GHTD-amide: A naturally occurring beta cell-derived peptide with hypoglycemic activity

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1. Introduction

Regulation of blood glucose primarily revolves around control of insulin secretion and action. In addition to insulin, beta cells produce a variety of bioactive peptides including amylin, pancreastatin, preptin, and kisspeptins. Amylin contributes to metabolic regulation through its actions on insulin secretion and insulin sensitivity [6,13], and feedback signals to the brain that reduce food intake [25]. Pancreastatin, the product of a proteolytic cleavage of chromogranin-A unique to beta cells [26,29], and

kisspeptins [21], both reduce glucose stimulated insulin release [11,21], whereas preptin, a cleavage product of proinsulin-like growth factor II, appears to amplify glucose stimulated insulin secretion [4]. Pancreatic peptides that originate from other cells types but also influence insulin secretion include Ghrelin, which is predominantly expressed in the gut mucosa but also found in islet alpha cells [8] and galanin, which is expressed in nerve endings surrounding the islets [28]. Both of these peptides inhibit insulin secretion [10,28,1,27]. Comprehensive knowledge of all factors controlling insulin secretion and action is required to complete our understanding of glucose homeostasis and its disturbance in disease states.

In the early 1970s a peptide containing preparation with insulin potentiating activity was isolated from human urine [19,32] but the active constituent remained chemically unidentified. Based on chromatographic properties and activity the urinary peptide was originally thought to be a fragment of growth hormone called acceleratory fragment of growth hormone or AcG that derived from pituitary extracts [19,20]. Improvements in liquid chromatography and mass spectrometry have now allowed the purification to near homogeneity of this peptide from the original semi-pure prepara-

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Abbreviations: BLAST, basic local alignment and search tool; ES, electrospray; FBG, fasting blood glucose; GHTD-amide, alpha amidated tetrapeptide with the sequence Gly-His-Thr-Asp; MALDI, matrix assisted laser desorption ionization; PDA, photodiode array; PMF, peptide mass fingerprint; TFA, trifluoroacetic acid; TOF, time of flight.

tion. Here we show that the urinary peptide is unrelated to growth hormone and has the sequence Gly-His-Thr-Asp-amide (GHTD-amide) that synthetic GHTD-amide promotes storage of glucose as glycogen and we provide evidence that endogenous GHTD-amide is produced by pancreatic beta cells.

2. Methods

2.1. Peptide purification and identification

Partial purification of peptide from urine was performed as described previously [20]. The lyophilized material was dissolved in 0.1% trifluoroacetic acid (TFA) and purified to near homogeneity by reverse phase HPLC chromatography on C18 and C3 columns. First, partially purified urinary peptide was eluted isocratically for 5 min followed by a linear gradient of 50% acetonitrile on a Vydac C18 column. Bioactive fractions were identified by measuring the incorporation of ^{14}C -glucose into glycogen in rat hemidiaphragm. Active fractions were pooled and further purified using a Vydac C3 column by isocratic elution in 5% acetonitrile, 1% TFA. This step was repeated a further 2 times, with bioactive fractions pooled, lyophilized and redissolved in 0.1% TFA after each step and then reappplied to the column. N-terminal sequence analysis was performed on an ASI 477 "pulse liquid" protein sequencer with an on-line ABI 120 A analyzer (Applied Biosystems, Inc. Model 475 A). The ES-MS and MS/MS measurements were performed on a micromass platform instrument.

2.2. Animal experiments

GHTD-amide was synthesized by standard solid phase Fmoc chemistry (Mimotopes, Melbourne, Australia). Male Wistar rats were from the Monash Animal Services facility and all procedures conformed to the NIH principles of laboratory animal care and were approved by the Monash University Animal Ethics Committee. Rats were given free access to food and water prior to overnight fasting. Male Wistar rats 6–8 weeks of age were fasted then injected intravenously in the tail vein with 60 mg/kg streptozotocin (stz). Rats with fasting blood glucose (FBG) concentrations greater than 11 mM 5 days after stz injection were included in the next stage of the experiment. Insulin deficiency was confirmed by RIA measurement of rat serum insulin (Linco, Millipore Biosciences, Australia).

Insulin-deficient rats were treated with GHTD-amide and/or insulin in a cross-over study. Rats ($n = 7$) were randomized into 2 treatment groups and received by intraperitoneal injection either 1 Unit/kg insulin (bovine insulin, Sigma) followed 2 days later by 1 U/kg insulin plus 4.27 mg/kg GHTD-amide or vice versa. The order of treatment had no effect on the experimental outcomes and the FBGs prior to each treatment were similar (16.5 ± 1.7 mM versus 17.3 ± 1.4 mM, mean $\pm$ SEM, $n = 7$). After injection, blood glucose was measured at regular time intervals over 2 h.

2.3. Glycogen synthesis

Glycogen synthesis was measured in HepG2 human hepatoma cells and C2C12 rat myotubes. Cells were maintained at 37 °C, 5% CO_2 in Dulbecco's modified Eagle medium (Gibco BRL) and 10% fetal bovine serum. C2C12 myoblasts were differentiated post confluence to myotubes by culturing in 5% horse serum for 3–4 days. Cells were serum starved overnight in media containing 5.5 mM glucose. The media was aspirated and replaced with fresh media supplemented with 0.4 $\mu\text{Ci/ml}$ D-[U- ^{14}C] glucose and containing insulin or GHTD-amide at the required concentrations. After incubation at 37 °C for 2 h the medium was removed and the cells were washed with ice-cold phosphate-buffered saline and

harvested. Glycogen was extracted according to the method of Huang et al. [14]. The radioactivity incorporated into glycogen was then measured by scintillation counting. Activity was corrected for protein content and the results expressed as the percentage of activity compared to the basal rate of incorporation.

2.4. Peptide detection in MIN6 cell culture

MIN6 cells were cultured at 37 °C, 5% CO_2 in DMEM with 10% FCS. For analysis of peptide secretion, a near confluent layer of cells was washed then incubated in serum free media for 24 h.

GHTD-amide in cell culture media was detected by reverse phase-HPLC chromatography with a Photo Diode Array (PDA) equipped Shimadzu 10A-VP system. Samples were centrifuged at $13,000 \times g$ for 5 min before loading onto Phenomenex Luna(2), 10.0 or 4.6 mm i.d. $\times$ 250 mm C18 columns that had been equilibrated with 10% buffer B (90% acetonitrile, 0.1%, v/v, H_3PO_4 , 2.5 mM octane sulfonic acid), at 2.5 (10.0 mm i.d.) or 1.0 (4.6 mm i.d.) ml/min. After sample injection buffer B was increased over a linear gradient of buffer A (milliQ water with 0.1%, v/v, H_3PO_4 and 2.5 mM octane sulfonic acid) to 100% over 25 min. The retention times of synthetic GHTD-amide were 16.89 (10.0 mm i.d.) or 16.29 (4.6 mm i.d.) minutes and a standard curve using known amounts of GHTD-amide was established for quantification of unknown amounts of peptide. PDA spectra and Matrix Assisted Laser Desorption Time Of Flight Mass Spectrometry (MALDI-TOF) were used to verify the molecular weight and peptide composition of the putative GHTD-amide in samples. MALDI-TOF and MALDI-peptide mass fingerprint (PMF) spectra were acquired using an Applied Biosystems 4700 MALDI-TOF/TOF mass spectrometer; data were analyzed using GPS explorer software.

2.5. Antibodies, radioimmunoassay and immunofluorescence

GHTD-amide conjugated to diphtheria toxoid via an amino terminal cysteine residue was used to immunize rabbits and mice (Institute of Medical and Veterinary Science, Adelaide, Australia). Serum was collected after primary inoculation and 3 booster injections.

An analogue of GHTD-amide containing a tyrosine (Y) at the amino terminus (YGHTD-amide) was labeled with Sodium ^{125}I iodide (Amersham) by chloramine-T oxidation. Labeled peptide was separated from unbound label using Sep-Pak C18 cartridges. Between 30 and 50,000 cpm of ^{125}I -YGHTD-amide per tube was mixed with rabbit anti-sera raised to GHTD-amide, the sample containing competing peptide and a buffer containing fish skin gelatin. After equilibration overnight at 4 °C the immune complexes were precipitated with a precipitating system for immune complexes (Linco, Millipore Biosciences, Australia) and centrifugation and the amount of ^{125}I in the precipitate was measured. Peptide analogues for competition were synthesized by standard methods (GL Biochem, Shanghai, China).

Indirect immunofluorescence was performed on formalin fixed, paraffin embedded tissue sections. Mouse tissue sections were cut to a thickness of 5 μm and mounted on a positively charged glass slides. A panel of human endocrine tissues was purchased from Abcam Ltd., Cambridge, UK. Tissues included were adrenal, maxillary gland, thyroid (fetal), brain (pituitary gland), parathyroid and pancreas. Antibodies were guinea pig anti-insulin (Dako), anti-GHTD-amide, rabbit anti-guinea pig FITC conjugate (Dako) and Alexafluor 568 conjugated anti-rabbit (Molecular Probes).

For immunogold electron microscopy rat pancreas was fixed in cacodylate-buffered 4% paraformaldehyde/0.5% glutaraldehyde and embedded in LR-white. Ultra-thin sections on nickel formvar coated grids were blocked with 0.1% fish skin gelatin for 1 h. Primary antibodies were 1:200 dilutions of preimmune or immune anti-

GHTD-amide rabbit sera and secondary antibody was 1:80 dilution of goat anti-rabbit conjugated to 10 nm gold (Aurion, Costerwegs, The Netherlands). Sections were stained with lead citrate before visualization by transmission electron microscopy. Gold particles decorating the dense core and halo of secretory granules were counted on 5 randomly selected beta cells from preimmune and immune serum incubations of each of 2 rat pancreases.

2.6. Statistics

Statistical analysis of data used GraphPad Prism software. Student's *t*-test was used for between group comparisons and group sizes and *p* values are as described in the figure legends. Animal data were baseline corrected and analyzed by Student's *t*-test.

3. Results

3.1. Purification and sequencing of the urinary peptide

The active constituent was purified from semi-purified urine extracts using a four-step reverse-phase HPLC procedure employing Vydac C18 and C3 columns with bioactivity confirmed at each step by assay of stimulation of glycogen synthesis in rat hemidiaphragm. ES mass spectrometry identified a single charged molecular ion species ($M+H^+$) with a *m/z* ratio of 429 Da. Amino acid sequencing and ES-MS/MS fragmentation identified an alpha-amidated tetrapeptide Gly-His-Thr-Asp.

3.2. Effects of GHTD-amide on glycogen synthesis

The tetrapeptide GHTD-amide was synthesized and tested for stimulation of glycogen synthesis in HepG2 cells and C2C12 myotubes. In HepG2 cells the peptide significantly increased α -[U- ^{14} C]-glucose incorporation into glycogen at concentrations ranging from 1 pM to 1 nM to levels similar to that induced by 10 nM insulin (Fig. 1A). At a concentration of 100 pM GHTD-amide was more potent than 1 nM insulin ($p < 0.05$). Evidence of high dose inhibition was apparent with 10 nM GHTD-amide showing significantly reduced activity compared to the optimally active concentration of 100 pM GHTD-amide ($p = 0.05$).

In C2C12 myotubes GHTD-amide stimulated glycogen synthesis but to a lesser degree and with lower potency than in HepG2 cells. α -[U- ^{14} C]-glucose incorporation into glycogen was significantly greater than basal at concentrations of 1 and 10 nM ($p < 0.05$) but not at 0.1 and 100 nM ($p > 0.2$) (Fig. 1B). Insulin (10 nM) increased glycogen synthesis by 80% and 117% in C2C12 and HepG2 cells respectively whereas GHTD-amide caused increases of 40% and 260% in the respective cell models, further reinforcing the greater potency of the peptide in HepG2 cells. There was no additive effect of GHTD-amide and insulin in C2C12 cells with the combined treatments not significantly different to GHTD-amide alone (Fig. 1B). Addition of GHTD-amide to 10 nM insulin caused an apparent decrease in glycogen synthesis compared to 10 nM insulin alone but this did not reach statistical significance ($p = 0.4-0.5$).

3.3. Hypoglycemic activity of GHTD-amide in insulin-deficient rats

Hypoglycemic activity in combination with insulin was tested in insulin-deficient Wistar rats (Fig. 2). This model was chosen to minimize complications due to endogenous insulin secretion. Injection of the rats with streptozotocin successfully ablated insulin production by the pancreatic beta cells as shown by a substantial decrease in serum insulin from (mean $\pm$ SEM) 2.2 ± 0.6 ng/ml prior to stz treatment to 0.28 ± 0.08 after treatment. Fasting blood glucose prior to insulin tolerance tests were 16.5 ± 1.7 and 17.3 ± 1.4 mM for insulin and insulin plus GHTD-amide

Fig. 1. Synthetic GHTD-amide promotes glycogen synthesis and enhances insulin-mediated glucose lowering activity. (A) Glycogen synthesis in HepG2 cells incubated with 1 or 10 nM insulin (white bars), 1 pM to 10 nM GHTD-amide (grey bars) or control (black bar). Both insulin and GHTD-amide significantly increased α -[U- ^{14} C] glucose incorporation into glycogen compared to basal ($p < 0.05$ for each treatment compared to basal, Student's *t*-test), $n = 9$ for each treatment and standard errors are shown by bars. (B) Glycogen synthesis in C2C12 myotubes incubated with 10 nM insulin (white bars), 0.1–100 nM GHTD-amide (grey bars), 10 nM insulin with 0.1–100 nM GHTD-amide (light grey bars) or control (black bar). Both insulin and GHTD-amide at 1 and 10 nM significantly increased α -[U- ^{14} C] glucose incorporation into glycogen compared to basal ($p < 0.05$ for each treatment compared to basal, Student's *t*-test), $n = 9$ for each treatment and standard errors are shown by bars.

respectively. Injection of the rats with 1 U/kg insulin caused a significant reduction in blood glucose with the largest average decrease of 8.3 mM occurring after 90 min (Fig. 2). When GHTD-amide was combined with the insulin injection blood glucose decreased by 11.1 mM after 90 min and was lower than for the insulin treatment throughout the time-course.

3.4. Immunochemical localization of GHTD-amide peptide

A rabbit anti-serum was raised to the amidated carboxyl-terminus of synthetic GHTD-amide conjugated to diphtheria

Fig. 2. Reductions in blood glucose after injection of insulin or insulin and GHTD-amide. Insulin-deficient male Wistar rats ($n = 7$) were injected with insulin at 1 U/kg (solid line) or insulin with 4.27 mg/kg GHTD-amide in a cross-over study and blood glucose measured at regular intervals for 2 h. The difference in blood glucose between treatments was significant at 90 and 120 min ($p < 0.05$, Student's *t*-test).

Fig. 3. The rabbit anti-serum raised to GHTD-amide recognizes the HTD-amide sequence. Radio-immunoprecipitation of ^{125}I -YGHTD was performed using a rabbit anti-serum raised to diphtheria toxoid-conjugated GSGSGHTD-amide. Competition with various peptides was performed at the concentrations shown (in microgram per ml). All competing peptides are alpha-amidated. The His, Thr and amidated Asp all contribute to the epitope.

toxoid. The specificity of the rabbit anti-serum was tested using an iodinated variant of the peptide, ^{125}I -YGHTD-amide, and competition for binding to the anti-GHTD-amide serum by tetrapeptide sequence variants of GHTD-amide (Fig. 3). All peptides containing HTD-amide effectively competed with ^{125}I -YGHTD-amide for binding to the anti-serum regardless of the amino acid in the first position. Substitution of His with Val, Asp with Asn or Thr with Ala each eliminated or significantly reduced competition, indicating that all three residues are required to form the epitope (Fig. 2). These results identify the epitope recognized by anti-GHTD-amide as HTD.

A range of tissues were screened by indirect immunofluorescence using anti-GHTD-amide including pancreas, liver, muscle, kidney and adipose tissue from mouse and the endocrine glands, adrenal, maxillary, thyroid (fetal), brain (pituitary gland), parathyroid and pancreas from human. Indirect immunofluorescence identified staining of pancreatic islets of both mouse and human (Fig. 4B and D). All other tissues showed no staining. In both mouse and human the rabbit anti-serum co-localized with an antibody to insulin (Fig. 4C, E and F), which identifies the GHTD-amide positive cells as pancreatic beta cells. There was no staining of islets apparent with pre-immune serum (not shown). To further confirm beta cell localization, mouse monoclonal antibodies were raised to

Fig. 4. Antibodies to GHTD-amide decorate pancreatic islets of mouse and human and co-localize with insulin. A mouse monoclonal antibody and rabbit anti-sera were raised to GHTD-amide conjugated to diphtheria toxoid. Tissue sections were stained with the GHTD-amide antibody and/or an anti-insulin antibody raised in guinea pig (Dako) and anti-rabbit or anti-mouse IgG conjugated to Alex568 (Molecular probes) and/or anti-guinea pig IgG conjugated to FITC (Dako). (A) Mouse pancreas stained with a monoclonal antibody that binds to GHTD-amide. (B and C) A mouse pancreatic islet stained with rabbit anti-GHTD-amide (B) and anti-insulin (C). (D–F) Human pancreas showing an islet with a central ductule (Dako) stained with anti-GHTD-amide (D), anti-insulin (E) and an overlay of D and E. (G–I) A single MIN6 mouse islet beta cell stained with anti-GHTD-amide (G), anti-insulin (H) and an overlay (I) showing co-localization in cytoplasmic vesicles. Scale bars for (C) and (E) are 50 µm and for (H) 25 µm.

Fig. 5. (A) Electron micrograph showing decoration of secretory granules by 10 nm gold particles marking binding by the anti-GHTD-amide rabbit anti-serum and (B) sparser numbers of gold particles on a section stained with the pre-immune serum. Magnification 25,000 $\times$. Scale bar 0.6 μ m. (C) The number of 10 nm gold particles decorating the dense core or halo of secretory granules from 10 randomly selected 12.8 μ m² images. Gold particles that could not be attributed to secretory granules were designated as "other".

the GHTD-amide diphtheria toxoid conjugate. Surprisingly all the monoclonals raised were of the IgM isotype. Supernatant from the best producing clone was used to stain mouse pancreas by indirect immunofluorescence and once again staining was restricted to pancreatic islets (Fig. 4A). The subcellular localization of anti-GHTD-amide immunoreactivity in pancreatic beta cells was explored further using the mouse islet beta cell line MIN6. Staining of MIN6 cells with antibodies to insulin and the anti-GHTD-amide anti-serum and confocal imaging revealed co-localization of anti-GHTD-amide and insulin binding in the cytoplasm of MIN6 cells (Fig. 4G-I). The overlay shows that while the relative staining intensity with insulin (green) and anti-GHTD-amide (red) varies the majority of vesicles in the cytoplasm stained with both antibodies (Fig. 4I).

Immunogold staining for GHTD-amide and electron microscopy was performed on rat pancreas to further define the site of GHTD-amide immune reactivity within beta cells. For sections stained with anti-GHTD-amide, there was a significant increase in the density of 10 nm gold particles decorating dense core secretory granules compared to pre-immune controls (Fig. 5) with an average of 61% of granules decorated compared to 33% for the preimmune serum (Fig. 5A and B). Gold particles were distributed throughout the cytoplasm with clusters apparent in the dense core of some secretory granules (Fig. 4A arrowed), whereas preimmune serum showed a lower density and more even distribution of gold particles (Fig. 5B). Counting of gold particles across 10 randomly selected 12.8 μ m² images showed that 50% of gold particles were associated with secretory granules divided into 35% associated with the dense core and 15% with the halo (Fig. 5C).

3.5. GHTD-amide in cell culture media conditioned by MIN6 beta cells

Detection of antigenic structures reactive with the GHTD-amide anti-serum in secretory granules of pancreatic beta cells suggested an islet beta cell origin for urinary GHTD-amide. This was tested

using the mouse beta cell line MIN6. Serum free media was conditioned by confluent MIN6 cells for 24 h. Media was collected and concentrated and analyzed for GHTD-amide by HPLC and mass spectrometry. A peptide species that absorbed at 214 nm with a retention time on C18 reverse phase HPLC (16.2 min) equivalent to that of synthetic GHTD-amide was identified in conditioned media but was absent in unconditioned media (Fig. 6). MALDI-TOF mass spectrometry on the 16–17 min fraction revealed a peptide species with average molecular weight of 429 Da and ms/ms produced fragment sizes consistent with GHTD-amide as assigned by the ion

Fig. 6. GHTD-amide is secreted by MIN6 cells. Serum free media conditioned for 24 h by a confluent layer of MIN6 cells was separated by reverse phase HPLC. Absorption at 214 nm for the conditioned media (solid line) and unconditioned serum free media (dashed line) identified the appearance of several peaks in conditioned media, including a species with a retention time equivalent to synthetic GHTD-amide (16.2 min, arrowed). This species was identified as GHTD-amide by MALDI-TOF mass spectrometry.

Table 1
MALDI-TOF fragmentation of GHTD-amide from media conditioned by MIN6 cells.

Mass (Da)	429	385	269.9	251.9	241.0	196.0	169.0	100.1	70.0
Assignment	Parent molecule	m-T1, a4	a3	d3b	HT	b2	a2	d2a	w1a

fragmentation tool of the GPS explorer software (Table 1). Thus GHTD-amide is produced by MIN6 beta cells confirming pancreatic islet beta cells as an endogenous source of the peptide.

3.6. A potential precursor of GHTD-amide is not in the annotated human proteome

A bioinformatics search was performed in an attempt to identify a precursor protein for GHTD-amide. The precursors of all known amidated peptides and proteins contain a Gly adjacent to the C-terminal amidated amino acid [12] and this is generally flanked by a basic residue that serves as a protease cleavage site. Alternatively, the amidated sequence may occur at the C-terminus of the precursor protein with a Gly as the last amino acid. Hence, the sequence GHTDG was used to BLAST search the NCBI non-redundant human protein sequence databases and Swissprot, which contain all characterized and predicted proteins from the human genome. Five proteins were identified that contain the sequence GHTDG. The first 4 of these proteins are members of an evolutionary conserved family of transducin-like enhancer of split transcriptional repressors (TLE1 through to TLE4) that contain WD domains characterized by a GH dipeptide followed 11–24 amino acids later by WD [5]. The GHTDG sequence occurs in a conserved WD domain in this TLE protein family. The other protein is transforming acidic coiled coil containing protein 2 (TACC2) [16]. These proteins are all widely expressed, restricted to the cell nucleus and lack potential protease cleavage sites adjacent to the C-terminal of the GHTDG sequence. There is a higher than expected chance of the GHTDG sequence occurring in these proteins due to the presence of several GH dipeptide motifs in TLE proteins and the high content of Gly (8.8%) and acidic residues, including Asp (5.5%), in TACC2. No other GHTDG containing proteins were found in the annotated human proteome.

4. Discussion

More than 30 years ago a peptide with insulin potentiating activity was isolated from human urine [19,20] but the technology to identify the peptide was not then available. Here we have shown that the active constituent is a tetrapeptide with the sequence GHTD-amide. Synthetic GHTD-amide stimulated glycogen synthesis in a hepatoma cell line and C2C12 myotubes and enhanced insulin-mediated glucose lowering in an insulin-deficient rat model of diabetes confirming that GHTD-amide is the active molecule originally described in human urine.

The original purification of the urinary peptide found the peptide to only be active in the presence of insulin [19,20,32]. In this study activation of glycogen synthesis in liver and muscle cell lines in the absence of insulin identifies an insulin-like activity attributable to GHTD-amide. This activity showed a high-dose inhibition effect, with reduced activity at concentrations above 100 pM in HepG2 cells. Reduced cellular responses at high concentrations are also frequently observed for insulin due to negative co-operativity in binding to the insulin receptor [9]. This may suggest that GHTD-amide is also operating through a receptor but as yet this has not been identified. Hypoglycemic activity in an insulin-deficient animal model may therefore be attributable to direct actions of the peptide or to the insulin potentiating activity originally described for the peptide preparations from urine.

Alpha-amidation of the carboxyl terminal amino acid is common in secreted peptide hormones, growth factors and neuropeptides [12]. However, alpha amidated aspartic acid appears to be uncommon with the only example being an opioid peptide from frog skin called deltorphin or dermenkephalin [2,23]. To our knowledge GHTD-amide is the first mammalian derived peptide described that contains alpha-amidated aspartic acid.

The rabbit anti-serum raised to a diphtheria toxoid/GHTD-amide conjugate bound to pancreatic islets and no other tissues. The specificity of the anti-serum for the C-terminus of the peptide, the unique nature of amidated Asp at the C-terminus, and the demonstration that MIN6 cells release GHTD-amide into cell culture media suggest that pancreatic beta cells are an endogenous source of this peptide. At this stage it is unclear whether GHTD-amide is co-secreted with insulin in response to glucose and amino acid stimuli since the detection methods currently available lack sufficient sensitivity to perform these experiments and the quantities released from MIN6 cells are small. A search for circulating endogenous GHTD-amide using the radioimmunoassay described here and a LC/MS/MS bioanalytical method both failed to detect the peptide in human or rodent plasma (data not shown) indicating that, if the peptide is present in the circulation, concentrations are below the lower limit of detection (0.5 ng/ml) of these methods. Co-localization with insulin in secretory granules of MIN6 cells and immunogold staining of dense core secretory granules suggest that further investigation of how GHTD-amide is released from MIN6 beta cells is warranted.

Peptides in urine usually derive from fragmented circulating plasma proteins and bioactive peptides [7]. It would be anticipated that GHTD-amide, like other alpha-amidated peptides, is processed from a larger precursor protein and released into the circulation, but no protein fulfilling the criteria for a precursor is present in the annotated human proteome. A precedent for endogenous alpha amidated tetrapeptides for which a precursor protein is not known is the endomorphins. Endomorphins were first isolated from brain and bind with high affinity and selectivity to mu opioid receptors [22,31]. A search for precursor proteins for endomorphin-1 (Tyr-Pro-Trp-Phe-amide) and endomorphin-2 (Tyr-Pro-Phe-Phe-amide) found no evidence of such proteins in the human proteome [30] and it has even been suggested that they may be synthesized non-ribosomally [24]. Many peptide hormone precursors are short polypeptides encoded by small messenger RNA that can be underrepresented in cDNA libraries and easily overlooked by gene prediction algorithms. There has been some success in identifying novel peptide hormone/neuropeptide-encoding genes using more complex algorithms and hidden Markov models [3,18]. However, these approaches are not guaranteed of success since the conserved bioactive regions encoded by some neuropeptide genes are interrupted by introns, as is seen in the neuropeptide Y family where the encoded RQRY-amide motif contains an intron in the codon for the penultimate residue in the RQRY peptide sequence [15,17]. Hence, it is possible that genes encoding precursor proteins for GHTD-amide and endomorphins exist but as yet have not been detected due to their small size or interruption of the encoded tetrapeptide sequences by introns. Resolution of this problem will require either purification of the unprocessed precursor proteins, extensive expression screening of small and rare cDNA clones from source tissues or a clear demonstration of non-ribosomal synthesis.

In conclusion, GHTD-amide is a novel islet beta cell peptide that stimulates glycogen synthesis in muscle and liver cells and enhances insulin-mediated blood glucose lowering in an insulin-deficient animal model of diabetes. While the mode of biosynthesis of the peptide remains unknown this activity and its production by pancreatic beta cells suggest a role in normal glucose homeostasis. Further study of the physiological role of GHTD-amide in health and disease will provide further insights into the regulation of blood glucose.

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References

- [1] Åhrén B, Pacini G, Wynick D, Wierup N, Sundler F. Loss-of-function mutation of the galanin gene is associated with perturbed islet function in mice. *Endocrinology* 2004;145:3190–6.
- [2] Amiche M, Sagan S, Mor A, Delfour A, Nicolas P. Dermenkephalin (Tyr-o-Met-Phe-His-Leu-Met-Asp-NH₂): a potent and fully specific agonist for the delta opioid receptor. *Mol Pharmacol* 1989;35:774–9.
- [3] Baggerman G, Liu F, Wets G, Schoofs L. Bioinformatic analysis of peptide precursor proteins. *Ann NY Acad Sci* 2005;1040:59–65.
- [4] Buchanan CM, Phillips ARJ, Cooper GJS. Preptin derived from proinsulin-like growth factor II (proIGF-II) is secreted from pancreatic islet b-cells and enhances insulin secretion. *Biochem J* 2001;360:431–9.
- [5] Chen G, Courey AJ. Groucho/TLE family proteins and transcriptional repression. *Gene* 2000;249:1–16.
- [6] Cooper G. Amylin compared with calcitonin gene-related peptide: structure, biology, and relevance to metabolic disease. *Endocr Rev* 1994;15:163–201.
- [7] Cutillas PR, Norden AGW, Cramer R, Burlingame AL, Unwin RJ. Detection and analysis of urinary peptides by on-line liquid chromatography and mass spectrometry: application to patients with renal Fanconi syndrome. *Clin Sci* 2003;104:483–90.
- [8] Date Y, Nakazato M, Hashiguchi S, Dezaki K, Mondal MS, Hosoda H, et al. Ghrelin is present in pancreatic α -cells of humans and rats and stimulates insulin secretion. *Diabetes* 2002;51:124–9.
- [9] De Meyts P, Roth J, Neville Gavin JR III J, Lesniak MA. Insulin interactions with its receptors: experimental evidence for negative cooperativity. *Biochem Biophys Res Commun* 1973;55:154–61.
- [10] Dezaki K, Sone H, Yada T. Ghrelin is a physiological regulator of insulin release in pancreatic islets and glucose homeostasis. *Pharmacol Therap* 2008;118:239–49.
- [11] Efendić S, Tatemoto K, Mutt V, Quan C, Chang D, Ostenson CG. Pancreastatin and islet hormone release. *Proc Natl Acad Sci USA* 1987;84:7257–60.
- [12] Eipper BA, Stoffers DA, Mains RE. The biosynthesis of neuropeptides: peptide α -amidation. *Annu Rev Neurosci* 1992;15:57–85.
- [13] Hettiarachchi M, Chalkley S, Furler SM, Choong YS, Heller M, Cooper GJ, et al. Rat amylin-(8–37) enhances insulin action and alters lipid metabolism in normal and insulin-resistant rats. *Am J Physiol* 1997;273:E859–67.
- [14] Huang D, Cheung AT, Parsons JT, Bryer-Ash M. Focal adhesion kinase (FAK) regulates insulin-stimulated glycogen synthesis in hepatocytes. *J Biol Chem* 2002;277:18151–60.
- [15] Larhammar D, Ericsson A, Persson H. Structure and expression of the rat neuropeptide Y gene (gene family/polymorphism/mRNA). *Proc Natl Acad Sci USA* 1987;84:2068–72.
- [16] Lauffart B, Gangisetty O, Still IH. Molecular cloning, genomic structure and interactions of the putative breast tumor suppressor TACC2. *Genomics* 2003;81:192–201.
- [17] Minth CD, Bloom SR, Polakt JM, Dixon JE. Cloning, characterization, and DNA sequence of a human cDNA encoding neuropeptide tyrosine. *Proc Natl Acad Sci USA* 1984;81:4577–81.
- [18] Mirabeau O, Perlas E, Severini C, Audero E, Gascuel O, Possenti R, et al. Identification of novel peptide hormones in the human proteome by hidden Markov model screening. *Genome Res* 2007;17:320–7.
- [19] Ng FM, Zimmel P, Bornstein J, Malinek M, Taft P. Isolation and characterization of a hypoglycaemic peptide from human urine. *Proc Endocr Soc Aust* 1971;14:51.
- [20] Ng FM, Zimmel PZ, Seiler G, Taft P, Bornstein J. Insulin potentiating action of a peptide fraction from urine. *Diabetes* 1974;23:950–6.
- [21] Ohtaki T, Shintani Y, Honda S, Matsumoto H, Hori A, Kanehashi K, et al. Metastasis suppressor gene KiSS-1 encodes peptide ligand of a G-protein-coupled receptor. *Nature* 2001;411:613–7.
- [22] Pan W, Kastin AJ. From MIF-1 to endomorphin: the Tyr-MIF-1 family of peptides. *Peptides* 2007;28:2411–34.
- [23] Richter K, Egger R, Kreil G. D-Alanine in the frog skin peptide dermorphin is derived from L-alanine in the precursor. *Science* 1987;238:200–2.
- [24] Ronai AZ, Szemenyei E, Kato E, Kocsis L, Orosz G, Al-Khrasani M, et al. Endomorphin synthesis in rat brain from intracerebroventricularly injected [3H]-Tyr-Pro: a possible biosynthetic route for endomorphins. *Regul Peptides* 2006;134:54–60.
- [25] Rushing PA, Hagan MM, Seeley RJ, Lutz TA, Woods SC. Amylin a novel action in the brain to reduce body weight. *Endocrinology* 2000;141:850–3.
- [26] Sanchez-Margalet V, Lucas M, Goberna R. Pancreastatin: further evidence for its consideration as a regulatory peptide. *J Mol Endocrinol* 1996;16:1–8.
- [27] Silvestre RA, Egido EM, Hernández R, Marco J. Kisspeptin-13 inhibits insulin secretion without affecting glucagon or somatostatin release: study in the perfused rat pancreas. *J Endocrinol* 2008;196:283–90.
- [28] Silvestre RA, Miralles P, Monge L, Moreno P, Villanueva ML, Marco J. Effects of galanin on hormone secretion from the in situ perfused rat pancreas and on glucose production in rat hepatocytes in vitro. *Endocrinology* 1987;121:378–83.
- [29] Tatemoto K, Efendić S, Mutt V, Makk G, Feistner GJ, Barchas JD. Pancreastatin, a novel pancreatic peptide that inhibits insulin-secretion. *Nature* 1986;324:476–8.
- [30] Terskiy A, Wannemacher KM, Yadav PM, Tsai M, Tian B, Howells RD. Search of the human proteome for endomorphin-1 and endomorphin-2 precursor proteins. *Life Sci* 2007;81:1593–601.
- [31] Zadina JE, Hackler L, Ge LJ, Kastin AJ. A potent and selective endogenous agonist for the mu-opiate receptor. *Nature* 1997;386:499–502.
- [32] Zimmel P, Ng F, Bornstein J, Taft P. Somantin(In-G), cataglycin (Ac-G) and glucose homeostasis in man. *Horm Metab Res* 1972;4:309–10.