Article

A VGF-Derived Peptide Attenuates Development of Type 2 Diabetes via Enhancement of Islet β-Cell Survival and Function

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SUMMARY

Deterioration of functional islet β-cell mass is the final step in progression to Type 2 diabetes. We previously reported that overexpression of Nkx6.1 in rat islets has the dual effects of enhancing glucose-stimulated insulin secretion (GSIS) and increasing β-cell replication. Here we show that Nkx6.1 strongly upregulates the prohormone VGF in rat islets and that VGF is both necessary and sufficient for Nkx6.1-mediated enhancement of GSIS. Moreover, the VGF-derived peptide TLQP-21 potentiates GSIS in rat and human islets and improves glucose tolerance in vivo. Chronic injection of TLQP-21 in prediabetic ZDF rats preserves islet mass and slows diabetes onset. TLQP-21 prevents islet cell apoptosis by a pathway similar to that used by GLP-1, but independent of the GLP-1, GIP, or VIP receptors. Unlike GLP-1, TLQP-21 does not inhibit gastric emptying or increase heart rate. We conclude that TLQP-21 is a targeted agent for enhancing islet β-cell survival and function.

INTRODUCTION

Pancreatic islet β-cells perform a critical role in the regulation of whole animal fuel homeostasis by secreting insulin in response to nutritional and hormonal signals. Deterioration of β-cell mass and function are key events in the development of type 2 diabetes (T2D) (Muoio and Newgard, 2008). Multiple pharmacotherapies exist for treating T2D, each with distinct impacts on β-cell mass and function. Sulfonylureas directly stimulate insulin release, but when given chronically may exacerbate β-cell failure (Efanova et al., 1998; Maedler et al., 2005). Insulin-sensitizing agents such as the thiazolidinediones (TZD) and metformin can delay and/or prevent loss of islet β-cell mass in rodents and humans, despite exerting their effects primarily in peripheral tissues (Finegood et al., 2001; Kahn et al., 2006; Ovalle and

Bell, 2002). Finally, emerging incretin-based therapies such as the stable GLP-1R agonists, exenatide and liraglutide, and the DPP-4 inhibitors sitagliptin and saxagliptin, enhance islet β-cell function and survival (Ahrén et al., 2000; Drucker and Nauck, 2006; Reimer et al., 2002; Stoffers et al., 2000; Xu et al., 1999). However, the ability of GLP-1 therapies to promote β-cell survival in the context of human T2D is not fully understood, in part due to the absence of methods for measuring human islet mass in situ. Also, β-cell secretagogues have traditionally suffered from a loss of efficacy over time; whether this will be a problem for GLP-1-based therapeutics has yet to be determined. Finally, GLP-1 and related peptides have significant side effects, including gastrointestinal effects that can be sufficiently severe to result in cessation of therapy in some patients. These unresolved issues encourage continued investigation into alternative pathways for preservation and enhancement of functional β-cell mass.

We previously demonstrated that overexpression of the homeodomain transcription factor Nkx6.1 in rat islets increases islet β-cell replication while simultaneously enhancing glucose-stimulated insulin secretion (GSIS) (Schisler et al., 2008, 2005). In the current study, we demonstrate that Nkx6.1 induces expression of the prohormone VGF (nonacronymic; unrelated to VEGF) and that this induction explains the enhancement of GSIS by Nkx6.1. We then show that the C-terminal VGF peptide, TLQP-21, potentiates GSIS in isolated rat islets and reduces glycemic excursion in Wistar rats following a glucose challenge. Chronic administration of TLQP-21 to Zucker Diabetic Fatty (ZDF) rat delays the onset of overt diabetes by preserving islet cell mass. Subsequent in vitro studies demonstrate that TLQP-21 is similar to exendin-4 in blocking islet β-cell apoptosis induced by two different agents. We demonstrate that TLQP-21 shares many of the antidiabetogenic properties of GLP-1R agonists (potentiates GSIS, improves glycemic control, reduces islet cell apoptosis) yet lacks some of the non-β-cell effects of GLP-1R agonists (inhibition of gastric emptying and stimulation of heart rate). Our studies suggest that TLQP-21 represents a specific agent for preserving β-cell mass and function during development of T2D.
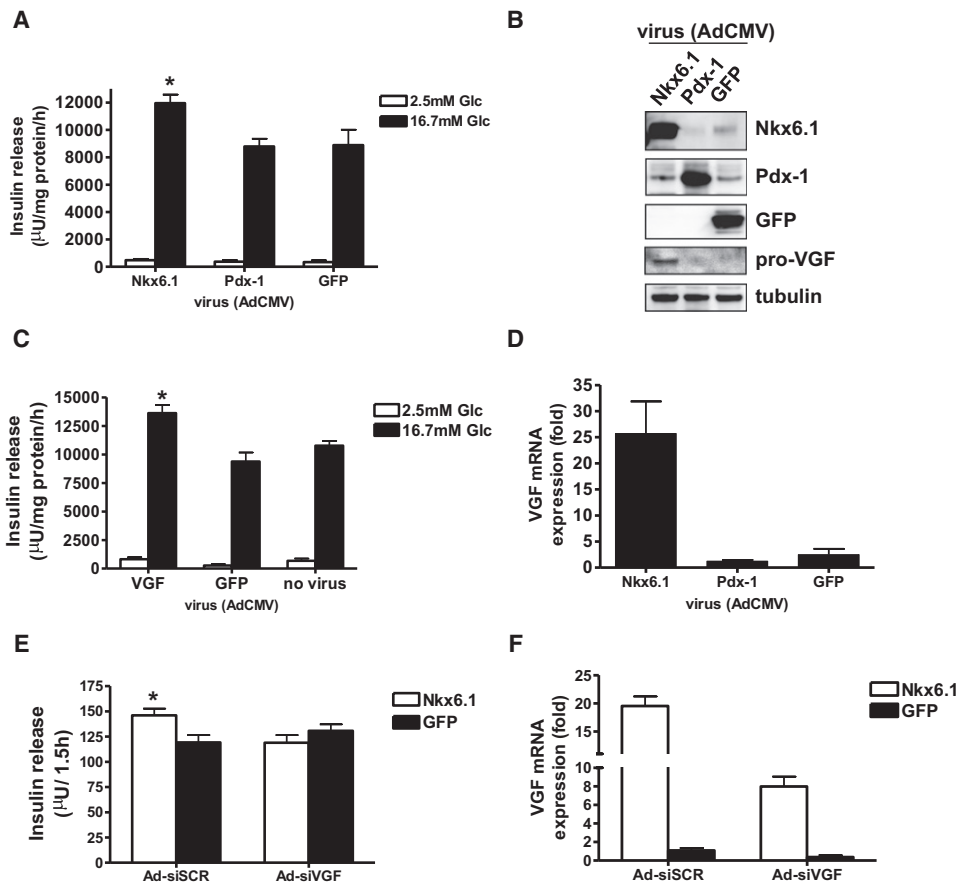
Figure 1. Overexpression of Nkx6.1, but Not Pdx-1 Enhances GSIS and Increases VGF Expression in Primary Rat Islets
(A–D) Rat islets were treated with recombinant adenoviruses (AdCMV) expressing Nkx6.1, Pdx-1, VGF or GFP as indicated and analyzed 72 hr postviral treatment. (A and C) Glucose-stimulated insulin secretion was measured by static incubation in media containing 2.5 mM Glc and 16.7 mM Glc for 1 hr each. (B) Immunoblot analysis of whole cell lysates. (D) Quantitative RT-PCR was used to measure VGF mRNA levels.
(E and F) Rat islets were treated with recombinant adenoviruses expressing Ad-siVGF or Ad-siSCR for 24 hr followed by treatment with AdCMV-Nkx6.1 or AdCMV-GFP for an additional 24 hr as indicated. Glucose-stimulated insulin secretion was measured by static incubation in media containing 11.2 mM Glc for 1.5 hr. In (A)–(F), data represent the mean ± SEM of 3 independent experiments. *p ≤ 0.01.

RESULTS

Overexpression of Nkx6.1, but Not Pdx-1 Enhances Glucose-Stimulated Insulin Secretion

We have previously demonstrated that overexpression of Nkx6.1 in adult rat islets has the rare effect of simultaneously promoting β-cell proliferation and enhancing GSIS (Schisler et al., 2008, 2005). Because Nkx6.1 and Pdx-1 are two prominent homeobox transcription factors that are preferentially expressed in β-cells, we compared their ability to enhance insulin secretion in the setting of the adult islet. Treatment of rat islets with adenoviruses containing the Nkx6.1 or Pdx-1 cDNAs caused increases of almost 10-fold in each protein relative to a GFP control (Figure 1B), but only Nkx6.1 overexpression caused a 35% increase in insulin secretion at stimulatory (16.7 mM) glucose (Figure 1A).

Based on the data shown in Figure 1A, we postulated that genes specifically regulated by Nkx6.1 but not Pdx-1 contribute to enhanced GSIS. We therefore performed cDNA microarray analysis of rat islets overexpressing Nkx6.1, Pdx-1, or β-galacto-

sidase (control). The Nkx6.1 array data was published previously (Schisler et al., 2008). Genes that were increased by ≥ 2-fold or reduced in expression by ≥ 50% by Nkx6.1 overexpression and not regulated by Pdx-1 relative to control islets were identified as potential regulators of islet β-cell function (Table S1). The nerve growth factor-inducible gene VGF (nonacronymic; unrelated to VEGF) was the most highly upregulated gene on this list. Confirming the results of the microarray data, we observed a 25-fold upregulation of VGF mRNA (Figure 1D) and a robust increase in pro-VGF protein levels (Figure 1B) in rat islets overexpressing Nkx6.1, but not Pdx-1 or GFP.

VGF Expression in Rat Islets Enhances GSIS

To determine if VGF overexpression can mimic the enhanced GSIS observed with Nkx6.1 overexpression, we generated a recombinant adenovirus containing the human VGF cDNA. For this experiment, we used adenovirus titers that gave comparable levels of VGF overexpression as obtained in response to Nkx6.1 overexpression (data not shown). Overexpression of VGF in primary rat islets resulted in a 46% increase in GSIS at

stimulatory glucose (16.7 mM Glc) relative to the GFP control, without affecting basal insulin secretion (2.5 mM Glc) (Figure 1C). Neither Nkx6.1 (Schisler et al., 2008) nor VGF overexpression changed insulin content in rat islets, and Nkx6.1 levels were not affected by manipulation of VGF expression (data not shown). These data demonstrate that VGF overexpression enhances GSIS in a manner similar to Nkx6.1 overexpression.

To determine if VGF upregulation is required for the Nkx6.1-mediated enhancement of GSIS, we used a recombinant adeno-virus to suppress VGF expression (Ad-siVGF) in rat islets. As shown in Figures 1E and 1F, Nkx6.1 overexpression resulted in a strong upregulation of VGF (~20-fold) and a corresponding increase in GSIS. siRNA-mediated suppression of VGF upregulation in Nkx6.1 overexpressing islets (Figure 1F) reduced GSIS to levels observed in islets treated with AdCMV-GFP (Figure 1E). In sum, data in Figures 1C, 1E, and 1F establish that upregulation of VGF is required for Nkx6.1-mediated enhancement of GSIS, and that increased expression of VGF is sufficient to drive the improved glucose response.

The C-Terminal VGF Peptide TLQP-21 Potentiates GSIS in Rat and Human Islets

VGF is expressed as a 67 kDa prohormone and processed by PC1/3 and PC2 to yield a number of distinct peptides (Garcia et al., 2005; Levi et al., 2004; Trani et al., 2002). In islet cells, these peptides are stored in large dense core granules and secreted via the regulated secretory pathway in response to glucose and other secretagogues (Possenti et al., 1999). C-terminal VGF-derived peptides, including TLQP-21 and AQEE-30 (shown in Figure 2A), have been previously documented to play distinct roles in the central nervous system (Bartolomucci et al., 2006; Hunsberger et al., 2007). These peptides have also been identified in islets (Cocco et al., 2007), but their biological functions have not been reported in islet cells.

To begin to investigate the potential impact of TLQP-21 on islet biology, we first studied the effect of acute administration of TLQP-21 on insulin secretion in primary rat islets, using the GLP-1R agonist, exendin-4, as a positive control. Addition of TLQP-21 to rat islets resulted in a concentration-dependent increase in GSIS, with significant effects observed with as little as 5 nM peptide, and a maximal 35% increase at 50 nM (Figure 2B). In comparison, 20 nM exendin-4 enhanced GSIS by 80%. Similar to exendin-4 and consistent with the VGF overexpression data (Figure 1C), TLQP-21 potentiated insulin release only at stimulatory (16.7 mM) glucose concentrations and did not raise basal (2.5 mM Glc) insulin secretion (Figure 2B). The concentrations of TLQP-21 required for enhancement of GSIS are consistent with those recently reported to enhance iso-proterenol-mediated lipolysis in 3T3 L1 adipocytes (Possenti et al., 2012). In contrast, a second C-terminal VGF peptide, AQEE-30, was unable to augment GSIS at any concentration studied (Figure S1A). To assess whether naturally occurring C-terminal extensions of the TLQP peptide could enhance the peptide's potency, we tested TLQP-30 and TLQP-42 (see Figure 2A). Both of these longer peptides potentiated GSIS to a similar extent as TLQP-21 (Figure S1B).

We also tested the effect of TLQP-21 in human islet preparations from four separate donors. Similar to rat islets, 50 nM

TLQP-21 enhanced insulin secretion by 42% at 16.7 mM glucose in human islets, as compared to an 82% enhancement by exen-din-4, with no effect on basal insulin release (Figure 2D). These results identify TLQP-21 as a VGF-derived peptide with the capacity to potentiate GSIS in both rat and human islets.

TLQP-21 Is Secreted from Human Islets in Response to Glucose

Based on these effects of TLQP-21, we wondered if islets produce significant amounts of the peptide in response to physiological stimuli. We therefore used an ELISA assay specific for human TLQP-21 to measure its secretion from human islets. We found that stimulatory (16.7 mM) glucose caused a significant 2.5-fold increase in TLQP-21 release from human islets relative to secretion at 2.5 mM glucose (Figure 2C), suggesting that local concentrations of TLQP-21 could change with appropriate dynamics for enhancement of insulin secretion. This observation is consistent with earlier reports of glucose-stimulated release of longer, C-terminal derived VGF peptides from insulinoma cells (Possenti et al., 1999), but is to our knowledge the first demonstration of regulated TLQP-21 secretion from human islets. Human islets secreted approximately 0.6 pg TLQP-21/mg islet protein/2 hr when exposed to high glucose. This rate of production is calculated to raise the TLQP-21 concentration in the media to approximately 0.1 nM over 2 hr, or 1.2 nM over 24 hr. The latter level approaches stimulatory concentrations in our rat islet studies. It should also be noted that this calculation does not measure higher concentrations of TLQP-21 that may exist at the cell surface when islet cells release the peptide. Since glucose does not stimulate secretion of somatostatin from delta cells or glucagon from alpha cells, we take our data to suggest that at least some of the islet-derived TLQP-21 is coming from β-cells. Total TLQP-21 content of human islets was determined to be 87.1 ± 6.1 pmol / mg protein, compared to insulin, at 15.6 ± 2.0 nmol / mg protein.

TLQP-21 Elevates cAMP Levels in Rat Islets Independent of Known Incretin Receptors

The receptor system(s) for VGF-derived peptides have not been described. To begin to investigate the mechanism by which TLQP-21 potentiates GSIS in rat islets, we measured islet cAMP levels. Treatment of rat islets with TLQP-21 resulted in a doubling of cAMP levels, whereas exendin-4 caused an almost 3-fold increase (Figure 2E), consistent with the more potent effects of exendin-4 on GSIS compared to TLQP-21 in both rat and human islets (Figures 2B and 2D).

GLP-1/exendin-4, glucose-dependent insulinotropic polypeptide (GIP), and vasoactive intestinal peptide (VIP) raise cAMP in islet cells through distinct GLP-1, GIP, and VIP receptors (GLP-1R, GIPR, and VIPR1/2, respectively). To test if TLQP-21 engages with any of these receptors to raise cAMP levels, we transfected HEK293 cells (which lack endogenous GLP-1, GIP, or VIP receptors) with plasmids expressing GLP-1R, GIPR, or VIPR2 and examined cAMP accumulation following exposure to various peptides, including TLQP-21 (Figure 2F). Exendin-4 and GIP strongly increased cAMP levels in HEK293 cells expressing their cognate receptors, but as expected, displayed no cross-reactivity. VIP potently elevated cAMP in cells expressing the VIPR2, but was also able to partially activate GLP-1R and
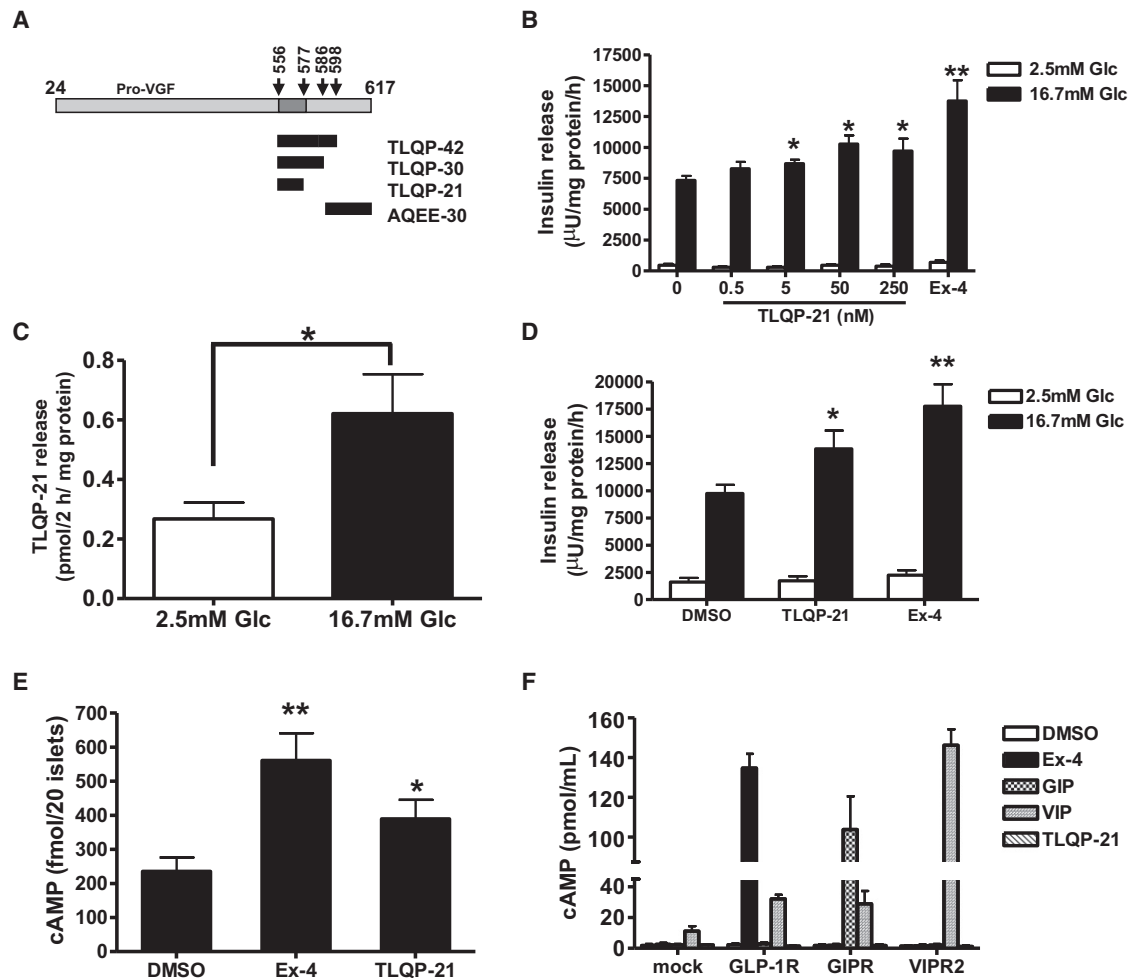
Figure 2. The C-Terminal VGF Peptide TLQP-21 Potentiates GSIS in Rat and Human Islets

(A) Schematic of the rat/mouse VGF proprotein highlighting the C-terminal peptides TLQP-21, TLQP-30, TLQP-42, and AQEE-30. The cleavage sites are indicated by the downward facing arrows with the amino acid positions labeled.

(B) Primary rat islets were assayed for insulin secretion by static incubation in media containing 2.5 mM Glc and then 16.7 mM Glc for 1 hr each. The indicated concentrations of the VGF-derived peptide TLQP-21 were added during both the basal and stimulatory glucose incubations. Exendin-4 (20 nM) was used as a positive control.

(C) Human islets from 3 separate donors were assayed for TLQP-21 secretion via static incubation in media containing 2.5 mM Glc and then 16.7 mM Glc for 2 hr each.

(D) Human islets from 4 separate donors were assayed for insulin secretion using the method described in (B) including DMSO (control), TLQP-21 (50 nM) or exendin-4 (20 nM).

(E) Rat islets were assayed for cAMP levels following a 30 min incubation in media containing 16.7 mM Glc with DMSO (control), TLQP-21 (50 nM), or exendin-4 (20 nM).

(F) HEK293 cells transfected with plasmids encoding the rat GLP-1R, the human GIPR, or human VIPR2 were incubated under basal conditions with DMSO (control), exendin-4 (20 nM), GIP (10 nM), VIP (10 nM) or TLQP-21 (50 nM) for 15 min and cAMP levels determined. In (A–F), data represent the mean ± SEM of three to five independent experiments. *$p \leq 0.01$; **$p \leq 0.002$.

GIPR. TLQP-21 failed to increase cAMP levels in cells expressing GLP-1R, GIPR, or VIPR2, indicating that TLQP-21 signaling is mediated by a receptor distinct from those involved in known incretin and VIP signaling pathways.

TLQP-21 Reduces Glycemic Excursion through Increased Insulin Secretion In Vivo

We next sought to determine the impact of TLQP-21 administration on glucose tolerance and insulin action. Normal Wistar rats were fasted overnight and TLQP-21 (4.5 mg/kg) or saline were delivered by intraperitoneal (i.p.) injection 30 min prior to a glucose bolus. TLQP-21 did not increase circulating insulin or induce hypoglycemia in fasted rats (Figures 3A and 3C, compare t = −30 min versus t = 0 min). Following the glucose bolus, animals injected with TLQP-21 exhibited a 20% reduction in the blood glucose peak (20 min) and a persistent decrease at later time points (Figure 3A), resulting in a 25% decrease in total glycemic excursion as assessed by area under the curve analysis (Figure 3B). The TLQP-21-mediated decrease in blood glucose was accompanied by a 25% increase in plasma insulin
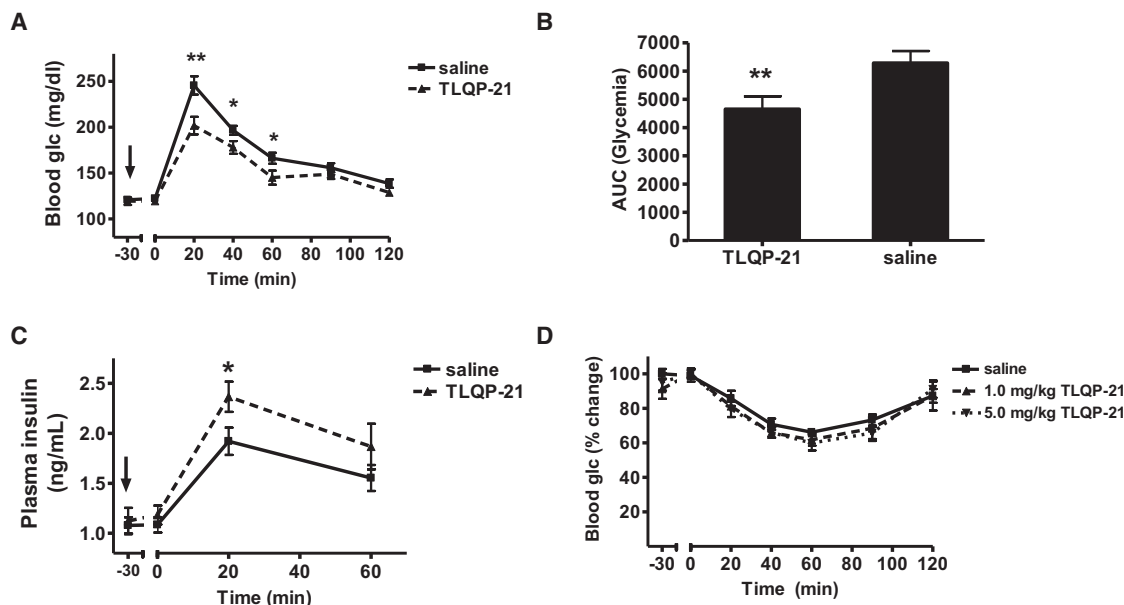
Figure 3. TLQP-21 Enhances Plasma Insulin Release and Reduces Glycemic Excursion in Male Wistar Rats
(A–C) Overnight (12 hr) fasted rats were injected i.p. with either vehicle (saline) or TLQP-21 (4.5 mg/kg) at 30 min prior to an i.p. glucose (1 g/kg) bolus. (A) Blood glucose was monitored at the indicated times. (B) Average area under the curve (AUC) analysis for the glucose tolerance test results. (C) Blood was sampled from the saphenous vein at the indicated times and assayed for insulin levels.
(D) Ad libitum fed rats were injected i.p. with saline, TLQP-21 (5 mg/kg) or TLQP-21 (1 mg/kg) at 30 min prior to an insulin (1 U/kg) challenge. Blood glucose was measured at the indicated times. Data represent the mean ± SEM (A–C), n = 12; (D), n = 5. *$p \leq 0.05$, **$p \leq 0.01$.

levels (Figure 3C) demonstrating that TLQP-21 potentiates glucose-stimulated insulin release in vivo as well as in isolated islets (Figures 2B and 2D). We further explored the activity of TLQP-21 at 3 doses (5 mg/kg, 1 mg/kg and 0.5 mg/kg) followed by a glucose challenge, and observed a dose-dependent reduction in glycemic excursion (Figure S2). Again, TLQP-21 did not reduce blood glucose in the fasted state (Figure S2, compare t = −20 min versus t = 0 min). To test for potential effects of TLQP-21 on insulin action, two doses of TLQP-21 were administered to ad libitum fed rats 30 min prior to injection of insulin (1 U/kg). Insulin-mediated decreases in blood glucose were indistinguishable in TLQP-21-injected versus control rats, indicating that TLQP-21 does not alter insulin sensitivity (Figure 3D). Also, treatment of rat islets with TLQP-21 for 24 or 48 hr did not alter total GLP-1 content, suggesting that the effects of TLQP-21 are not mediated by changes in islet GLP-1 (data not shown). Overall, our studies demonstrate that TLQP-21 enhances glucose tolerance in normal rats by enhancing GSIS.

TLQP-21 Treatment Does Not Reduce Gastric Emptying or Increase Heart Rate
Having established that TLQP-21 is similar to the GLP-1R agonist exendin-4 in its ability to enhance insulin secretion in a glucose-dependent manner and lower blood glucose, but via a GLP-1R-independent mechanism, we next determined if TLQP-21 shares two prominent side effects of GLP-1/ exendin-4-inhibition of gastric emptying and stimulation of heart rate (Barragán et al., 1994; Yamamoto et al., 2002). To examine gastric emptying, we performed an acetaminophen absorption test, which is based on the observation that acetaminophen is largely absorbed in the duodenum (Hatanaka et al., 1994).

Fasted animals were injected with either TLQP-21 (4.5 mg/kg), a scrambled peptide of identical amino acid composition to TLQP-21 (SCR-21; 4.5 mg/kg), exendin-4 (10 μg/kg), or saline at 20 min prior to delivery of an oral bolus of acetaminophen (100 mg/kg) given as a sugar-rich solution (children's painkiller). Whereas exendin-4-treated animals exhibited a profound delay in acetaminophen appearance in peripheral blood relative to the control groups, TLQP-21 injection had no effect on this variable relative to saline or SCR-21 injection, indicating that TLQP-21 does not alter gastric emptying (Figure 4A). In this same experiment, TLQP-21 and exendin-4 increased circulating insulin levels to a similar extent (Figure 4B), demonstrating normal potentiation of GSIS by TLQP-21 despite its lack of effect on the gut. To further substantiate these findings, we used a separate experimental model of gastric emptying in which dry stomach contents of fasted/refed animals were compared. Once again, TLQP-21 treated animals behaved similarly to control (saline and SCR-21-treated) animals, all with approximately 20% of stomach contents remaining 3 hr post feeding, whereas exendin-4 treatment resulted in a profound delay in solid food clearance from the stomach, with 66% retained (Figure 4C).

We next examined changes in resting heart rate in animals subjected to the various treatments (saline, TLQP-21, SCR-21, Ex-4). Exendin-4 treatment caused a rapid increase in heart rate (HR) of approximately 10%–12% that was sustained for the duration of the study (Figure 4D) consistent with previously published reports in rodents (Barragán et al., 1994; Yamamoto et al., 2002). In contrast, TLQP-21 and the control treatments (saline and SCR-1) had no effect on heart rate at any time point. Taken together these data indicate that TLQP-21 does not cause
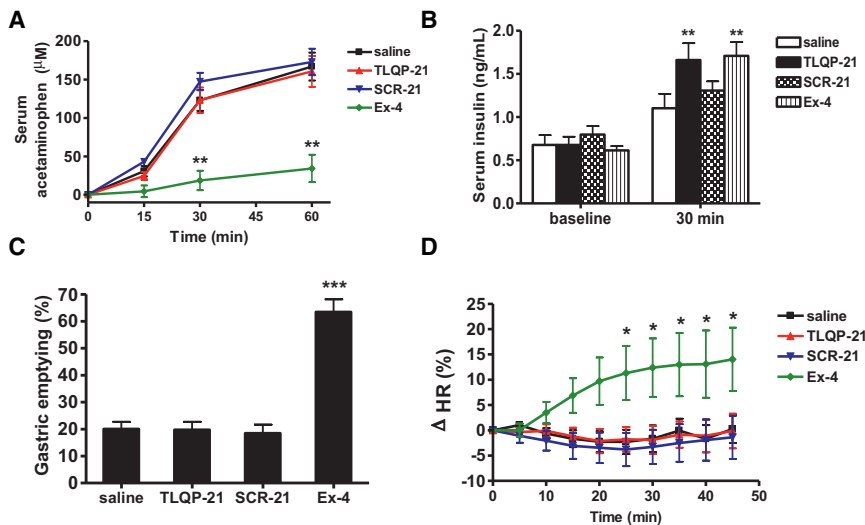
Figure 4. TLQP-21 Does Not Alter Gastric Emptying or Elevate Resting Heart Rate

(A–D) Male Wistar rats were fasted overnight (12 hr). (A and B) Rats were injected i.p. with saline, TLQP-21 (4.5 mg/kg), SCR-21 (4.5 mg/kg; scrambled peptide of identical amino acid composition to TLQP-21), or exendin-4 (10 μg/kg). At 20 min post peptide injection animals received an oral gavage of acetaminophen (100 mg/kg). Blood was sampled at the indicated times and serum levels of (A) acetaminophen and (B) insulin were determined via colorimetric assay and ELISA, respectively. (C) Fasted/ refed rats were injected (i.p.) with saline, TLQP-21 SCR-21, or exendin-4 at 1 hr post feeding. At 3 hr post peptide injection stomach contents were retrieved. The percentage food content remaining in the stomach was determined as the dry weight of stomach contents divided by the total amount of food consumed per animal x 100. (D) Male Wistar rats were sedated using avertin (250 mg/kg) and resting heart rate (HR) established using continuous ECG recordings (∼5 min intervals). Animals then received an i.p. injection of saline, TLQP-21, SCR-21, or exendin-4 and heart rate was monitored continuously for up to 50 min. Data are presented as the mean ± SEM (n = 7–8). *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.

two of the prominent gastrointestinal and cardiovascular side-effects of GLP-1/ exendin-4 in rodents.

Chronic Treatment with TLQP-21 Slows Development of Hyperglycemia in Prediabetic ZDF Rats

To evaluate the potential of TLQP-21 as an antidiabetogenic agent, we performed a chronic (4 wk) treatment study in male Zucker Diabetic Fatty (ZDF; *fa/fa*) rats. In this study, 7 wk old animals were injected with 5 mg/kg TLQP-21 or SCR-21 control peptide, or an equivalent volume of saline, at a frequency of every other day for 4 wks. Note that at this stage, ZDF animals are insulin resistant, mildly glucose intolerant, and hyperinsulinemic, but have normal fasting glucose levels. At the conclusion of the 4 week treatment period, rats injected with TLQP-21 had a decrease in blood glucose in the fed state of 150 mg/dl compared to rats treated with SCR-21 peptide or saline (Figure 5A). The TLQP-21-treated animals also had a striking improvement in their response to a glucose challenge compared to either control group (Figure 5B). In addition, fasting blood glucose was ∼150 mg/dl lower in TLQP-21 treated animals compared to control animals (Figure 5B, t = 0 min). TLQP-21-treated animals exhibited no decrease in body weight (Figure 5C). Plasma insulin levels obtained from fasted animals sampled at the end of the study were markedly higher in TLQP-21-treated animals than in either of the control groups (Figure 5D). It is important to note that animals did not receive peptide within 24 hr of the glucose tolerance test. These studies document a clear effect of TLQP-21 to delay onset of T2D and improve glucose homeostasis in a rodent model, an effect that appears to be independent of changes in body weight.

We postulated that the improvement in glycemic control and increased fasting insulin levels in the TLQP-21-treated animals may be due to a preservation of islet β-cell mass. Histological examination of pancreas sections from the 3 treatment groups revealed that TLQP-21-treated ZDF rats exhibited a 40% increase in area of insulin positive cells (a surrogate measure of β-cell mass) compared to the two control groups

(Figure 5E). Analysis of DNA fragmentation via TUNEL staining showed that the difference in islet β-cell mass was due, at least in part, to a decrease in β-cell apoptosis in TLQP-21 treated animals as compared to SCR-21-treated control animals (Figure 5F). We note that overnight exposure of isolated rat islets to the VGF peptide TLQP-21 did not enhance islet cell proliferation as measured by [³H]-thymidine incorporation (Figure S1C), suggesting that replication was not a major contributor to TLQP-21-induced differences in islet β-cell mass in the ZDF model.

TLQP-21 Reduces Islet Cell Death Induced by Cytotoxins

We next sought to determine if TLQP-21 has a direct antiapoptotic effect in rat islets, as suggested by the reduced TUNEL staining in TLQP-21 treated ZDF rats (Figure 5F), and a previous report of antiapoptotic activity of TLQP-21 in cerebellar granular cells deprived of serum (Severini et al., 2009). To test this hypothesis in islets, we evaluated whether pretreatment of isolated islets with TLQP-21 could suppress islet cell apoptosis induced by the ER stress agent, thapsigargin. For these experiments (Figures 6A–6C), isolated islets were pretreated with DMSO or TLQP-21 for 24h followed by overnight treatment (18 hr) with thapsigargin. Islets were then dispersed and apoptotic cells identified by TUNEL staining. As expected, treatment of rat islets with thapsigargin caused a marked increase in the number of TUNEL positive cells compared to the vehicle (DMSO) control (Figure 6C); however, pretreatment of rat islets with TLQP-21 reduced the number of thapsigargin-induced TUNEL positive cells by greater than 50% (compare Figure 6A with Figure 6B; quantification in Figure 6C). TLQP-21 also caused a significant suppression of apoptosis caused by chronic (72 hr) exposure of rat islets to the DNA damage-inducing agent etoposide, and the magnitude of this effect was again comparable to the effect of exendin-4 (Figure 6D). These experiments employed an alternative methodology (Annexin V staining/ flow cytometry) for measurement of apoptosis. In experiments with either apoptosis inducer, protection
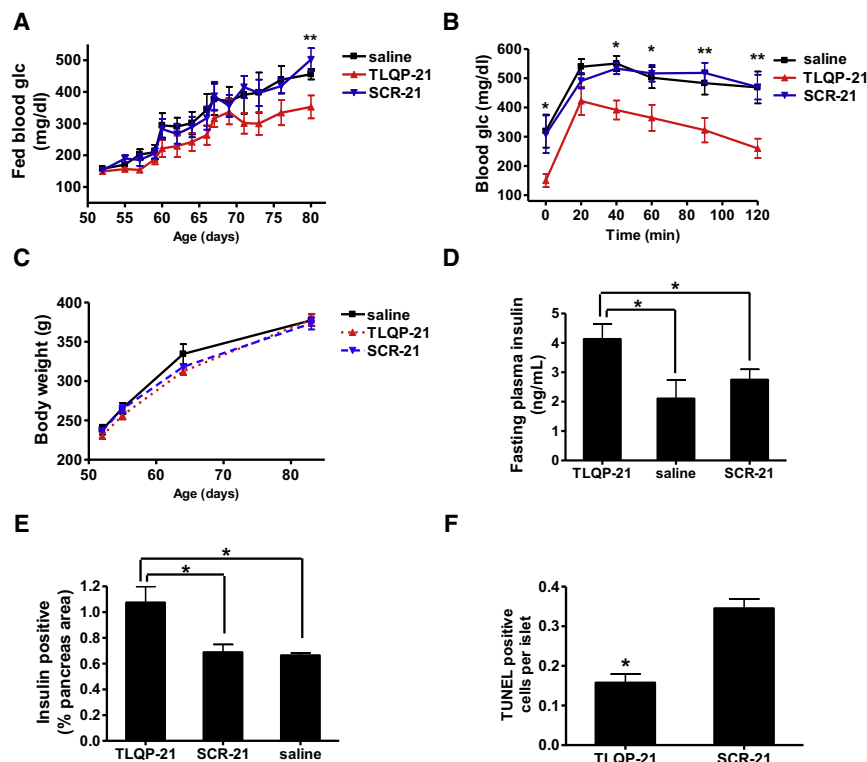
Figure 5. Chronic Treatment of ZDF Rats with TLQP-21 Improves Glucose Homeostasis and Preserves Islet β-Cell Mass

(A–F) Male Zucker Diabetic Fatty (*fa/fa*) rats were injected i.p. with TLQP-21 (5 mg/kg), SCR-21 (5 mg/kg), or saline every other day for 4 wks beginning at ~7 wks of age. (A) Ad libitum fed blood glucose. (B) Overnight (12 hr) fasted rats were given a glucose (1 g/kg) challenge (i.p.) and blood glucose monitored at the indicated times. (C) Body weight was measured at the indicated times. (D) Plasma insulin levels were assayed from overnight (12 hr) fasted rats at the end of the treatment period. (E) β-cell mass was calculated as the percentage of insulin staining area relative to the total area of pancreas section from 4 independent sections per animal. (F) Quantification of the average number of TUNEL positive, insulin positive cells per islet. Data are presented as the mean ± SEM (n = 6–8). *$p \leq 0.05$, **$p \leq 0.01$.

DISCUSSION

Type 2 diabetes (T2D) occurs as a result of several distinct metabolic lesions, including impaired insulin action in fat and muscle, a failure to control hepatic glucose output, and slow development of insulinopenia due to loss of β-cell mass and function. Because β-cell decompensation is often the last metabolic lesion to appear and the one that heralds diabetes onset, interest in identifying new strategies for preserving and/or restoring functional β-cell mass is high.

The role of the VGF-derived peptide TLQP-21 in preserving islet β-cell mass and function described herein emerged through our studies of the effects of the homeobox transcription factor Nkx6.1 in adult islets (Schisler et al., 2008, 2005). In comparing the effects of Nkx6.1 and Pdx-1 overexpression in rat islets, we noticed that only Nkx6.1 is able to enhance GSIS (Figure 1). VGF emerged from this analysis as the most strongly Nkx6.1-upregulated and Pdx-1-unaffected gene (Table S1). Our subsequent experiments demonstrate that upregulation of VGF is required for Nkx6.1-mediated enhancement of GSIS, and that VGF overexpression is sufficient to drive the improved glucose response.

VGF was originally described as a growth factor-regulated transcript in PC12 cells (Salton, 1991; Salton et al., 1991), and subsequently shown to be expressed in multiple neuroendocrine tissues in the central nervous system, including the hypothalamus and hippocampus (Ferri et al., 1992; van den Pol et al., 1989) as well as in pancreatic islet cells (Cocco et al., 2007), including β-cells. VGF is processed by the subtilisin-like proteases PC1/3 and/or PC2. Similar to pro-opiomelanocortin and proglucagon, depending upon the prevalence of these processing enzymes within VGF expressing cells, distinct VGF peptides with potentially separate biological functions may be generated. Much of our current understanding of VGF function(s) has been derived from the targeted deletion of VGF in mice (Hahm et al., 1999; Watson et al., 2005). These animals display improved

afforded by treatment with 50–100 nM TLQP-21 was similar to that observed in islets treated with 20 nM exendin-4 (Figures 6C and 6D). Taken together, these data clearly demonstrate that TLQP-21 can suppress apoptosis induced by multiple agents, thereby providing a likely mechanism by which this peptide preserves β-cell mass in the context of the ZDF model of T2D.

Given that TLQP-21 treatment of rat islets causes an increase in cAMP levels, we reasoned that its ability to suppress islet cell death may be dependent on the cAMP-dependent kinase, PKA. Additionally, Akt has been implicated as a component of GLP-1R-mediated cell survival, and was therefore also considered as a candidate mediator of the TLQP-21 protective mechanism (Wang et al., 2004). Treatment of rat islets with the PKA inhibitor H89, or the PI3 kinase inhibitor LY294002 blocked the ability of TLQP-21 to protect against ER stress-related (thapsigargin-induced) cell death (Figure 7A). In contrast, the MEK inhibitor U0126 had no effect on TLQP-21-mediated protection. Chronic GLP-1R signaling can indirectly activate Akt via enhanced IGF-1R signaling (Cornu et al., 2009; Jhala et al., 2003). This is thought to occur via PKA activation of CREB and subsequent CREB-mediated upregulation of the IGF-1R and/ or IRS-2 (Figure 7B). Pretreatment of rat islets with the IGF-1R/ insulin receptor inhibitor OSI 906 abrogated both TLQP-21- and exendin-4-mediated protection against thapsigargin-induced apoptosis (Figure 7C). However, we were unable to attribute changes in IGF-1R signaling mediated by TLQP-21 or Ex-4 to increases in IGF-1R or IRS-2 protein expression (Figure S3), as previously suggested (Cornu et al., 2010; Jhala et al., 2003). Nevertheless, we were able to demonstrate the ability of chronic TLQP-21 and Ex-4 treatments to enhance glucose-stimulated Akt activation (Figure S3) as previously documented for Ex-4.
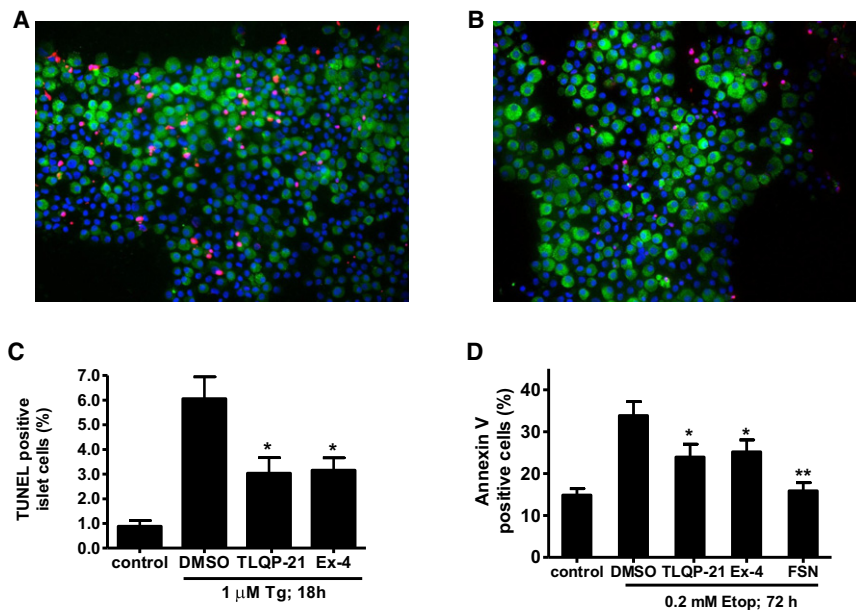
Figure 6. TLQP-21 Reduces Apoptosis Caused by Multiple Agents

(A–C) Primary rat islets were pretreated for 24 hr with DMSO, TLQP-21 (100nM) or Ex-4 (20nM) followed by overnight incubation with thapsigargin (1 μM). Non-thapsigargin treated (control) islets were also included. Following incubation, islet cells were dispersed onto coverslips and stained for nicked DNA using TUNEL (red). Insulin (green) and DAPI (blue) were used as counter stains. Representative micrographs of islets that were pretreated with either DMSO (A) or TLQP-21 (B) followed by thapsigargin treatment are shown.

(C) Percentage of TUNEL positive nuclei were determined (∼1700 nuclei per experiment) using ImageJ software.

(D) Rat islets were treated with etoposide (0.2 mM) for 72 hr. DMSO, TLQP-21 (50 nM), exendin-4 (20 nM), or forskolin (5 nM) were added to the media (daily) for the duration of the experiment (72 hr). Islet cells were assayed for Alexa Fluor 488-conjugated Annexin V staining via flow cytometry. (C and D) Data represent the mean ± SEM from four to six independent experiments. *p ≤ 0.05, **p < 0.01.

insulin sensitivity, and have reduced islet cell mass, normal islet cell architecture, and a significant decrease in circulating insulin levels. More specific studies of VGF peptides such as TLQP-21 or AQEE-30 have mainly evaluated their effects when administered centrally. For example, intracerebroventricular (i.c.v.) infusion of TLQP-21 was shown to cause weight loss and protect against diet-induced obesity (Bartolomucci et al., 2006), with these effects ascribed variously to decreases in food intake and gastric emptying (Jethwa et al., 2007) or increased energy expenditure (Bartolomucci et al., 2006; Watson et al., 2009) by different groups. The complex effects of VGF on peripheral metabolism and energy balance make it impossible to determine from the available data if peptides derived from this prohormone also have cell-intrinsic effects in pancreatic islets.

The current study was therefore designed to assess the direct effects of TLQP-21 on islet biology. Here, we show that the VGF peptide TLQP-21 is able to potentiate GSIS in isolated rat and human pancreatic islets. Similarly, peripheral (i.p.) administration of TLQP-21 to Wistar rats increases circulating insulin in response to a glucose challenge, thereby enhancing glucose tolerance. TLQP-21 stimulation of insulin secretion occurs in concert with a rise in cAMP, suggesting that the potentiating effect of TLQP-21 is mediated via downstream targets of activated PKA, as with other cAMP-raising agents such as GLP-1, GIP, PACAP, and VIP. A role for PKA in the TLQP-21 signaling pathway is supported by our observation (Figure 7A) that inhibition of PKA blocks the ability of TLQP-21 to suppress thapsigargin-induced islet cell death. Notably, TLQP-21 does not directly engage the incretin (GLP-1R, GIPR) or VIP (VIPR2) receptors. In addition, it is unlikely that TLQP-21 engages with these receptors in vivo, because peripheral administration of TLQP-21 is not accompanied by a reduction in gastric emptying rate or elevated heart rate in rodents, two common effects observed with GLP-1R agonists. Although there have been reports of TLQP-21 directly stimulating contraction of foregut muscle strips in culture, animal studies show that the effects of TLQP-21 on gastric emptying occur only with central administration (i.c.v. infusion) and not peripheral injection (Severini et al., 2009), consistent with the findings reported here. Taken together, our data demonstrates similarities of the TLQP-21 signaling pathway with those used by other β-cell secretagogues such as GLP-1, but also shows clearly that TLQP-21 operates via a unique receptor system. Our findings also imply that peripherally administered TLQP-21 does not effectively cross the blood/brain barrier to engage central signaling pathways. Consistent with this idea, chronic peripheral injection of TLQP-21 did not alter body weight in our ZDF rat study, nor did acute injection alter insulin sensitivity, suggesting that the effects of TLQP-21 on these metabolic variable reported by others were reliant on the central mode of administration (Bartolomucci et al., 2009; Bartolomucci et al., 2006; Watson et al., 2005).

In addition to the acute potentiating effect of TLQP-21 on GSIS and protection against ER stress and DNA damage-related cell death in vitro, chronic peripheral injection of TLQP-21 was able to preserve islet cell mass and slow the onset of hyperglycemia in a rodent model of T2D. While full understanding of the therapeutic implications of our findings will require further investigation, our demonstration that TLQP-21 operates via a distinct receptor system relative to the incretins suggest that it may ultimately represent a new antidiabetogenic agent. It may seem surprising that the antidiabetogenic effects of TLQP-21 observed in ZDF rats occurred with a regimen of every other day injection. In this regard it should be noted that similar long-lasting effects of GLP-1/exendin-4 have been also reported despite its very short half-life. For example, once daily injection of exendin-4 in diabetic db/db mice caused sustained lowering of blood glucose, and a similar regimen improved glucose tolerance in Zucker rats (Greig et al., 1999; Szayna et al., 2000). Similarly, a 48 hr infusion of GLP-1 into ZDF rats resulted in sustained improvement of glucose tolerance for as long as 3 weeks after peptide administration (Hui et al., 2002).

At the current state of technology, an apparent hurdle to considering TLQP-21 as a therapeutic agent is the rather high
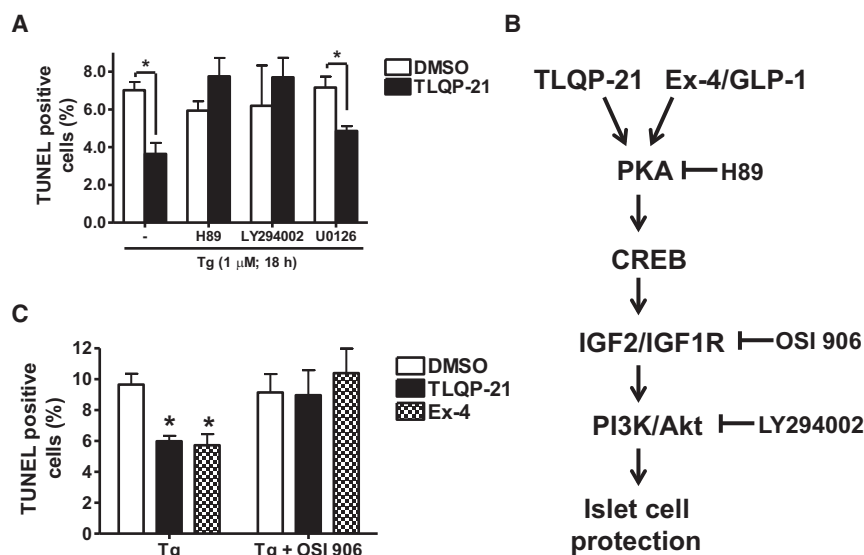
A

C

B

TLQP-21   Ex-4/GLP-1

↓   ↓

PKA ⊣ H89

↓

CREB

↓

IGF2/IGF1R ⊣ OSI 906

↓

PI3K/Akt ⊣ LY294002

↓

Islet cell protection

Figure 7. Inhibition of PKA and Akt Impair TLQP-21-Mediated Protection against Islet Cell Death

(A and C) Primary rat islets were pretreated for 6 hr with DMSO or TLQP-21 (100nM) in the presence of H89 (10 nM), LY294002 (10 nM), or U0126 (5 nM) (A) or OSI 906 (100nM) (C) as indicated followed by overnight (18 hr) incubation with thapsigargin (1 μM). (A and C) Following incubation, islet cells were dispersed onto coverslips and stained for nicked DNA using TUNEL. Percentage of TUNEL positive nuclei were determined (∼2000 nuclei per experiment) using ImageJ software. Data represent the mean ± SEM from four to seven independent experiments. *p ≤ 0.05.

(B) Proposed model of the GLP-1/Ex-4 and TLQP-21 antiapoptotic signaling pathway.

dose required (∼2 μmol/kg) to observe effects on insulin secretion in vivo as compared to GLP-1/ Ex-4 (1–10 nmol/kg). This is in contrast to the similar concentrations of TLQP-21 and GLP-1 required to potentiate GSIS and promote islet cell survival in vitro (50 nM TLQP-21 versus 20 nM Ex-4). A possible explanation for this discrepancy is a rapid turnover of TLQP-21 in plasma, such that a large bolus is required to reach the target tissue (i.e., pancreatic islets). Elucidation of the degradation pathway of TLQP-21, with particular focus on inactivating proteolytic cleavage events, will be required in order to understand the pharmacokinetics of this peptide. Identification of the DPP-4-catalyzed cleavage event proved to be a major milestone in the development of methods for detecting active forms of GLP-1, and for development of new analogs that are resistant to enzymatic cleavage (Deacon et al., 1995; Kieffer et al., 1995). We suspect that it will ultimately be possible to design more stable TLQP-21 analogs, and that these agents will be useful for defining the full therapeutic potential of this molecule. Alternatively, it may be possible to take advantage of the expression and secretion of VGF/TLQP-21 from the islets themselves. In this regard, we show here that glucose is able to stimulate secretion of TLQP-21 from human islets. This finding suggests that it may be possible to define strategies for enhancing production of TLQP-21 from endogenous islets as a means of protecting islet mass and enhancing islet function in the prediabetic state. Further studies focused on defining the role of locally produced TLQP-21 on islet biology will be important for development of such strategies.

EXPERIMENTAL PROCEDURES

Note that some of the reagents and common procedures used in this study are described in Supplemental Experimental Procedures.

Glucose-Stimulated Insulin Secretion

Islets were isolated from male Wistar rats at 12–16 weeks of age as described (Schisler et al., 2008). Insulin secretion was measured by static incubation of 3 groups of 20 islets per condition as described previously (Schisler et al., 2008, 2005), in secretion assay buffer (SAB) containing 2.5mM Glc for 1 hr at 37°C

(basal) followed by incubation in SAB containing 16.7 mM Glc for 1 hr (stimulatory). Hormones (exendin-4, TLQP-21/30/42, AQEE-30) were included as indicated. Insulin was measured in SAB using the Coat-a-Count kit (Siemens). Islets were lysed in RIPA buffer and total protein determined by BCA (Pierce), and insulin content determined as described (Schisler et al., 2008, 2005).

TLQP-21 Secretion and Content

Media was collected from human islets following static incubations of 5–6 groups of 50 islets in SAB containing 2.5 mM Glc for 2 hr at 37°C (basal), followed by incubation in SAB containing 16.7 mM Glc for 2 hr (stimulatory). Islets were lysed in RIPA buffer and total protein determined by BCA (Pierce). Secreted TLQP-21 was assayed from the media, and total TLQP-21 content was determined from diluted islet cell lysates, using a human TLQP-21-specific ELISA (Bachem). Human islets were obtained from the Integrated Islet Distribution Program (IIDP).

cAMP Determination and Receptor Transfection Studies

For cAMP measurements, primary rat islets were prepared as for insulin secretion assays, except the incubation at stimulatory glucose was for 30 min in the presence of 0.1 mM isobuytlmethylxanthine (IBMX). HEK293 cells were transiently transfected with plasmids containing GLP-1R, GIPR, VIPR2, or mock (no plasmid), and were preincubated in SAB for 1.5 hr followed by a 15 min incubation in fresh SAB containing DMSO, exendin-4 (20 nM), GIP (10 nM), VIP (10 nM) or TLQP-21 (50 nM) in the presence of IBMX. Islet and HEK293 cell extracts were assayed using the cAMP EIA kit from Biomedical Technologies, Inc.

Gastric Emptying Studies

For the acetaminophen absorption test, overnight (12 hr) fasted male Wistar rats received a 100 mg/kg oral gavage of acetaminophen (children's pain killer) 20 min postinjection (i.p.) of saline or peptide (4.5 mg/kg TLQP-21, 4.5 mg/kg of a scrambled peptide of identical amino acid composition as TLQP-21 [SCR-21], or 10 ug/kg exendin-4). Blood was sampled from either the tail or saphenous veins at the indicated times. Serum was assayed for acetaminophen and insulin using a colormetric assay (Genzyme) and ELISA (Millipore), respectively. For the solid-phase gastric clearance test, fasted animals were refed for 1 hr and then injected i.p. with saline or one of the 3 peptides described above. At 3 hr postinjection, animals were sacrificed, stomach contents retrieved and dried in an oven. The percentage food content remaining in the stomach was determined from the dry weight of stomach contents divided by the total amount of food consumed per animal multiplied by 100.

Heart Rate Measurements

Male Wistar rats were sedated using 250 mg/kg tribromoethanol (Avertin). Continuous ECG recording were obtained with subcutaneously placed 29-gauge needle electrodes in both forelimbs in a Lead I configuration using the

ML138 Octal Bioamp (ADInstruments Colorado Springs, CO) connected to a Powerlab 16/30 acquisition system (ADInstruments). After establishment of baseline heart rate recordings (5–10 min), animals received i.p. injections of either saline or peptide (4.5 mg/kg TLQP-21, 4.5 mg/kg SCR-21, or 10 μg/kg exendin-4). ECG data was analyzed for heart rate using LabChart Pro 7.2 Software (AD Instruments).

Chronic Peptide Treatment Study
Seven-week-old male Zucker Diabetic Fatty (fa/fa) rats were randomly assigned to 1 of 3 groups receiving TLQP-21, SCR-21, or saline. 5 mg/kg peptide or an equivalent volume of saline were injected at a frequency of every other day for 4 wks. Glucose was measured in blood collected weekly from ad lib fed rats via the saphenous vein in the morning. Insulin and glucagon levels were determined using a rat/mouse insulin ELISA (Millipore) and rat glucagon RIA (Millipore), respectively.

In Vitro Apoptosis Assay
Pools of 50 islets were treated with TLQP-21 (100 nM), exendin-4 (20 nM), H89 (10 nM), LY294002 (10 nM), U0126 (5 nM), OSI 906 (100 nM) or vehicle (DMSO) for 6–24 hr followed by overnight (18 hr) incubation with thapsigargin (1 μM). Islets were dispersed with trypsin/EDTA and attached to poly-D-lysine coated coverslips (BD). For detection of apoptosis, islet cells were stained using the In situ Cell Death detection kit (Roche). Insulin and DAPI were used as counter stains. Five to seven images containing ~200 nuclei per image per slide were evaluated for TUNEL staining using ImageJ software. Percentage of TUNEL positive cells was determined as the ratio of TUNEL stained cells to total nuclei x 100. In a separate series of studies, pools of 50 islets were treated with DMSO or etoposide (0.2 mM) for 72 hr in the presence of TLQP-21 (50 nM), exendin-4 (20 nM), or forskolin (5 nM). Islets were dispersed using trysin/EDTA and stained with Alexa Fluor 488-conjugated Annexin V (Invitrogen) and propidium iodide (Sigma). Data were collected using a FACScan analyzer and analyzed using WinMDI 2.9 software.

Statistical Analysis
Data are presented as the mean ± SEM. For statistical significance determinations, data were analyzed by the two-tailed paired, Student's t test or by ANOVA with Bonferonni post hoc analysis for multiple group comparisons.

Abbreviations
Area under the curve (AUC); glucagon-like peptide-1 (GLP-1); glucose-dependent insulinotropic polypeptide (GIP); glucose-stimulated insulin secretion (GSIS); Heart rate (HR); intracerebroventricular (i.c.v.); intraperitoneal (i.p.); intraperitoneal glucose tolerance test (IPGTT); secretion assay buffer (SAB); terminal deoxynucleotide transferase-mediated 2′-deoxyuridine 5′-triphosphate nick-end labeling (TUNEL); Type 2 diabetes (T2D); vasoactive intestinal peptide (VIP); a scrambled peptide of identical amino acid composition as TLQP-21 (SCR-21); Zucker Diabetic Fatty (ZDF) rats.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, Supplemental References, three figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2012.05.011.

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