

# Evaluation of the degradation and metabolic effects of the gut peptide xenin on insulin secretion, glycaemic control and satiety

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## Abstract

Recently, glucagon-like peptide 1 (GLP1) and glucose-dependent insulinotropic polypeptide (GIP) have received much attention regarding possible roles in aetiology and treatment of type 2 diabetes. However, peptides co-secreted from the same enteroendocrine cells are less well studied. The present investigation was designed to characterise the *in vitro* and *in vivo* effects of xenin, a peptide co-secreted with GIP from intestinal K-cells. We examined the enzymatic stability, insulin-releasing activity and associated cAMP production capability of xenin *in vitro*. In addition, the effects of xenin on satiety, glucose homeostasis and insulin secretion were examined *in vivo*. Xenin was time dependently degraded ( $t_{1/2} = 162 \pm 6$  min) in plasma *in vitro*. In clonal BRIN-BD11 cells, xenin stimulated insulin secretion at 5.6 mM ( $P < 0.05$ ) and 16.7 mM ( $P < 0.05$  to  $P < 0.001$ ) glucose levels

compared to respective controls. Xenin also exerted an additive effect on GIP, GLP1 and neurotensin-mediated insulin secretion. In clonal  $\beta$ -cells, xenin did not stimulate cellular cAMP production, alter membrane potential or elevate intra-cellular  $Ca^{2+}$ . In normal mice, xenin exhibited a short-acting ( $P < 0.01$ ) satiety effect at high dosage (500 nmol/kg). In overnight fasted mice, acute injection of xenin enhanced glucose-lowering and elevated insulin secretion when injected concomitantly or 30 min before glucose. These effects were not observed when xenin was administered 60 min before the glucose challenge, reflecting the short half-life of the native peptide *in vivo*. Overall, these data demonstrate that xenin may have significant metabolic effects on glucose control, which merit further study.

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## Introduction

Xenin was originally identified in human gastric mucosa as a mammalian equivalent to the amphibian octapeptide xenopsin, a peptide homologous in structure to neurotensin (Feurle *et al.* 1992). It is a naturally occurring 25 amino acid peptide secreted from intestinal K cells into the bloodstream in response to feeding (Anlauf *et al.* 2000). Early studies identified a contraction and relaxation effect of xenin in the jejunum, and a relaxation effect in the colon of guinea pigs (Feurle *et al.* 1996). Subsequent studies have shown xenin to elicit dose-dependent contractions in dog gall bladder and non-dose-dependent contractions in the jejunum (Kamiyama *et al.* 2007).

More recent advances in the understanding of xenin physiology have focused on anorectic and satiety actions, because of its discovery in the hypothalamus (Alexiou *et al.* 1998). Thus, a concentration-dependent inhibitory effect on food intake has since been demonstrated in chicks and rats (Cline *et al.* 2007, Nandar *et al.* 2008, Cooke *et al.* 2009). In addition, *i.c.v.* and *i.p.* xenin injection has been shown to reduce food intake in mice (Leckstrom *et al.* 2009). Similar to other anorectic gastrointestinal peptides, levels of circulating xenin appear to increase after a meal, suggesting that

xenin may regulate food intake by acting as a satiety factor (Feurle *et al.* 2003). In addition, Cooke *et al.* (2009) demonstrated that *i.c.v.* administration of xenin reduced food intake in rats. Furthermore, the same study also reported that acute, but not chronic, peripheral administration of xenin reduced food intake in mice and rats (Cooke *et al.* 2009). Indeed, it has recently been shown that xenin reduces feeding in part by acting through signalling pathways in the hypothalamus, which are independent of those used by leptin or melanocortin (Leckstrom *et al.* 2009).

However, little is known about the physiological significance of xenin secretion and action. Xenin probably appeared early in evolution as its amino acid sequence is conserved from yeast to mammals (Hamscher *et al.* 1996). Despite this, its exact role in mammalian physiology remains elusive. Since xenin is structurally similar to neurotensin, it is thought to bind and activate the neurotensin receptor subtype 1 (Clemens *et al.* 1997). Interestingly, the feeding-suppressive effect of xenin is abolished in neurotensin receptor-deficient mice, thus supporting a role for this receptor in the anorectic effect of xenin (Kim & Mizuno 2010). However, additional biological effects are apparent, but identification of a specific 'xenin receptor' is still outstanding.

For example, in dogs, i.v. infusion of xenin stimulated exocrine pancreatic secretion and also evoked a rise in insulin and glucagon levels as well as an increase in vasoactive intestinal polypeptide and pancreatic polypeptide levels (Feurle *et al.* 1997). Interestingly, a C-terminally truncated form of xenin, xenin-8, has been shown to exert possible insulinotropic and glucagonotropic actions in the perfused rat pancreas, but lacked somatostatin-releasing effects (Silvestre *et al.* 2003). Furthermore, given that plasma xenin concentrations were significantly elevated during feeding (Feurle *et al.* 2003), and that it is secreted from the same K cell as the incretin hormone glucose-dependent insulinotropic polypeptide (GIP), the possibility of xenin functioning in an incretin-like context is also conceivable. Therefore, the present study has examined the plasma stability and satiety effects of xenin, and further characterised the glucose-lowering and insulinotropic effects of xenin both alongside GIP, glucagon-like peptide-1 (GLP1) and neurotensin.

## Materials and Methods

### *Degradation of xenin in murine plasma*

Degradation profile of xenin was assessed in pooled plasma from fasted normal mice using HPLC analysis as described previously (Gault *et al.* 2002). Briefly, xenin (50 µg; GL Biochem Ltd, Shanghai, China) was incubated at 37 °C in 50 mM triethanolamine-HCl (pH 7.8) with mouse plasma (50 µl) for 0, 30, 60, 120, 240 and 360 min. The incubation was repeated in the presence of the DPP IV inhibitor vildagliptin (10 µM). This concentration has been shown in our laboratory to inhibit DPP IV-mediated degradation of GIP and GLP1. Reactions were terminated by the addition of 10% (v/v) trifluoroacetic acid/water. Intact xenin was separated from the major degradation products by HPLC and peaks collected manually before matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) analysis. HPLC peak area data were used to calculate percentage-intact peptide remaining at various time points throughout incubation.

### *In vitro biological activity*

For cAMP measurements, BRIN-BD11 cells were collected, seeded into 96-well plates ( $3 \times 10^4$  cells per well) and grown for 16 h. Cells were washed twice in Hanks-buffered saline (HBS) buffer and incubated (20 min; 37 °C) with varying concentrations of xenin ( $10^{-12}$ – $10^{-6}$  M) in HBS buffer containing 1 mM 3-isobutyl-1-methylxanthine in the presence of 5.6 or 16.7 mM glucose. Medium was subsequently removed, cells were lysed and cAMP levels in the lysate were measured using an HTS chemiluminescent immunoassay kit (Millipore, Watford, UK).

For the assessment of insulin release, BRIN-BD11 cells were seeded into 24-well plates ( $10^5$  cells per well) and allowed

to attach overnight at 37 °C. The original characteristics of these cells have been reported previously (McClenaghan *et al.* 1996). Before acute tests, cells were pre-incubated (40 min; 37 °C) in Krebs-Ringer bicarbonate buffer (KRBB) (pH 7.4) supplemented with 0.5% (w/v) BSA and 1.1 mM glucose. Test incubations were performed in the presence of 5.6 or 16.7 mM glucose with a range of concentrations of xenin alone or in the presence of native GIP, GLP1 or neurotensin ( $10^{-8}$  M). After incubation (20 min; 37 °C), buffer was removed from each well and aliquots (200 µl) were stored at –20 °C before measurement of insulin.

For measurement of membrane potential and intra-cellular  $[Ca^{2+}]_i$  ([Ca<sup>2+</sup>]<sub>i</sub>), BRIN-BD11 cells were utilised as described previously (McClenaghan *et al.* 2009). Briefly, cells were seeded into 96-well blackwalled clear-bottom microplates at a density of  $1.0 \times 10^5$  cells/well and allowed to attach overnight before replacing the culture medium with either standard culture medium or culture medium supplemented with various concentrations of xenin in the presence of 5.6 or 16.7 mM glucose. After culture for 18 h, the medium was replaced with 100 µl of KRBB and monolayers were incubated for 20 min, after which 100 µl of either Flex membrane potential assay kit reagent (McClenaghan *et al.* 2009) or Flex calcium assay kit reagent (Molecular Devices, Sunnyvale, CA) were added to the wells at 37 °C as described previously (McClenaghan *et al.* 2009). Fluorometric data were acquired using the FlexStation scanning fluorimeter and integrated fluid transfer workstation (Molecular Devices). The cells were exposed to excitation from a xenon-arc flash lamp at a wavelength of 530 nm (membrane) or 485 nm ([Ca<sup>2+</sup>]<sub>i</sub>), and subsequent fluorescence emission was measured at 565 nm (membrane) or 525 nm ([Ca<sup>2+</sup>]<sub>i</sub>). Emission cut-off filters were set at 550 nm for membrane potential or 515 nm for [Ca<sup>2+</sup>]<sub>i</sub>.

### *Animals*

Normal mice derived from the colony originally maintained at Aston University, UK (Flatt & Bailey 1981), were used at 16–20 weeks of age. Animals were housed individually in an air-conditioned room at  $22 \pm 2$  °C with a 12 h light:12 h darkness cycle. Drinking water and standard rodent maintenance diet (Trouw Nutrition, Cheshire, UK) were freely available. Groups of six to eight animals were used for individual tests. All animal experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986.

### *In vivo studies*

For food intake studies, mice were fasted for 12 h before s.c. injection of 50, 100 or 500 nmol/kg xenin. Mice were then allowed free access to normal chow. Cumulative food intake was measured at the time points indicated in figure 3. For glucose-homoeostatic and insulin-releasing assessment of xenin, groups of overnight (18 h) fasted mice were injected

s.c. with glucose alone (18 nmol/kg) or in combination with xenin (25 nmol/kg). For duration of biological action studies, groups of mice were similarly injected with xenin (25 nmol/kg) or vehicle (0.9% w/v, NaCl) 30 or 60 min before glucose load (18 mmol/kg).

### Biochemical analyses

Blood samples were taken from the cut tip of the tail vein of conscious mice at times indicated in the figures. Blood glucose was measured directly using a hand-held Ascencia Contour blood glucose meter (Bayer Healthcare). For insulin analysis, blood samples were collected from the cut tip on the tail vein of conscious mice into chilled fluoride/heparin glucose microcentrifuge tubes (Sarstedt, Numbrecht, Germany). Plasma was separated immediately by centrifugation (30 s at 13 000 *g*) using a Beckman microcentrifuge (Beckman Instruments, Galway, Ireland) and stored at  $-20^{\circ}\text{C}$  before insulin analysis. Insulin was determined using a modified dextran-coated charcoal RIA as described previously (Flatt & Bailey 1981).

### Statistical analysis

Data are expressed as mean  $\pm$  S.E.M., and values were compared using repeated-measures ANOVA or one-way ANOVA, followed by the Student–Newman–Keuls *post hoc* test, as appropriate. Area under the curve (AUC) analyses were calculated using the trapezoidal rule with baseline subtraction.  $P < 0.05$  was considered to be statistically significant.

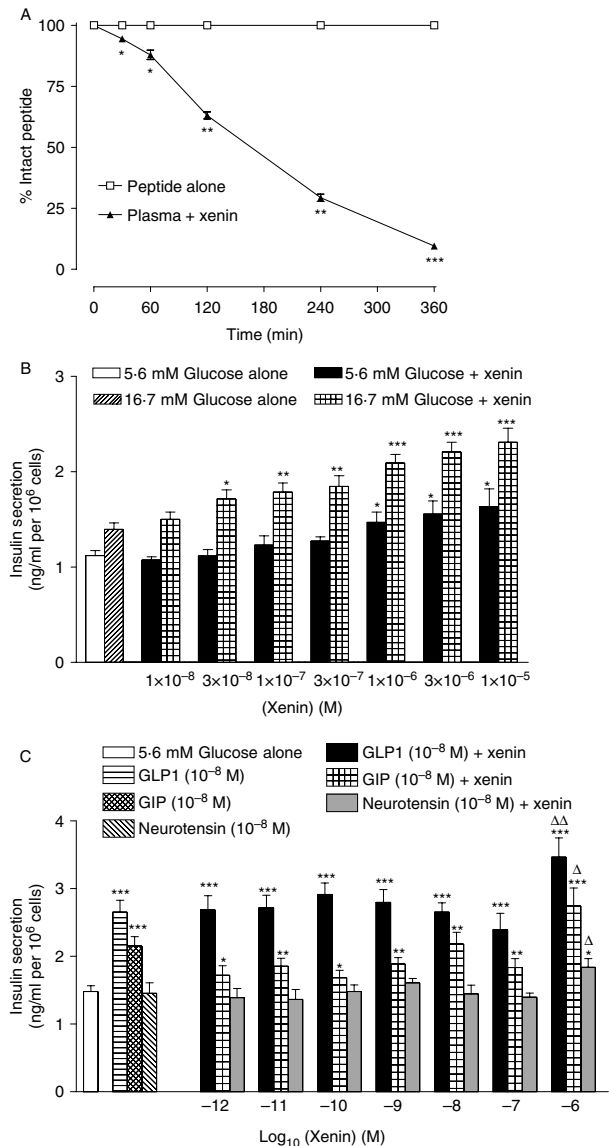
## Results

### Degradation of xenin by murine plasma and identification of products

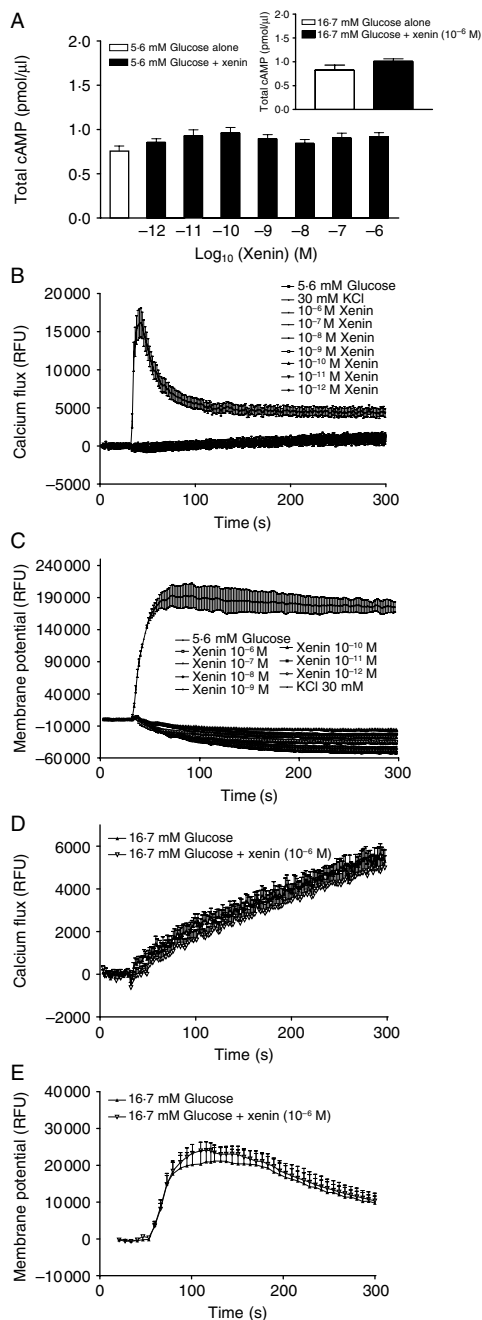
Xenin was rapidly degraded by murine plasma over the 6 h period as determined by HPLC and MALDI-TOF analysis (Fig. 1A). Thus, native xenin was degraded by 7% at 30 min, 14% at 60 min, 37% at 120 min, 72% at 240 min and 91% ( $P < 0.001$ ) at 360 min. The  $t_{1/2}$  for xenin under these conditions was calculated to be  $162 \pm 6$  min. Incubation of peptide without plasma did not produce a significant change in peptide peak area at any of the time points measured. Interestingly, incubation with the DPP IV inhibitor vildagliptin did not prevent xenin degradation (data not shown).

### In vitro actions of xenin

Xenin induced a significant ( $P < 0.05$ ) augmentation of insulin release from BRIN-BD11 cells at concentrations of  $10^{-6}$  M and above in the presence of 5.6 mM glucose (Fig. 1B). Moreover, the insulinotropic action of xenin was



**Figure 1** Plasma degradation profile of xenin and insulin secretory effects in BRIN-BD11 cells. (A) Resistance of xenin to degradation by plasma was measured ( $n=3$ ) after 0, 30, 60, 120, 240 and 360 min incubations. Reaction products were subsequently analysed by HPLC and degradation expressed as a percentage of intact peptide relative to the major degradation fragments. (B) BRIN-BD11 cells were exposed to various concentrations of xenin at 5.6 and 16.7 mM glucose concentrations for 20 min and insulin release measured using RIA. (C) BRIN-BD11 cells were incubated with a range of concentrations of xenin in the presence of stimulatory GIP, GLP1 or neurotensin ( $10^{-8}$  M) for 20 min and insulin release measured using RIA. Values represent means  $\pm$  S.E.M. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  compared with respective control.  $\Delta P < 0.05$  and  $\Delta\Delta P < 0.01$  compared with respective stimulatory effect of GIP, GLP1 or neurotensin.



**Figure 2** Effects of xenin on  $\beta$ -cell signalling pathways. (A) BRIN-BD11 cells were exposed to various concentrations of xenin at 5.6 and 16.7 mM (inset) glucose for 20 min ( $n=4$ ) and cAMP production subsequently measured using ELISA. (B and D) BRIN-BD11 cells were exposed to various concentrations of xenin at (B) 5.6 mM and (D) 16.7 mM glucose for 20 min ( $n=4$ ), and membrane potential was measured using the FlexStation scanning fluorimeter. (C and E) BRIN-BD11 cells were exposed to various concentrations of xenin at (C) 5.6 mM and (E) 16.7 mM glucose for 20 min ( $n=4$ ), and intra-cellular  $\text{Ca}^{2+}$  was measured using the FlexStation scanning fluorimeter. Values represent means  $\pm$  S.E.M.

significantly ( $P<0.05$  to  $P<0.001$ ) superior at 16.7 mM glucose, achieving significance at  $3 \times 10^{-8}$  M xenin. In addition, xenin ( $10^{-6}$  M) significantly ( $P<0.05$  to  $P<0.01$ ) enhanced the insulinotropic response of GLP1, GIP and neurotensin (Fig. 1C). However, xenin did not stimulate the production of cAMP in BRIN-BD11 cells over the concentration range ( $10^{-12}$ – $10^{-6}$  M) tested (Fig. 2A). In addition, xenin did not have any significant effect on membrane potential,  $[\text{Ca}^{2+}]_i$  or cAMP at 5.6 or 16.7 mM glucose levels (Fig. 2A–E).

#### Effects of xenin on feeding

Xenin did not induce a significant change in cumulative food intake in overnight fasted mice when administered at a dose of 50 or 100 nmol/kg (Fig. 3). However, when mice received a dose of 500 nmol/kg, xenin induced a 60% decrease ( $P<0.01$ ) in food intake at 30 min post injection compared to controls (Fig. 3). This satiety effect was not evident at the later time points examined (Fig. 3).

#### Acute glucose-lowering and insulinotropic effects of xenin in normal mice

Administration of xenin to 18 h fasted mice in combination with glucose significantly ( $P<0.05$  to  $P<0.001$ ) lowered individual and overall glucose levels in overnight fasted mice (Fig. 4A). This was associated with a significant ( $P<0.05$ ) elevation of overall insulin release compared to controls (Fig. 5A).

#### Persistent glucose-lowering and insulinotropic effects of xenin in normal mice

Administration of xenin 30 min earlier significantly ( $P<0.05$ ) decreased individual plasma glucose levels after s.c. glucose injection in mice (60 min post injection; Fig. 4B). This was associated with a decreased overall glycaemic excursion (Fig. 4B). The overall insulin release as assessed by AUC measures was significantly elevated in overnight fasted mice compared to respective controls (Fig. 5B). Administration of xenin 60 min before glucose challenge annulled the glucose-lowering and insulin-releasing effects of xenin (Figs 4C and 5C).

## Discussion

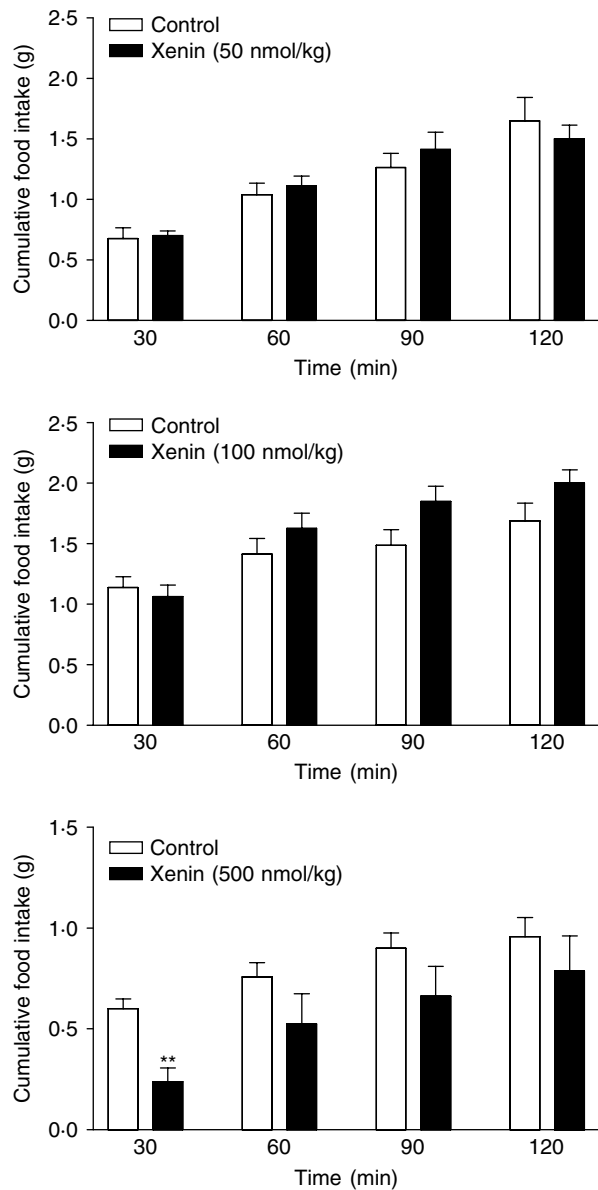
Since its discovery in 1992, xenin has received attention as an effector of gut motility (Feurle *et al.* 1992, 2003). In addition, xenin is now thought to be involved in the regulation of calorie intake (Leckstrom *et al.* 2009) and possibly insulin secretion (Silvestre *et al.* 2003). Thus, the aim of the current study was to evaluate the effects and role of xenin in food intake, glucose homeostasis and insulin secretion.

Initially, we undertook *in vitro* studies in BRIN-BD11 cells to confirm earlier reports that xenin-related peptides

stimulate insulin release (Silvestre *et al.* 2003). To our knowledge, this is the first time that the *in vitro* insulin-releasing action of the full-length form of xenin (25 amino acids) has been assessed. We confirmed that xenin possesses strong insulin secretory actions under normoglycaemic and particularly hyperglycaemic conditions. The effects were more pronounced at higher glucose levels raising the possibility of glucose-dependent actions, akin to the effects of GIP and GLP1 (Green & Flatt 2007). Nonetheless, it should be noted that in contrast to xenin, GLP1 induces changes in membrane electrical activity and intra-cellular  $Ca^{2+}$  concentrations at elevated glucose concentrations (Yada *et al.* 1993, Gromada *et al.* 1997, Fernandez & Valdeolmillos 1999). However, this glucose-dependent increase in insulin secretion is comparable to the findings of Silvestre *et al.* (2003), where xenin-8 displayed similar effects in the perfused rat pancreas. Furthermore, we have shown an additive insulinotropic effect of xenin on GIP- and GLP1-mediated insulin secretion, suggesting possibly synergistic interactions with the incretin hormones.

Given that xenin-mediated effects are thought to stem, in part, from binding and activating the neurotensin receptor (Clemens *et al.* 1997) involving calcium-dependent potassium channels (Feurle *et al.* 1996), we thought it prudent to investigate the mechanism of xenin-induced insulin release observed. However, no significant changes in membrane potential or intra-cellular  $Ca^{2+}$  were observed even with the highest concentration of xenin ( $10^{-6}$  M) tested under normo- or hyperglycaemic conditions. This suggests that xenin does not elicit its insulinotropic actions through binding the neurotensin receptor; however, further studies are needed to confirm this. Furthermore, xenin was devoid of effects on cAMP production in BRIN-BD11 cells, implying that unlike the incretin hormones GIP and GLP1 (Green & Flatt 2007), it does not utilise this second messenger pathway in mediating its insulin secretory response. Thus, clarification of the mechanism of xenin-induced insulin release is necessary, possibly focusing on effects mediated through action of phospholipase C. Possible activation of this alternative signal regulation pathway would be consistent with additive effects of xenin when combined with either GIP or GLP1. However, it has been shown earlier that xenin does not exert an additive effect on carbachol-induced insulin release (Silvestre *et al.* 2003). Thus, taken together, additional work is needed to clarify the direct and indirect actions of xenin on insulin release. Furthermore, it has already been shown that xenin has effects on exocrine pancreatic secretions (Nustede *et al.* 1999).

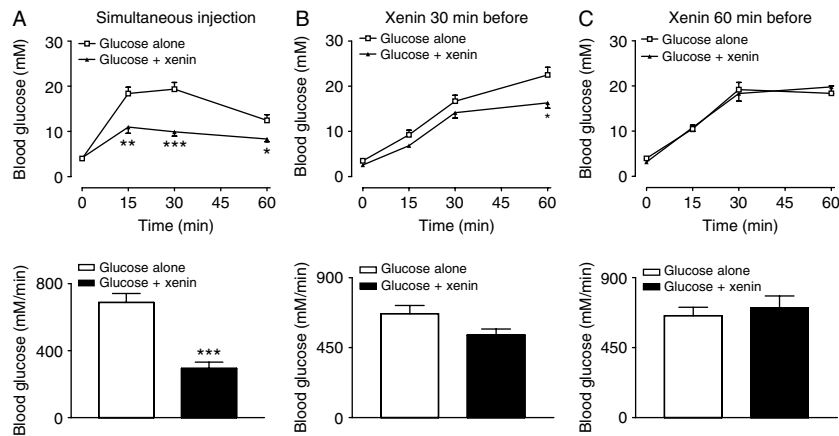
Consistent with earlier reports (Cline *et al.* 2007, Nandar *et al.* 2008, Cooke *et al.* 2009, Leckstrom *et al.* 2009), we were able to demonstrate a satiety effect of xenin in normal mice. The dose required to induce such effects was high (500 nmol/kg), but this is in agreement with others (Leckstrom *et al.* 2009). However, knowledge of normal physiological circulating concentrations of xenin is required to aid interpretation. The short duration of action of satiety effects is in agreement with our *in vitro* studies on enzymatic



**Figure 3** Effects of xenin on cumulative food intake in 12 h fasted normal mice. Cumulative food intake was measured after s.c. administration of 50 nmol/kg, 100 nmol/kg or 500 nmol/kg xenin. Values represent means  $\pm$  s.e.m. for eight mice. \*\* $P < 0.01$  compared to control.

stability, revealing xenin to be rapidly degraded to constituent metabolic fragments within 30 min. To the authors' best knowledge, this is the first documentation of plasma-mediated xenin degradation. As such, the identity and biological activity of these degradation fragments require consideration and may reveal further insights into the physiological significance of xenin. However, it appears that unlike GIP and GLP1, DPP IV does not play a role in xenin degradation.

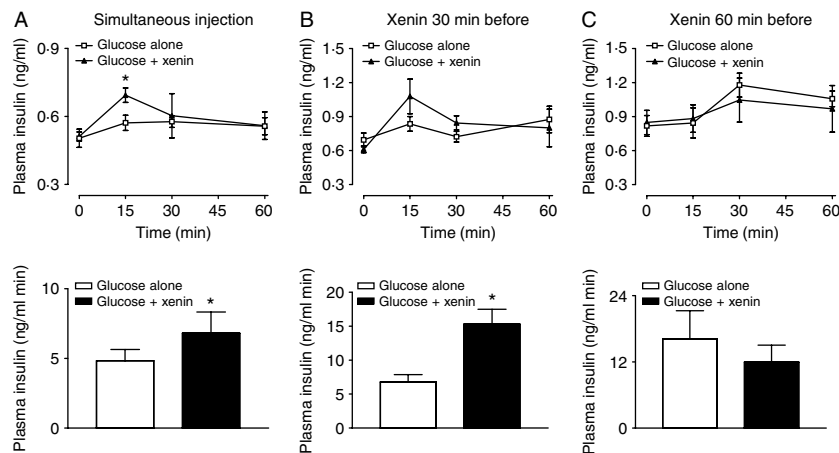
Given the *in vitro* insulinotropic effects of xenin in the current study, we evaluated the glucose-lowering and



**Figure 4** (A) Acute and (B and C) persistent glucose-lowering effects of xenin in 18 h fasted normal mice. (A) Blood glucose concentrations were measured before and after s.c. administration of glucose alone (18 mmol/kg) or in combination with xenin (25 nmol/kg). (B and C) Xenin (25 nmol/kg) was administered (B) 30 min or (C) 60 min before glucose load (18 mmol/kg). Blood glucose area under the curve (AUC) values for 0–60 min post injection are also shown. Values represent means  $\pm$  s.e.m. for eight mice. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  compared to control.

insulin-releasing actions of a bolus injection of xenin in normal mice. Xenin administration was associated with lowered glycaemic and enhanced insulin levels during glucose tolerance tests. Furthermore, the effects were still evident when xenin was administered 30 min before glucose challenge. However, the short duration of biological action of native xenin was evidenced from the lack of biological effect when administered 60 min before glucose. Nonetheless, it is clear that in addition to the well documented effect of xenin on feeding, this peptide may also function as a gluco-regulatory hormone.

In conclusion, these data indicate that xenin is a short-acting peptide with potential important metabolic effects on blood glucose control. Further study of the biological effects of xenin in animal models of diabetes and or obesity will help to delineate any physiological or potential therapeutic role. In addition, investigation of the metabolic significance of xenin enzymatic degradation products is required. Generation of specific xenin antagonists or longer acting xenin mimetics may be useful in assessing the overall biological consequence of xenin-mediated actions.



**Figure 5** (A) Acute and (B and C) persistent insulin-releasing effects of xenin in 18 h fasted normal mice. (A) Plasma insulin concentrations were measured before and after s.c. administration of glucose alone (18 mmol/kg) or in combination with xenin (25 nmol/kg). (B and C) Xenin (25 nmol/kg) was administered (B) 30 min or (C) 60 min before glucose load (18 mmol/kg). Plasma insulin area under the curve (AUC) values for 0–60 min post injection are also shown. Values represent means  $\pm$  s.e.m. for eight mice. \* $P < 0.05$  compared to control.



## 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

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