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Mechanisms of action of the antidiabetic peptide [S4K]CPF-AM1 in *db/db* mice

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Abstract

The antidiabetic effects and mechanisms of action of an analogue of a frog skin host-defence peptide belonging to the caerulein-precursor fragment family, [S4K]CPF-AM1 were investigated in *db/db* mice with a genetically inherited form of degenerative diabetes-obesity. Twice-daily treatment with the peptide (75 nmol/kg body weight) for 28 days significantly decreased blood glucose ($P < 0.01$) and HbA1c ($P < 0.05$) and increased plasma insulin ($P < 0.05$) concentrations with no effect on body weight, energy intake, body composition or plasma lipid profile. Peptide administration improved insulin sensitivity and intraperitoneal glucose tolerance. Elevated biomarkers of liver and kidney function associated with the *db/db* phenotype were significantly lowered by [S4K]CPF-AM1 administration. Peptide treatment significantly ($P < 0.05$) increased pancreatic insulin content and improved the responses of isolated islets to established secretagogues. Elevated expression of genes associated with insulin signalling (*Slc2a4*, *Insr*, *Irs1*, *Akt1*, *Pik3ca*, *Ppm1b*) in the skeletal muscle of *db/db* mice were significantly downregulated by peptide treatment. Genes associated with insulin secretion (*Abcc8*, *Kcnj11*, *Slc2a2*, *Cacn1c*, *Glp1r*, *Gipr*) were significantly upregulated by treatment with [S4K]CPF-AM1. Studies with BRIN-BD11 clonal β -cells demonstrated that the peptide evoked membrane depolarisation, increased intracellular Ca^{2+} and cAMP and activated the protein kinase C pathway. The data indicate that the antidiabetic properties of [S4K]CPF-AM1 mice are mediated by direct insulinotropic action and by regulation of transcription of genes involved in both the secretion and action of insulin.

Key Words

- ▶ diabetes type II
- ▶ gene transcription
- ▶ insulin action
- ▶ insulin signalling

Journal of Molecular
Endocrinology
(2021) **66**, 115–128

Introduction

A sedentary lifestyle, consumption of energy-dense foods and physical inactivity has led to an epidemic of obesity and dramatic increases in the prevalence, mortality and morbidity associated with Type 2 diabetes mellitus (T2DM). This situation means that T2DM is considered to be one of the major global health care challenges of the 21st century (Basu *et al.* 2013). Existing glucose-lowering drugs are often associated with poor glycaemic control and do not prevent severe and often life-threatening

complications. This has necessitated the development of novel antidiabetic agents that improve impaired glycaemic responses and delay the related complications in the diabetic patient. The success of the antidiabetic peptide exendin-4 (generic name exenatide) that was first isolated from the venom of the Gila monster lizard *Heloderma suspectum* and functions as a long-acting agonist at the glucagon-like peptide-1 (GLP-1) receptor (Parkes *et al.* 2013) has intensified the search for other peptides with

antidiabetic properties from animal sources. Antidiabetic agents whose mechanism of action is different from those of existing drug classes are of particular interest.

Skin secretions of frogs have proved to be a rich source of peptides with therapeutic potential. Amphibian host-defence peptides have demonstrated anti-bacterial, anti-fungal, anti-viral, anti-cancer, wound healing and immunomodulatory activities (reviewed in [Conlon *et al.* 2014](#), [Xu & Lai 2015](#), [Pantic *et al.* 2017](#)). In addition, several such peptides that were first identified on the basis of their antimicrobial properties have subsequently been shown to stimulate insulin release *in vitro* using BRIN-BD11 rat clonal β -cells and mouse pancreatic islets and improve glycaemic responses in both lean and high fat-fed mice (reviewed in [Conlon *et al.* 2018, 2019](#)). In particular, peptides belonging to the caerulein precursor fragment (CPF) family of peptides, found in skin secretions of frogs belonging to the genus *Xenopus* ([Conlon & Mechkarska 2014](#), [Conlon *et al.* 2019](#)), display both insulinotropic properties *in vitro* and a range of antidiabetic properties in animal models of T2DM ([Srinivasan *et al.* 2013, 2015](#)). For example, CPF-SE1 from *Xenopus epitropicalis* stimulates the *in vitro* release of insulin from BRIN-BD11 cells at low concentrations that do not compromise the integrity of the plasma membrane of the cell. Long-term administration of the peptide (twice-daily administration of 75 nmol/kg body weight for 28 days) to high fat-fed mice significantly increased circulating insulin and decreased plasma glucagon and triglyceride concentrations, pancreatic glucagon content and total body fat ([Srinivasan *et al.* 2015](#)). Thus, it was concluded that CPF-SE1 showed therapeutic promise for development into an agent for the treatment of patients with obesity-related T2DM.

Major disadvantages of naturally occurring frog skin peptides as therapeutic agents, particularly for systemic administration, are their relatively low potency, short half-life in the circulation and their toxicity to mammalian cells. The strategy of increasing cationicity of these peptides by substituting one or more acidic or neutral amino acids by D- or L-lysine has led to the development of more stable analogues with increased antimicrobial potency and reduced haemolytic activity ([Conlon *et al.* 2007, 2019](#)). Application of this strategy to the development of potential peptide-based antidiabetic drugs has led to the design of analogues of alyteserin-2a ([Ojo *et al.* 2013a](#)) brevinin-2-related peptide ([Abdel-Wahab *et al.* 2010](#)), esculentin-2CHa (1-30) ([Vasu *et al.* 2017](#)), hymenochirin 1B ([Owolabi *et al.* 2016a,b](#)), PGLa-AM1 ([Owolabi *et al.* 2017](#)), pseudin-2 ([Abdel-Wahab *et al.* 2008](#))

and tigerinin-1R ([Srinivasan *et al.* 2014](#), [Ojo *et al.* 2016](#)) with enhanced cationicity that exhibit both increased insulinotropic activity *in vitro* and improved glucose-lowering properties *in vivo* compared with the naturally occurring peptides.

Previous studies have reported broad-spectrum antimicrobial properties of CPF-AM1 (GLGSVLGKALKIGANLL.NH₂), first isolated from skin secretions of the Volcano clawed frog *Xenopus amieti* ([Conlon *et al.* 2010](#)), including activity against oral pathogens ([McLean *et al.* 2014](#)). Further investigations showed that the peptide produced concentration-dependent increases in insulin release from BRIN-BD11 cells and GLP-1 release from GLUTag enteroendocrine cells without disrupting the integrity of the cell plasma membrane ([Ojo *et al.* 2013b](#)). Preliminary data indicated that the [L-Lys⁴]-substituted analogue of CPF-AM1 was more potent than the naturally occurring peptide in stimulating insulin-releasing activity in BRIN-BD11 cells and improved glycaemic responses in lean mice against a glucose challenge (B Owolabi & V Musale, unpublished observations). Based on these promising results, the present study examined the effects of twice-daily administration of [S4K]CPF-AM1 (75 nmol/kg body weight) for 28 days on glycaemic control, insulin concentration, lipid profile, islet morphology and function and expression of key genes in muscle and islets involved in glucose homeostasis in genetically obese *db/db* mice with degenerative diabetes. The effects of the peptide were compared with those of the established antidiabetic agent exenatide.

Materials and methods

Peptide synthesis

[S4K]CPF-AM1 (GLGKVLGKALKIGANLL.NH₂) was supplied in crude form by Synpeptide Co. Ltd. (Shanghai, China) and purified to near homogeneity (>98% purity) by reversed-phase HPLC as previously described ([Srinivasan *et al.* 2015](#)). The identity of purified peptide was confirmed by matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry using a Voyager DE PRO instrument (Applied Biosystems) (observed molecular mass 1664.2 Da, calculated molecular mass 1664.1 Da).

Animals

All animal experiments were carried out in compliance with the UK Animals (Scientific Procedures) Act 1986

and EU Directive 2010/63EU for animal experiments and approved by Ulster University Animal Ethics Review Committee. Male C57.BLKsJ *db/db* mice (BKS.Cg-*Leprdb*/*Leprdb*/OlaHsd strain) together with male, non-diabetic C57BLKsJ $^{+/+}$ control mice (BKS.Cg-(Lean)/OlaHsd strain) were supplied by Envigo (Huntingdon, UK) and housed individually with access to water and standard laboratory chow (10% fat, 30% protein, 60% carbohydrate; total energy 12.99 kJ/g; Trouw Nutrition, Cheshire, UK) in an air-conditioned room ($22 \pm 2^\circ\text{C}$) with a 12 h light:12 h darkness cycle. All necessary steps were taken to prevent any animal discomfort. Mice were kept for a 1 week acclimation period and were 8-week-old at the start of the experiment. Mice were allocated into four groups of eight animals with matching average body weights and blood glucose levels: (A) *db/db* mice treated with [S4K]CPF-AM1 (75 nmol/kg body weight), (B) *db/db* mice treated with exenatide (25 nmol/kg body weight), (C) control *db/db* mice treated with saline (0.9%, w/v NaCl) and (D) control lean $^{+/+}$ mice treated with saline.

Effects of twice-daily peptide administration on body weight, metabolic parameters and islet morphology

Before initiation of treatment, mice were injected intraperitoneally twice-daily with saline for 3 days to facilitate adaptation to handling and injection stress. Mice were injected twice-daily (09:30 and 17:00 h) with either [S4K]CPF-AM1, exenatide or saline over a 28-day period. Body weight, food intake, water intake, blood glucose and plasma insulin concentrations were monitored at 3-day intervals. After the treatment period, intraperitoneal glucose tolerance (18 mmol/kg body weight), and insulin sensitivity (25 U/kg body weight) tests were performed as previously described (Owolabi *et al.* 2016b, Graham *et al.* 2020). Insulin sensitivity was assessed by determination of the Quantitative Insulin Sensitivity Check Index (QUICKI) (Katz *et al.* 2000). After all, tests were completed, mice were killed by cervical dislocation and body fat composition and bone mineral density were measured using DEXA scanning (PIXImus densitometer, USA; O'Harte *et al.* 2018).

For immunohistochemical studies, pancreatic tissues were fixed and processed as described previously (Vasu *et al.* 2014). Pancreata and skeletal muscle were snap-frozen in liquid nitrogen and stored at -80°C . For insulin and glucagon staining of pancreata, mouse monoclonal anti-insulin antibody (ab6995, 1:400; Abcam) and guinea-pig anti-glucagon antibody (PCA2/4, 1:50; raised

in-house) were used. Following treatment with secondary antisera (Alexa Fluor 488 goat anti-guinea pig IgG (1:400) and Alexa Fluor 594 goat antimouse IgG (1:400)), slides were observed under a fluorescent microscope (Olympus system microscope, model BX51) with the DP70 camera adapter system. Islet parameters (islet number, islet area, β -cell area, α -cell area and islet size distribution) were analysed using Cell[^]F image analysis software (Olympus Soft Imaging Solutions, GmbH) as previously described (Parthasarathy *et al.* 2016).

In vitro insulin secretion studies with islets from *db/db* mice

Pancreatic islets were isolated from each of the groups of mice by collagenase digestion (Gotoh *et al.* 1985) and the insulin responses to incubations with well-established secretory agents (10 mmol/L alanine, 20 mmol/L KCl, 10 mmol/L arginine, 1 $\mu\text{mol/L}$ human GLP-1 and 1 $\mu\text{mol/L}$ human glucose-dependent insulinotropic polypeptide, GIP) were determined as previously described (Vasu *et al.* 2017). Following 60-min pre-incubation with 1.4 mmol/L glucose, islets were incubated at 37°C with Krebs-Ringer bicarbonate buffer (500 $\mu\text{L/tube}$) supplemented with 0.5% (w/v) bovine serum albumin, 16.7 mmol/L glucose and the test agents. After 60 min, the supernatants were collected and stored at -20°C for insulin RIA (Flatt & Bailey 1981).

Gene expression studies

Islets and skeletal muscle for gene expression studies were stored at -72°C . mRNA, extracted using TriPure Isolation Reagent (Sigma-Aldrich), was converted to cDNA using SuperScript[™] II Reverse Transcriptase (Invitrogen Life Technologies) (Vasu *et al.* 2014). Gene amplification was carried out by real-time PCR using an MJ Mini Thermal Cycler (Bio-Rad Laboratories). PCR reaction mixture (containing 4.5 μL of QuantiFast SYBR green PCR mix (Qiagen), 1 μL of forward and 1 μL reverse primers, 3 μL of cDNA and 1 μL of nuclease-free water) was added to the PCR tube strips. The forward and reverse primers nucleotide sequences have been provided previously (Ojo *et al.* 2016, Owolabi *et al.* 2016b). All the genes were normalised to β -actin (Actb) expression and analysed using the $\Delta\Delta\text{Ct}$ method.

Biochemical analyses

Blood samples were collected in fluoride/heparin microcentrifuge tubes (Sarstedt, Numbrecht, Germany) by tail bleeding and centrifuged for 30 s at 13,000 *g*.

Glucose concentrations were measured using an Ascenacia Counter Blood Glucose Meter (Bayer). Blood HbA1c concentrations were measured using an A1cNow⁺ kit (PTS Diagnostics, Indianapolis, IN, USA) following the manufacturer's instructions. Hepatic and renal toxicity was assessed by measuring plasma aspartic acid transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (AP) activities and creatinine concentrations, using commercially available kits (Randox Laboratories, Crumlin, UK) following manufacturer's instructions. Plasma cholesterol concentrations were determined using an I-lab 650 automated clinical chemistry analyser (Diamond Diagnostics, Holliston, MA, USA). LDL-cholesterol concentrations were calculated using the de Cordova equation: $3/4$ (total cholesterol – HDL-c) (Martins *et al.* 2015). Plasma amylase activity was determined using a commercially available assay kit following manufacturer's instructions (Abcam).

Mechanistic studies with BRIN-BD11 clonal β -cells

The effects of [S4K]CPF-AM1 (1 μ mol/L) on membrane potential and intracellular calcium in BRIN-BD11 cells were determined fluorimetrically using a FLIPR Membrane Potential Assay Kit and a FLIPR Calcium 5 Assay Kit (Molecular Devices, Sunnyvale, CA, USA) as previously described (Ojo *et al.* 2013a). Control incubations were carried out using 5.6 mmol/L glucose only, 5.6 mmol/L glucose+30 mmol/L KCl and 5.6 mmol/L glucose+10 mmol/L alanine. Incubations to assess effects on insulin secretion were carried out in the presence of known modulators of insulin release (the K⁺ channel activator, diazoxide (300 μ mol/L) and the blocker of L-type voltage-dependent Ca²⁺ channels, verapamil (50 μ mol/L) and in Ca²⁺ media containing 1 mmol/L EGTA.

Effects of the peptide (1 μ mol/L) on cAMP production in BRIN-BD11 cells was determined as previously described (Owolabi *et al.* 2017) using an R&D Systems Parameter assay kit (Abingdon, UK) following the manufacturer's recommended protocol. Effects of downregulation of the protein kinase A (PKA) and protein kinase C (PKC) pathways on [S4K]CPF-AM1 (1 μ mol/L)-mediated insulin release were determined in BRIN-BD11 cells that had been incubated overnight with either or both forskolin (25 μ mol/L), an activator of PKA, or phorbol 12-myristate 13-acetate (PMA; 10 nmol/L, an activator of PKC, as previously described (Owolabi *et al.* 2017). Control incubations with GLP-1 (10 nmol/L), CCK-8 (10 nmol/L), forskolin (25 μ mol/L), PMA (10 nmol/L) and forskolin (25 μ mol/L)+PMA (10 nmol/L) were also carried out.

Effects of [S4K]CPF-AM1 on GLP-1 release from GLUTag cells

The effects of [S4K]CPF-AM1 (10^{-12} – 10^{-6} mol/L) on the release of GLP-1 from GLUTag enteroendocrine cells were determined as previously described (Ojo *et al.* 2013b). Glutamine (10 mmol/L) was used as a positive control and GLP-1 concentrations were measured using an assay kit (Millipore) according to the manufacturer's instructions.

Statistical analysis

Data were compared using either one-way ANOVA or two-way ANOVA as appropriate with Bonferroni *post hoc* test and unpaired Student's t-test (non-parametric, with two-tailed *P* values and 95% CI) as appropriate (GraphPad PRISM Version 5.0). Area under the curve (AUC) analysis was performed using the trapezoidal rule with baseline correction. Data are presented as mean $\pm$ S.E.M. Where appropriate, outliers greater than two S.D. from the mean were removed from the data set.

Results

Effects of [S4K]CPF-AM1 administration on body weight, energy intake, blood glucose, and plasma insulin concentrations

Prior to the start of treatment, body weight (Fig. 1A and B), energy intake (Fig. 1C and D), non-fasting blood glucose (Fig. 1E and F) and plasma insulin (Fig. 1G and H) were significantly greater ($P < 0.001$) in all groups of *db/db* mice compared with lean mice. After 28 days of treatment with [S4K]CPF-AM1, no significant differences in body weight and energy intake were observed compared with control *db/db* mice treated with saline. In contrast, energy intake was significantly ($P < 0.001$) decreased in the exenatide-treated group although there was no change in body weight. This apparent contradiction suggests that energy expenditure may have been greater in this group. [S4K]CPF-AM1 administration produced a small but significant ($P < 0.05$ – $P < 0.01$) decrease in blood glucose concentration throughout the treatment period but the magnitude of the decrease was appreciably less than that produced by exenatide. The hyperinsulinemia associated with the *db/db* phenotype progressively declined during the 28-day treatment period in control mice consistent with failing β -cell function. [S4K]CPF-AM1 treatment delayed the fall of circulating insulin concentrations but to a lesser extent than in mice treated with exenatide.

Figure 1

Effects of 28-day administration of [S4K]CPF-AM1 (75 nmol/kg) or exenatide (25 nmol/kg) on (A and B) body weight, (C and D) energy intake, (E and F) non-fasting blood glucose and (G and H) plasma insulin concentrations in *db/db* mice. The black horizontal bar represents the treatment period. Values are mean $\pm$ S.E.M. for eight mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with lean mice and $\Delta P < 0.05$, $\Delta\Delta P < 0.01$, $\Delta\Delta\Delta P < 0.001$ compared with control *db/db* mice.

Effects of [S4K]CPF-AM1 administration on glucose tolerance and insulin sensitivity

Following an intraperitoneal glucose challenge, plasma glucose concentrations and integrated responses (AUC) were elevated until 60 min in *db/db* control mice compared with those in lean mice (Fig. 2A and C). [S4K]CPF-AM1 treatment significantly ($P < 0.05$ – $P < 0.001$) decreased plasma glucose concentrations at all time points compared with *db/db* controls. The corresponding plasma insulin concentrations (Fig. 2B) and integrated insulin response (Fig. 2D) were also elevated ($P < 0.05$) in [S4K]CPF-AM1 treated mice but to a lesser extent than in animals treated with exenatide. Computing the insulin response in terms of the integrated change in insulin from 0 to 30 min divided by the change in glucose over the same period gives a ratio of 0.095 ± 0.037 for the [S4K]CPF-AM1-treated *db/db* mice and a comparable ratio of 0.128 ± 0.031 for exenatide-treated *db/db* mice. These values are significantly greater than the ratio for saline-treated *db/db* control mice (0.023 ± 0.004).

Administration of [S4K]CPF-AM1 over a 28-day period significantly decreased blood HbA1c concentrations from 80.0 ± 7.6 mmol/mol in *db/db* control mice to 57.2 ± 4.9 mmol/mol ($P < 0.05$) in [S4K]CPF-AM1 treated mice and to 41.8 ± 5.3 mmol/mol ($P < 0.01$) in exenatide treated mice (Fig. 3A). In order to evaluate effects on insulin

Figure 2

Effects of 28-day administration of [S4K] CPF-AM1 (75 nmol/kg) or exenatide (25 nmol/kg) on intraperitoneal glucose tolerance in *db/db* mice. Panels show (A) non-fasting plasma glucose concentration, (B) integrated glucose concentrations (area under the curve (AUC)), (C) plasma insulin concentrations and (D) integrated insulin concentrations. Values are mean $\pm$ S.E.M. for eight mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with lean mice and $\Delta P < 0.05$, $\Delta\Delta P < 0.01$, $\Delta\Delta\Delta P < 0.001$ compared with *db/db* control mice.

sensitivity, exogenous insulin was administered to mice intraperitoneally. The rate of glucose clearance from the circulation was significantly ($P < 0.05$) greater in [S4K]CPF-AM1 treated mice compared with *db/db* animals treated with saline (Fig. 3B and C). Similarly, QUICKI values, indicative of insulin sensitivity, were significantly ($P < 0.05$) improved in peptide treated mice compared to *db/db* controls (Fig. 3D).

Effects of [S4K]CPF-AM1 administration on *in vitro* insulin secretion

Islets from *db/db* mice were associated with impaired release of insulin in response to glucose (1.4 mmol/L, 5.6 mmol/L, 16.7 mmol/L) and other established insulin secretagogues (Fig. 3E). Twenty-eight-day treatment with

either [S4K]CPF-AM1 or exenatide significantly ($P < 0.001$) increased the secretory responses relative to those of islets from the *db/db* control group but not to the full extent of the responses of islets from the lean group.

Effects of [S4K]CPF-AM1 administration on liver enzyme and amylase activities, creatinine concentration, lipid profile and body composition

Activities of ALT, AST and ALP were significantly ($P < 0.001$) elevated in the plasma of saline-treated *db/db* mice (76, 246 and 105%, respectively) compared to their non-diabetic littermates (Fig. 4A, B and C). Treatment with either [S4K]CPF-AM1 or exenatide significantly ($P < 0.05$ – $P < 0.001$) lowered these parameters compared to *db/db* controls. Elevated plasma creatinine concentrations in

Figure 3

Effects of 28-day administration of [S4K]CPF-AM1 (75 nmol/kg) or exenatide (25 nmol/kg) on (A) blood HbA1c concentrations ($n = 4$), (B and C) plasma glucose concentrations and integrated responses after exogenous insulin administration ($n = 8$) and (D) Quantitative Insulin Sensitivity Check Index (QUICKI) ($n = 8$) in *db/db* mice and (E) insulin release from pancreatic islets in the presence of established secretagogues ($n = 4$). All data are represented as mean $\pm$ S.E.M.

* $P < 0.05$, * $P < 0.01$, *** $P < 0.001$ compared with lean mice and $\Delta P < 0.05$, $\Delta\Delta P < 0.01$, $\Delta\Delta\Delta P < 0.001$ compared with *db/db* control mice.

Figure 4

Effects of 28-day administration of [S4K]CPF-AM1 (75 nmol/kg) or exenatide (25 nmol/kg) on plasma (A) aspartic acid transaminase, (B) alanine transaminase, (C) alkaline phosphatase activities, (D) creatinine, (E) cholesterol, (F) triglyceride, (G) HDL and (H) LDL concentrations and (I) amylase activity in *db/db* mice. Values are mean $\pm$ S.E.M. for eight mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with lean mice and ^ $P < 0.05$, ^^ $P < 0.01$, ^^ $P < 0.001$ compared with control *db/db* mice.

db/db mice were also decreased by peptide treatment (Fig. 4D). The *db/db* phenotype was associated with elevated concentrations of plasma triglycerides (Fig. 5F) and low-density lipoprotein (LDL) (Fig. 5H) but these parameters were not significantly reduced by [S4K]CPF-AM1 treatment. Similarly, plasma cholesterol (Fig. 4E) and high-density lipoprotein (HDL) (Fig. 5G) concentrations were not significantly altered by treatment with the peptide. Plasma amylase was slightly but significantly elevated in mice treated with [S4K]CPF-AM1 compared with *db/db* controls ($P < 0.01$) and with lean mice ($P < 0.05$) (Fig. 5I). Treatment of *db/db* mice with either [S4K]CPF-AM1 or exenatide produced no significant

change in bone mineral density, bone mineral content, lean body mass amount of body fat and body fat expressed a percentage of total body mass compared with *db/db* control mice (Fig. 5A, B, C, D, E and F).

Effects of [S4K]CPF-AM1 administration on pancreatic insulin content and islet histology

Pancreatic insulin content was significantly increased ($P < 0.01$) in mice treated with [S4K]CPF-AM1 relative to the *db/db* control group but not to the level found in the lean group (Fig. 6A). In saline treated *db/db* mice, number of islets/mm² (Fig. 6B) and β -cell area (Fig. 6D)

Figure 5

Effects of 28-day administration of [S4K]CPF-AM1 (75 nmol/kg) or exenatide (25 nmol/kg) on (A) bone mineral density (g/cm³), (B) bone mineral content (g/cm³), (C) bone area, (D) body lean mass (g), (E) body fat mass (g), (F) % body fat in *db/db* mice. Values are mean $\pm$ S.E.M. for eight mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with lean mice.

Figure 6

Effects of 28-day administration of [S4K]CPF-AM1 (75 nmol/kg) or exenatide (25 nmol/kg) on (A) pancreatic insulin content, (B) number of islets/mm², (C) islet area (μm²), (D) β-cell area (μm²), (E) α-cell area (μm²) and (F) islet size distribution (% of total area) in *db/db* mice. Values are mean ± S.E.M. **P* < 0.05, ***P* < 0.01 compared with lean mice and Δ*P* < 0.05, ΔΔ*P* < 0.01, ΔΔΔ*P* < 0.001 compared with control *db/db* mice. A minimum of 50 islets per mouse were analysed using Cell⁺F analysis software.

were significantly decreased and α-cell area significantly increased (Fig. 6E) compared with lean mice (Fig. 6E). There was no significant difference in islet area (Fig. 6C). Treatment with [S4K]CPF-AM1 produced no significant changes in these parameters compared with the *db/db* control group but an increase in the number of large- and medium-size islets was observed (Fig. 6F).

Effects of [S4K]CPF-AM1 administration on gene expression in skeletal muscle and pancreatic islets

Expression of genes associated with insulin signalling [*Slc2a4* (glucose transporter 4, GLUT4), *Insr* (insulin receptor), *Irs1* (insulin receptor substrate 1) *Pik3ca* (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha), *Akt1* (protein kinase B alpha) and *Ppm1b* (protein phosphatase 1B)] in the skeletal muscle of *db/db* mice was elevated relative to lean mice. This increase was completely reversed by [S4K]CPF-AM1 treatment in all cases (Fig. 7A, B, C, D, E and F). The magnitude of these

effects was comparable to those observed in exenatide treated mice.

Expression of the insulin I gene *Ins1* and genes involved in insulin secretions *Abcc8* (ATP binding cassette subfamily C member 8), *Kcnj11* (potassium voltage-gated channel subfamily J member 11), *Slc2a2* (glucose transporter 2), *Cacna1c* (calcium voltage-gated channel subunit alpha 1C) and *Gck* (glucokinase) were significantly (*P* < 0.001) decreased in *db/db* mice compared with lean mice (Fig. 8A, B, C, D, E and F). In all cases, treatment with [S4K]CPF-AM1 significantly (*P* < 0.01 – *P* < 0.001) upregulated gene expression compared with the *db/db* control animals but generally to a lesser extent than the effects produced by exenatide.

[S4K]CPF-AM1 and exenatide treatments also reversed the downregulation of the *Gipr* (gastric inhibitory polypeptide receptor), *Glp1r* (glucagon-like peptide-1 receptor), *Gcg* (glucagon) and *Pdx1* (pancreatic and duodenal homeobox 1) genes that was observed in the islets of *db/db* mice (Fig. 8G, H, I and J). The significantly

Figure 7

Effects of 28-day administration of [S4K]CPF-AM1 (75 nmol/kg) or exenatide (25 nmol/kg) on mRNA expression (arbitrary units) of (A) *Slc2a4*, (B) *Insr*, (C) *Irs1*, (D) *Akt*, (E) *Pik3ca* and (F) *Ppm1b* genes in skeletal muscle from *db/db* mice. Values are mean ± S.E.M. (*n* = 4). **P* < 0.05, ***P* < 0.01 ****P* < 0.001 compared with lean mice and Δ*P* < 0.05, ΔΔ*P* < 0.01, ΔΔΔ*P* < 0.001 compared to control *db/db* mice.

Figure 8

Effects of 28-day administration of [S4K]CPF-AM1 (75 nmol/kg) or exenatide (25 nmol/kg) on mRNA expression (arbitrary units) of (A) *Ins1*, (B) *Abcc8*, (C) *Kcnj11*, (D) *Slc2a2*, (E) *Cacna1c*, (F) *Gck*, (G) *Gpr*, (H) *Glp1r*, (I) *Gcg*, (J) *Pdx1* and (K) *Stat1* genes in islets from *db/db* mice. Values are mean $\pm$ s.e.m. ($n = 4$).

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with lean mice and $\Delta P < 0.05$, $\Delta\Delta P < 0.01$, $\Delta\Delta\Delta P < 0.001$ compared with control *db/db* mice.

upregulated expression of the *Stat1* (signal transducer and activator of transcription 1) gene that was observed in islets from *db/db* mice was also reversed by peptide treatment (Fig. 8K).

Effects of [S4K]CPF-AM1 on membrane depolarisation, intracellular Ca^{2+} and cAMP concentration in BRIN-BD11 cells

As shown in Fig. 9A, [S4K]CPF-AM1, at a concentration of 1 $\mu\text{mol/L}$, stimulated the rate of insulin release from BRIN-BD11 cells (McClenaghan *et al.* 1996) in the presence of 1.1 and 5.6 mmol/L glucose and the effect was maintained at high (16.7 mmol/L) glucose. The insulinotropic effect of the peptide was significantly ($P < 0.05$) attenuated in the presence of the K^+ channel activator, diazoxide but not significantly so in the presence of the blocker of L-type voltage-dependent Ca^{2+} channels, verapamil (Fig. 9A). The rate of insulin release was stimulated by a 30 mmol/L KCl depolarising stimulus and this increase was

significantly ($P < 0.001$) augmented by [S4K]CPF-AM1. When incubations were carried out in Ca^{2+} -free medium at 5.6 mmol/L glucose concentration, the ability of the peptide to stimulate release was attenuated but not completely abolished (Fig. 9B). Incubation of BRIN-BD11 cells with the peptide (1 $\mu\text{mol/L}$) produced a rapid and sustained increase in membrane potential (Fig. 9C) and an increase in intracellular Ca^{2+} concentrations (Fig. 9D).

In the presence of 200 $\mu\text{mol/L}$ 3-isobutyl-1-methylxanthine (IBMX), [S4K]CPF-AM1 (1 $\mu\text{mol/L}$) produced a significant ($P < 0.05$) increase in cAMP production in BRIN-BD11 cells compared with the response at 5.6 mmol/L glucose alone (Fig. 10A). The magnitude of the increase was less than that produced by 10 nmol/L GLP-1. After downregulation of the PKA pathway by overnight incubation with forskolin, the insulin stimulatory activity of GLP-1, but not [S4K]CPF-AM1 and CCK-8, was strongly attenuated. In contrast, downregulation of the PKC pathway by overnight incubation with PMA was without effect on the stimulatory activity of GLP-1 but the effects of

Figure 9

Effects of [S4K]CPF-AM1 on (A) insulin release from BRIN-BD11 cells in the presence of established modulators of insulin secretion ($n = 6$), (B) insulin release from BRIN-BD11 cells in the absence of extracellular calcium ($n = 6$), (C) membrane potential in BRIN-BD11 cells expressed as a relative fluorescence units (RFU) ($n = 6$) and (D) intracellular Ca²⁺ concentrations in BRIN-BD11 cells expressed as RFU ($n = 6$). All data are represented as mean $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with 5.6 mmol/L glucose alone, ++ $P < 0.01$, +++ $P < 0.001$ compared with 5.6 mmol/L glucose in the presence of the peptide, $\Delta P < 0.05$, $\Delta\Delta P < 0.01$, $\Delta\Delta\Delta P < 0.001$ compared with incubation in the absence of the peptide.

[S4K]CPF-AM1 and CCK-8 were abolished. Downregulation of both the PKA and PKC pathways by forskolin+PMA abolished the stimulatory responses of all peptides tested (Fig. 10B). The data suggest an involvement of the PKC pathway in the [S4K]CPF-AM-mediated stimulation of insulin release.

Effects of [S4K]CPF-AM1 on GLP-1 release from GLUTag cells

Incubation of GLUTag enteroendocrine cells with [S4K]CPF-AM1 at a concentration of 1 μ mol/L produced a small but significant ($P < 0.05$) increase in the rate of GLP-1 release (Fig. 10C).

Discussion

Previous studies from the laboratory have investigated the antidiabetic and anti-obesity effects of acute and

long-term administration of frog skin-derived peptides and their analogues in both lean mice and high fat-fed mice exhibiting obesity, insulin resistance and impaired glucose tolerance (reviewed in Conlon *et al.* 2018, 2019). These studies have now been extended to a study of the effect of an analogue of the frog skin peptide CPF-AM1 in genetically obese and diabetic *db/db* mice. The leptin receptor deficient *db/db* mouse is an animal model of degenerative T2DM characterised by early obesity, progressive hyperglycaemia and hyperinsulinaemia which on C57BL/KsJ genetic background is followed by a phase of β -cell destruction and severe hyperglycaemia (Yang *et al.* 2015).

In T2DM, a progressive decline in insulin biosynthesis and secretion results in increasing hyperglycaemia (Butler *et al.* 2003). In *db/db* mice, the progressive decrease in non-fasting plasma insulin concentrations was delayed significantly by treatment with [S4K]CPF-AM1.

These findings correlate with enhanced pancreatic insulin content which illustrates the beneficial effects of the analogue on β -cell content and function. Consistent with previous studies in *db/db* mice (Wang & Brubaker 2002, Park *et al.* 2007), exenatide both delayed and

attenuated the progressive decline in circulating insulin concentrations.

The onset of secondary complications in T2DM can be prevented by maintaining strict glycaemic control (UK Prospective Diabetes Study (UKPDS) Group 1998). In the present study, [S4K]CPF-AM1 treatment of *db/db* mice significantly decreased circulating glucose concentrations without impacting body weight or energy intake. Glycated haemoglobin (HbA1c), reflecting average blood glucose concentration over several weeks, was also lowered by treatment with the analogue (29 %) but to a lesser extent than produced by exenatide (52%).

As early as 6 weeks of age, *db/db* mice show impaired glycaemic responses to a glucose load which deteriorate with age (Wang & Brubaker 2002). There have been previous reports of several frog skin peptides and their analogues showing improved glucose tolerance in high fat-fed mice (Srinivasan *et al.* 2015, Ojo *et al.* 2016, Owolabi *et al.* 2016b, Vasu *et al.* 2017). In the present study, *db/db* mice treated with saline only showed an impaired glycaemic response to an intraperitoneal glucose load. After 28-day treatment with [S4K]CPF-AM1, the response to a glucose challenge was improved significantly as was found in mice treated with exenatide. The improvement of glycaemic response in the [S4K]CPF-AM1 treated group correlated with improved insulin sensitivity as evidenced by a higher QUICKI index. Long-term administration of the structurally related peptide CPF-SE1 to high fat-fed mice using the same protocol as in the present study also did not affect body weight or energy intake but, along with decreased circulating glucose and increased insulin concentrations, decreased body fat and plasma triglyceride concentrations (Srinivasan *et al.* 2015). However, treatment of *db/db* mice with [S4K]CPF-AM1 had no significant effect on adiposity or lipid profile whereas exenatide treatment produced significant decreases in circulating triglyceride and LDL concentrations. The present study suffers from the limitation that the effects of an only single dose of peptide administered over 28 days were investigated but further studies using additional doses over different time periods will be performed to optimise therapy and that these may reveal superior effects.

Expression of the *Slc2a4*, *Insr*, *Irs1*, *Akt1*, *Pik3ca* and *Ppm1b* genes, associated with insulin action, was upregulated in skeletal muscle of *db/db* control mice compared with expression in *+/+* lean controls. These observations are consistent with previous findings that an increase in *Akt1* expression was observed in the liver and kidney of *db/db* mice (Feliers *et al.* 2001). Similarly, the activities of enzymes in the proximal insulin signalling

Figure 10

Effects of [S4K]CPF-AM1 on (A) cAMP concentrations in BRIN-BD11 cells ($n = 6$), (B) insulin release from BRIN-BD11 cells in the presence or absence of 25 μ mol/L forskolin, 10 nmol/L PMA and 25 μ mol/L forskolin + 10 nmol/L PMA ($n = 8$), (C) GLP-1 release from GLUTag cells ($n = 4$). All data are represented as mean $\pm$ s.e.m. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with 5.6 mmol/L glucose alone, $\Delta\Delta\Delta P < 0.001$ compared with incubation under normal culture conditions. In panel C, $\phi P < 0.05$, $\phi\phi\phi P < 0.001$ compared with 2.0 mmol/L glucose alone.

cascade are increased in the insulin-resistant skeletal muscle of mice with experimentally induced liver cirrhosis (Jessen *et al.* 2006). It is tempting to speculate that this increase in insulin signalling activity and the increase expression of *Slc2a4* could be an attempt to compensate for an impaired ability in GLUT4 translocation which is a primary defect in the skeletal muscles of T2DM patients (DeFronzo & Tripathy 2009). Consistent with improved insulin sensitivity in [S4K]CPF-AM1 treated mice, the expression of these genes was normalised to the same extent as observed with exenatide.

Impaired insulin secretion is a significant hallmark of long-standing T2DM (Kahn *et al.* 2006). As found in the study of Do *et al.* (2014), islets isolated from *db/db* mice exhibited impaired insulin release in response to glucose and established insulin secretagogues. In [S4K]CPF-AM1 and exenatide treated mice, insulin secretion was improved significantly. The positive effects of the analogue on β -cell function were further demonstrated by its effects on islet gene expression. Consistent with other studies in *db/db* mice (Shimoda *et al.* 2011, Poitout 2013), genes associated with insulin secretory activity (*Pdx1*, *Glp1r*, *Gipr*, *Abcc8*, *Kcnj11*, *Gck*, *Cacna1c* and *Gcg*) were significantly downregulated and the *Stat1* gene, which regulates expression of multiple of genes involved with cell survival, was significantly upregulated compared to lean control mice. These diabetes-induced changes were attenuated by both [S4K]CPF-AM1 and exenatide treatments. Effects on islet morphology were relatively minor but immunohistochemical analysis of pancreatic sections revealed that both peptides prevented the substantial decline of the large and medium-size islets seen in *db/db* mice.

In clinical practice, hepatic parameters such as ALT, AST and ALP activities are measured to monitor the progression of disease and toxicity of drugs. In T2DM, the normal function of the liver is compromised by the accumulation of lipid, which induces hypertrophic changes in hepatocytes, as indicated by elevated levels of these enzymes (Son *et al.* 2015, Bora *et al.* 2016). In the present study, elevated activities of the liver enzymes associated with the *db/db* phenotype were reversed by both [S4K]CPF-AM1 and exenatide treatments. Furthermore, creatinine concentrations, a molecular marker of renal function, were markedly decreased in both treatment groups. These observations suggest an absence of toxicity of the analogue at the dose used and possible beneficial effects on liver and kidney function. However, the effects on the lipid profile produced by exenatide administration were superior to

the corresponding effect produced by the frog peptide. Exenatide treatment significantly lowered the elevated concentrations of triglycerides and LDL associated with the *db/db* group whereas [S4K]CPF-AM1 had no effect on these parameters. In *db/db* mice treated with either [S4K]CPF-AM1 or exenatide, a small increase in amylase activity was observed compared with saline-treated *db/db* controls suggesting a potential issue with exocrine pancreatic function. Increased plasma amylase activities have been reported for T2DM patients treated with GLP-1 mimetics but a correlation with the development of acute pancreatitis has not been established (Filippatos *et al.* 2014, Steinberg *et al.* 2017).

In common with a number of other frog skin-derived peptides (reviewed in Conlon *et al.* 2018), [S4K]CPF-AM1 at a concentration of 1 μ M produced sustained membrane depolarisation and elevation of intracellular Ca^{2+} in BRIN-BD11 clonal β -cells. The insulin-releasing activities of the peptide were attenuated in Ca^{2+} -free media and in the presence of the potassium channel activator, diazoxide suggests that the effects of the peptides are mediated, at least in part, by the KATP channel-dependent pathway. However, the increase in insulin release produced by a depolarising stimulus (30 mM KCl) is augmented by the peptide suggesting that the peptides may also be acting by a KATP channel-independent pathway.

Overall, these results demonstrate that [S4K]CPF-AM1 exhibits a broad spectrum of actions in *db/db* mice with severe degenerative diabetes which are similar, although in most cases less pronounced, than the established antidiabetic drug exenatide. Such changes appear to be mediated by direct actions on the β -cell involving increases in intracellular Ca^{2+} and cAMP concentrations together with activation of the PKC pathway leading to increased release of insulin as well as by changes in expression of key signalling genes in both islets and skeletal muscle leading to increased insulin sensitivity. Despite the fact that [S4K]CPF-AM1 and exenatide display no similarity in primary structure, the peptides demonstrate rather similar biological activities in *db/db* mice although the frog peptide is generally less effective. The actions of exenatide are mediated through activation of the GLP-1 receptor (Thorens *et al.* 1993) and it is noteworthy that [S4K]CPF-AM1 treatment markedly increases expression of *Glp1r* and modestly stimulates GLP-1 release from GLUTag cells. The future therapy of T2DM will increasingly involve agents with the ability to activate multiple pathways. This is well illustrated by the successful development of unimolecular dual or triple agonists acting at GLP-1, GIP and glucagon

receptors (Brandt *et al.* 2018). In this light, future studies will investigate the antidiabetic action of fusion peptides comprising [S4K]CPF-AM1 covalently linked to GLP-1, exenatide, or GIP which may overcome some of the disadvantages such as poor glycaemic control and undesirable side effects associated with the use of the mono agonist peptides alone. In addition, actions of long-acting analogues of such peptides containing albumin-binding moieties such as palmitate will be investigated.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by a project grant from Diabetes UK (Grant Number 12/0004457), the Northern Ireland Department of Education and Learning (DEL), and Ulster University Strategic Funding.

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Received in final form 23 November 2020

Accepted 25 November 2020

Accepted Manuscript published online 27 November 2020