cJUN N-terminal kinase (JNK) activation mediates islet amyloid-induced beta cell apoptosis in cultured human islet amyloid polypeptide transgenic mouse islets

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Received: 21 March 2011 / Accepted: 12 September 2011 / Published online: 26 October 2011
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Abstract

Aims/hypothesis Aggregation of human islet amyloid polypeptide (hIAPP) as islet amyloid is associated with increased beta cell apoptosis and reduced beta cell mass in type 2 diabetes. Islet amyloid formation induces oxidative stress, which contributes to beta cell apoptosis. The cJUN N-terminal kinase (JNK) pathway is a critical mediator of beta cell apoptosis in response to stress stimuli including oxidative stress and exogenous application of hIAPP. We determined whether amyloid formation by endogenous hIAPP mediates beta cell apoptosis through JNK activation and downstream signalling pathways.

Methods hIAPP transgenic and non-transgenic mouse islets were cultured for up to 144 h in 16.7 mmol/l glucose to induce islet amyloid in the presence or absence of the amyloid inhibitor Congo Red or a cell-permeable JNK inhibitor. Amyloid, beta cell apoptosis, JNK signalling and activation of downstream targets in the intrinsic and extrinsic apoptotic pathways were measured.

Results JNK activation occurred with islet amyloid formation in hIAPP transgenic islets after 48 and 144 h in culture. Neither high glucose nor the hIAPP transgene alone was sufficient to activate JNK independent of islet amyloid. Inhibition of islet amyloid formation with Congo Red reduced beta cell apoptosis and partially decreased JNK activation. JNK inhibitor treatment reduced beta cell apoptosis without affecting islet amyloid. Islet amyloid increased mRNA levels of markers of the extrinsic (*Fas*,

Fadd) and intrinsic (*Bim* [also known as *Bcl2l11*]) apoptotic pathways, caspase 3 and the anti-apoptotic molecule *Bclxl* (also known as *Bcl2l1*) in a JNK-dependent manner.

Conclusions/interpretation Islet amyloid formation induces JNK activation, which upregulates predominantly pro-apoptotic signals in both extrinsic and intrinsic pathways, resulting in beta cell apoptosis.

Keywords Beta cell apoptosis · hIAPP · Islet amyloid · JNK activation

Abbreviations

BAD	BCL2-associated agonist of cell death
BCL2	B-cell leukaemia/lymphoma 2
BCLXL	BCL2-like protein 1
BH3	BCL2 homology domain 3
BIM	BCL2-interacting mediator of cell death
CASP3	Caspase 3
ER	Endoplasmic reticulum
FADD	FAS-associated death domain
hIAPP	Human islet amyloid polypeptide
IAPP	Islet amyloid polypeptide
JNK	cJUN N-terminal kinase
p-cJUN	Phosphorylated cJUN
p-JNK	Phosphorylated JNK

Introduction

Islet amyloid formation is a pathological hallmark of type 2 diabetes occurring in the majority of patients [1], and is associated with decreased beta cell mass and function [2, 3]. The unique polypeptide constituent of these amyloid deposits is islet amyloid polypeptide (IAPP), which is a normally soluble secretory product of the beta

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cell released with insulin [4–6]. Human IAPP (hIAPP) is amyloidogenic in contrast to rodent IAPP, due to differences at several critical amino acid residues [7].

The process of amyloid formation involves aggregation of IAPP monomers to form oligomers and then fibrils, ultimately leading to insoluble amyloid deposits [2]. Several studies have shown that early hIAPP aggregates are cytotoxic, resulting in beta cell apoptosis in cultured cells and islets [8–13]. Oxidative stress has been shown to be a mediator of hIAPP/amyloid-induced beta cell apoptosis. In human autopsy samples of patients with type 2 diabetes, islet amyloid was associated with oxidative stress markers [14]. Furthermore, in an in vitro culture model of islet amyloid using isolated islets from hIAPP transgenic mice, we showed that amyloid formation induces oxidative stress, which time-dependently potentiates amyloid formation and contributes to increased beta cell apoptosis [15]. The relationship between islet amyloid and endoplasmic reticulum (ER) stress is less clear. Human autopsy samples have shown that type 2 diabetes is associated with ER stress [16], but that islet amyloid is not [17]. Amyloid formation was not associated with ER stress in our in vitro model [17]. However, studies in mouse and rat models of amyloid formation with transgenic overproduction of hIAPP have shown that under conditions of increased hIAPP levels, ER stress is a potential mediator of hIAPP-induced beta cell apoptosis [18, 19].

The signalling pathways that mediate beta cell apoptosis downstream of islet amyloid formation from endogenously produced hIAPP have yet to be fully characterised. The cJUN N-terminal kinase (JNK) pathway is an important central pro-apoptotic pathway in the beta cell, which is activated in response to several stress stimuli including high glucose [20], NEFA [21], pro-inflammatory cytokines [22, 23], and both oxidative and ER stress [21, 24]. JNK also mediates beta cell apoptosis in cultured cell lines or isolated islets exposed to acute, supraphysiological (micromolar) concentrations of synthetic hIAPP [25–27]. Furthermore, the FAS-associated death receptor pathway and caspase 3 (CASP3) have been implicated downstream of JNK signalling in exogenous hIAPP-mediated cytotoxicity [28]. However, whether the JNK pathway also mediates beta cell apoptosis due to amyloid formed from endogenous hIAPP produced at physiological concentrations is the focus of this study.

The intricacies of the apoptotic pathways and specifically those downstream of JNK, although well studied in other cell types, have not been well characterised in the beta cell [29]. In neurons, a cell with similarities to the beta cell [30], JNK signalling plays a critical role in the extrinsic and intrinsic pathways of apoptosis by regulating the transcription of pro-apoptotic molecules and post-translational modification of both pro- and anti-apoptotic molecules. Thus, in this study, we sought to identify JNK-dependent candidate signals in the extrinsic and intrinsic pathways of apoptosis that are

activated by endogenously produced hIAPP during amyloid formation and may contribute to beta cell apoptosis.

Methods

Transgenic mice Hemizygous hIAPP transgenic mice [31] on an F1 C57BL/6×DBA/2J background were used in this study with non-transgenic littermates as controls. Polymerase chain reaction was used to determine transgenic status as previously described [32]. The study was approved by the Institutional Animal Care and Use Committee at the VA Puget Sound Health Care System.

Islet isolation and culture Islets were isolated from 10-week-old male and female mice as previously described [33]. After overnight recovery, islets were cultured for up to 144 h in medium containing 11.1 or 16.7 mmol/l glucose in the presence or absence of the amyloid inhibitor Congo Red (200 µmol/l) [15], cell-permeable JNK inhibitor peptide (JNK inhibitor 1, L-form; 10 µmol/l; EMD Chemicals, Gibbstown, NJ, USA) [20, 26, 34] or a negative control peptide (JNK inhibitor 1-negative control, L-form; 10 µmol/l; EMD Chemicals). JNK inhibitor 1 contains the minimal 20-amino acid inhibitory domain of islet brain-1 protein linked to a 10-amino acid HIV-TAT sequence that rapidly translocates into the cell and has been shown to reduce JNK-mediated activation of cJUN [34]. The 10 µmol/l dose of JNK inhibitor was chosen based on efficacy to reduce phosphorylated (p)-cJUN levels with tested doses ranging from 1 to 10 µmol/l.

Histological assessment of islet amyloid and beta cell apoptosis Islets were fixed in 4% (wt/vol.) phosphate-buffered paraformaldehyde and embedded in paraffin. Ten micrometer sections were stained with thioflavin S, insulin antibody and Hoechst 33258 to visualise amyloid deposits, beta cells and nuclei, respectively [33]. Beta cell apoptosis was quantified by staining islets with propidium iodide and anti-insulin antibody [15]. Histological assessment of both islet amyloid and beta cell apoptosis was carried out by a single investigator who was blinded to both genotype and experimental treatment for each sample. An average of 16 islets per experimental condition was analysed. Amyloid severity (per cent of islet area occupied by amyloid) was computed using Image Pro Plus (Media Cybernetics, Bethesda, MD, USA) by determining the thioflavin S and insulin-positive areas within each islet cross-section [33]. The proportion of apoptotic beta cells was calculated by manually counting the number of condensed and fragmented nuclei in insulin-positive cells [15].

Protein extraction and assessment of JNK activation Islets (200 per condition) were lysed (Bio-Plex Cell Lysis Kit,

Bio-Rad Laboratories, Hercules, CA, USA) and sonicated. Supernatants were collected and protein concentration was determined using the bicinchoninic acid assay.

p-cJUN and total cJUN were measured in islet lysates (250 µg/ml) using the Bio-Rad phosphoprotein immunoassay kit and Bio-Plex 200 suspension array system (Bio-Rad Laboratories). For western blots, islet protein (20 µg) was loaded on a 12% sodium dodecyl sulphate-polyacrylamide gel transferred to polyvinylidene difluoride membrane and probed with anti-p-JNK, anti-p-cJUN or total cJUN (1:500; Cell Signaling, Danvers, MA, USA), followed by goat anti-rabbit immunoglobulin/horseradish peroxidase (HRP) (1:50,000; Dako, Carpinteria, CA, USA). Membranes were stripped (Restore PLUS Western Blot Stripping Buffer Kit, Thermo Scientific, Rockford, IL, USA) and reprobed with anti-β-actin (1:2,000; Sigma, St Louis, MO, USA) as a loading control, followed by goat anti-rabbit immunoglobulin/HRP (1:75,000, Dako). Immunoreactive bands were detected by enhanced chemiluminescence as described by the manufacturer (Thermo Scientific) and quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The densities of p-JNK, p-cJUN and total cJUN were expressed as a ratio of β-actin.

RNA isolation and real-time quantitative reverse transcription-PCR Total islet RNA was isolated from 25 islets per condition (High Pure RNA Isolation Kit, Roche Applied Science, Indianapolis, IN, USA) and reverse transcribed (High Capacity cDNA Archive Kit, Applied Biosystems, Foster City, CA, USA) [33]. mRNA levels of *cJun*, the pro-apoptotic genes *Fas*, *Fadd*, *Bim* (also known as *Bcl2l1l*) and *Casp3*, and the anti-apoptotic genes *Bclxl* (also known as *Bcl2l1*) and *Bcl2* were measured in triplicate by real-time PCR using TaqMan assays on demand (ABI Prism 7500; Applied Biosystems) [15, 33]. As an endogenous control, 18S ribosomal RNA levels were used. mRNA levels were expressed relative to non-transgenic islets cultured in 16.7 mmol/l glucose alone using the $\Delta\Delta C_t$ method.

Statistical analyses Data are expressed as mean±SEM. Data were compared by analysis of variance with post hoc analysis or with Mann–Whitney *U* test if they were not normally distributed. A $p<0.05$ was considered statistically significant.

Results

Glucose dependence of islet amyloid formation, beta cell apoptosis and JNK activation Culture of *hIAPP* transgenic islets in 11.1 mmol/l glucose for 48 h was associated

with minimal amyloid formation, whereas culture in 16.7 mmol/l glucose induced islet amyloid formation (Fig. 1a). As expected, non-transgenic islets did not form amyloid.

Islet amyloid formation over 48 h in 16.7 mmol/l glucose was associated with increased beta cell apoptosis (Fig. 1b) and JNK activation, the latter shown as increases in both p-JNK (Fig. 1c, d) and p-cJUN levels (Fig. 1e) compared with non-transgenic islets cultured in 16.7 mmol/l glucose. Both *hIAPP* transgenic islets cultured in 11.1 mmol/l glucose and non-transgenic islets cultured in 16.7 mmol/l glucose demonstrated similar levels of p-JNK and p-cJUN to non-transgenic islets cultured in 11.1 mmol/l glucose (Fig. 1c–e).

Fig. 1 Islet amyloid severity (per cent islet area occupied by amyloid) (a) and beta cell apoptosis (per cent apoptotic beta cells/total cells) (b) in non-transgenic (NT; white bars) and *hIAPP* transgenic (T; black bars) islets cultured in 11.1 and 16.7 mmol/l glucose for 48 h. Non-transgenic islets did not form amyloid. p-JNK and p-cJUN levels were measured by western blot in non-transgenic and *hIAPP* transgenic islets using β-actin as a loading control and representative western blots are shown in (c). Quantification of p-JNK/β-actin and p-cJUN/β-actin are shown in (d) and (e), respectively. *cJun* mRNA levels are shown in (f). $n=4-6$; * $p<0.05$ and ** $p<0.01$ vs *hIAPP* transgenic islets cultured in 11.1 mmol/l glucose. † $p<0.05$ and †† $p<0.01$ vs non-transgenic islets cultured in 16.7 mmol/l glucose

cJun mRNA levels increased by 60% in *hIAPP* transgenic islets cultured in 16.7 mmol/l glucose compared with those cultured in 11.1 mmol/l glucose and non-transgenic islets (Fig. 1f).

Time dependence of islet amyloid formation and JNK activation Islet amyloid formation in *hIAPP* transgenic islets increased over the 144 h culture period (Fig. 2a). p-cJUN and total cJUN levels did not change in non-transgenic islets over time (Fig. 2b, c). p-cJUN levels were higher in *hIAPP* transgenic islets compared with non-transgenic islets as early as 48 h and this increase persisted at 144 h (Fig. 2b). Although total cJUN levels were not significantly increased in *hIAPP* transgenic islets following 48 h of culture, they were significantly increased after 144 h of culture (Fig. 2c). *cJun* mRNA levels increased in *hIAPP* transgenic compared with non-transgenic islets cultured for 144 h (Fig. 2d).

Fig. 2 Representative amyloid (green), insulin (red) and nuclear (blue) staining (**a**) in non-transgenic and *hIAPP* transgenic islets cultured in 16.7 mmol/l glucose over 144 h. p-cJUN (**b**) and total cJUN (**c**) levels at 48 and 144 h were measured by Bio-Plex immunoassay in both non-transgenic (white bars) and *hIAPP* transgenic (black bars) islets. Data are expressed in arbitrary units relative to non-transgenic islets cultured for 48 h. *cJun* mRNA levels measured in non-transgenic and *hIAPP* transgenic islets after 144 h are shown in (**d**). $n=4-6$; ** $p<0.01$ vs non-transgenic islets cultured for 48 h. † $p<0.05$ and †† $p<0.01$ vs non-transgenic islets cultured for 144 h

Effect of the islet amyloid inhibitor Congo Red on islet amyloid severity, beta cell apoptosis and JNK activation Congo Red treatment inhibited amyloid formation in *hIAPP* transgenic islets (Fig. 3a), and this was associated with a reduction in beta cell apoptosis in *hIAPP* transgenic islets to levels similar to those in non-transgenic islets (Fig. 3b). Congo Red treatment also partially decreased p-cJUN levels (Fig. 3c), an observation that was confirmed by western blot (data not shown).

Effect of JNK inhibition on p-cJUN levels, islet amyloid severity and beta cell apoptosis JNK inhibition did not change p-cJUN levels in non-transgenic islets (Fig. 4a), whereas in *hIAPP* transgenic islets it resulted in a 45% reduction in p-cJUN levels, making them similar to those in non-transgenic islets (Fig. 4a). Negative control peptide treatment (10 $\mu\text{mol/l}$; $n=3$) resulted in no differences in p-cJUN levels in either non-transgenic islets (1.05 ± 0.22 vs 1.00 ratio of p-cJUN/ β -actin expressed as fold over non-transgenic islets cultured in 16.7 mmol/l glucose; $p=0.83$) or *hIAPP* transgenic islets (1.75 ± 0.38 vs 3.02 ± 1.43 ratio of p-cJUN/ β -actin expressed as fold over non-transgenic islets cultured in 16.7 mmol/l glucose; $p=0.36$).

Co-culture of *hIAPP* transgenic islets with the JNK inhibitor had no effect to reduce islet amyloid formation (Fig. 4b). Similarly, the negative control peptide had no effect on amyloid formation ($0.90\pm0.14\%$ vs 0.58 ± 0.05 ; $p=0.17$). JNK inhibition resulted in no significant difference in

Fig. 3 Islet amyloid severity (**a**), beta cell apoptosis (**b**) and p-cJUN levels measured by Bio-Plex immunoassay (**c**) in non-transgenic (white bars) and *hIAPP* transgenic (black bars) islets cultured in 16.7 mmol/l glucose for 48 h in the absence or presence of Congo Red (200 $\mu\text{mol/l}$), an amyloid inhibitor. Non-transgenic islets did not form amyloid. $n=4$; * $p<0.05$ vs *hIAPP* transgenic islets cultured in 16.7 mmol/l glucose. †† $p<0.01$ vs non-transgenic islets cultured in 16.7 mmol/l glucose

Fig. 4 Effect of JNK inhibitor treatment (10 μ mol/l) on p-cJUN levels measured by western blot (**a**), islet amyloid severity (**b**) and beta cell apoptosis (**c**) in non-transgenic (NT, white bars) and hIAPP transgenic (black bars) islets cultured in 16.7 mmol/l glucose for 48 h. p-cJUN levels were quantified and expressed as a ratio p-cJUN/β-actin. Non-transgenic islets did not form islet amyloid. $n=6$; * $p<0.05$ and ** $p<0.01$ vs non-transgenic islets cultured in 16.7 mmol/l glucose. † $p<0.05$ and †† $p<0.01$ vs hIAPP transgenic islets cultured in 16.7 mmol/l glucose

levels of beta cell apoptosis in non-transgenic islets. In contrast, JNK inhibition in hIAPP transgenic islets resulted in a 72% reduction in beta cell apoptosis (Fig. 4c). Negative control peptide treatment did not affect beta cell apoptosis as compared with no treatment in either non-transgenic ($0.14 \pm 0.02\%$ vs $0.10 \pm 0.05\%$; $p=0.46$) or hIAPP transgenic islets ($0.59 \pm 0.02\%$ vs $0.48 \pm 0.14\%$; $p=0.28$).

Islet amyloid-induced, JNK-dependent gene targets in the extrinsic and intrinsic apoptotic pathways mRNA levels of pro- and anti-apoptotic signals were measured in hIAPP transgenic and non-transgenic islets cultured in 16.7 mmol/l glucose for 48 h alone or in the presence of Congo Red or the JNK inhibitor. In the extrinsic pathway, *Fas* and *Fadd* were upregulated in hIAPP transgenic islets (Fig. 5a, b, respectively). In hIAPP transgenic islets, co-culture with Congo Red resulted in a 74% reduction in *Fas* mRNA levels with a non-significant decrease in *Fadd* levels. Co-culture with JNK inhibitor resulted in 74% and 48% reductions in *Fas* and *Fadd* mRNA levels, respectively. Significant reductions of *Fas* mRNA levels were observed in non-transgenic islets cultured in the presence of Congo Red and JNK inhibitor.

In the intrinsic pathway, *Bim* was upregulated in hIAPP transgenic islets (Fig. 5c). Co-culture with Congo Red or JNK inhibitor resulted in 30% and 48% reductions in *Bim*

levels, respectively (Fig. 5c). mRNA levels of *Casp3* were upregulated in hIAPP transgenic islets compared with non-transgenic islets, and were found to be reduced in the presence of Congo Red and JNK inhibitor (Fig. 5d). Levels of the anti-apoptotic factor *Bclxl* were upregulated in hIAPP transgenic islets compared with non-transgenic islets, which was abolished by co-culture with Congo Red and JNK inhibitor (Fig. 5e). In contrast, whereas *Bcl2* was upregulated in hIAPP transgenic islets compared with non-transgenic islets, treatment with Congo Red and JNK inhibitor had no effect on *Bcl2* mRNA levels (Fig. 5f).

Discussion

In this study, we sought to determine the mechanism(s) by which islet amyloid formation, under conditions of endogenous hIAPP production, results in beta cell apoptosis. We chose to examine the role of JNK signalling, a pro-apoptotic pathway that mediates beta cell apoptosis in response to several stressors including exogenous hIAPP treatment [25]. We found that JNK signalling is activated during the process of islet amyloid formation, and this occurs in part downstream of amyloid formation. We have also shown that the JNK pathway is a critical mediator of islet amyloid-induced beta cell apoptosis and identified potential downstream mediators in both the extrinsic (*Fas* and *Fadd*) and intrinsic (*Bim*) apoptotic pathways. Last, we observed that the anti-apoptotic molecule *Bclxl* is upregulated in a JNK-dependent manner downstream of islet amyloid formation.

Culture of hIAPP transgenic islets in high glucose (16.7 mmol/l) results in light microscopy-visible amyloid deposition and associated beta cell apoptosis [15, 33]. We observed JNK activation, manifest as increased levels of p-JNK and p-cJUN protein and *cJun* mRNA in hIAPP transgenic islets only under amyloidogenic (16.7 mmol/l glucose) conditions, indicating that the observed JNK activation was not merely secondary to the expression of the hIAPP transgene, but rather was dependent on the process of amyloid formation. It has been previously shown that culture of human islets in increased glucose (33.3 mmol/l) for 18 h induces JNK activation and beta cell apoptosis [20]. All human islets have the propensity to develop islet amyloid, although this was not examined in the study by Maedler et al. [20], making it difficult to discern whether the observed JNK activation was secondary to high glucose or the presence of amyloid. To control for the potential effect of glucose independent of amyloid, we cultured non-transgenic islets in 11.1 and 16.7 mmol/l glucose and observed no difference in either p-JNK or p-cJUN protein levels or *cJun* mRNA levels, demonstrating that increased glucose alone was insufficient for JNK activation. In fact, under increased glucose conditions

Fig. 5 mRNA levels of pro-apoptotic genes *Fas* (a), *Fadd* (b), *Bim* (c) and *Casp3* (d), and anti-apoptotic genes *Bclxl* (e) and *Bcl2* (f) in non-transgenic (white bars) and hIAPP transgenic (black bars) islets cultured in 16.7 mmol/l glucose in the absence or presence of Congo

Red (200 μ mol/l) or JNK inhibitor (10 μ mol/l). $n=6$; * $p<0.05$ vs non-transgenic islets cultured in 16.7 mmol/l glucose. † $p<0.05$ vs hIAPP transgenic islets cultured in 16.7 mmol/l glucose

in non-transgenic islets there was a tendency for a decrease in the activation of JNK that did not coincide with a reduction in phosphorylation of cJUN, an observation that would not be in line with the canonical mechanism of JNK. Thus, we conclude that the observed JNK activation occurs only in the presence of islet amyloid formation.

There are several potential mechanism(s) that underlie islet amyloid-induced JNK activation including oxidative stress, which we have shown to be an important mediator of islet amyloid-induced cytotoxicity [15]. Another possible mechanism is inflammation. Culture of human islets under high glucose, a potentially amyloidogenic condition, stimulates IL-1 β production, which mediates beta cell apoptosis [35]. Our recent study demonstrated that fibrillar hIAPP elicits an inflammatory response (including IL-1 β production) from bone marrow-derived dendritic cells and showed IL-1 β co-localisation with islet amyloid deposits in vivo [36]. It is therefore certainly possible that islet amyloid formation could also induce IL-1 β production and JNK activation in beta cells, a scenario requiring further study.

As the process of islet amyloid formation is time dependent [33], we also determined whether JNK activation persists with increasing amyloid formation over time. We demonstrated that JNK is activated in the early stages of

amyloid formation (48 h), and remains elevated under more chronic conditions of amyloid deposition (144 h). In contrast, non-transgenic islets demonstrated no increase in JNK activation over time. At 48 h, JNK signalling manifested as increased p-cJUN without a significant increase in total cJUN, although *cJun* mRNA is increased at this time point. Under the condition of prolonged amyloid formation, increases in p-cJUN, total cJUN and *cJun* mRNA were observed. This pattern of activation is somewhat consistent with that reported in 16 h studies in which micromolar concentrations of hIAPP were applied to beta cell lines and increased phosphorylation of cJUN preceded increases in total cJUN [25]. To capture the kinetics of JNK activation in relation to the process of islet amyloid formation in our model, it would have been ideal to examine islets prior to the formation of light microscopy-visible amyloid deposits (prior to 24 h in culture). However, it is well documented that the islet isolation procedure itself induces stress and JNK activation, precluding this early time point [37]. As isolated islets recover to a low basal rate of JNK signalling following 48 h in culture [37], we controlled for the stress of isolation by using an overnight recovery period, followed by a minimum of 48 h in culture prior to making any stress signalling measurements. The

low, reproducible p-cJUN levels observed in non-transgenic islets suggest that this recovery period is sufficient to control for the confounding effects of the stress of isolation and culture.

To determine whether the JNK pathway is activated upstream or downstream of islet amyloid formation, we interrogated the culture system with the amyloid inhibitor Congo Red. This compound inhibits islet amyloid formation resulting in a rate of beta cell apoptosis in hIAPP transgenic islets similar to that in non-transgenic islets [15]. Treatment with Congo Red resulted in a significant but incomplete reduction in p-cJUN levels. We propose two possible explanations for this observation. First, the JNK signalling pathway is activated in part downstream of islet amyloid but there may be an additional upstream role of JNK signalling. This upstream JNK signalling may (1) play a role in mediating amyloid formation itself or (2) mediate other cytotoxic effects of hIAPP that are potentially amyloid independent. We believe the former is unlikely given that hIAPP transgenic islets treated with a JNK inhibitor demonstrated no change in amyloid severity. The latter explanation is plausible, although it would be a challenging paradigm to explore in our model in which amyloid is induced as early as 24 h in culture, and studies prior to this time point are subject to the confounding effects of the stress of isolation.

We have shown that JNK signalling is a critical mediator by which islet amyloid induces beta cell apoptosis. JNK inhibition resulted in reduced beta cell apoptosis in hIAPP transgenic islets to levels identical to those in non-transgenic islets. Furthermore, JNK signalling under conditions of amyloid formation upregulated mRNA levels of the pro-apoptotic signalling molecules *Fas* and *Fadd* in the extrinsic pathway, *Bim* in the intrinsic pathway and, as expected, the terminal effector caspase, *Casp3*, through which both the extrinsic and intrinsic pathways converge to induce apoptosis. The critical role of CASP3 in mediating the toxicity of islet amyloid has been underscored by a recent in vivo study in hIAPP transgenic mice, which demonstrated that prevention of CASP3 activation protected beta cells from amyloid-induced apoptosis and resulted in preservation of beta cell mass and improved beta cell function [38].

The extrinsic pathway is typically initiated by cell surface death receptors, which includes the FAS ligand receptor, resulting in downstream JNK activation and caspase 8 activation. A recent study has shown that exogenous hIAPP treatment of beta cell lines over an acute culture period of 8 h results in upregulation of FAS and FAS-associated death domain (FADD), both at the mRNA and protein levels, with associated increases in beta cell apoptosis [28]. Furthermore, this study demonstrated that hIAPP induces FAS through an interaction at the cell surface, which results in JNK activation. JNK signalling is then able to feedback to

upregulate levels of FAS, perpetuating a cycle of pro-apoptotic signalling. These observations are consistent with those in our model of endogenous hIAPP production and islet amyloid formation in which we observe JNK-dependent upregulation of *Fas* and *Fadd*.

The intrinsic pathway of apoptosis involves a dynamic interplay between pro-apoptotic B-cell leukaemia/lymphoma 2 (BCL2) homology domain 3 (BH3)-only proteins and anti-apoptotic BCL2-like proteins. In response to pro-apoptotic stimuli, BH3-only proteins bind anti-apoptotic BCL2-like proteins, resulting in the release of BCL2-associated agonist of cell death (BAD) and/or BCL2 agonist killer 1 (BAK), which induce changes in mitochondrial membrane permeability and membrane potential causing cytochrome *c* release and activation of effector caspases to induce apoptosis [39, 40]. We show for the first time that the process of islet amyloid formation and its resultant toxicity activates the pro-apoptotic BH3-only molecule *Bim*, in a JNK-dependent manner. The involvement of the intrinsic pathway of apoptosis in the cytotoxicity of islet amyloid is consistent with our previous findings that islet amyloid formation is associated with induction of oxidative stress [15], and oxidative stress is known to activate the intrinsic pathway [41]. In islets, BCL2-interacting mediator of cell death (BIM) has also been shown to be a critical mediator of glucotoxicity and is induced with cytokine treatment, underscoring its importance in mediating apoptosis in response to other types of stress in the islet [40, 42]. Furthermore, exogenous hIAPP treatment of a beta cell line has been shown to (1) upregulate several BH3-only proteins including BCL2-associated protein X (BAX), BAD, p53-upregulated modulator of apoptosis (PUMA) and truncated BH3-interacting domain death agonist (t-BID), (2) induce cytochrome *c* release and (3) activate caspase 7 and 9 [43].

The role of the anti-apoptotic proteins and how their dynamic regulation impacts the fate of the beta cell in response to the classic stressors of diabetes is largely unrecognised. Given that we have shown that islet amyloid formation results in beta cell apoptosis, we hypothesised that we may observe downregulation in the anti-apoptotic BCL2-like family of proteins as a potential mechanism of islet amyloid-induced beta cell death. Interestingly, we demonstrated no significant change in the levels of *Bcl2*, and found a JNK-dependent increase in *Bclxl* in the presence of islet amyloid formation. This is in contrast to studies with exogenous hIAPP treatment, which have found no difference in BCL2-like protein 1 (BCLXL) levels and a small decrease in BCL2 protein levels [43]. The increase in *Bclxl* in our study may represent a compensatory pro-survival mechanism by which the beta cell is attempting to offset the toxicity of amyloid formation. The induction of anti-apoptotic factors in response to beta cell stress has been observed in islets treated with cytokines [44] and islets

up to 1 week post transplantation [45]. Although the JNK signalling pathway has classically been thought to be pro-apoptotic, there is growing evidence that JNK signalling can induce pro-survival pathways in certain cell types, including neurons, T cells and B lymphocytes [46]. The anti-apoptotic response in islets challenged with cytokines is JNK3-dependent, which is in contrast to the JNK1 and JNK2 isoforms, which are pro-apoptotic [30]. By using a non-specific JNK inhibitor, we were unable to differentiate between the contributions of the different JNK isoforms. The significance of JNK-dependent *Bclxl* upregulation in the presence of islet amyloid as a potential compensatory protective mechanism will require further exploration, and could represent a potential therapeutic target that could be modulated to protect the beta cell from amyloid-induced cytotoxicity.

We used Congo Red, an amyloid inhibitor, which we have shown reproducibly inhibits islet amyloid formation and reduces associated beta cell apoptosis [15]. Interestingly, we observed changes in mRNA levels of some pro-apoptotic genes in our non-transgenic islets, suggesting that Congo Red may have amyloid-independent effects in islets. Whether this would have long-term deleterious effects is unclear, although a recent report in *Caenorhabditis elegans* described an extension of lifespan and a slowing of ageing with another amyloid-binding dye, Thioflavin T [47]. Other amyloid inhibitors, including WAS-406 (2-acetamido-1,3,6-tri-*O*-acetyl-2,4-dideoxy- α -D-xylo-hexopyranose), although effective at reducing amyloid deposition, also have amyloid-independent effects and may impact islet cell viability, and as such are limited in their utility [15]. Thus, a consideration in the future development of amyloid inhibitors would be to maximise specificity for amyloid while minimising potential off-target effects.

In summary, we have demonstrated that JNK signalling is a critical mediator of islet amyloid-induced beta cell apoptosis and that pro-apoptotic molecules in the extrinsic and intrinsic pathway are induced by JNK signalling downstream of amyloid formation. Based on these findings, we believe that future studies should evaluate JNK signalling in relation to oxidative stress and the relative contribution and importance of the downstream signalling molecules in the extrinsic and intrinsic pathways in mediating islet amyloid-induced beta cell apoptosis. Interventions targeted to prevent the activation of the JNK pathway and/or downstream mediators induced by islet amyloid may offer therapeutic benefit in minimising toxicity of islet amyloid and the preservation of beta cells in type 2 diabetes.

Acknowledgements We thank B. Barrow, C. Braddock, M. Cone, C. Forsyth, M. Peters, M. Watts and J. Willard for their excellent technical support. This work was supported by the Department of Veterans Affairs and National Institutes of Health grants DK-075998 (S. E. Kahn), DK-074404 (R. L. Hull), DK-080945 (S. Zraika), DK-

007247 and DK-017047. S. Subramanian and K. Aston-Mourney were supported by an American Diabetes Association Mentor-Based Fellowship. K. Aston-Mourney was also supported by a University of Washington McAbee Fellowship. J. Udayasankar was supported by a Juvenile Diabetes Research Foundation Postdoctoral Fellowship.

Contribution statement SLS, RLH, SZ, KA-M, JU and SEK conceived and designed the study, and participated in the analysis and interpretation of the data. SLS drafted the manuscript and all other authors revised it critically for intellectual content. All authors approved the final version of the paper.

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

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