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Isolation and characterisation of GLP-1 7–36 amide from rat intestine

Elevated levels in diabetic rats

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Glucagon-like peptide-1 (GLP-1) was purified to homogeneity by HPLC and anion-exchange chromatography. A molecular mass of 3297.4 Da was obtained by FAB mass spectrometry which corresponded exactly to GLP-1 7–36 NH_2, providing evidence that amidation occurs at an arginine residue during the post-translational processing of GLP-1. The distribution of GLP-1 7–36 NH_2-like immunoreactivity (GLP-1 7–36 NH_2 IR) was determined in the rat gastrointestinal tract. Highest concentrations were found in terminal ileum and colon. Streptozocin-induced diabetic rats, who showed a significant increase in food intake, had a significant increase of GLP-1 7–36 NH_2 IR in the colon.

Glucagon-like peptide-1; Chromatography; Mass spectrometry; Diabetes mellitus; Peptide termination factor; (Rat)

1. INTRODUCTION

Mammalian proglucagon consists of 160 amino acid residues and is synthesized in the islets of Langerhans, intestine and brain [1,2]. As well as for glucagon, it also encodes for two glucagon-like peptides (GLP-1 1–37 or proglucagon sequence 72–108, and GLP-2 or proglucagon sequence 126–158) [3]. Paired basic amino acids were considered to be the enzymatic cleavage points. However, the arginine residue at position 6 of GLP-1 was a possible monobasic cleavage site [4]. The isolation and partial sequence analysis of the first 17 amino acids of pig intestinal GLP-1 confirmed that cleavage does indeed occur at this residue 6 [5]. However, the proglucagon sequence 104 to 110 is Val-Lys-Gly-Arg-Gly-Arg-Arg. Thus several molecular forms are possible at the C-terminus of GLP-1, i.e.: GLP-1 7–33, 7–34, 7–34 NH_2, 7–35, 7–36, 7–36 NH_2 and 7–37.

The aim of this study was therefore to purity rat intestinal GLP-1 and to determine its precise molecular weight by mass spectrometry and thus to deduce the sequence of the main naturally occurring molecular form.

Synthetic GLP-1 7–36 NH_2 greatly enhances glucose-induced insulin release in man at physiological concentrations and may be the most potent insulin-releasing hormone yet identified [6]. We therefore also determined the distribution of GLP-1 7–36 NH_2-like immunoreactivity (GLP-1 7–36 NH_2 IR) in rat intestine of normal and of streptozocin-induced diabetic (STZ-D) rats, a commonly employed experimental model of diabetes mellitus.

2. MATERIALS AND METHODS

2.1. *Radioimmunoassay*

GLP-1 7–36 NH_2-like immunoreactivity (IR) and glucagon-like immunoreactivity (GLI) were measured as described [6,7].

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The GLP-1 7–36 NH_2 assay reacts 100% with GLP-1 1–36 NH_2. The GLI assay cross-reacts with glicentin and oxyntomodulin, the two main molecular forms of gut glucagon processing.

2.2. *Extraction of rat intestine*

Wistar rats (n = 6) (400–450 g) were killed by suffocation in a CO_2 chamber. The colon and the distal half of the small intestine were removed and cleaned with ice cold water. The tissue was then extracted in boiling water (500 ml) for 10 min. The supernatant was decanted and the tissue re-extracted with an equal volume of boiling 0.5 M acetic acid for a further 10 min. The solutions were allowed to cool down, 25 g of diatomaceous earth (Sigma, Poole, England) added to each, then stirred, and passed through a Whatman filter paper size no.113 (Whatman, Maidstone, England). Fractions (100 ml) of these solutions were then concentrated by passing through 5 activated C 18 Sep-Pak cartridges (Waters Associates, Milford, MA, USA), connected in series, and eluted with 10 ml of 60% acetonitrile (ACN) (v/v). The eluents from both water and acid extract were pooled together separately and lyophilised.

2.3. *Chromatography*

Before each chromatographic step, the samples were centrifuged for 10 min at 11300 × g in a microcentrifuge. The first chromatographic step utilised a poly fluorocarbon column (Poly F®, Du Pont, Stevenage, England) on a high pressure liquid chromatography (HPLC) system (Waters Associates) with a gradient from 0–60% solvent B (solvent A: H_2O/0.1% TFA; solvent B: ACN/0.1% TFA). The flow rate was 1 ml/min and 2 min fractions were collected.

The fractions showing GLP-1 7–36 NH_2 IR were pooled together, lyophilised and reconstituted in buffer A for the anion-exchange chromatography. This was carried out on a FPLC system (Pharmacia, Milton Keynes, England) with a Mono Q column (HR 5/5) (buffer A: 25% aqueous ACN/20 mmol ethanolamine, pH 9.5; buffer B: buffer A plus 1 M NaCl, pH 9.5). Acid and water extracts were run separately. The flow rate was 1 ml/min and 2 min fractions were collected. The peak fraction of GLP-1 7–36 NH_2 IR from the water and acid extracts were pooled together and dried down to a final volume of 0.5 ml before re-injection onto a Pep RPC C_{18} reverse-phase column (Pharmacia) on a FLPC system. A mixture of isocratic and linear gradient of solvent B (ACN/0.1% TFA) in solvent A (H_2O/0.1% TFA) was used: 0–10 min: 0–20%; 10–130 min: 20–30%; 130–190 min: isocratic 30%; 190–310 min: 30–40%; 310–330 min: 40–60%. The final purification was monitored by UV absorption at 214 nm.

2.4. *Mass spectrometry*

The analysis was carried out at M-Scan Ltd, Ascot, England. The sample was dissolved in 5% aqueous acetic acid and 2 μl were loaded onto a stainless steel FAB target previously smeared with 2–4 μl of meta-nitrobenzyl alcohol. Fast atom bombardment analysis was carried out as described in [8] on a M-Scan's VG Analytical ZAB high field mass spectrometer operating at V_{acc} = 8 kV for 15000 mass range at full sensitivity. A cesium ion gun operating at 30 kV was used to generate spectra which were recorded using a PDP 11–250 J data system. Calibration was carried out using cesium iodide.

2.5. *STZ-D rats*

Male Wistar rats (n = 25) weighing 190–230 g were fed standard rat food (PRD pellets, Labsure, London, England) and water ad libitum. One group (n = 13) received STZ (55 mg/kg in 0.2 ml buffer, Upjohn, Kalamazoo, MI, USA) and the other 12, acting as control animals, were given 0.2 ml isotonic saline and sodium citrate solution at the same time. Eight weeks after inducing diabetes mellitus the rats were exsanguinated by cardiac puncture while under light ether anaesthesia. Blood was collected into fluoride oxalate tubes for measurement of plasma glucose concentration by a glucose oxidase-based autoanalyzer. The gut was removed, dissected, and cleaned with ice cold water and extracted in boiling 0.5 M acetic acid (10 vol per g wet wt tissue). Tissue extracts were subjected to anion-exchange chromatography on the above described system with a linear gradient of 0–20% buffer B (buffer A: 0.02 M ethanolamine and 0.1% polyoxyethylene sorbitan mono-oleate, pH 9.5; buffer B: buffer A plus 1 M NaCl) over 60 min at a flow rate of 1 ml/min.

2.6. *Statistical analyses*

Results were expressed as mean and standard error of the mean and statistical significance analysed by unpaired Student's t-test. P values of <0.05 were considered statistically significant.

3. RESULTS

3.1. *Purification and mass spectrometry*

The results of the purification procedures are shown in fig.1. A single peak of GLP-1 7–36 NH_2 IR eluted from the Poly F column (fig.1A) and was further purified by anion-exchange chromatography. Anion-exchange chromatography gave similar profiles for both water and acid extracts (fig.1B). The peak fractions were therefore run together for the final purification by HPLC. The major peak after HPLC on a Pep RPC C-18 column contained 93% of the GLP-1 7–36 NH_2 IR (fig.1C). A second peak with a 15 min longer retention time corresponded to 3.5% (fig.1C). The remaining GLP-1 7–36 NH_2 IR eluted in 6 small peaks (<0.3 pmol/tube) with retention times between 151 and 192 min.

The FAB mass spectrometry of the major peak gave a molecular mass of 3297.4 ± 1 Da corresponding exactly to the molecular mass of GLP-1 7–36 amide. Due to the low amounts of material in the other fractions we were unable to obtain reliable m/z ratios for them.

3.2. *STZ-D rats*

The body mass of the STZ-D rats was significantly decreased (STZ-D rats: 288 ± 12 g, control: 387 ± 7 g; $P < 0.001$) whereas food intake

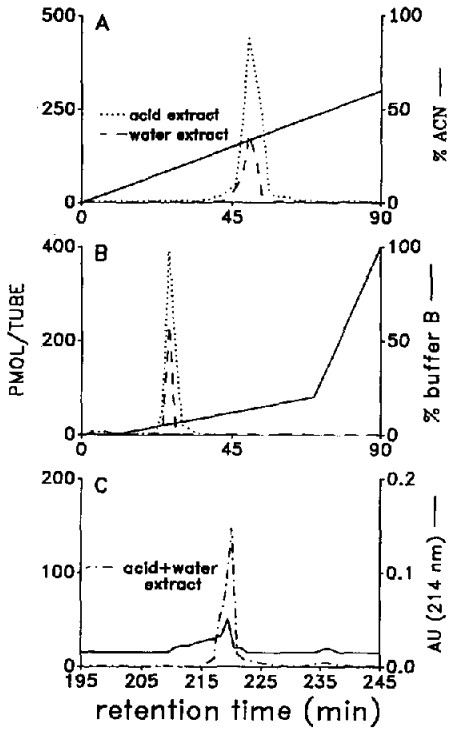

Fig.1. Profiles of GLP-1 7–36 NH_2 IR during the purification procedures. Details of each chromatographic step are described in section 2. (A) HPLC with a Poly F column. (B) Anion-exchange chromatography. (C) HPLC with a PEP RPC C-18 column.

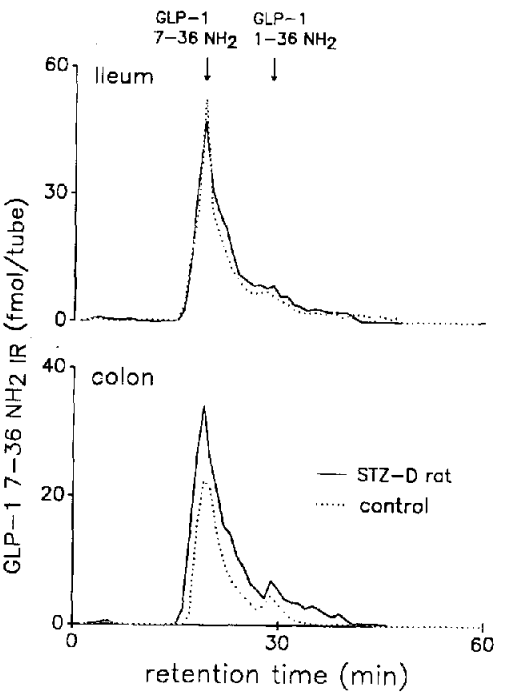

Fig.2. GLP-1 7–36 NH_2 IR profiles after anion-exchange chromatography of ileal and colonic extracts of STZ-D rats and control rats. The arrows indicate the retention times of synthetic GLP-1 7–36 NH_2 and GLP-1 1–36 NH_2, run under identical conditions.

Table 1

Absolute (pmol/g wet wt) and relative concentration (pmol/g wet wt per 100 g body weight) of GLP-1 7–36 NH_2 IR and GLI in gut of STZ-D rats (n = 13) and normal rats (n = 12)

	Stomach	Upper small intestine	Terminal ileum	Colon
GLP-1 7–36 NH_2 IR				
(pmol/g)				
Control	0.3 ± 0.05	5.4 ± 0.4	188 ± 10	100 ± 10
STZ-D rats	0.4 ± 0.05	3.8 ± 0.3	171 ± 9.8	136 ± 9.7*
(pmol/g per 100 g body wt)				
Control	0.08 ± 0.01	1.4 ± 0.1	48.6 ± 4.4	26 ± 2
STZ-D rats	0.14 ± 0.01	1.3 ± 0.1	59.3 ± 5.6	47 ± 4.3*
GLI				
(pmol/g)				
Control	0.6 ± 0.04	8.1 ± 0.4	247 ± 14	144 ± 10
STZ-D rats	0.8 ± 0.1	7.0 ± 0.6	239 ± 10	212 ± 15*
(pmol/g per 100 g body wt)				
Control	0.2 ± 0.01	2.1 ± 0.2	63 ± 5.9	37 ± 3.1
STZ-D rats	0.3 ± 0.02	2.4 ± 0.2	82 ± 7.6	73 ± 6.5*

* $P < 0.02$

and non-fasted plasma glucose concentrations were significantly increased (food intake (g/day per rat): STZ-D rats: 49 ± 2; control: 35 ± 3; glucose (mmol/l): STZ-D rats: 28.3, control: 8.6 ± 0.2; $P < 0.001$). In both the STZ-D rats and the normal controls highest concentrations of GLP-1 7–36 NH_2 IR and GLI were found in the terminal ileum (table 1). A significant increase of colonic GLP-1 7–36 NH_2 IR and GLI was observed in the diabetic rats compared to the normal controls, whereas in the ileum, even when calculated relative to body mass, no significant change was found (table 1). Anion-exchange chromatography showed a similar profile of colonic and ileal tissue extract of STZ-D rats and control rats (fig.2). The majority of the immunoreactive material eluted at the position of synthetic GLP-1 7–36 NH_2.

4. DISCUSSION

The isolation and molecular mass determination of GLP-1 7–36 NH_2 IR from rat intestine demonstrated for the first time that GLP-1 is indeed C-terminal cleaved at the pairs of basic amino acids (proglucagon sequence 109, 110) and that the terminal glycine is processed to amidate the arginine residue. Only two similar arginine α-amidations have been reported so far: the pancreatic GLP of anglerfish proglucagon and a highly active snake neurotoxin [9,10]. The differences in the molar content of gut GLI and GLP-1 7–36 NH_2 IR would indicate that not all GLP-1 of the intestine is processed to GLP-1 7–36 NH_2. In addition to detecting GLP-1 7–36 NH_2 several other minor immunoreactive peaks were also detected. These indicate the presence of other molecular forms of GLP-1 albeit present in low concentrations. The low amount of immunoreactivity agrees with their corresponding small size of peak on the UV profile. It seems therefore very likely that GLP-1 7–36 NH_2 is the major form produced in the gastrointestinal tract. The increase of colonic GLP-1 7–36 NH_2 IR and GLI in the STZ-D rats may be due to hyperphagia and the consequently increased food residue in the gut in this animal model of diabetes mellitus. Similar significant increases of GLI have also been reported in hypothermia-induced hyperphagia rats and in lactating rats, another model of hyperphagia [11]. No change was observed in the processing of GLP-1 7–36 NH_2 between the diabetic animals and their controls. GLP-1 7–36 NH_2 is a potent insulin-releasing hormone and it may be that the synthesis of proglucagon derived peptides in the gut is regulated by negative feedback mechanism in which diminished insulin levels result in increased synthesis of proglucagon in the intestine.

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