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GIP plus xenin-25 indirectly increases pancreatic polypeptide release in humans with and without type 2 diabetes mellitus

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Abstract

Xenin-25 (Xen) is a 25-amino acid neurotensin-related peptide that activates neurotensin receptor-1 (NTSR1). We previously showed that Xen increases the effect of glucose-dependent insulinotropic polypeptide (GIP) on insulin release 1) in hyperglycemic mice via a cholinergic relay in the periphery independent from the central nervous system and 2) in humans with normal or impaired glucose tolerance, but not type 2 diabetes mellitus (T2DM). Since this blunted response to Xen defines a novel defect in T2DM, it is important understand how Xen regulates islet physiology.

On separate visits, subjects received intravenous graded glucose infusions with vehicle, GIP, Xen, or GIP plus Xen. The pancreatic polypeptide response was used as an indirect measure of cholinergic input to islets. The graded glucose infusion itself had little effect on the pancreatic polypeptide response whereas administration of Xen equally increased the pancreatic polypeptide response in humans with normal glucose tolerance, impaired glucose tolerance, and T2DM. The pancreatic polypeptide response to Xen was similarly amplified by GIP in all 3 groups. Antibody staining of human pancreas showed that NTSR1 is not detectable on islet endocrine cells, sympathetic neurons, blood vessels, or endothelial cells but is expressed at high levels on PGP9.5-positive axons in the exocrine tissue and at low levels on ductal epithelial cells. PGP9.5 positive nerve fibers contacting beta cells in the islet periphery were also observed. Thus, a neural relay, potentially involving muscarinic acetylcholine receptors, indirectly increases the effects of Xen on pancreatic polypeptide release in humans.

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Author contributions

Burton M. Wice was responsible for the overall conception and design of the study, data analysis and interpretation, writing and editing the manuscript. Sara Chowdhury was involved in data analysis and interpretation, writing and editing the manuscript. Songyan Wang was involved in performing assays and antibody staining, data management, and editing the manuscript. Dominic N. Reeds supervised the administration of the clinical study visits and edited the manuscript. Bruce W. Patterson was involved in data analysis and editing the manuscript. All authors have approved the final manuscript.

Conflicts of interest

Washington University is pursuing a patent related to the use of xenin-25 to treat T2DM. In the future, this could lead to personal financial benefit to Burton M. Wice and the University. No other potential conflicts of interest to this article were reported.

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Keywords

Xenin; GIP; Cholinergic; Pancreatic Polypeptide; Neurotensin Receptor; Neuronal Relay

1. Introduction

Pancreatic islet dysfunction, including impaired insulin secretion, is one of the hallmark features of type 2 diabetes mellitus (T2DM). Glucose-dependent insulintropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are incretin hormones produced by enteroendocrine cells located in the proximal and distal intestine, respectively [1–3]. Both peptides are released into the circulation after meal ingestion in response to nutrients present in the lumen of the gut, but not to nutrients in the blood [3,4]. Circulating incretins then potentiate glucose-stimulated insulin secretion. Orally-derived glucose elicits a much greater insulin secretory response than comparable levels of intravenously administered glucose which is called the incretin effect. In addition to GIP and GLP-1, numerous neuropeptides and neurotransmitters regulate insulin release [5].

It has been known for many years that the incretin response, but not incretin release, is blunted in humans with T2DM [6,7]. In spite of this, exogenously administered GLP-1 remains active in T2DM and forms the rationale for incretin-based pharmacotherapies that increase GLP-1 receptor signaling [8,9]. Although it has been generally felt that the effects of GIP on insulin secretion are blunted in T2DM [10–12], we recently demonstrated that the *magnitude* of the insulin secretory response to exogenously administered GIP is similar in humans with normal glucose tolerance (NGT), impaired glucose tolerance (IGT), and T2DM [13]. Thus, humans with T2DM exhibit a blunted insulin secretory response to endogenously released, but not exogenously administered, GIP and GLP-1. The basis for this difference is unknown.

Xenin-25 (Xen) is a 25-amino acid neurotensin-related peptide originally reportedly produced by a subset of GIP-producing cells [14]. Although Xen is longer than the 13-amino acid neurotensin (Fig 1A), only 6 (neurotensin) or 8 (Xen) C-terminal amino acids are required for biological activity. Both peptides require an unblocked C-terminal leucine for biological activity [15,16]. We previously showed that in mice, Xen increases the effects of GIP on insulin release but has little effect alone [17]. This in vivo response to Xen: 1) was not recapitulated with isolated islets, insulin-producing cell lines, or the in situ perfused pancreas; 2) was inhibited by atropine sulfate (crosses the blood-brain barrier) and atropine methyl bromide (does not cross the blood-brain barrier); and 3) was not associated with increased c-fos expression in regions of the brain involved in afferent and efferent signaling [17]. In contrast to Xen, carbachol potentiated the effects of GIP on insulin secretion in the in vitro/situ systems. Thus, Xen increases GIP-mediated insulin release in mice via a cholinergic relay in the periphery, possibly independent from parasympathetic neurons that innervate the islets. Interestingly, Kirchgeßner and Gershon [18,19] have described an extensive network of myenteric neurons in the enteric nervous system that directly connect the stomach/duodenum to the pancreas. These interneurons function independently from the central nervous system and can modify pancreatic endocrine function. We have recently shown that Xen increases cytosolic free calcium levels in a subset of myenteric neurons isolated from guinea pig duodenum [15]. Thus, Xen-responsive neurons may regulate islet function.

We recently demonstrated using intravenous graded glucose infusions (ivGGIs) that Xen, in combination with GIP but not alone, rapidly and transiently increased insulin and glucagon secretion in humans with NGT and IGT, but not T2DM [13]. This response over the first 40

minutes of the ivGGIs occurred in the absence of significant changes in plasma glucose levels. Since this blunted response to Xen defines a novel defect in T2DM, it is important to understand how Xen regulates islet physiology. Several studies have reported that islets within the human pancreas are innervated by both cholinergic and non-cholinergic neurons [20–22] suggesting that as in mice, a neural relay could mediate the Xen signal to beta cells in humans. However, it has recently been reported that human islets are poorly innervated and islet-derived acetylcholine is released from alpha cells rather than neurons [23,24]. As a first step in understanding how Xen regulates islet function in humans, it is important to determine: 1) if Xen increases cholinergic input to islets in humans with and without T2DM and 2) which cells in the human pancreas express receptors for Xen.

Pancreatic polypeptide (PP) is a 36-amino acid peptide produced only by islet PP cells [25–27]. Classically, PP release in response to insulin-induced hypoglycemia has been used to indirectly assess cholinergic input to islets in humans. However, it has been shown that following an 8-hour fast, intravenous infusion of neurotensin also stimulates PP release in humans and this response is completely blocked by atropine [28]. Genetic and pharmacologic studies have shown that neurotensin receptor-1 (NTSR1) mediates the effects of Xen [15,29–33]. Our previous ivGGI study was conducted following a 10-hour fast and plasma glucose levels remained at basal levels for at least 40-minutes. Thus, the PP response (PPR) over this early portion of the ivGGI represents a surrogate measure for Xen-induced cholinergic input to islets. We now show that 1) the PP response to Xen is not impaired in humans with T2DM and 2) NTSR1 is expressed on pancreatic neurons but not on islet endocrine cells. Thus, a neural relay, potentially involving muscarinic acetylcholine receptors, indirectly increases the effects of Xen on islet endocrine cell function in humans.

2. Materials and Methods

2.1. Study Design and Protocols

All protocols were approved by Washington University's Human Research Protection Office and the FDA (IND#103,374) and are registered with ClinicalTrials.gov (NCT00798915). Studies were performed in the Clinical Research Unit of the Institute of Clinical and Translational Sciences of Washington University after obtaining written informed consent. The ivGGI involves step wise increases in intravenous glucose infusion rates every 40 minutes to progressively raise plasma glucose levels over a 240 minute period (Figs 1B, C). Importantly, this protocol bypasses potentially confounding effects that could arise from release of endogenous incretins in response to oral glucose. Briefly, subjects were assigned to their respective group (NGT, IGT, or T2DM) based on their 2-hour glucose value during a 75-g oral glucose tolerance test (Table 1). With respect to T2DM, hemoglobin A_{1c} levels were required to be < 9.0% in all subjects. Subjects using insulin to control their diabetes were excluded. Subjects treated with oral anti-diabetic medications were enrolled if the agent(s) could be safely discontinued for 48-hours preceding each study visit. These criteria for T2DM were designed to identify subjects with mild T2DM and to exclude subjects with advanced beta cell failure and to identify participants with residual insulin secretion who have a better potential to respond to incretins.

The ivGGIs were administered to subjects following a 10 hour fast and the results reported in this paper analyze the same cohort of participants studied in a previous report [13]. On each of 4 occasions separated by at least 2 weeks, subjects (blinded to treatment) received an ivGGI along with an intravenous infusion of albumin alone (Alb), GIP alone, Xen alone, or the combination of GIP plus Xen (G+X). Following a 10-min priming dose, each peptide(s) was continuously administered at a dose of 4 pmol/kg/min. The rationale for peptide dosing was based on published studies [11,12,34,35] as previously discussed [13]. Screening, group characteristics, experimental details, and glucose, insulin, C-peptide, glucagon, GIP, Xen,

and insulin secretion rate (ISR) determinations were previously reported [13] and PP levels reported in the present study were measured in plasma samples collected in this earlier study.

2.2 Assays

PP was measured in plasma by an ELISA (Millipore Corp, St. Charles, MO). Intra- and inter-assay variations are 4.3% and 6.2%, respectively. The PPR is defined as the PP level at the indicated time minus basal PP levels and was calculated for each subject at each study visit. Acetonitrile-extracted heparinized plasma was used to measure fasting PP levels. To accomplish this, 150 μ L plasma was vigorously mixed with 225 μ L acetonitrile, incubated at room temperature for 10–30 minutes, and then centrifuged at 17,000 $\times$ g for 5 minutes. The supernatant was transferred to a fresh tube, dried in a speed-vac, resuspended in assay buffer, and then stored at –80 degrees until assayed. Standards prepared in 2X charcoal-stripped heparinized plasma (Bioreclamation LLC, Hicksville, NY) were extracted in parallel with samples. Recovery of PP (%) following extraction of heparinized plasma was 90 ± 2.6 whereas recovery from EDTA plasma was lower and more variable (63 ± 8.8). For comparison of PP levels with other studies, 1 pg/mL = 0.24 pM.

2.3. Statistics

The PPRs were first calculated for each individual during each infusion and then used to calculate group averages. Incremental areas under the curves (iAUCs) were determined by trapezoid rule for PP concentrations above basal level. One-way ANOVA was used to determine if mean values between groups were different. Physiologic data within each group were analyzed using the mixed effects model with subject as a random effect and peptide treatment as a fixed effect. Pairwise comparisons were limited to evaluating effects of 1) Xen versus Alb; 2) GIP versus Alb; and 3) G+X versus Xen. Two-tailed t-tests were used to compare effects of peptides on the PP response.

2.4. Immunochemical Studies

Paraffin-embedded blocks of human pancreas were obtained from our Department of Pathology. Sections were deparaffinized, subjected to antigen retrieval as required (see below), blocked using CAS-Block (Invitrogen Corporation, Frederick, MD) and incubated with primary antibodies overnight at 4 degrees C (see below) as previously described [15,36]. After washing, bound primary antibodies were detected following incubation for 45 minutes at room temperature with the appropriate conjugated secondary antibodies. Nuclei were counterstained with hematoxylin or bis-benzimide as indicated in figure legends. For double-label studies, single tissue sections were incubated with both primary antibodies and multiple single color images merged using Adobe Photoshop. All antibodies were diluted in Da Vinci Green Diluent (Biocare Medical, Concord, CA). The following antibodies including source, catalog number, dilution, and antigen retrieval technique are: goat anti-NTSR1 [Santa Cruz Biotechnology, Inc., Dallas, TX; #SC-7596; 1:100; EDTA or DIVA (Biocare Medical)], rabbit anti-PGP9.5 (Millipore Corp.; #AB1761; Diva, Citrate, or EDTA), mouse anti-glucagon (Sigma Chemical Company, St. Louis, MO; #G2654; 1:50; None or EDTA), mouse anti-tyrosine hydroxylase (Immunostar, Hudson, WI; #22941; 1:500; EDTA), mouse anti-smooth muscle actin (Sigma Chemical; #A5228; 1:200; EDTA or DIVA); mouse anti-CD-39 (PE-CAM; Cell Signaling Technology, #3528; 1:100; EDTA or DIVA) and/or guinea pig anti insulin (Dako, Carpinteria, CA; #A0564; 1:50; DIVA). Minimal cross reacting, horseradish peroxidase-, Alexa Fluor 488-, and Alexa Fluor 549-conjugated donkey anti-mouse, guinea pig, goat, or rabbit antibodies were obtained from Jackson ImmunoResearch (West Grove, PA) and used at a 1:500 dilution.

3. Results

3.1. Subject Characteristics

Groups were well-matched and as anticipated, the 2-hour and fasting plasma glucose and HbA_{1c} levels progressively increased in groups with IGT and T2DM compared to NGT (Table 1). Subjects with T2DM did not have gastroparesis or clinically evident peripheral neuropathies. Five diabetics were taking metformin and 2 were also on a sulfonylurea. No subjects were receiving incretin-based therapies. Three subjects with T2DM received insulin to lower basal glucose levels before 1, 1 and 2 of their four ivGGIs.

3.2. Validation of PP assay

The initial effects of Xen on GIP-mediated insulin release in our previous study occurred during the first 40 minutes making this a critical period to study. Because heparinized, but not EDTA, plasma was prepared at the 10, 20, and 30 minute time points, initial studies were conducted to compare the use of heparinized versus EDTA plasma for the PP ELISA. For each of 25 subjects, PP values were measured in plasma samples prepared before and 40 minutes after infusion with Alb, GIP, Xen, or G+X was initiated. PP levels were higher for each subject in their respective heparinized versus EDTA plasma (not shown). However, linear regression analysis (Fig 1D) showed that the PPR from 0 to 40 minutes of the ivGGIs were highly correlated for each infusion and every subject using unextracted EDTA or heparinized plasma samples. Thus, unextracted heparinized plasma was used to assess changes in, but not absolute levels of, PP. Unextracted plasma from 5 subjects had fasting levels above the upper limit of detection in the assay that did not decrease with plasma dilution. Since this indicates interfering compounds in the plasma of these subjects, their data was excluded from this comparison.

Next, studies were conducted to determine if fasting PP levels could be measured in acetonitrile-extracted plasma. Standards were prepared according to manufacturer's instructions as well as in 2X-charcoal-stripped heparin or EDTA plasma. As shown in Figure 1E, the measured PP levels in the unextracted standards as well as in the extracted heparinized samples, were essentially identical over a wide range of concentrations. However, at low concentrations, PP levels in the extracted EDTA samples were much lower than expected. Thus, acetonitrile-extracted heparinized plasma was used to measure fasting PP levels.

3.3. Fasting PP levels are not different in humans with NGT, IGT, and T2DM

Fasting PP levels were determined in heparinized plasma following extraction with acetonitrile. As shown in figure 1F, fasting PP levels (in pg/mL) were not statistically different ($p=0.56$ by one-way ANOVA) in subjects with NGT (31.8 ± 5.1), IGT (45.0 ± 8.8), and T2DM (46.2 ± 14.9). This result agrees with another study showing that fasting PP levels are not different in humans with and without mild T2DM [37].

3.3. Glucose reduces the pancreatic polypeptide response

As shown in figure 1C, the stepwise increases in glucose infusion rates (in the absence of peptides) caused progressive increases in plasma glucose levels in all 3 groups. However, plasma glucose levels reached increasingly higher levels in the order from NGT to IGT to T2DM. In contrast, the ivGGI itself (i.e. during infusion with Alb) had relatively little effect on PPRs over the entire 240 minute study (Fig 2A, D, and G) in any group. During the first 40 minutes of the ivGGI, the PPRs were essentially unchanged in all 3 groups (Figs 2B, E, and H). Thereafter, there was a small but steady decrease in the PP response in all 3 groups of subjects as plasma glucose levels progressively increased during the entire 240-minute

glucose infusion. However, the mean *decrease* in the PPR (Δ pg/mL per 240-minutes) was progressively greater in the group with NGT ($-16,042 \pm 3362$) versus IGT (-5937 ± 4045) versus T2DM (-1096 ± 1890). These differences were statistically different between all three groups ($p = 0.01$ by one-way ANOVA). A post hoc analysis further indicated that the difference between the means were significant when comparing groups with NGT and T2DM. Thus, there is a small but significant inverse relationship between plasma glucose levels and the PPR. However, when normalized to plasma glucose levels, the group differences in PPR were not significant (Not Shown).

3.4. GIP plus Xen increases the PP response equally well in humans with NGT, IGT, and T2DM

In striking contrast to albumin, administration of G+X caused rapid increases in the PPRs in all 3 groups that remained elevated for 40 minutes (Fig 2A, D, and G). Thereafter, the PPRs decreased but stayed significantly higher than those during infusion with Xen, as shown by the iAUCs from 0–40 minutes (Fig 2B,E, and H) and 0–240 minutes (Fig 2C,F, and I) for all three groups. With infusion of G+X, the respective peak PPRs, iAUCs (0–40 min), and iAUCs (0–240 min) were not statistically different between groups. Infusion with Xen elicited a rapid, but smaller and more transient, increase in the PPRs (Fig 2A, D, and G) which was only significant in subjects with IGT and T2DM for the first 40 minutes (Fig 2E and H). The PPR was not different ($p > 0.05$) with Xen infusion compared to the Alb control from 0–240 minutes for all three groups. Infusion with GIP (Fig 2A, D, and G), caused a comparatively small and somewhat delayed increase in the PPRs in all 3 groups. There was no significant difference in PPR from 0–40 minutes with GIP as determined by iAUC for all three groups (Figure 2B,E and H). Only subjects with NGT had a small, significant increase in PPR from 0–240 minutes with GIP infusion (Fig 2C,F, and I). Within each group, the PPR to G+X was significantly greater than that to Xen during both the early period and throughout the entire infusion. Thus, infusion with G+X, but not glucose, increases the PPR equally well in humans with NGT, IGT, and T2DM.

3.5. Receptors for xenin-25 are detectable on neurons, but not on islet endocrine cells

To determine if Xen could act directly on islet endocrine cells, paraffin-embedded sections of human pancreas were stained with antibodies to NTSR1. NTSR1 immunoreactivity was detected at high levels on axons in the interlobular space (Fig 3A) and lower levels on the basal membrane of ductal epithelial cells (Fig 3B). In contrast, NTSR1 was not detectable on cells within the islet (Fig 3C). Double-label immunofluorescence studies were conducted to further define the expression pattern of NTSR1. PGP9.5 is a marker for enteric and other neurons and in humans, is also expressed by islet beta cells [38,39]. As shown in figure 4, NTSR1 was expressed on a subset of PGP9.5-positive pancreatic neurons. As expected, islets were intensely stained with antibodies to insulin (Fig 5B) and PGP9.5 (Fig 5C). Insulin negative, PGP9.5-positive nerve fibers can also be seen approaching and surrounding the islet. Importantly, some of these fibers appear to be contacting double-labeled islet beta cells (Fig 5D and E). Moreover, NTSR1 is expressed on a nerve fiber running adjacent to an islet but is not detectable on nearby glucagon-positive or other surrounding islet endocrine cells (Fig 6). NTSR1 was not co-expressed with tyrosine hydroxylase (Fig 7), smooth muscle actin (not shown), or PE-CAM (not shown) which are respective markers of sympathetic neurons, blood vessels, and endothelial cells.

4. Discussion

We previously showed that within the first 40 minutes of ivGGIs, infusion with GIP, but not Xen, rapidly and transiently increased insulin secretion in humans with NGT, IGT, and T2DM [13] which is in striking contrast to the dogma that the effects of GIP on insulin

secretion are blunted in T2DM. This initial GIP response occurred with little change in plasma glucose levels and was further increased by co-infusion with Xen in subjects with NGT and IGT, but not T2DM. Thus, beta cell sensitivity to a Xen-initiated signal was greatest in subjects with IGT and severely blunted in those with T2DM. These observations suggest that increased beta cell sensitivity to Xen defines an adaptive response in humans with IGT to maintain insulin secretion in the face of worsening beta cell function and loss of this adaptive response is associated with development of T2DM. To define this defect in humans with T2DM, it is critical to understand how Xen regulates islet physiology.

We previously showed in mice that Xen indirectly increases the effects of GIP on insulin release via a cholinergic relay in the periphery [13]. In the current study, we have used the PPR as an indirect measure of cholinergic input to islets. Fasting levels of PP are known to vary by both age and gender [40] and are also likely to be different when measured using different assays (e.g. ELISA versus RIA) and/or extraction techniques. In spite of this, fasting PP levels measured in our samples are similar to those reported by others [28,41–43]. Moreover, by employing a host of validation techniques, the PP responses for our subjects can be compared even if absolute values are slightly different from those obtained in other studies. In the absence of peptide administration, changes in PP levels were inversely related to plasma glucose levels in all 3 groups. However, these negative responses were relatively small in all 3 groups when compared to positive PPRs measured during peptide infusions. Importantly, we now show that co-infusion of Xen and GIP rapidly and profoundly increases the PPR equally well in humans with NGT, IGT, and T2DM in the absence of significant changes in plasma glucose levels (i.e. the first 40 minutes of the ivGGI). Thus, this early response is glucose independent. We are currently performing ivGGIs in humans to determine the effects of atropine sulfate, an inhibitor of muscarinic acetylcholine receptors, on the PPR (ClinicalTrials.gov Identifier NCT01951729). Preliminary findings with 2 subjects with IGT indicate that atropine completely blocks the PPR to GIP plus Xen. Thus, the PPR is a valid, albeit indirect, measure of cholinergic input to islets in humans under the experimental conditions used for this study. Importantly, the PPR to GIP plus Xen is neither increased in subjects with IGT nor impaired in T2DM. Thus, changes in Xen-mediated cholinergic input to islets cannot account for the increased or blunted insulin secretory responses to Xen in IGT or T2DM, respectively. It is interesting to speculate that beta cells acquire resistance to cholinergic input as humans progress from IGT to T2DM. It should also be noted that the current study was conducted with a single combinatorial dose of GIP and xenin-25 and it is unknown if these are optimal doses of each peptide. Thus, it is possible that effects of GIP plus xenin-25 could be even larger in response to optimal doses of peptides.

Others have reported that neurotensin receptor expression is very high in ductal pancreatic adenocarcinoma but very low or not detectable in normal human pancreas [44–46]. Immunohistochemical studies in this paper showed that NTSR1 was not detectable on any islet endocrine cells in the human pancreas. In contrast, NTSR1 was detected at very high levels on PGP9.5-positive axons and nerve fibers in the pancreas. Importantly, PGP9.5 nerve endings making contact with beta cells were also noted. Although we cannot exclude the possibility that low, but not detectable, levels of NTSR1 are sufficient to activate islet endocrine cells, it seems unlikely that Xen increases GIP-mediated insulin release by acting directly on beta cells or by activating a cholinergic relay initiated by alpha cells. Our antibodies against markers of cholinergic neurons did not work in the paraffin-embedded sections of pancreas. Thus, it is unknown if the PGP9.5 positive nerve fibers are also cholinergic. However, our preliminary studies show that atropine completely blocks the PPR to infusion of GIP plus Xen. In spite of this, it is important to note that our studies do not exclude the possibility that neurotransmitters or peptides other than acetylcholine mediate the effects of Xen on insulin and glucagon, but not pancreatic polypeptide, release.

We previously showed that NTSR1 is also expressed on cholinergic and non-cholinergic myenteric neurons in the human proximal small intestine [15] and that Xen can increase intracellular cytosolic free calcium levels in a subset of duodenal myenteric neurons [15]. A subset of enteric neurons connecting the stomach and duodenum to the pancreas function independently from the CNS and can modify islet function [18,19,47]. Our mouse studies suggest that these neurons may relay the Xen signal to the islets [17]. Results in this, as well as our previous paper, are consistent with the mouse studies and indicate that in humans: 1) Xen increases cholinergic input to islets; 2) Xen does not act directly on beta cells to increase the effects of GIP on insulin release; and 3) a neural relay utilizing non-sympathetic neurons may initiate Xen signaling to islet endocrine cells. Extensive islet innervation would not be required to propagate the Xen-initiated signal because islet endocrine cells are electrically coupled. Although Xen increases cholinergic input to islets in humans, additional studies will be required to determine if as in mice, increased cholinergic signaling augments the effects of Xen on GIP-mediated insulin release. However, results from our and other laboratories suggest that altered cholinergic signaling plays an important role in regulating insulin release. Consistent with our results showing that the effects of Xen on GIP-mediated ISRs are increased in subjects with IGT compared to NGT [13], induction of mild hyperglycemia for 48-hours in humans with NGT caused a compensatory increase in C-peptide secretion which was attenuated by atropine [48]. Thus, increased cholinergic input may be an adaptive response in humans with IGT (i.e. mild hyperglycemia) to augment the effects of GIP on ISR. Consistent with our earlier study showing that the effects of Xen on insulin release are blunted in those with T2DM [13], many antipsychotic drugs associated with increased T2DM exert off-target antagonism to M3 muscarinic acetylcholine receptors [49], the same receptors that mediate the effects of acetylcholine on insulin release [50]. Thus, altered beta cell sensitivity to acetylcholine rather than GIP may play a critical role in the progression from NGT to IGT to T2DM.

Glucagon also plays a central role in regulating insulin secretion and blood glucose levels and the glucagon response is dysregulated in humans with T2DM [51]. Infusion of GIP transiently increased plasma glucagon levels in healthy humans during euglycemia [52] but not hyperglycemia [53]. In our earlier study, GIP also transiently increased plasma glucagon levels during the first 40 minutes of the ivGGI (before plasma glucose levels increased) in humans with NGT, IGT, and T2DM [13]. Like ISRs, the glucagon response increased further during infusions with GIP plus Xen in subjects with IGT and this response was then blunted in subjects with T2DM [13]. M3 muscarinic acetylcholine receptors also regulate glucagon release [50]. Thus, alpha cells in humans with IGT may also exhibit enhanced sensitivity to acetylcholine which is then blunted in T2DM.

4.1. Conclusions

This study was not designed to determine if Xen or myenteric neurons actually regulate cholinergic input to islets under physiologic conditions. Rather, Xen was used as a pharmacologic agent to study islet physiology *in vivo*. Our data clearly show that GIP plus Xen increases cholinergic input to islets equally well in humans with NGT, IGT, and T2DM. Moreover, antibody staining demonstrated that NTSR1, which mediates the effects of Xen, is expressed on pancreatic neurons, but not islet endocrine cells. Thus, Xen signaling to islets is indirect and may be mediated by a neuronal relay as previously demonstrated in mice [17]. However, it remains to be seen whether this cholinergic input to islets actually mediates the effects of Xen on insulin and glucagon release. As shown in this paper, sympathetic neurons are unlikely to relay the effects of xenin-25 on islet function in humans. We are currently conducting a series of clinical studies to determine if atropine sulfate (NCT01951729) and/or truncal vagotomy (NCT01951716) inhibit the effects of xenin-25 on GIP-mediated PP, insulin and glucagon release in humans with IGT. Collective results from

these studies will define the role(s) of cholinergic versus non-cholinergic relays and parasympathetic versus non-parasympathetic neurons for mediating the effects of xenin-25 on PP, insulin, and glucagon release. If neither parasympathetic nor sympathetic neurons mediate the effects of xenin-25 on specific aspects of islet function, this would suggest that enteric neurons may play an underappreciated yet important role in regulating islet function in humans. However, it seems likely that in humans with IGT, islet alpha and beta cells may develop hypersensitivity to cholinergic input as an adaptive response to worsening glucose homeostasis, and in T2DM alpha and beta cells may no longer respond to increased cholinergic input. Importantly, altered responses to cholinergic rather than GIP input may play an important role in the progression from NGT to IGT to T2DM. Once we understand the physiologic mechanism for xenin-25 action in vivo, complementary in vitro studies can be used to further define specific signaling pathways in islets that mediate these effects and thus, are defective in humans with T2DM.

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Abbreviations

Alb	albumin alone
GLP-1	glucagon-like peptide-1
GIP	glucose-dependent insulintropic polypeptide
G+X	GIP plus Xen
IGT	impaired glucose tolerance
ISR	insulin secretion rate
ivGGIs	intravenous graded glucose infusions
NTSR1	neurotensin receptor 1
NGT	normal glucose tolerance
PP	pancreatic polypeptide
PPR	pancreatic polypeptide response
T2DM	type 2 diabetes mellitus
Xen	xenin-25

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Highlights

- The pancreatic polypeptide response was used to assess cholinergic input to islets
- Xen plus GIP increased cholinergic input to islets in humans with/without T2DM
- Antibody staining showed Xen receptors are present on pancreatic neurons
- Xen receptors were not detected on islet endocrine cells
- Effects of Xen may be mediated by a neural relay in humans

Figure 1. Fasting PP levels are similar in humans with NGT, IGT, and T2DM

Panel A. Amino acid sequences for neurotensin-related peptides are shown. Note that the amino termini of neurotensin and xenopsin (<Q) contain a cyclic modification of glutamine. **Panel B.** Following a 10-hour overnight fast, subjects were administered ivGGIs at the indicated glucose and peptide infusion rates. **Panel C.** Plasma glucose levels were measured at the indicated times during the ivGGIs without peptide administration. Group mean $\pm$ SEM for each time point is shown for subjects with NGT (green circles), IGT (yellow squares), and T2DM (red triangles). Data are taken from ref 13. **Panel D.** Changes in PP levels from 0 to 40 minutes of the ivGGI were measured in EDTA and heparinized plasma during infusion with Alb, GIP, Xen, or the combination of G+X. Values measured in the heparin versus EDTA samples from 25 subjects are shown. **Panel E.** PP standards were prepared in buffer supplied by the manufacturer (black circles) or charcoal-stripped heparinized (red squares) or EDTA (green triangles) plasma. Only the plasma samples were extracted with acetonitrile before PP measurements. Measured values are plotted versus the theoretical concentration in the diluted standards. **Panel F.** Fasting PP levels were measured in heparinized plasma samples extracted with acetonitrile. Average PP levels $\pm$ SEM are shown for each group.

Figure 2. The combination of GIP plus Xen similarly increases the PP response in humans with NGT, IGT, and T2DM

ivGGIs were administered as outlined in Fig 1A during infusion of Alb(yellow triangles), GIP (red squares), Xen (green inverted triangles), or G+X (blue circles). The PPR for the NGT, IGT, and T2DM groups at the indicated times are shown in panels A, D, and G. iAUCs for the PPR were calculated for each infusion and for each individual. Group average iAUC $\pm$ SEM over the first 40 minutes (panels B, E, and H) and the entire 240 minute ivGGI (panels C, F, and I) are shown. Note that unextracted plasma from some subjects contained compounds that interfered with PP measurements and their data was not included.

Figure 3. NTSR1 is not detectable on islet endocrine cells

Panels A–C: A single paraffin-embedded section of normal human pancreas was incubated with antibodies to NTSR1. To avoid potential artifacts due to autofluorescence of endocrine cells, bound primary antibodies were visualized using horseradish peroxidase conjugated secondary antibodies followed by incubation with diaminobenzidine. Nuclei were counterstained with hematoxylin. An intensely labeled axon (Panel A), a low expressing duct (Panel B), and an islet with undetectable levels of NTSR1 (Panel C) are shown.

Figure 4. NSTR1 is expressed on neurons in the pancreas

Panel A–D: A single paraffin-embedded section of normal human pancreas was incubated with antibodies to NTSR1 plus antibodies to PGP9.5. Nuclei were counterstained blue using bis-benzimide (Panel A). Bound primary antibodies were visualized using ALEXA 549 (Panel B; red) and ALEXA 488 (Panel C; green) secondary antibodies. Panel D is a merged image of Panels A–C. The open and solid arrows indicate PGP9.5 labeled axons that do and do not co-express NTSR1, respectively.

Figure 5. Neurons contact human islets

Panels A–E: A single paraffin-embedded section of normal human pancreas was stained with antibodies to PGP9.5 plus antibodies to insulin as described in Fig 4. Panels A, B, and C show staining only for nuclei (blue), insulin (green), and PGP9.5 (red), respectively. Panel D is a merged image of Panels A–C. Panel E is enlarged from the boxed region in panel D. Open arrows point to representative PGP9.5 positive nerve fibers. The closed arrow points to a nerve fiber contacting a double-labeled beta cell.

Figure 6. NTSR1 is not detectable on alpha cells

A single paraffin-embedded section of normal human pancreas was stained with antibodies to insulin plus antibodies to PGP9.5 as described in Fig 4. Panels A, B, and C show staining only for nuclei (blue), insulin (green), and PGP9.5 (red), respectively. Panel D is a merged image of Panels A–C. Panel E is enlarged from the boxed region in panel D. The open and closed arrows point to representative axons and alpha cells, respectively.

Figure 7. NTSR1 is not detectable on sympathetic neurons

A single paraffin-embedded section of normal human pancreas was stained with antibodies to tyrosine hydroxylase (TH) plus antibodies to NTSR1 as described in Fig 4. Panels A, B, and C show staining only for nuclei (blue), tyrosine hydroxylase (green), and NTSR1 (red), respectively. Panel D is a merged image of Panels A–C.

Table 1³. Group Characteristics.

	NGT (n=10)	IGT (n=10)	T2DM (n=9)
<i>2 hour Glucose (mg/dL)</i> ¹	117 ± 6.1 (67 – 132)	175 ± 4.9 (141 – 193)	255 ± 12.7 (195 – 303)
<i>Fasting Glucose (mg/dL)</i> ²	93 ± 1.3 (88 – 102)	103 ± 2.2 (91 – 113)	122 ± 6.3 (97 – 163)
<i>HbA_{1c} (%)</i> ²	5.5 ± 0.1 (4.9 – 5.9)	5.9 ± 0.15 (5.1 – 6.7)	6.6 ± 0.14 (6.1 – 7.2)
<i>Sex (Males/Females)</i>	6/4	6/4	3/6
<i>Age (yrs)</i>	48.8 ± 3.1 (29 – 62)	55.9 ± 1.7 (46 – 63)	55.2 ± 3.0 (37 – 64)
<i>BMI (kg/m²)</i>	30.9 ± 2.2 (21 – 42)	29.2 ± 1.4 (24 – 37)	32.8 ± 1.3 (28 – 41)

Values represent the mean ± SEM. The group means for the 2-hour glucose, fasting glucose, and HbA_{1c} were statistically different.

¹ Indicates *p* values for all pair wise comparisons <0.001.

² Indicates *p* value for NGT versus IGT is >0.05 and *p* <0.01 for the other pair wise comparisons.

³ Table is taken from ref 13 and is copyrighted by the American Diabetes Association (2012).