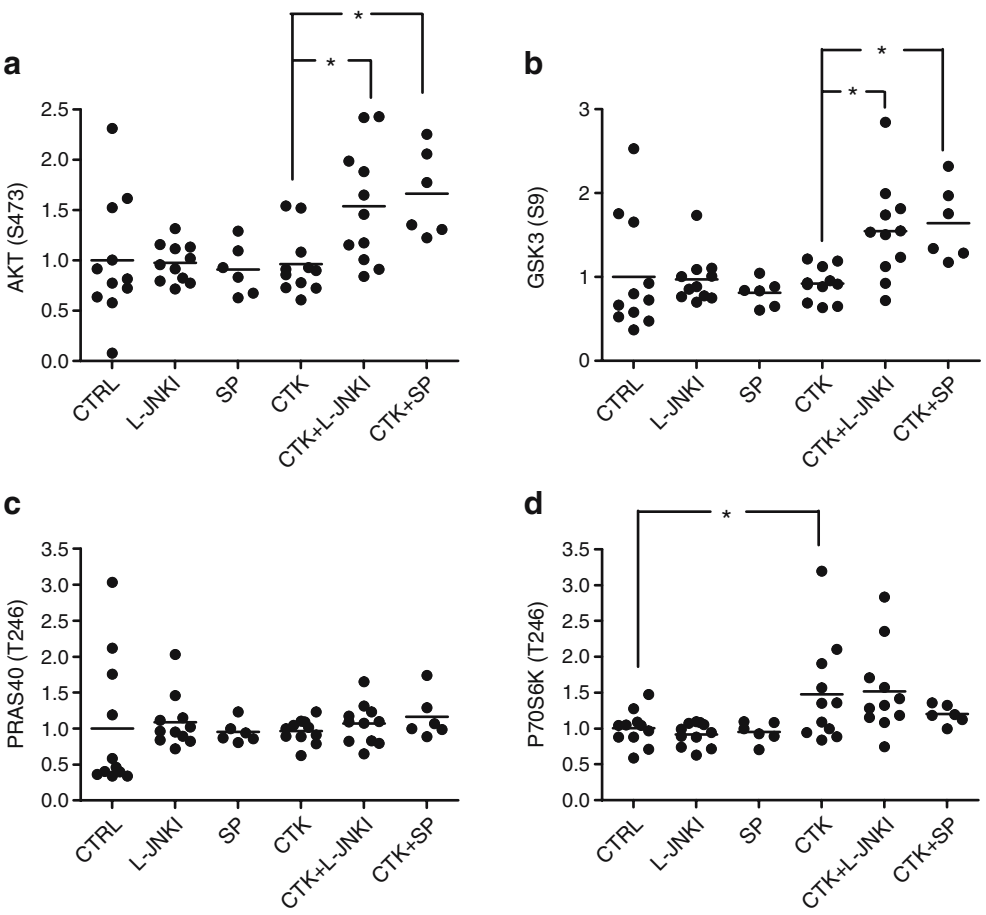

Fig. 4 Effect of cytokines and L-JNKI on AKT signalling pathway. We studied 25 μg of cell lysates for each condition. Data are expressed as units of phosphorylated protein/μg protein. Different preparations (n= 6–11) were used. Islets were treated with L-JNKI and SP600125 (SP; an ATP competitive inhibitor of JNK). **a** Both L-JNKI and SP increased AKT phosphorylation at Ser473 when administered in the presence of cytokines. **b** A similar pattern of phosphorylation was observed for GSK3B at Ser9, resulting in GSK3B inhibition, while PRAS40 (**c**) was not affected by any of the JNK inhibitors used. **d** Cytokines (CTK) alone were able to phosphorylate p70S6K, but neither L-JNKI nor SP600125 could prevent this effect. *p<0.05

Mice receiving L-JNKI-treated islets received daily intraperitoneal injection of L-JNKI or saline for 16 days and at 2 h prior to transplantation. Preliminary experiments confirmed the transduction of a TAT-FITC peptide 24 h after human islets were treated with TAT-FITC and transplanted under the kidney capsule (Fig. 5).

When we combined L-JNKI treatment of both freshly isolated islets and of transplant recipients (n=5), diabetes reversal was comparable to that of controls (n=4), with a mean reversal time of 11.8±13.1 and 23.5±44.3 days, respectively (NS). One of the four animals in the control group did not achieve normoglycaemia (>100 days). All animals receiving L-JNKI reverted to a non-diabetic state within 30 days of transplant (Fig. 6). Despite comparable restoration of normoglycaemia in animals in both groups, L-JNKI lead to a much improved glucose tolerance test at day 16 after transplant in the normoglycaemic mice (n=3 per group), in which glycaemic values (at 1 and 4 min) and AUC were improved compared with normoglycaemic controls (Fig. 7a,d), This effect was partially lost 45 days after interruption of L-JNKI treatment (post-transplant day 60; Fig. 7b,d) and no longer evident by 120 days after transplant (Fig. 7c,d). Comparison of AUC values performed within the same group at different time points revealed no differences.

Transplantation of islets treated in vitro with L-JNKI in untreated mice did not result in improved diabetes reversal or glucose tolerance test profiles, when compared with recipients of untreated islets (Table 2).

We also tested whether a 7 day treatment with L-JNKI had any effect on glucose metabolism in animals bearing functional human islet grafts. In one experimental setting, animals (n=3) underwent IVGTT before initiation and after completion of the L-JNKI therapy. L-JNKI had no effect on the IVGTT profiles in this setting (Fig. 7e). In additional experiments, two groups of mice (five to six animals per condition) receiving or not the 7-day L-JNKI treatment were tested in parallel. Glycaemic profiles during IVGTT were comparable between groups (111.28±5.27 and 85.6±16.59 [mmol×min] l^{-1} in control and L-JNHI-treated, respectively; NS).

The absence of a specific effect on islet function independent of islet mass was also tested in vitro with dynamic GSIR. We did not observe improved insulin release after stimulation with either 11 mmol/l glucose or 25 mmol/l KCl as assessed by dynamic perfusion studies (Fig. 7f). Addition of glucagon-like peptide-1 (GLP-1) at 30 min before and/or during perifusion did not modify the perifusion profiles (data not shown).

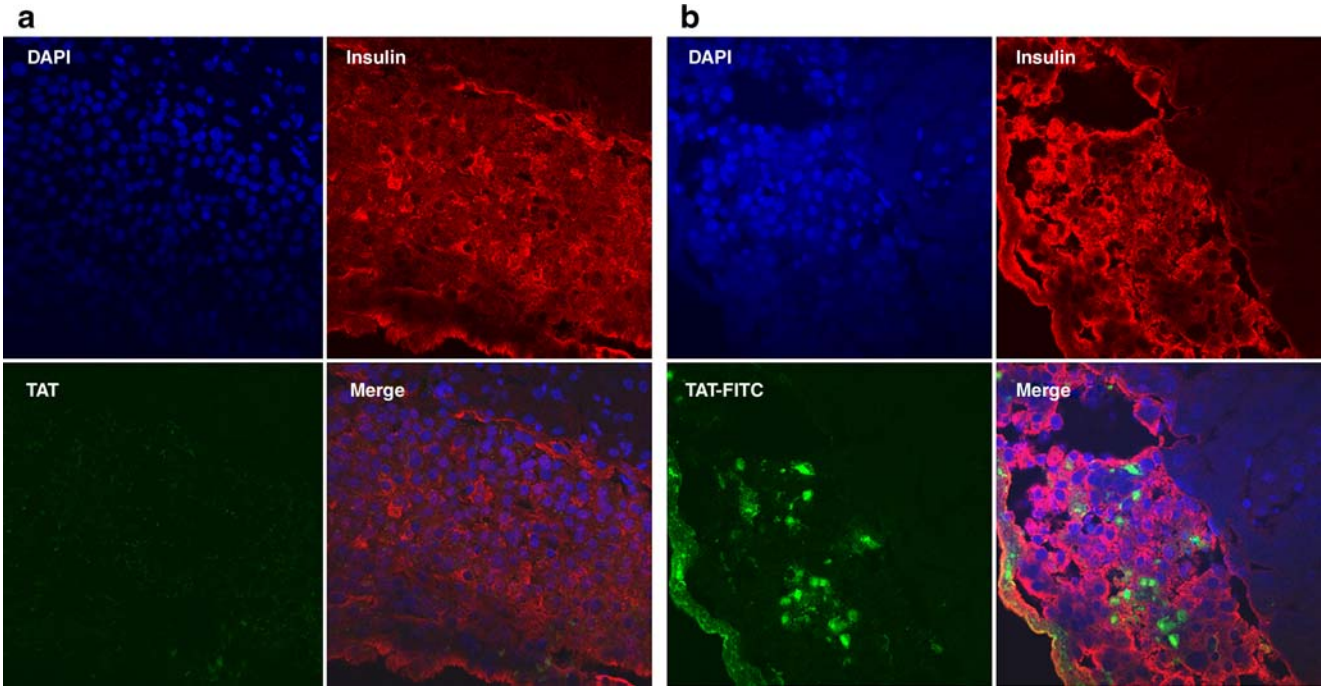

Fig. 5 Transduction of TAT-FITC to islets graft. TAT-FITC (10 μmol/l) was added to the culture media of islets prior to transplantation and was administered to graft recipient intraperitoneally to achieve the same concentration. Representative frozen section of the kidney bearing the graft, stained for DAPI (blue), insulin (red) and analysed for TAT-FITC localisation (green) after TAT alone (**a**) or TAT-FITC (**b**) treatment. Mice were killed 24 h after treatment

Discussion

The inflammatory response activated in the pancreas at the time of isolation and implantation is secondary to multiple events, such as donor brain death, pancreas procurement, organ preservation, islet isolation and early stages of transplantation [30–32]. Interestingly, recent data suggest that JNK may represent a key target factor of such an inflammatory response [1, 33, 34], since activation of JNK during islet isolation and engraftment is detrimental to islet cell survival [4, 5, 9, 24]. Both ATP-competitive and non-competitive JNK inhibitors have shown biological activity in pancreatic islets [4, 9], although we and others have shown that some cell-permeable peptides may be toxic to islet cells [5, 8, 12].

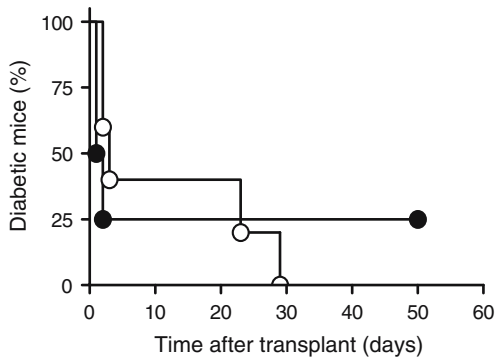

Fig. 6 Reversal of diabetes in *Nu/Nu* mice receiving human islets. Chemically induced diabetic nude mice received human islets (1,000 IEQ per mouse) under the kidney capsule. Animals received L-JNKI-treated islets and L-JNKI intraperitoneal treatment for 16 days (white circles; $n=5$). Control mice received untreated islets and intraperitoneal saline injections (black circles; $n=4$). The proportion of diabetic animals and time to diabetes reversal (glycaemic values <11 mmol/l) after transplantation are indicated

We first compared the activity of L-JNKI and of an irrelevant TAT-peptide on the protection of islet mass, consistent with results reported in porcine and murine islets [8, 9]. TAT peptide alone was used as control, because our previous observation did not show any difference in toxicity between TAT alone and the L-isoform of TAT-JNKI in porcine islets [8]. L-JNKI showed the expected protective effects on islet mass (Fig. 1a). Interestingly, this was not associated with an improvement of beta cell viability (Fig. 1b) or with higher insulin content. This suggests that the protective effect of JNK in the first overnight culture might be very different from the one observed after cytokine exposure in culture and might involve factors responsible for the protection of islet disruption. It is conceivable that the observed effects of L-JNKI could be markedly improved by use of the protease-resistant D-JNKI, which has recently been shown to be safe and effective in human islets even when used at higher concentrations [6].

While activation of JNK is high in the early phases during and immediately after isolation, it becomes almost undetectable in culture [28]. Thus, activation of JNK in vitro would occur only in the presence of stress stimuli, among which

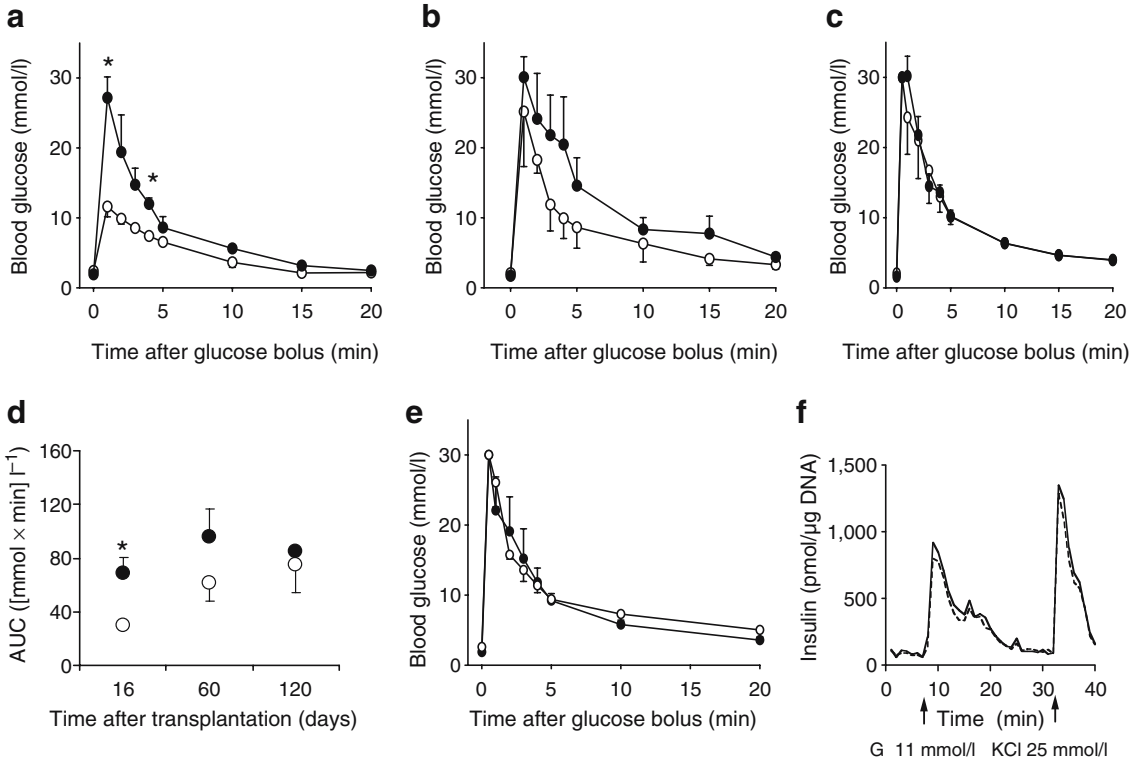

Fig. 7 Effect of L-JNKI treatment on islet performance. Nude mice were transplanted with human islets treated with L-JNKI and treated with L-JNKI (10 mg/kg, open symbols) or TAT peptide alone (solid symbols) 2 h before transplant and then daily for 16 days after transplantation. **a**–**c** Intravenous glucose tolerance tests were performed on mice with functional grafts (i.e. with non-fasting glycaemia <11 mmol/l) at 16 (**a**), 60 (**b**) and 120 (**c**) days after transplantation. In the control group the same three animals were tested each time. In the JNKI-treated group, three normoglycaemic animals were tested at 16 days and four at post-transplant days 60 and 120. Improved glucose clearance was observed in L-JNK treated mice on day 16 post transplant (**a**) *$p<0.05$ at indicated times. **d** The area under the glycaemic curve was reduced at 16 days after transplantation ($p=0.027$); this difference was reduced at 60 (NS) and lost at 120 days. **e** Comparison of IVGTT profiles in mice bearing established human islet grafts before (black circles) and after (white circles) a 7 day treatment with L-JNKI ($n=4$ per group). No differences in glucose disposal were observed (NS). **f** Effect of L-JNKI on glucose-stimulated insulin release. The profiles of insulin production in response to 11 mmol/l glucose (G) and 25 mmol/l KCl were obtained from six different human islet preparations. Graph shows a representative perifusion profile of untreated islets (control; continuous line) compared with islets pretreated with L-JNKI (dotted line). No statistically significant difference was observed in glucose-stimulated insulin release when islets were treated with L-JNKI vs control, even when GLP-1 was added throughout the experiment

Table 2 Reversal of diabetes in chemically diabetic *Nu/Nu* mice transplanted with human islets

Graft size (IEQ)	Control		L-JNKI	
	Number (*n*)	Day of reversal	Number (*n*)	Day of reversal
2,000	3	1	1	1
1,000	7	2[a], 3[a], 9 [b]	3	1, 3, 3
500	2	15, >75	2	6, >75

Nu/Nu mice recipients of untreated or L-JNKI-treated islets (with three different graft sizes of 500, 1,000 or 2,000 IEQ per mouse) were followed after transplantation and screened for time to reversal of diabetes. Time to diabetes reversal was similar in mice recipients of TAT alone treated islets (Control) or of L-JNKI treated islets (L-JNKI) Number of mice in the same group with same time to reversal of diabetes:
[a] 3
[b] 1

we focused our attention on inflammatory cytokines (IL-1β, TNFα and INFγ), which have been shown to be relevant to islet viability and function [35–37] and are markedly activated in early islet transplantation even when islets are transplanted under the kidney capsule [38]. We first confirmed that JNK is markedly phosphorylated in cultured human islets by a cocktail of inflammatory cytokines and that increased phosphorylation is associated with increased JNK activity (Fig. 2a,b). It is important to note that inflammatory cytokines can upregulate other mediators of cell death such as nuclear factor kappa-B and p-38 [28, 39], but we deliberately focused our attention on JNK. Interestingly, there was a high degree of variability in the susceptibility of different islet preparations to cytokines. However, analysis of donor-related characteristics (see ESM Table1) failed to identify a factor responsible for such variability. Among the islet preparations with at least a twofold induction of JNK

phosphorylation by cytokines, the cytokine IL-1β was mainly responsible for such activation (Fig. 2c).

In order to test the hypothesis that L-JNKI specifically protects beta cells, we evaluated the mitochondrial membrane potential of both beta cell and non beta cell subsets of dissociated human islets, since dissipation of the mitochondrial membrane potential is a feature of apoptotic and necrotic beta cell damage [23]. We found that cytokines predominantly affect beta cells and that pretreatment with L-JNKI prevents this phenomenon (Fig. 3a), consistent with the percentage of beta cells observed by LSC in cytokine-treated islets exposed to L-JNKI (Table 1). The observed modulation of the mitochondrial transmembrane potential by cytokine exposure could be the result of excessive nitric oxide production resulting in inhibition of mitochondrial complex 4 [1, 40] and potentiation of JNK activity [41]. When we selectively analysed the NG^- subpopulation of cells, we found that cytokines markedly reduced the mitochondrial membrane potential in those cells as well, but that L-JNKI had no effect (Fig. 3b). Thus, quantitative analysis of cellular composition by LSC was used to determine whether the population of NG^- cells affected by cytokines were non-beta islet cells or exocrine cells. We found that cytokine treatment had no significant effects on glucagon, somatostatin or PP positive cells (Table 1), suggesting that contaminant exocrine cell death may account for the 25% reduction in $TMRE^+$, NG^- and $7AAD^-$ cells observed after cytokine exposure.

Intact expression of IR and its downstream substrates has been reported in human islets [42]. Since the integrity of the insulin signalling cascade in beta cells is responsible for preservation of beta cell mass and for insulin secretion [43], and since JNK inhibition affects IRS-1/AKT in peripheral tissues [13–15], we tested the effect of L-JNKI in human islet insulin pathway with our main focus on AKT and its substrates. Among those substrates, GSK-3B and p70S6K participate in the regeneration of beta cell mass via increased mitogenesis [44, 45], while PRAS40 is also activated by insulin in peripheral tissues and is implicated in protection of neurons against ischaemic injury [46, 47]. We found that JNK inhibition in human islets treated with either L-JNKI or SP600125 specifically stimulated the phosphorylation of AKT and GSK-3B (Fig. 4a,b). However, this effect was observed only when islets were stimulated with cytokines, although cytokines alone did not affect either AKT or GSK-3. The activation of both AKT and GSK3 by cytokines is interesting, since it has been shown in haemopoietic cells that cytokine-induced phosphorylation of GSK-3 is AKT-independent [48]. Overall, these findings suggest that cytokines normally stimulate both the AKT/GSK3 and JNK pathways, the latter usually being predominant and preventing a significant increase in AKT phosphorylation. It is possible that high doses of insulin and IGF-1 in the islet media may affect islet viability by overcoming the activation of JNK, as suggested elsewhere [28]. L-JNKI did not affect PRAS40 either at baseline or after cytokine exposure. This was surprising, since the AKT-mediated protection against apoptosis of neurons exposed to an ischaemic event has been shown to be mediated by PRAS40 [47]. Similarly, L-JNKI did not affect p70S6K, although cytokines activated p70S6K similarly to results reported in adipocytes exposed to IL-1β [49]. The lack of regulation of IRS (S312) phosphorylation by L-JNKI was also unexpected. However, multiple other serine sites could represent the target of L-JNKI, but were not assessed in this study.

We then studied whether the beneficial effect of L-JNKI in islets may persist in vivo. We first verified that a TAT-FITC cell-permeable peptide can transduce human islets (Fig. 5). To assess the effect of L-JNKI in vivo, we evaluated the proportion of animals with diabetes reversal and performed IVGTT in mice recipients of islets exposed to L-JNKI. We found that L-JNKI was able to improve IVGTT profiles in vivo only when administered to cultured islets and to recipient mice in the peri-transplantation period (Fig. 7a,d), but not when islets alone were treated with L-JNKI (Table 2) or when treatment was given to animals bearing established human islet grafts (Fig. 7e). These results suggest that L-JNKI did not exert an independent activity on established graft function, although early islet performance was improved by the JNK inhibitor's ability to reduce local inflammation at the graft site. To further analyse an independent ability of L-JNKI to affect islet function, we tested the ability of L-JNKI to improve GSIR in whole islets in vitro (Fig. 7f). We found that L-JNKI administered prior to stimulation with 11 mmol/l glucose did not lead to an increase in insulin secretion. When the same experiments were performed in the presence of GLP-1, no significant increase in insulin secretion was observed (data not shown). This is consistent with findings in betaTC3 cells treated with an L-JNKI [7], although an ATP-competitive JNKI was shown to improve insulin secretion by human islets in the presence of 3-isobutyl-1-methylxanthine (IBMX), a cAMP phosphodiesterase inhibitor [4]. Thus, the better glucose tolerance test profiles observed in diabetic mice treated with L-JNKI and receiving islets treated with L-JNKI probably reflects an L-JNKI-induced improvement of islet early engraftment and performance. Since our in vitro studies pointed to the protective effect of JNK inhibition on islet survival in the presence of a proinflammatory environment, the beneficial effect of L-JNKI treatment in vivo appears to be consistent with local cytoprotection of the graft early after transplantation. However, the systemic anti-inflammatory properties of JNK inhibition may have contributed to the observed protection [50]. We ruled out the possibility that the improvement of the AUC in Fig. 7d could be explained by a systemic improvement in insulin sensitivity [14], since administration

of a JNK inhibitor in mice with an established graft did not show any benefit. The relative contribution of the local versus systemic effects of JNK inhibition to the improved graft performance, as well as the loss of benefit of L-JNKI treatment upon drug discontinuation, warrant further investigation.

In conclusion, we have shown that L-JNKI is functional in human islets and protects functional beta cell mass both in vitro and in vivo in the early post transplant period. The protective effect of L-JNKI in human islets following acute cytokine exposure is associated and probably mediated by phosphorylation of AKT and GSK-3B. Thus, the introduction of JNK inhibitors in clinical islet isolation and transplantation may allow for a better preservation of islet mass and for improvement of islet engraftment.

Acknowledgements We acknowledge valuable assistance from Juvenile Diabetes Research Foundation (JDRF)-supported Preclinical Cell Processing and Imaging Cores (JDRF grant 4-2004-361). We also acknowledge the superb work of our current Good Manufacturing Practices (cGMP) facility. The current study was supported by grants from the National Institute of Health NIH/NIDDK (DK-59993 to R. L. Pastori and DK-25802-21 to C. Ricordi), from the NIH/NCRR (U42 RR016603 and M01RR16587 to C. Ricordi) and from the Stanley Glaser Foundation (to A. Fornoni), as well as by the Diabetes Research Institute Foundation (diabetesresearch.org).

Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

References

1. Abdelli S, Ansite J, Roduit R et al (2004) Intracellular stress signaling pathways activated during human islet preparation and following acute cytokine exposure. Diabetes 53:2815–2823
2. Ammendrup A, Maillard A, Nielsen K et al (2000) The c-Jun amino-terminal kinase pathway is preferentially activated by interleukin-1 and controls apoptosis in differentiating pancreatic beta-cells. Diabetes 49:1468–1476
3. Rosenberg L, Wang R, Paraskevas S, Maysinger D (1999) Structural and functional changes resulting from islet isolation lead to islet cell death. Surgery 126:393–398
4. Aikin R, Maysinger D, Rosenberg L (2004) Cross-talk between phosphatidylinositol 3-kinase/AKT and c-jun NH2-terminal kinase mediates survival of isolated human islets. Endocrinology 145: 4522–4531
5. Noguchi H, Nakai Y, Ueda M et al (2007) Activation of c-Jun NH (2)-terminal kinase (JNK) pathway during islet transplantation and prevention of islet graft loss by intraportal injection of JNK inhibitor. Diabetologia 50:612–619
6. Abdelli S, Abderrahmani A, Hering BJ, Beckmann JS, Bonny C (2007) The c-Jun N-terminal kinase JNK participates in cytokine- and isolation stress-induced rat pancreatic islet apoptosis. Diabetologia 50:1660–1669
7. Bonny C, Oberson A, Negri S, Sauser C, Schorderet DF (2001) Cell-permeable peptide inhibitors of JNK: novel blockers of beta-cell death. Diabetes 50:77–82
8. Fornoni A, Cobianchi L, Sanabria NY et al (2007) The l-isoform but not d-isoforms of a JNK inhibitory peptide protects pancreatic beta-cells. Biochem Biophys Res Commun 354:227–233
9. Noguchi H, Nakai Y, Matsumoto S et al (2005) Cell permeable peptide of JNK inhibitor prevents islet apoptosis immediately after isolation and improves islet graft function. Am J Transplant 5:1848–1855
10. Ribeiro MM, Klein D, Pileggi A et al (2003) Heme oxygenase-1 fused to a TAT peptide transduces and protects pancreatic beta-cells. Biochem Biophys Res Commun 305:876–881
11. Klein D, Ribeiro MM, Mendoza V et al (2004) Delivery of Bcl-XL or its BH4 domain by protein transduction inhibits apoptosis in human islets. Biochem Biophys Res Commun 323:473–478
12. Cardozo AK, Buchillier V, Mathieu M et al (2007) Cell-permeable peptides induce dose- and length-dependent cytotoxic effects. Biochim Biophys Acta 1768:2222–2234
13. Hirosumi J, Tuncman G, Chang L et al (2002) A central role for JNK in obesity and insulin resistance. Nature 420:333–336
14. Kaneto H, Nakatani Y, Miyatsuka T et al (2004) Possible novel therapy for diabetes with cell-permeable JNK-inhibitory peptide. Nat Med 10:1128–1132
15. Lee YH, Giraud J, Davis RJ, White MF (2003) c-Jun N-terminal kinase (JNK) mediates feedback inhibition of the insulin signaling cascade. J Biol Chem 278:2896–2902
16. Bernal-Mizrachi E, Wen W, Stahlhut S, Welling CM, Permutt MA (2001) Islet beta cell expression of constitutively active Akt1/PKB alpha induces striking hypertrophy, hyperplasia, and hyperinsulinemia. J Clin Invest 108:1631–1638
17. Tuttle RL, Gill NS, Pugh W et al (2001) Regulation of pancreatic beta-cell growth and survival by the serine/threonine protein kinase Akt1/PKBalpha. Nat Med 7:1133–1137
18. Aikin R, Rosenberg L, Maysinger D (2000) Phosphatidylinositol 3-kinase signaling to Akt mediates survival in isolated canine islets of Langerhans. Biochem Biophys Res Commun 277:455–461
19. Ricordi C, Strom TB (2004) Clinical islet transplantation: advances and immunological challenges. Nat Rev Immunol 4:259–268
20. Ichii H, Pileggi A, Molano RD et al (2005) Rescue purification maximizes the use of human islet preparations for transplantation. Am J Transplant 5:21–30
21. Latif ZA, Noel J, Alejandro R (1988) A simple method of staining fresh and cultured islets. Transplantation 45:827–830
22. Ricordi C, Gray DW, Hering BJ et al (1990) Islet isolation assessment in man and large animals. Acta Diabetol Lat 27:185–195
23. Ichii H, Inverardi L, Pileggi A et al (2005) A novel method for the assessment of cellular composition and beta-cell viability in human islet preparations. Am J Transplant 5:1635–1645
24. Lukowiak B, Vandewalle B, Riachy R et al (2001) Identification and purification of functional human beta-cells by a new specific zinc-fluorescent probe. J Histochem Cytochem 49:519–528
25. Brendel MD, Kong SS, Alejandro R, Mintz DH (1994) Improved functional survival of human islets of Langerhans in three-dimensional matrix culture. Cell Transplant 3:427–435
26. Berney T, Molano RD, Cattan P et al (2001) Endotoxin-mediated delayed islet graft function is associated with increased intra-islet cytokine production and islet cell apoptosis. Transplantation 71: 125–132
27. Pileggi A, Molano RD, Berney T et al (2001) Heme oxygenase-1 induction in islet cells results in protection from apoptosis and improved in vivo function after transplantation. Diabetes 50: 1983–1991
28. Paraskevas S, Aikin R, Maysinger D et al (2001) Modulation of JNK and p38 stress activated protein kinases in isolated islets of Langerhans: insulin as an autocrine survival signal. Ann Surg 233: 124–133
29. Aikin R, Hanley S, Maysinger D et al (2006) Autocrine insulin action activates Akt and increases survival of isolated human islets. Diabetologia 49:2900–2909
30. Lakey JR, Burridge PW, Shapiro AM (2003) Technical aspects of islet preparation and transplantation. Transpl Int 16:613–632

31. Toyama H, Takada M, Suzuki Y, Kuroda Y (2003) Activation of macrophage-associated molecules after brain death in islets. Cell Transplant 12:27–32
32. Noguchi H, Matsumoto S (2006) Protein transduction technology: a novel therapeutic perspective. Acta Med Okayama 60:1–11
33. Eckhoff DE, Smyth CA, Eckstein C et al (2003) Suppression of the c-Jun N-terminal kinase pathway by 17beta-estradiol can preserve human islet functional mass from proinflammatory cytokine-induced destruction. Surgery 134:169–179
34. Paraskevas S, Aikin R, Maysinger D et al (1999) Activation and expression of ERK, JNK, and p38 MAP-kinases in isolated islets of Langerhans: implications for cultured islet survival. FEBS Lett 455:203–208
35. Kutlu B, Cardozo AK, Darville MI et al (2003) Discovery of gene networks regulating cytokine-induced dysfunction and apoptosis in insulin-producing INS-1 cells. Diabetes 52:2701–2719
36. Scarim AL, Heitmeier MR, Corbett JA (1997) Irreversible inhibition of metabolic function and islet destruction after a 36-hour exposure to interleukin-1beta. Endocrinology 138:5301–5307
37. Eizirik DL, Mandrup-Poulsen T (2001) A choice of death—the signal-transduction of immune-mediated beta-cell apoptosis. Diabetologia 44:2115–2133
38. Montolio M, Biarnes M, Tellez N, Escoriza J, Soler J, Montanya E (2007) Interleukin-1beta and inducible form of nitric oxide synthase expression in early syngeneic islet transplantation. J Endocrinol 192:169–177
39. Cardozo AK, Heimberg H, Heremans Y et al (2001) A comprehensive analysis of cytokine-induced and nuclear factor-kappa B-dependent genes in primary rat pancreatic beta-cells. J Biol Chem 276:48879–48886
40. Sarti P, Arese M, Bacchi A et al (2003) Nitric oxide and mitochondrial complex IV. IUBMB Life 55:605–611
41. Storling J, Binzer J, Andersson AK et al (2005) Nitric oxide contributes to cytokine-induced apoptosis in pancreatic beta cells via potentiation of JNK activity and inhibition of Akt. Diabetologia 48:2039–2050
42. Muller D, Huang GC, Amiel S, Jones PM, Persaud SJ (2006) Identification of insulin signaling elements in human β-cells: autocrine regulation of insulin gene expression. Diabetes 55: 2835–2842
43. Leibiger IB, Leibiger B, Berggren PO (2002) Insulin feedback action on pancreatic beta-cell function. FEBS Lett 532:1–6
44. Mussmann R, Geese M, Harder F et al (2007) Inhibition of GSK3 promotes replication and survival of pancreatic beta cells. J Biol Chem 282:12030–12037
45. Amaral ME, Cunha DA, Anhe GF et al (2004) Participation of prolactin receptors and phosphatidylinositol 3-kinase and MAP kinase pathways in the increase in pancreatic islet mass and sensitivity to glucose during pregnancy. J Endocrinol 183:469–476
46. Nascimento EB, Fodor M, van der Zon GC et al (2006) Insulin-mediated phosphorylation of the proline-rich Akt substrate PRAS40 is impaired in insulin target tissues of high-fat diet-fed rats. Diabetes 55:3221–3228
47. Saito A, Narasimhan P, Hayashi T, Okuno S, Ferrand-Drake M, Chan PH (2004) Neuroprotective role of a proline-rich Akt substrate in apoptotic neuronal cell death after stroke: relationships with nerve growth factor. J Neurosci 24:1584–1593
48. Vilimek D, Duronio V (2006) Cytokine-stimulated phosphorylation of GSK-3 is primarily dependent upon PKCs, not PKB. Biochem Cell Biol 84:20–29
49. He J, Usui I, Ishizuka K et al (2006) Interleukin-1alpha inhibits insulin signaling with phosphorylating insulin receptor substrate-1 on serine residues in 3T3-L1 adipocytes. Mol Endocrinol 20: 114–124
50. Fahmy RG, Waldman A, Zhang G et al (2006) Suppression of vascular permeability and inflammation by targeting of the transcription factor c-Jun. Nat Biotechnol 24:856–863