Characterisation of the biological activity of xenin-25 degradation fragment peptides

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Abstract

Xenin-25, a peptide co-secreted with the incretin hormone glucose-dependent insulinotropic polypeptide (GIP), possesses promising therapeutic actions for obesity-diabetes. However, native xenin-25 is rapidly degraded by serum enzymes to yield the truncated metabolites: xenin 9–25, xenin 11–25, xenin 14–25 and xenin 18–25. This study has examined the biological activities of these fragment peptides. *In vitro* studies using BRIN-BD11 cells demonstrated that native xenin-25 and xenin 18–25 possessed significant ($P<0.05$ to $P<0.001$) insulin-releasing actions at 5.6 and 16.7 mM glucose, respectively, but not at 1.1 mM glucose. In addition, xenin 18–25 significantly ($P<0.05$) potentiated the insulin-releasing action of the stable GIP mimetic (D-Ala^2)GIP. In contrast, xenin 9–25, xenin 11–25 and xenin 14–25 displayed neither insulinotropic nor GIP-potentiating actions. Moreover, xenin 9–25, xenin 11–25 and xenin 14–25 significantly ($P<0.05$ to $P<0.001$) inhibited xenin-25 (10^{-6} M)-induced insulin release *in vitro*. I.p. administration of xenin-based peptides in combination with glucose to high fat-fed mice did not significantly affect the glycaemic excursion or glucose-induced insulin release compared with controls. However, when combined with (D-Ala^2)GIP, all xenin peptides significantly ($P<0.01$ to $P<0.001$) reduced the overall glycaemic excursion, albeit to a similar extent as (D-Ala^2)GIP alone. Xenin-25 and xenin 18–25 also imparted a potential synergistic effect on (D-Ala^2) GIP-induced insulin release in high fat-fed mice. All xenin-based peptides lacked significant satiety effects in normal mice. These data demonstrate that the C-terminally derived fragment peptide of xenin-25, xenin 18–25, exhibits significant biological actions that could have therapeutic utility for obesity-diabetes.

Key Words

- glucose-dependent insulinotropic polypeptide (GIP)
- high fat-fed mice
- bioactivity
- xenin-25

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Introduction

Xenin-25 is a 25 amino acid gastrointestinal hormone secreted from the same enteroendocrine K-cells from which the incretin hormone glucose-dependent insulinotropic polypeptide (GIP) is secreted (Anlauf *et al.* 2000). Key advances in the understanding of xenin-25 physiology have demonstrated that xenin-25 not only affects gut motility and feeding behaviour (Cline *et al.* 2007, Leckstrom *et al.* 2009) but also acts as an independent insulinotropic agent (Taylor *et al.* 2010) and a potentiator of GIP-induced insulin secretion (Wice *et al.* 2010, Martin *et al.* 2012). However, there is still a dearth of information regarding the biological profile of

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xenin-25, despite its amino acid sequence being highly conserved through evolution (Hamscher *et al.* 1996). Notwithstanding this, the various biological actions of xenin-25 established to date have aroused significant interest in the possible therapeutic action of stable, enzyme-resistant, xenin-based analogues for the therapy of obesity-diabetes (Martin *et al.* 2012). This is particularly applicable given that xenin-25 could restore the well-documented compromised insulin-releasing action of GIP in type 2 diabetes (Nauck *et al.* 1993).

Similar to most regulatory peptides, native xenin-25 is efficiently degraded by plasma enzymes once secreted into the bloodstream (Taylor *et al.* 2010, Martin *et al.* 2012). Recently, the degradation products and enzymatic cleavage sites for native xenin-25 have been determined through the use of electrospray ionisation tandem mass spectrometry (ESI-MS/MS) sequencing, algorithms and databases (Martin *et al.* 2012). Thus, the plasma enzymatic degradation fragment peptides of native xenin-25 include xenin 9–25, xenin 11–25, xenin 14–25 and xenin 18–25 (Martin *et al.* 2012). However, the physiological importance of these degradation fragments is largely unknown, and it is conceivable that they could possess important biological functions. In this regard, xenin 18–25 (also named xenin-8) was originally identified in dogs (Feurle *et al.* 1997) and demonstrated to have insulinotropic effects in the perfused rat pancreas (Silvestre *et al.* 2003). Despite this, a novel, fully enzymatic resistant form of native xenin-25 was shown to have significantly improved biological activity compared with the native peptide, including enhanced insulin-releasing actions (Martin *et al.* 2012).

Since therapeutic applications may be found for xenin-25 in the future, it is essential to determine the biological significance of the degradation fragment peptides. In light of this, the present study has attempted to elucidate the bioactive domain(s) within the xenin-25 molecule. Accordingly, we have examined the *in vitro* effects of xenin 9–25, xenin 11–25, xenin 14–25 and xenin 18–25 on insulin secretion, both alone and in the presence of stimulatory GIP and xenin-25. In addition, the effects of xenin fragment peptides on food intake, anti-hyperglycaemic and insulinotropic activity *in vivo* were also evaluated.

Materials and methods

Peptides synthesis

All peptides were obtained from GL Biochem (Shanghai, China) and characterised using matrix-assisted laser desorption ionisation time-of-flight mass spectrometry, as described previously (Martin *et al.* 2012). The experimentally derived masses of xenin 9–25, xenin 11–25, xenin 14–25 and xenin 18–25 were 1991.3, 1833.3, 1450.4 and 1045.8 Da, respectively, which corresponded closely to the theoretical masses of 1992.4, 1834.3, 1450.3 and 1046.3 Da respectively.

In vitro insulin secretion

BRIN-BD11 cells were harvested with the aid of trypsin/EDTA (Gibco), seeded into 24-multiwell plates (Nunc, Roskilde, Denmark) at a density of 1.0×10^5 cells/well, and allowed to attach overnight at 37 °C. Acute tests for insulin release were preceded by 40 min preincubation at 37 °C in 1.0 ml Krebs–Ringer bicarbonate buffer (115 mM NaCl, 4.7 mM KCl, 1.28 mM $NaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 10 mM $NaHCO_3$, 0.5% (w/v) BSA, pH 7.4) supplemented with 1.1 mM glucose. Test incubations were performed ($n=8$) in the presence of 1.1, 5.6 or 16.7 mM glucose, as appropriate, over a range of concentrations (10^{-12} to 10^{-6} M) of xenin fragment peptides in the presence or absence of 10^{-7} M (D-Ala[2])GIP or 10^{-6} M xenin-25. After 20 min incubation, the buffer was removed and used for the measurements of insulin by RIA (Flatt & Bailey 1981).

Animals

Male NIH Swiss mice (Harlan, Blackthorn, Oxfordshire, UK) had free access to drinking water and standard rodent maintenance (10% fat, 30% protein and 60% carbohydrate, Trouw Nutrition, Cheshire, UK) or high fat (45% fat, 35% carbohydrate and 20% protein, Special Diet Services, Witham, Essex, UK) diet as appropriate. Before commencement of glucose homoeostasis and insulin secretory studies, high fat-fed mice were maintained on high-fat diet from 6 weeks of age for 140 days. Obesity, hyperglycaemia and insulin resistance were clearly manifested, as judged by body weight, plasma glucose and insulin analyses. All experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986. The animals were housed individually in an air-conditioned room at 22±2 °C with a 12 h light:12 h darkness cycle (lights off between 0930 to 2130 h).

Glucose-lowering and insulinotropic activity *in vivo*

The glucose homoeostatic and insulin secretory effects of xenin-25 and related fragment peptides (25 nmol/kg body weight; i.p. injection), both alone and in the presence of

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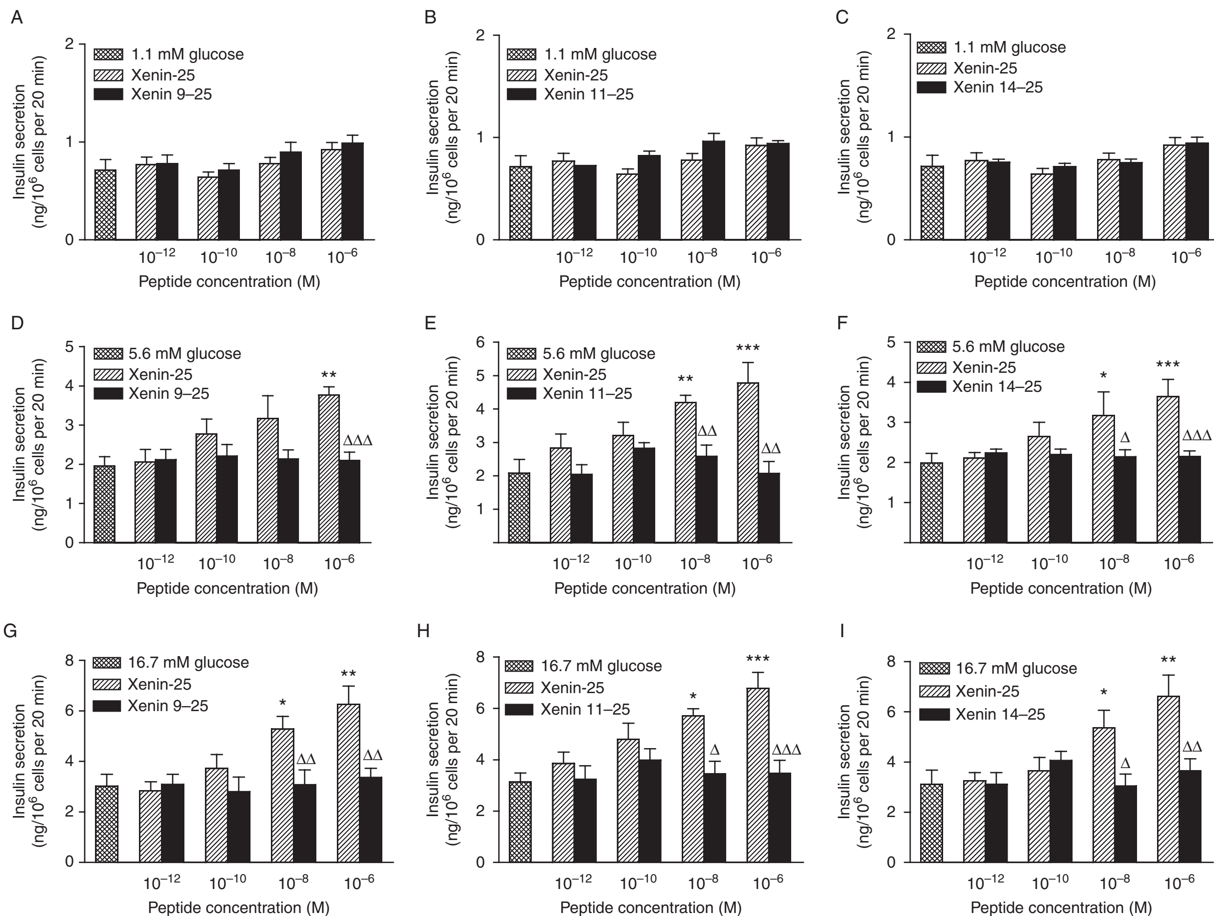

Figure 1

Insulinotropic effects of xenin-25, xenin 9–25, xenin 11–25 and xenin 14–25 in BRIN-BD11 cells. Cells were exposed to various concentrations of xenin peptides for an acute 20 min period in the presence of (A, B and C) 1.1, (D, E and F) 5.6 or (G, H and I) 16.7 mM glucose. Values represent mean ± s.e.m. for eight separate observations. $*P<0.05$, $**P<0.01$, $***P<0.001$ compared with respective glucose control. $^{\Delta}P<0.05$, $^{\Delta\Delta}P<0.01$ and $^{\Delta\Delta\Delta}P<0.001$ compared with native xenin-25 at the same concentration.

(D-Ala[2])GIP (25 nmol/kg body weight; i.p. injection), were examined in combination with i.p. glucose injection (18 mmol/kg body weight) in non-fasted high fat-fed mice. These doses were chosen based on our extensive experience with xenin and GIP peptides in this animal model (Gault *et al.* 2002, Taylor *et al.* 2010, Porter *et al.* 2011, Irwin *et al.* 2012, Martin *et al.* 2012). The high fat-fed mouse model was selected in order to establish whether the xenin-related peptides could enhance GIP-mediated effects in a type 2 diabetic model known to have compromised GIP action (Martin *et al.* 2012). Blood samples, collected from the cut tip of the tail vein of conscious mice at the times indicated in the figures, were immediately centrifuged using a Beckman microcentrifuge (Beckman Instruments, High Wycombe, UK) for 30 s at 13 000 ***g***. The resulting plasma was then aliquoted into fresh Eppendorf tubes and stored at −20 °C until analyses for glucose and insulin.

Effects on food intake *in vivo*

For food intake experiments, lean NIH mice were fasted for 12 h before i.p. injection of (500 nmol/kg) of native xenin-25 or related analogues. This dose was chosen based on previous food intake dose–response studies with xenin-25, where 500 nmol/kg was the lowest effective dose when administered i.p. (Taylor *et al.* 2010). Mice were then allowed free access to normal chow. Cumulative food intake was measured at 15, 30, 60, 90 and 120 min post-injection.

Plasma glucose and insulin assays

Plasma glucose was assayed by an automated glucose oxidase procedure using a Beckman Glucose Analyzer II. Plasma insulin was assayed by a dextran-charcoal RIA as described previously (Martin *et al.* 2012).

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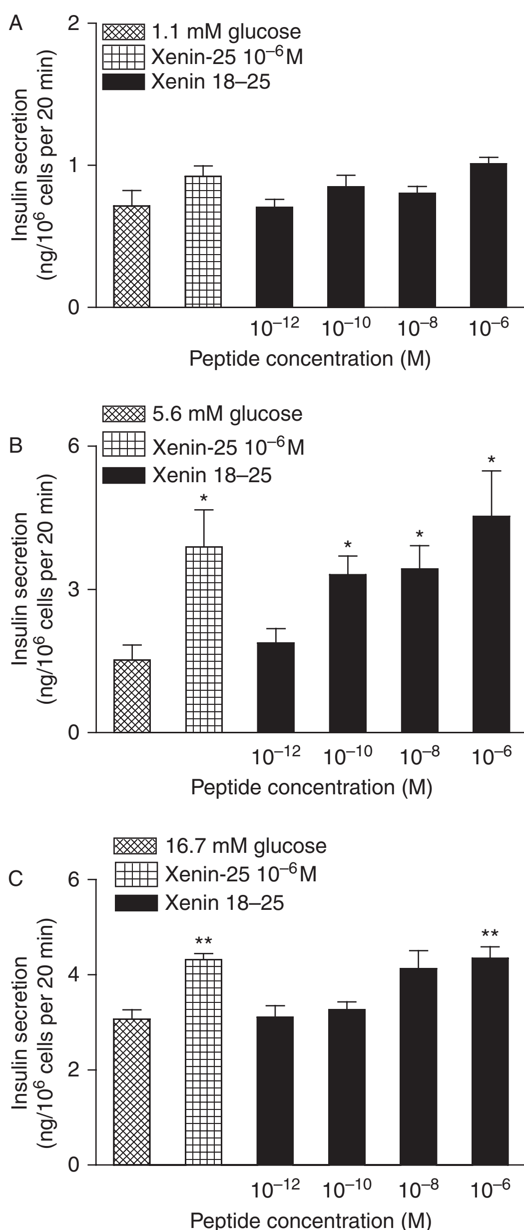

Figure 2
Insulinotropic effects of xenin-25 and xenin 18–25 in BRIN-BD11 cells. Cells were exposed to various concentrations of xenin peptides for an acute 20 min period in the presence of (A) 1.1, (B) 5.6 or (C) 16.7 mM glucose. Values represent mean ± S.E.M. for eight separate observations. $^*P<0.05$ and $^{**}P<0.01$ compared with respective glucose control.

Statistical analysis

Results are expressed as mean ± S.E.M., and data were compared using unpaired Student's *t*-test. Where appropriate, data were compared using one-way ANOVA, followed by the Student–Newman–Keuls *post hoc* test. Groups of data from both were considered to be significantly different if $P<0.05$.

Results

Actions of xenin-25 and related fragment peptides on insulin secretion in clonal BRIN-BD11 cells

Xenin-25 did not induce insulin release at 1.1 mM glucose (Fig. 1A, B and C); however, it enhanced insulin release in a concentration-dependent manner at 5.6 and 16.7 mM glucose levels (Fig. 1D, E, F, G, H and I). In a similar fashion, xenin 18–25 significantly ($P<0.05$ to $P<0.01$) augmented insulin secretion at both 5.6 and 16.7 mM glucose concentrations, but not at 1.1 mM glucose (Fig. 2). In contrast, xenin 9–25, xenin 11–25 and xenin 14–25 had no observable insulinotropic actions (Fig. 1A, B, C, D, E, F, G, H and I). Of the xenin fragments examined, only xenin 18–25 significantly ($P<0.05$) augmented (D-Ala^2)GIP-induced (10^{-7} M) insulin secretion (Fig. 3A). When incubated in the presence of native xenin-25, xenin 9–25 and xenin 14–25 significantly ($P<0.05$ to $P<0.001$) decreased xenin-25-generated insulin secretion at the concentrations of 10^{-10} M and above (Fig. 3B). Xenin 11–25 also significantly ($P<0.001$) reduced xenin-25-stimulated insulin secretion, but only at 10^{-6} M (Fig. 3B). In contrast, when incubated in the presence of xenin-25, xenin 18–25 (10^{-6} M) enhanced ($P<0.05$) the overall insulin secretory response from BRIN-BD11 cells when compared with xenin-25 alone (Fig. 3B).

Antihyperglycaemic and insulin-releasing activity of xenin-25 and related fragment peptides in high fat-fed mice

The relative glucose-lowering and insulinotropic abilities of xenin-25, xenin 9–25, xenin 11–25, xenin 14–25 and xenin 18–25 in high fat-fed mice are shown in Fig. 4. Injection of glucose alone resulted in a rapid and marked increase in individual and overall plasma glucose levels (Fig. 4). Xenin-25 and xenin 18–25 had a tendency to reduce plasma glucose levels and increase plasma insulin concentrations, but this failed to reach significance over the 60 min observation period (Fig. 4). As such, an increased sample size may have improved the statistical power of this experiment. However, all xenin-based peptides significantly ($P<0.01$ to $P<0.001$) lowered the overall glycaemic excursion in high fat-fed mice when injected concomitantly with (D-Ala^2)GIP (Fig. 5A and B), although the magnitude of reduction was similar to administration of (D-Ala^2)GIP alone (Fig. 5A and B). This was associated with significantly ($P<0.01$ to $P<0.001$) increased plasma insulin concentrations 15 min post-injection in all treatment groups (Fig. 5C). However,

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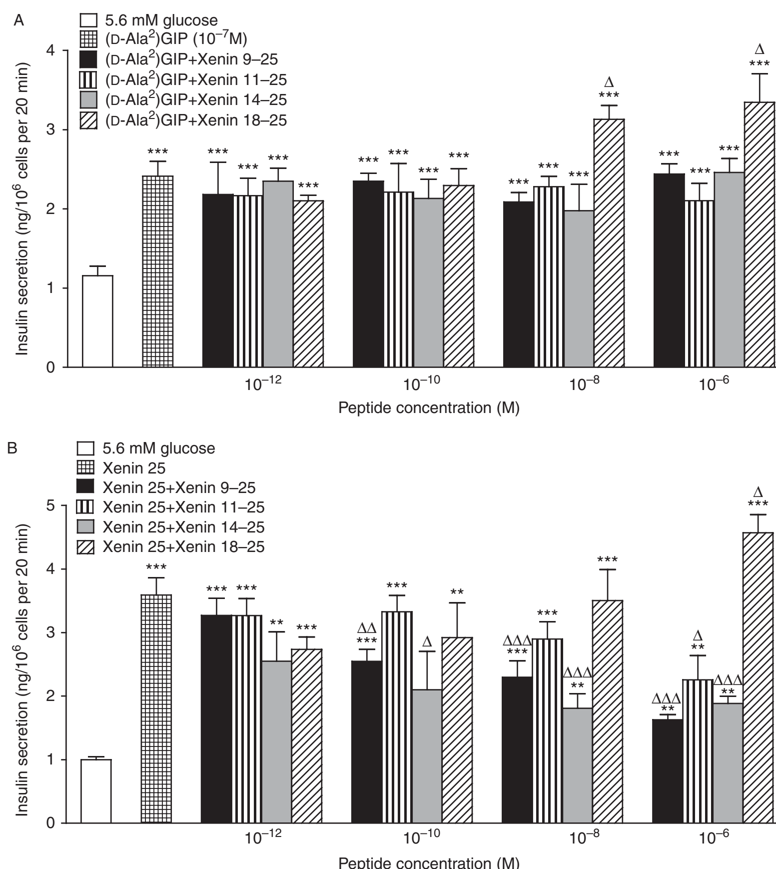

Figure 3
Insulinotropic effects of xenin 9–25, xenin 11–25, xenin 14–25 and xenin 18–25 in the presence of (A) (D-Ala^2)GIP and (B) xenin-25 in BRIN-BD11 cells. Cells were exposed to various concentrations of xenin peptides in the presence of (A) (D-Ala^2)GIP (10^{-7} M) and (B) xenin-25 (10^{-6} M) in BRIN-BD11 cells for an acute 20 min period in the presence of 5.6 mM glucose. Values represent mean ± S.E.M. for eight separate observations. $^{**}P<0.01$ and $^{***}P<0.001$ compared with 5.6 mM glucose control. $^{\Delta}P<0.05$, $^{\Delta\Delta}P<0.01$ and $^{\Delta\Delta\Delta}P<0.001$ compared with (D-Ala^2)GIP (10^{-7} M) or xenin-25 (10^{-6} M) as appropriate.

corresponding 0–60-min overall glucose-induced insulin values were significantly ($P<0.05$) elevated only in mice treated with xenin-25 or xenin 18–25 in combination with (D-Ala^2)GIP when compared with controls (Fig. 5D).

Satiety effects of xenin-25 and related fragment peptides in normal mice

None of the peptides examined had any significant effects on feeding behaviour compared with saline-treated controls over the 120 min period (Fig. 6). However, native xenin-25 did have a clear non-significant tendency to reduce food intake at each observation point (Fig. 6).

Discussion

Xenin-25 is considered as having potential for future treatment of type 2 diabetes based on encouraging findings from cellular and animal models (Taylor *et al.* 2010, Wice *et al.* 2010, Martin *et al.* 2012). However, the rapid degradation of xenin-25 by plasma enzymes and consequent short biological half-life (Taylor *et al.* 2010) is a major factor that hampers possible therapeutic use of the peptide. Accordingly, we have recently shown that an enzyme-resistant fatty acid-derivatised analogue of xenin-25, xenin-25(Lys^{13}PAL), has improved biological activity compared with the native peptide (Martin *et al.* 2012). Despite this, there is a possibility that the recently characterised enzymatic degradation fragments of xenin-25 (Martin *et al.* 2012) may have a physiological role in relation to the overall biological effects of xenin-25. As such, xenin 18–25 was demonstrated to possess insulin-releasing actions in the perfused rat pancreas (Silvestre *et al.* 2003).

Consistent with previous observations using clonal pancreatic BRIN-BD11 cells (Taylor *et al.* 2010,

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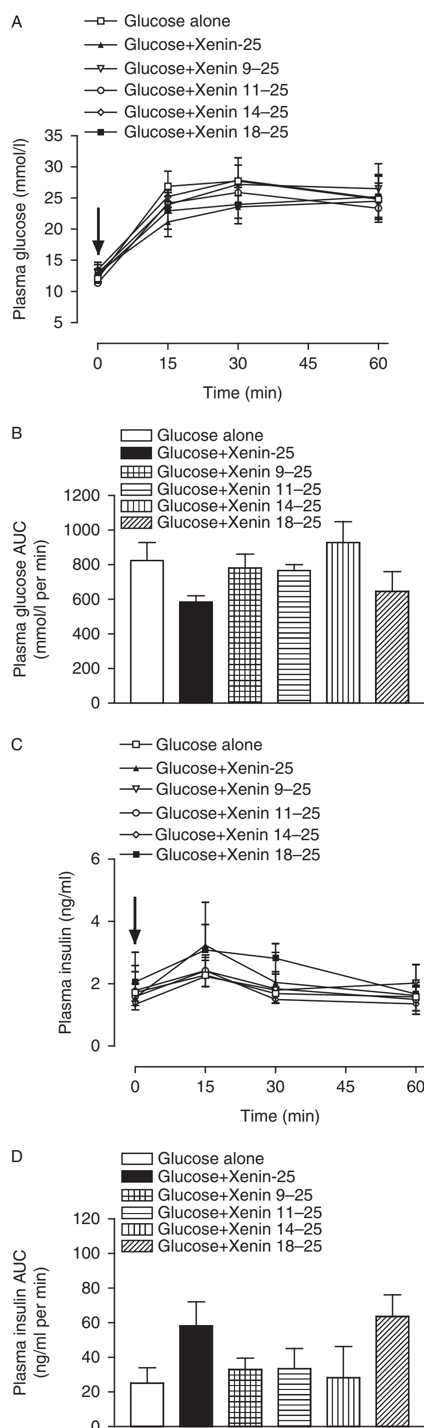

Figure 4
Effects of xenin-25, xenin 9–25, xenin 11–25, xenin 14–25 and xenin 18–25 on glucose homeostasis and insulin concentrations in high fat-fed mice. Plasma (A) glucose and (C) insulin concentrations were measured before and after i.p. administration of glucose alone (18 mmol/kg body weight), or in combination with xenin-related peptide (each at 25 nmol/kg). The time of injection is indicated by the arrow. Plasma (B) glucose and (D) insulin area under the curve (AUC) values for 0–60 min post injection are also shown. Values represent mean±S.E.M. for eight mice.

Martin *et al.* 2012), xenin-25 induced a concentration-dependent increase in insulin release at physiological and supraphysiological glucose concentrations. Importantly, there was no observable augmentation of insulin release at 1.1 mM glucose levels, indicating a glucose-dependent nature of the insulinotropic actions of xenin-25. In contrast, all xenin-25 fragment peptides, barring xenin 18–25, lacked insulinotropic activity either alone or in the presence of (D-Ala^2)GIP, a stable and potent GIP agonist (Porter *et al.* 2011). In addition, when incubated with stimulatory xenin-25 (10^{-6} M), these non-insulin-releasing fragment peptides significantly countered the ability of the native peptide to enhance insulin secretion, albeit at high concentrations. Thus, xenin 9–25, xenin 11–25 and xenin 14–25 might have a role in the regulation of the physiological actions of the parent peptide by interference or inhibition of the insulinotropic effects of xenin-25. Indeed, it has previously been shown that the major degradation fragment of GIP, GIP(3–42), can induce antagonism at the GIP receptor (Gault *et al.* 2002). However, further *in vivo* studies are required to elucidate whether the interactions observed in the present study occur at physiologically relevant concentrations.

To evaluate the biological actions of xenin fragment peptides on glucose homeostasis *in vivo*, we employed high fat-fed mice. This is an extensively studied animal model displaying several abnormal characteristic of type 2 diabetes, including obesity, insulin resistance and moderate hyperglycaemia (Irwin *et al.* 2012). The present results corroborate the previous findings that xenin-25 displayed notable insulinotropic and glucose lowering activity when administered in conjunction with (D-Ala^2)GIP to high-fat mice (Martin *et al.* 2012). Thus, type 2 diabetes is characterised by a reduced response to the biological actions of GIP (Nauck *et al.* 1993), making high fat-fed mice an excellent model for the current investigations. Moreover, in agreement with the lack of biological activity *in vitro*, all fragment peptides, besides xenin 18–25, displayed no improved glucose homeostatic or insulin-releasing actions when administered alone or in combination with (D-Ala^2)GIP in high-fat mice. This lack of *in vivo* biological action of the fragment peptides was mirrored by studies examining feeding behaviour, as we were unable to demonstrate any satiety effects in normal mice. Consistent with earlier reports (Cline *et al.* 2007, Cooke *et al.* 2009, Leckstrom *et al.* 2009), a mild satiating effect of native xenin-25 was observed *in vivo*, corroborating use of this model to investigate the biological actions of xenin-25 fragment peptides. Indeed, it has recently been shown that xenin-25 induces appetite-suppressive

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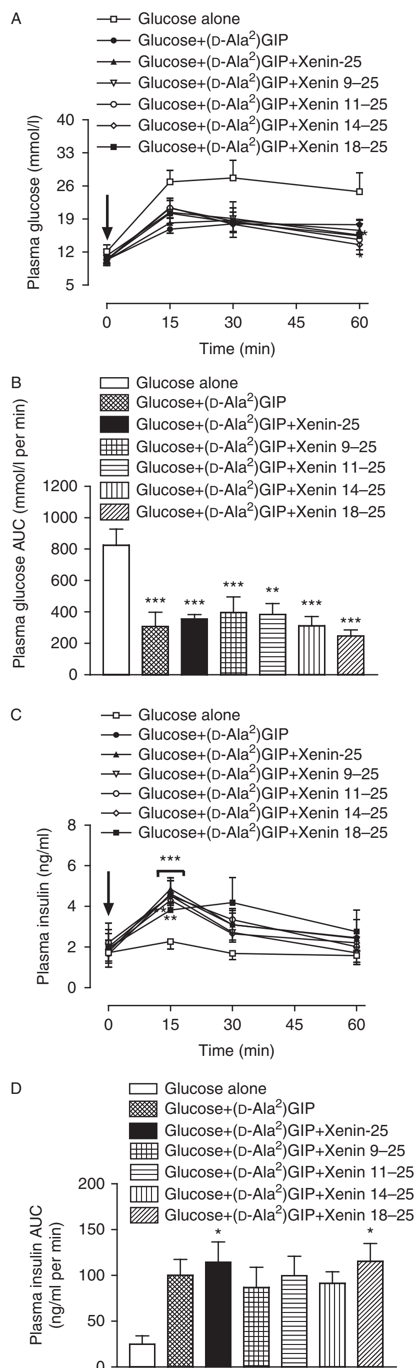

Figure 5
Glucose homoeostatic and insulinotropic effects of xenin-25, xenin 9–25, xenin 11–25, xenin 14–25 and xenin 18–25 in the presence of (D-Ala^2)GIP in high fat-fed mice. Plasma (A) glucose and (C) insulin concentrations were measured before and after i.p. administration of glucose (18 mmol/kg body weight), or together with (D-Ala^2)GIP (25 nmol/kg) alone and in combination with xenin-related peptide (each at 25 nmol/kg). The time of injection is indicated by the arrow. Plasma (B) glucose and (D) insulin area under the curve (AUC) values for 0–60 min post injection are also shown. Values represent mean±S.E.M. for eight mice. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ compared with glucose alone.

effects through delayed gastric emptying and activation of cells in the nucleus of the solitary tract (Kim & Mizuno 2010). Moreover, administration of suprapharmacological doses of the xenin peptides, and particularly xenin-25, may have yielded more significant observations on appetite suppression.

The importance of the C-terminus of xenin-25 in terms of biological activity has been indicated previously. As such, xenin 18–25 was shown to have direct effects on pancreatic hormone secretion (Silvestre *et al.* 2003). We have confirmed these findings in the present study, where xenin 18–25 had equipotent insulinotropic effects to the native peptide. Moreover, xenin 18–25 and xenin-25 displayed synergistic-like effects on the insulin-releasing actions of (D-Ala^2)GIP *in vivo* (Taylor *et al.* 2010, Martin *et al.* 2012). Furthermore, when incubated in combination with xenin-25, xenin 18–25 did not perturb the insulinotropic effect of the native peptide, unlike the larger degradation fragment peptides. Indeed, combined incubation of xenin-25 and xenin 18–25 resulted in markedly enhanced insulinotropic actions. These observations of beneficial *in vitro* effects were confirmed by studies assessing the metabolic effects of xenin 18–25 in high fat-fed mice. Xenin 18–25 did not affect feeding behaviour, which may suggest altered passage of this fragment peptide through the blood–brain barrier (Kim & Mizuno 2010); however, further studies are required to confirm this.

In conclusion, the present study has characterised the bioactivity of all xenin-25 degradation fragment peptides (Martin *et al.* 2012). The results show that xenin 9–25, xenin 11–25 and xenin 14–25 exhibit no distinct biological actions, but may act to regulate the activity of

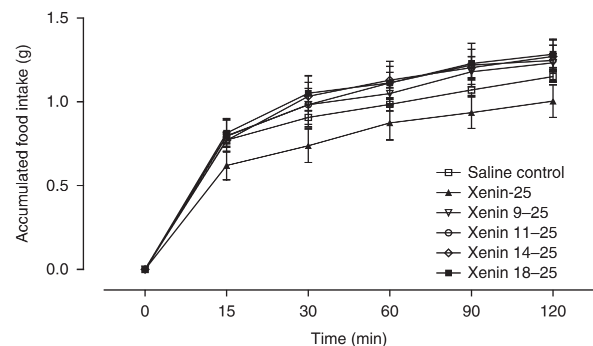

Figure 6
Effects of xenin-25, xenin 9–25, xenin 11–25, xenin 14–25 and xenin 18–25 on cumulative food intake in normal mice. Cumulative food intake was measured after i.p. administration of each peptide (500 nmol/kg). Values represent mean±S.E.M. for eight mice.

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the parent peptide. Conversely, xenin 18–25 had essentially similar biological actions as native xenin-25, but lacked significant satiety effects. Development of shorter bioactive forms of xenin-25, based on xenin 18–25, could be conceivable in the future, which would reduce production costs and possibly facilitate non-injectable drug delivery.

Declaration of interest

V A G, P R F and N I hold shares with Diabetica Ltd, which has patents for exploitation of peptide therapeutics.

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