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A Novel Role for Somatostatin in the Survival of Mouse Pancreatic Beta Cells

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Key Words
Somatostatin • Beta cell survival • Lipotoxicity • Cellular stress • Intra-islet communication • Type 2 diabetes

Abstract

\textbf{Background/Aims:} Cross-talk between different pancreatic islet cell types regulates islet function and somatostatin (SST) released from pancreatic delta cells inhibits insulin secretion from pancreatic beta cells. In other tissues SST exhibits both protective and pro-apoptotic properties in a tissue-specific manner, but little is known about the impact of the peptide on beta cell survival. Here we investigate the specific role of SST in the regulation of beta cell survival in response to physiologically relevant inducers of cellular stress including palmitate, cytokines and glucose. \textbf{Methods:} Pancreatic MIN6 beta cells and primary mouse islet cells were pre-treated with SST with or without the \textit{G}_{i/o} signalling inhibitor, pertussis toxin, and exposed to different cellular stress factors. Apoptosis and proliferation were assessed by measurement of caspase 3/7 activity, TUNEL and BrdU incorporation, respectively, and expression of target genes was measured by qPCR. \textbf{Results:} SST partly alleviated upregulation of cellular stress markers (\textit{Hspa1a} and \textit{Ddit3}) and beta cell apoptosis in response to factors such as lipotoxicity (palmitate), pro-inflammatory cytokines (IL1\textit{\beta} and TNF\textit{\alpha}) and low glucose levels. This effect was mediated via a \textit{G}_{i/o} protein-dependent pathway, but did not modify transcriptional upregulation of the specific NF\kappa B-dependent genes, \textit{Nos2} and \textit{Ccl2}, nor was it associated with transcriptional changes in SST receptor expression. \textbf{Conclusion:} Our results suggest an underlying protective effect of SST which modulates the beta cell response to ER stress and apoptosis induced by a range of cellular stressors associated with type 2 diabetes.

E. L. Damsteegt and Z. Hassan contributed equally to this work.

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Introduction

The development of type 2 diabetes is characterized by insulin resistance as well as an increased impairment of pancreatic beta cell function with a corresponding decline in insulin release. This has been ascribed to beta cell loss associated with accelerated levels of apoptosis [1] and is thought to be a consequence of cellular stress caused by a range of factors including gluco- and lipotoxicity and a pro-inflammatory environment characteristic of early type 2 diabetes.

Insulin-secreting beta cells are located within the three-dimensional anatomical structures of the pancreatic islets together with other cell types, the most important of which are glucagon-producing alpha cells and somatostatin-secreting delta cells. It is now clear that the intra-islet environment influences beta cell function and the effect of homotypic crosstalk between beta cells, and of heterotypic interactions between alpha and beta cells, has been studied in some detail by us and others [2-7]. Less attention has been paid to the intra-islet roles of delta cell-derived somatostatin (SST).

We have previously demonstrated that SST released locally from the delta cell exerts a tonic inhibitory effect on hormone secretion from both alpha and beta cells [8]. Recent single-cell transcriptome analysis of mouse and human islet cell types have in addition suggested a pivotal role for the islet delta cell and SST in receiving and mediating external signals from leptin, ghrelin and dopamine signalling pathways [9-11], suggesting a central role for this cell type in the overall integration of central and peripheral inhibitory cues regulating islet secretory function.

In contrast, little information is currently available about the impact of SST on beta cell survival. In addition to release from the islets, SST is also synthesized and secreted by neuroendocrine cells in the central nervous system and in the gastrointestinal and immune systems [12, 13]. In other tissues SST is reported to inhibit proliferation and promote apoptosis in a number of cancer cells including lung, gastric, colorectal and brain [14, 15]. In contrast, protective and anti-apoptotic properties of SST and analogues have also been shown in a range of contexts, such as in ischemia- and diabetes-induced retinopathies [16-20], following hepatectomy [21] and in models of neurodegenerative diseases such as Alzheimer's Disease [22] and Multiple Sclerosis [23]. The impact of SST on cell survival therefore appears to be tissue specific.

Our recent study of mouse pancreas remodelling during the first three weeks of life showed that the absence of SST had a negative impact on a range of islet mass parameters, leading to decreased proliferation and increased apoptosis [24], thus suggesting a role of the peptide in the regulation of beta cell mass in early life. Although foetal and early life events are crucial for the functional capacity of the beta cell in adult life (e.g. [25]), a significant change in islet size is not seen in adult SST-deficient mice compared to control animals under normal conditions [8]. We therefore suggest that a potential role of SST in beta cell survival is likely to become apparent under conditions of significant organ remodelling such as those seen in early life as reported above, or under conditions of increased physiological stresses, such as during the development of type 2 diabetes. The aim of this study was therefore to investigate the role of SST in beta cell survival with particular focus on the response to physiologically relevant conditions of cellular stress such as lipotoxicity and cytokine-induced inflammation, with subsequent impact on ER stress and cell survival.
Materials and Methods

Tissue culture
MIN6 beta cells were kindly provided by Professor J-I Miyazaki, University of Tokyo, Japan, and INS1 beta cells by Dr Natasha Hill, Kingston University London, UK. Reagents were from Sigma (Poole, UK) unless otherwise stated. All cell lines were routinely tested for mycoplasma contamination.

MIN6 beta cells (passages 23-40) were maintained in DMEM (10% [vol/vol] foetal bovine serum, 2 mmol/l L-glutamine, 100 U/ml penicillin/0.1 mg/ml streptomycin, 25 mmol/l glucose) and INS1 beta cells (passage 17-25) in RPMI 1640 medium (10% [vol/vol] foetal bovine serum, 2 mmol/l L-glutamine, 100 U/ml penicillin/0.1 mg/ml streptomycin, 1 mmol/l sodium pyruvate, 10 mmol/l HEPES buffer, 0.05 mmol/l β-mercaptoethanol and 11 mmol/l glucose). Cells were incubated at 37°C and 5% CO₂. The medium was changed every 3 days and the cells were passaged and used for experiments when 70-80% confluent. For some experiments (as indicated) MIN6 beta cells were maintained in supplemented DMEM containing 7.5 mmol/l glucose for 4-8 weeks prior to experimental use.

Islet isolation, maintenance and dispersal
Prior to islet isolations male ICR mice (30g, Charles River Laboratories, Margate, UK) were housed on a 12-h light/dark cycle and had access to food and water ad libitum in accordance with the UK Home Office Regulations. Mouse islets were isolated by collagenase digestion (1 mg/ml, type XI) and separated from exocrine pancreatic tissue on a histopaque gradient, as described [8]. Islets were incubated overnight at 37°C (5% CO₂) in RPMI 1640 medium (10% [vol/vol] foetal bovine serum, 2 mmol/l L-glutamine, 100 U/ml penicillin/0.1 mg/ml streptomycin, 11 mmol/l glucose) prior to experiments. For islet cell dispersal, islets were washed in PBS and incubated with Accutase (0.2 ml per 2-300 islets) for 8-10 min with intermittent dispersal by gentle pipetting. The reaction was stopped by the addition of PBS and cells were resuspended in RPMI and seeded onto coverslips (50-100,000 cells/coverslip) in 12-well plates. Incubation studies were commenced after 1h as described below.

Incubation studies with cellular stressors
MIN6 cells were trypsinized and seeded into 96-well plates for measurement of apoptosis, viability or proliferation (15,000 cells/well) and into 6 cm tissue culture petri dishes (1.5x10⁶ cells/dish) for assessment of mRNA expression. Cells were left to adhere overnight after which they were incubated in supplemented DMEM (10% FBS) with or without SST14 (Bachem AG, Bubendorf, Switzerland) or pertussis toxin (PTX, 100 ng/ml, Bio-Techne Ltd, Abingdon, UK) as indicated for 48h. The cells were maintained for an additional 20 or 48h in supplemented DMEM (2% FBS) with or without SST14, PTX and cellular stressors as indicated in the results section. Similar procedures were performed using INS1 cells, isolated islets and dispersed islet cells using supplemented RPMI. Prior to experiments, palmitate was dissolved in 50% ethanol (vol/vol) and heated at 70°C for 10 min, after which it was diluted 1:10 with 1% BSA and 0.5% ethanol. Control media contained 1% BSA and 0.5% ethanol. Please see Fig. 1A-B and 4A-B for optimisation of palmitate and cytokine concentrations.

Assessment of apoptosis
Apoptosis was induced in MIN6 and INS1 cells by exposure to the cellular stressors palmitate (0.5 mmol/l, 0.95% BSA) with or without 20 or 25 mmol/l glucose or cytokines (50 U/ml IL1β and 1000 U/ml TNFα, PeproTech EC Ltd, London, UK). It was measured using a Caspase-Glo assay (Promega, Southampton, UK) according to manufacturer’s instructions and read on a GloMax Navigator luminometer (Promega, Southampton, UK). In experiments using 5.5 mmol/l glucose cells were co-treated with SST14 whilst maintained in 5.5 mmol/l glucose for 48h prior to measurement of programmed cell death. In separate experiments whole islets were exposed to different glucose concentrations as indicated for 20h prior to assessment of apoptosis. Apoptosis induced in dispersed islet cells was assessed using a TdT (TUNEL) In Situ Apoptosis Detection Kit (DAB) according to manufacturer’s instructions (R&D systems, Bio-Technie Ltd, Abingdon, UK). Results shown are representative data from a minimum of three independent studies with 4-8 replicates per treatment group within experiments.
Cell viability

MIN6 and INS1 beta cell viability was assessed by measurement of cellular ATP content following treatment with SST14 and/or cellular stressors as described above using a CellTiter-Glo Luminescent Cell Viability Assay (Promega, Southampton, UK).

Proliferation

DNA synthesis was assessed as a marker of cell proliferation by measuring 5-bromo-2′-deoxyuridine (BrdU) incorporation over 3h in MIN6 cells following treatment with SST14. A commercially available kit (Cell Proliferation ELISA, BrdU, Colorimetric, Roche Applied Science, Mannheim, Germany) was used according to the manufacturer’s instructions. Results shown are representative data from three independent studies with 8 replicates per treatment group in individual experiments.

qPCR

Islets were harvested or MIN6 cells trypsinised and washed twice in ice-cold PBS. Total RNA was extracted from different treatment groups of islets or MIN6 cells according to the manufacturer’s protocol using an RNeasy kit (Qiagen, Sussex, UK) and quantified on a NanoDrop ND-1000 UV/Vis spectrophotometer. Following DNAse treatment (RNase-Free DNase set, Qiagen, Sussex, UK), reverse transcription of RNA to cDNA was performed using Applied Biosystems High-Capacity cDNA Reverse Transcription kit (Fisher Scientific UK Ltd, Loughborough, UK). Quantitative (q)RT-PCR was performed with Light cycler 480 (Roche, UK) and StepOne Plus (Applied Biosystems, UK) real time PCR systems using QuantFast SYBR Green PCR Kit (Qiagen, Sussex, UK) with 40 cycles of amplification as described previously [26]. Specificity of primer pairs was confirmed by melting curve analyses. Each sample value was normalized to the reference genes Ppia and Hprt and relative expression of mRNAs calculated by the 2^(-ΔΔCt) method [27]. Ddit3 (QT01749748), Hspa1a (QT01660664), Ppia (QT00247709), Hprt (QT00166768), Sst (QT00167832), Ccl2 (QT00167832), Nos2 (QT00100275) and Ins2 (QT00114289) primers were obtained from Qiagen (Mt: 60°C) and primers for Sstr1-5 were designed as previously described [8]. Results shown are means ± SEM of a minimum of three separate experiments.

Data analysis

Data are expressed as means ± SEM and analysed statistically using one way ANOVA and Student’s t test or Bonferroni’s multiple comparisons test, as appropriate. Differences between treatments were considered significant at P<0.05.

Results

Effect of SST14 on beta cell survival in response to lipotoxicity

Due to the composite nature of islets consisting of different cell types, the pancreatic beta cell line MIN6 was used as a research model to assess the impact of somatostatin on beta cell survival, thereby excluding any paracrine influences from non-beta cells in the islet, including endogenous SST. Since SST14 is the isoform produced locally in the pancreatic islets, SST14 rather than SST28 was used as an exogenous peptide throughout the study.

It has previously been established that the saturated fatty acid, palmitate, is a diabetogenic factor which induces beta cell death and this was confirmed in MIN6 beta cells (Fig. 1A-D). Pre-incubation with SST14 for 48h significantly reduced palmitate-induced apoptosis as shown in Fig. 1C whereas no significant change was observed when cells were maintained under non-stressed, basal conditions. SST receptors signal via both G_i/o protein-dependent and independent pathways, but the observed reduction in caspase 3/7 activity was mediated via G_i/o signalling, since co-treatment with the G_i/o signalling inhibitor, pertussis toxin, abolished the observed effect (Fig. 1D).

Palmitate induces ER stress and upregulation of the unfolded protein response in beta cells (UPR, [28, 29]). Accordingly, mRNA expression of Hspa1a, which codes for the inducible early stress response protein, heat shock protein 70 (HSP70), and Ddit3, coding for the ER stress-responsive, pro-apoptotic transcription factor, CHOP, were upregulated in MIN6 beta cells (Fig. 1E-H).
Fig. 1. MIN6 beta cell survival following exposure to palmitate with or without SST14. To optimise the palmitate concentration used for assessment of apoptosis MIN6 beta cells were initially incubated for 20h with increasing concentrations of palmitate (PAL) in DMEM containing 2 or 10% FBS as indicated (panel A). In a separate set of experiments MIN6 cells were exposed to 0.5 or 1 mmol/l palmitate or oleate (OL), respectively, with 2% FBS (panel B). Based on the results, a concentration of 0.5 mmol/l palmitate was subsequently used to induce beta cell apoptosis. MIN6 beta cells were pre-treated for 48h with SST14 followed by 20h incubation with or without palmitate (PAL, black bars) and apoptosis following treatment was assessed by measurement of 3/7 caspase activity (panel C). The observed reduction in apoptosis following SST14 (1 μmol/l) pre-treatment was reversed by co-incubation with the G\textsubscript{i/o} inhibitor, pertussis toxin (PTX, 100 ng/ml, panel D). mRNA levels of Hspa1a (panel E) and Ddit3 (panel F) were assessed by qPCR as an indication of cellular and ER stress, respectively, and expression of both were significantly reduced by SST14. Panel A and B: **P<0.01, ***P<0.001 vs 10% FBS control or ∆∆∆P<0.001 vs 2% FBS control, n=8. Panel C-F: *P<0.05, **P<0.001 vs control without SST14, ∆∆∆P<0.001 SST14 vs SST14 + PTX (one way ANOVA and Bonferroni’s multiple comparisons test (A-D) or Student’s t test (E and F)). Panel C and D representative of 3-7 experiments. Panel E and F: mean ± SEM of 4-6 separate experiments.
cells following exposure to palmitate (Fig. 1E). Consistent with the effect of SST14 on MIN6 beta cell survival, palmitate-induced mRNA expression of both Hspa1a and Ddit3 were significantly reduced following pre-treatment with SST14 (Fig. 1E-F). Apoptosis was measured by counting of DAB-positive cells following TUNEL staining. Data representative of three separate experiments. **P<0.01 vs treatment with palmitate only (one way ANOVA and Bonferroni’s multiple comparisons test).

Intact islets contain endogenous SST but islet dispersal leads to a reduction in endogenous Stt mRNA expression by 88% (n=2) in populations of dispersed islet cells compared to intact islets, suggesting a loss of SST-secreting delta cells during the dispersal procedure. We therefore assessed the effect of SST14 on apoptosis in dispersed islet cells. Consistent with findings in MIN6 beta cells, palmitate-induced apoptosis was significantly reduced in dispersed islet cells by SST14 after 20h (Fig. 2).

SST has previously been reported to exert anti-proliferative effects particularly in cancer cells, but we did not observe any reduction in rate of proliferation or overall cell viability of MIN6 cells following incubation with SST14 for 48h under normal culture conditions when assessed by measurement of BrdU incorporation or cellular ATP content, respectively (Fig. 3A and B).

**Effect of SST on beta cell survival in response to other cellular stress factors**

**Pro-inflammatory cytokines.** To assess whether the observed effect of SST14 on beta cell survival was specific to palmitate-induced ER stress and apoptosis, MIN6 beta cells were exposed to other cellular stress factors of physiological relevance. Fig. 4C shows that cytokine-induced apoptosis (50 U/ml IL1β and 1000 U/ml TNFα) was significantly reduced...
when cells were pre-treated with increasing concentrations of SST14. Cytokines have been reported to induce upregulation of Ddit3 mRNA (e.g. [30, 31]), but in our hands Ddit3 levels were unchanged following 20h exposure to a combination of IL1β and TNFα (P>0.2), although these levels were significantly reduced in the presence of SST14 (1 μmol/l: 75±3% control, P<0.01). In contrast, 20h exposure of the MIN6 beta cells to IL1β and TNFα led to upregulation of the NFκB-dependent genes Nos2 and Ccl2, which encode for the enzyme inducible nitric oxide synthase 2 and the chemokine Ccl2, respectively, from undetectable (Ct values >35) to clearly measurable levels (Nos2: Ct=25.4±0.9, Ccl2: 27.1±0.4). However, no significant change in the upregulation of their mRNA expression was detected when cells were treated with SST14 prior to cytokine exposure (Nos2: 100 nmol/l SST14: 117±17% cytokine control only, 1μmol/l SST14: 127±22%; Ccl2: 100 nmol/l SST14: 112±11% cytokine control only, 1μmol/l SST14: 136±10%, P>0.05), suggesting that the observed effect of SST14 is unlikely to be mediated via direct modulation of NFκB expression and/or activation.

Consistent with previous reports [32], 20h exposure to palmitate did not induce upregulation of Nos2 and Ccl2 mRNA expression in MIN6 cells, and mRNA for both genes was indeed undetectable under both control and palmitate-induced conditions.

**Hyperglycaemia and glucolipo toxicity.** Under standard conditions, the MIN6 cell line does not lend itself to studies on the impact of hyperglycaemia on beta cell survival given the recommended culture conditions of 25 mmol/l glucose [33], so we investigated the effect of high glucose in MIN6 beta cells maintained in media containing 7.5 mmol/l glucose for 4-8 weeks prior to experiments and in the rat beta cell line INS1, which is maintained at 11 mmol/l glucose. Under these conditions MIN6 cells maintained their proliferative capacity

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**Fig. 4.** Role of SST14 in beta cell survival following exposure to pro-inflammatory cytokines. To identify an effective exposure time and cytokine combination for assessment of apoptosis, MIN6 cells were initially exposed to TNFα and IL1β alone or in combination for 6, 16 or 20h as well as to TNFα, IL1β and IFNγ combined (1000 U/ml, 50U/ml and 1000 U/ml, respectively, panel A and B, n=4). Based on the results, apoptosis was subsequently assessed in MIN6 beta cells pre-treated with SST14 for 48h prior to 20h incubation with 50 U/ml IL1β and 1000 U/ml TNFα panel C, data representative of three separate experiments. ***P<0.001 vs control without cytokines as appropriate, **P<0.001 SST14 vs control (one way ANOVA and Bonferroni’s multiple comparisons test).
(Fig. 5A) and the cells did not exhibit significantly altered gene expression of *Ddit3*, *Hspa1a* or *Ins2, Sstr1, Sstr2, Sstr3* and *Sstr5* compared to cells maintained in 25 mmol/l (Table 1). In both experimental settings we found that 20 or 48h exposure to high concentrations of glucose did not lead to increased caspase 3/7 activity (MIN6: 20h exposure to 25 mmol/l glucose: 107±7% of caspase 3/7 activity at 7.5 mmol/l glucose; 48h: 104±16%; INS1: 20h...
exposure to 20 mmol/l glucose: 118±14% of activity at 11 mmol/l glucose, P>0.1, Fig. 5B, E) nor did it cause a decrease in cell viability of INS1 cells (Fig. 5C-D). These findings are consistent with reports suggesting that a combination of lipo- and glucotoxicity is a more potent beta cell stressor in beta cells and islets than glucose alone [34], and MIN6 cells were therefore exposed to a combination of 25 mmol/l glucose and 0.5 mmol/l palmitate for 20h. Under these conditions 48h pre-treatment with SST14 partly reduced the detrimental effect of palmitate and glucose on cell survival in MIN6 cells (Fig. 5B) although the combination of glucose and palmitate did not further increase caspase 3/7 activity compared to 0.5 mmol/l palmitate alone (P>0.1). As was the case with cells maintained at 25 mmol/l glucose, cytokine-induced apoptosis was reduced following pre-treatment with SST14 (Fig. 5B).

**Low glucose and serum.** We have previously reported that increasing glucose concentrations (5-25 mmol/l) can exhibit a protective, rather than a detrimental, effect on beta cell survival and reduce caspase activity levels induced by prolonged exposure to low glucose in MIN6 beta cells (48h, 2.5 mmol/l, [35]). Efanova et al. [36] similarly found that 40h incubation with 5 mmol/l glucose or below led to increased cell death in rodent islets compared to 11 mmol/l glucose, a concentration which is generally accepted as being optimal for maintaining rodent islets in culture [36, 37]. In the present study we also observed an increase in caspase 3/7 activity of islets exposed to low glucose concentrations for 20h (5 mol/l glucose: 259±34% caspase 3/7 activity at 11 mmol/l glucose, 0 mmol/l glucose: 226±34%, P<0.05). Consistent with these findings, 48h exposure of MIN6 beta cells to 5.5 mmol/l glucose significantly increased beta cell apoptosis compared to controls and this effect was abolished by 48h co-incubation with SST14 (Fig. 6A).

The PERK-ATF4 signalling branch of the unfolded protein response (UPR) is reportedly upregulated by low glucose concentrations, leading to increased expression of pro-apoptotic ATF4 target genes, including Ddit3 [37]. Ddit3 mRNA expression was not, however, further increased by exposure to 5.5 mmol/l glucose compared to controls (Fig. 6B). This may reflect the fact that 48h exposure to control media containing 2% FBS compared to 10% FBS led to a significant two-fold increase in Ddit3 mRNA levels on its own, suggesting that prolonged reduction in serum levels itself constitutes a stress factor (Fig. 6B). A similar, although not statistically significant, trend of increased expression was seen in MIN6 cells after 20h culture at 2% FBS (150±29% of caspase activity at 10% FBS), although these findings did not manifest themselves in increased levels of caspase activity. Thus exposure to decreasing concentrations of serum for 20 or 48h did not induce apoptosis at concentrations used routinely in this study (Fig. 6C-D). Nevertheless, under all these conditions pre-treatment with 1 μmol/l SST14 significantly reduced Ddit3 mRNA expression (Fig. 1F and 6B).

**SST receptor expression under conditions of cellular stress**

MIN6 cells express Str1, 2, 3 and 5 mRNA with Sstr3 in particular being highly expressed, as has also recently been reported for beta cells in single islet cell transcriptome studies ([9, 10], Fig. 7A). Previous reports suggest that G-protein-coupled receptor (GPCR) mRNA expression including that of SSTRs can be altered in response to various forms of cellular stress factors such as inflammation and hyperglycaemia [23, 38]. Sstr mRNA expression was therefore determined following exposure to palmitate and cytokines at concentrations and durations investigated above.

**Table 1.** Gene expression in MIN6 cells maintained for 4-8 weeks in 25 or 7.5 mmol/l glucose. Target genes of interest were expressed relative to the house keeping gene Ppia. Results are shown as means±SEM of 3-4 separate cell preparations, where statistical significance was tested using Student's t-test.

<table>
<thead>
<tr>
<th>Gene</th>
<th>25 mmol/l glucose</th>
<th>7.5 mmol/l glucose</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ddit3</td>
<td>0.1±0.008</td>
<td>0.09±0.003</td>
<td>0.29</td>
</tr>
<tr>
<td>Hspa1a</td>
<td>3.85±0.80±10^-4</td>
<td>2.12±0.18±10^-4</td>
<td>0.06</td>
</tr>
<tr>
<td>Ins2</td>
<td>64.6±4.42</td>
<td>63.07±4.51</td>
<td>0.82</td>
</tr>
<tr>
<td>Sstr1</td>
<td>1.60±0.27±10^-4</td>
<td>1.48±0.34±10^-4</td>
<td>0.81</td>
</tr>
<tr>
<td>Sstr2</td>
<td>1.15±0.38±10^-4</td>
<td>1.29±0.19±10^-4</td>
<td>0.73</td>
</tr>
<tr>
<td>Sstr3</td>
<td>10.87±3.10±10^-3</td>
<td>7.98±1.47±10^-3</td>
<td>0.40</td>
</tr>
<tr>
<td>Sstr5</td>
<td>4.20±2.17±10^-3</td>
<td>2.29±0.28±10^-3</td>
<td>0.35</td>
</tr>
</tbody>
</table>
Culture for 20h with palmitate or cytokines did not alter Sstr mRNA levels compared to controls in a consistent manner (Fig. 7B-E), nor did 2% FBS on its own have any effect (data not shown). However, Sstr3 expression was reduced in the presence of cytokines (P<0.05, Fig. 7D) and Sstr1 and 2, but not Sstr3 and 5 mRNA expression was significantly upregulated by prolonged (48h) exposure to 2% FBS (Fig. 8A-D).

Overall, pre-incubation with SST14 prior to conditions of stress did not significantly change receptor expression although under conditions of prolonged exposure to low serum levels Sstr1 and 5 mRNAs were down-regulated (Fig. 8A and D). These results suggest that in our experimental setting, Sstr transcription was not universally up- or downregulated in response to stress factors such as palmitate or cytokines, although serum deprivation impacted on mRNA levels in a divergent manner which was dependent on receptor subtype. The observed effect of SST14 on beta cell survival is therefore unlikely to be a result of transcriptional changes at receptor level.
The importance of intra-islet SST in the regulation of pancreatic hormone secretion is well-established, but at present there is little information regarding a potential role of the peptide in the regulation of beta cell survival. Here we report the novel finding that the level of beta cell apoptosis following exposure to physiologically relevant cellular stressors is reduced by SST14. Our results indicate that the action of SST14 is likely to involve an underlying protective mechanism rather than being linked to specific conditions of cellular stress and subsequent cell death.

**Fig. 7.** Effect of SST14 on SSTR receptor expression under conditions of lipid and cytokine toxicity. Sstr mRNA expression was assessed in MIN6 beta cells by qPCR under normal culture conditions (panel A) or following pre-treatment with 1 μmol/l SST14 for 48h followed by 20h exposure to 0.5 mmol/l palmitate (PAL) or cytokines (50 U/ml IL1β and 1000 U/ml TNFα), with or without SST14. Panels B-E show results for Sstr 1, 2, 3, and 5, respectively. *P<0.05, vs control (one way ANOVA and Bonferroni's multiple comparisons test). Data presented as means ± SEM of 6-8 separate experiments.

**Discussion**

The importance of intra-islet SST in the regulation of pancreatic hormone secretion is well-established, but at present there is little information regarding a potential role of the peptide in the regulation of beta cell survival. Here we report the novel finding that the level of beta cell apoptosis following exposure to physiologically relevant cellular stressors is reduced by SST14. Our results indicate that the action of SST14 is likely to involve an underlying protective mechanism rather than being linked to specific conditions of cellular stress and subsequent cell death.
We initially chose to investigate the impact of SST treatment on cellular responses to lipotoxicity since this is a key contributor to beta cell dysfunction and damage in the development of type 2 diabetes. The saturated free fatty acid, palmitate, exerts a number of detrimental effects which under chronic conditions leads to beta cell dysfunction and apoptosis [32, 39]. This was confirmed in our study by the induction of cellular stress as reflected in increased mRNA expression of *Hspa1a* coding for Hsp70, by upregulation of the ER stress-responsive, pro-apoptotic transcription factor, *Ddit3*, and ultimately by upregulation of caspase 3/7 activity indicating onset of apoptosis. Assessment of these parameters confirmed a protective role of SST14 in response to lipotoxicity in the pancreatic beta cell line MIN6.

The study of SST14 in primary islets is complicated by the presence within islets of different cell types including SST14-secreting delta cells that are likely to be associated with high local concentrations of endogenous SST14. In our hands dispersal of islets into single cell suspensions led to substantial reduction in SST expression and we were thus able to confirm our findings on the protective effects of SST14 from MIN6 cells in primary islet cells.

Both type 1 and type 2 diabetes are associated with an inflammatory insult of the beta cells and lipotoxicity itself also leads to the generation of inflammatory factors from adipose tissue [40]. We therefore assessed whether the observed protective effect of SST14 extended to this type of cellular stress. The detrimental impact of circulating fatty acids or cytokines on beta cells is mediated via a range of mechanisms, overlapping as well as diverging [30, 32, 39]. Thus, both these stress conditions can for example induce ER stress [28, 41] although
potentially by different means. Our study confirmed a protective effect of SST14 on beta cell survival following cytokine as well as palmitate exposure although we did not observe an upregulation of the ER stress responsive transcription factor Ddit3 in response to cytokines. This does not, however, exclude potential involvement of other arms of the UPR, ER stress and apoptotic pathways. NFkB is another key transcription factor which upon activation initiates cytokine-induced apoptosis via transcriptional regulation of downstream target genes such as Nos2 and the chemokine Ccl2. Excessive NO production by inducible nitric oxide synthase 2 leads to ER stress [42] and expression of both Ccl2 and Nos2 mRNAs were increased in MIN6 cells following cytokine treatment. SST14 did not, however, modify the upregulation of these target genes, thus questioning an impact of SST14 on NFkB. The specific underlying pathways being modulated by SST14 therefore remain to be further investigated.

Hyperglycaemia had little detrimental effect on caspase 3/7 activity in both INS1 and MIN6 cells and our results suggest that lipotoxicity rather than glucoxicity is the main stress factor in our experimental models, although it is possible that high glucose concentrations negatively affect beta cell survival via caspase 3/7 independent pathways. Regardless, the observed protective effect of SST14 on beta cell apoptosis was maintained in MIN6 beta cells under these conditions as was also the case in response to the negative, although less severe effects of prolonged exposure to low glucose levels which could be important in times of fasting. Overall our results suggest that prolonged exposure to SST14 partly protects beta cells against a range of physiologically relevant cellular stressors. We note in particular that the use of different surrogate markers for beta cell stress and apoptosis resulted in varied outcomes depending on the type of stressor involved, suggesting that these markers are not necessarily linked in a causal manner. Nevertheless, the overall effect of SST14 was consistently that of beta cell protection.

We have previously found that hyperglycaemia leads to changes in the expression in islets of another GPCR, the muscarinic M3 receptor, with subsequent consequences for islet function [38]. In a recent single cell transcriptome study SSTR mRNA expression was increased in islet cells from human type 2 diabetes islet samples compared to healthy controls [11] and another study reported of SSTR2 and 4 upregulation in human brain endothelial cells in response to cytokines and lipopolysaccharides (LPS, [23]). Although we observed some minor changes in individual receptor expression in response to cytokines and 48h culture in low serum, these changes were not uniform in response to the different types of cellular stresses both with regards to receptor subtype and to transcriptional up- or down-regulation. Importantly, the observed minor changes were not consistently counteracted by SST14 treatment (which in other experiments alleviated beta cell apoptosis in response to a range of cellular stress factors), suggesting that the impact of SST14 on beta cell apoptosis is unlikely to be dependent on transcriptional changes of the SSTRs.

Our data indicate that SST14 mediates its effect on beta survival via pertussis toxin sensitive G_{i/o} protein coupled signalling. It is beyond the scope of this study to identify specific components of the intracellular transduction pathway(s) involved, particularly given the complexity of receptor subtypes and formation of potential homo- or hetero-oligomeric receptor complexes with differential links to the intracellular signalling machinery. In the beta cell SST inhibits insulin secretion via inhibition of adenylate cyclase, K_{ATP} channels, Ca^{2+} influx as well as via action at distal sites of exocytosis [43, 44]. SST also inhibits the release of cytokines from a range of immune and endothelial cells [45] and islets similarly have the capacity to release a vast secretome of cytokines. It is therefore conceivable that SST mediates its effect on survival via a similar inhibitory action on releasable factors (other than NO and Ccl2) involved in the apoptotic response of the beta cell.

SST14 had little effect on cell viability, proliferation and survival under non-stressed conditions. This is consistent with previous findings in 8-12 week old Sst-deficient mice, where no significant change in islet size was observed compared to control animals under normal conditions [8]. In contrast, lack of SST led to increased apoptosis during pancreas remodelling in early life [24] and our current results confirm the suggestion that the protective effect of SST is particularly relevant under conditions of beta cell stress such as
for example during the development of type 2 diabetes. Consistent with these findings a recent study by Luque et al. [46] showed that Sst-deficient mice fed a low fat diet exhibited a normal metabolic phenotype, whereas a high fat diet for 12-14 weeks led to gender-specific impairment of glucose metabolism. Thus, male Sst-deficient mice exhibited increased fasting glucose levels and impaired glucose clearance whereas there were no observed changes in insulin tolerance compared to wild type mice fed a high fat diet.

Increases in the number of delta cells have been observed in type 1 diabetes [47, 48] whereas there are diverging reports regarding changes in delta cell mass of both patients and animal models of type 2 diabetes [48-51]. Functionally, glucose-induced SST secretion appears to be impaired in both types of the condition (reviewed in [52]) and it is therefore conceivable that imbalances in SST release not only impact insulin secretion in the development of type 2 diabetes, but may also directly affect the overall survival of the beta cell during this period via separate mechanisms. This hypothesis is supported by our present findings and suggests that intra-islet communication between pancreatic beta cells and non-beta cells such as the delta cell is critical for optimal maintenance of a functional beta cell mass, which in turn has implications for the onset of type 2 diabetes.

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Disclosure Statement

The authors have nothing to disclose.

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