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

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ABSTRACT

In the early 1970s, a peptide fraction with insulin potentiating activity was purified from human urine but the identity and origins of the active constituent remained unknown. Here we identify the active component and characterize its origins. The active peptide was identified as an alpha amidated tetrapeptide with the sequence GHTD-amide. The peptide was synthesized and tested for stimulation of glycogen synthesis and insulin potentiation by insulin tolerance testing in insulin-deficient rats, which confirmed GHTD-amide as the active peptide. Tissue localization using a peptide-specific anti-serum and epifluorescent and confocal microscopy showed decoration of pancreatic islets but not other tissues. Confocal microscopy revealed co-localization with insulin and immunogold and electron microscopy showed localization to dense core secretory granules. Consistent with these observations GHTD-amide was found in media conditioned by MIN6 islet beta cells. Sequence database searching found no annotated protein in the human proteome encoding a potential precursor for GHTD-amide. We conclude that the insulin potentiating activity originally described in human urine is attributable to the tetrapeptide GHTD-amide. GHTD-amide is a novel peptide produced by pancreatic beta cells and no precursor protein is present in the annotated human proteome. Stimulation of glycogen synthesis and co-localization with insulin in beta cells suggest that GHTD-amide may play a role in glucose homeostasis by enhancing insulin action and glucose storage in tissues.

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.

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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 Vydec 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 reapplied 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 μCi/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. × 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}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-