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Detection of Glycated Gastric Inhibitory Polypeptide within the Intestines of Diabetic Obese (*ob/ob*) Mice

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Gastric inhibitory polypeptide (GIP) is produced within endocrine cells of the small intestine and released into the circulation upon nutrient ingestion. This study has quantified the levels of this insulinotropic peptide in the intestines of lean and diabetic obese *ob/ob* mice and estimated the proportion that is glycated. The total intestinal GIP concentration and content of the diabetic mice were significantly greater ($p < 0.01$) than that of control animals. Affinity chromatographic separation and side-viewing GIP radioimmunoassay demonstrated that approx 20% of the GIP extracted from intestines of *ob/ob* mice was present in glycated form. Less than 2% of intestinal GIP was glycated in lean mice. In conclusion substantial quantities of glycated GIP exist within the intestines of diabetic *ob/ob* mice, suggesting that this may be a contributing factor to the physiological disarray of this syndrome.

Key Words: Glycation; gastric inhibitory polypeptide; obese mice; hyperglycemia.

Introduction

Gastric inhibitory polypeptide (GIP) is released into the circulation from endocrine K-cells present in the duodenum and proximal jejunum following ingestion of glucose, fat, or protein *(1)*. Fat and glucose constitute the most potent stimuli for the release of GIP, and secretion is dependent upon absorption and not just the mere presence of nutrients within the gut *(2)*. GIP together with glucagon-like peptide-1(7–36) amide (tGLP-1) are generally accepted to account for the full incretin effect whereby hormones released from the gut potentiate glucose-induced insulin release *(3)*. In addition to insulinotropic activity, GIP has been shown to enhance insulin-dependent inhibition of glycogenolysis in liver *(4)* and to have potent effects on glucose uptake and metabolism in isolated mouse diaphragm muscle *(5)*. Functional GIP receptors have been detected on adipocytes *(6)*, and GIP has been demonstrated to stimulate glucose transport *(7)*, increase fatty acid synthesis *(8)*, and stimulate lipoprotein lipase activity in adipose tissue *(9)*.

The process of glycation has long been associated with the hyperglycemic disarray of diabetes and the development of the chronic pleiotropic complications of this disease *(10)*. Glycation involves a non-enzymatic condensation reaction between a sugar aldehyde and a free amino group of a protein *(11)*. Although most interest in glycation has centered on the modification of a range of structural proteins such as lens crystallin *(12)*, collagen *(13)*, albumin *(14)*, myelin *(15)*, sciatic nerve tubulin *(16)*, and osteocalcin *(17)*, other functional proteins are known to be glycated in vivo under prevailing hyperglycemic conditions. Increased levels of glycated hemoglobin *(18)* and glycated IgG *(19)* and IgM *(20)* are present in diabetic individuals. A whole series of enzymes including glucose-6-phosphate dehydrogenase *(21)*, aldehyde reductase *(22)*, glutathione reductase *(23)*, and Cu,Zn-superoxide dismutase *(24)* have also been shown to be glycated. Previous studies have demonstrated that a number of key regulatory peptides including tGLP-1 *(25)*, cholecystokinin-8 *(26)*, insulin *(27)*, and GIP *(28)* undergo glycation during in vitro incubation under hyperglycemic conditions.

Much of the existing knowledge on the in vivo glycation of proteins has come from the use of various experimental animal models of diabetes *(29)*. The obese hyperglycemic *ob/ob* mouse exhibits a genetically transmitted form of type-2 diabetes due to an autosomal recessive mutation on chromosome 6, which produces a truncated, non-functional form of leptin *(29)*. Leptin plays an important role in long-term body weight homeostasis and in the regulation of energy balance *(30)*. As there is a complete lack of feedback from leptin in the *ob/ob* mouse, hypothalamic neuropeptide Y content and secretion are elevated *(31)*. The resulting hyperphagia, decreased energy utilization, obesity, and hyperinsulinemia culminate in an obese phenotype, which is characterized by extreme insulin resistance, glucose intolerance, and hyperglycemia *(29)*. The present study was undertaken to determine whether glycation of GIP occurs in vivo within the endocrine cells of the small intestine of

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Table 1
Body Weight, Plasma Glucose,
Small Intestinal Weight, and GIP in Intestines of Lean and Obese Mice[a]

Total intestinal GIP content	Body weight	Plasma glucose	Small intestine weight	Intestinal GIP concentration	
				g	m*M*
Obese mice *n* = 8	72.3 ± 0.4***	22.4 ± 2.9***	2.4 ± 0.1***	443 ± 64*	1064 ± 136**
Lean mice *n* = 6	41.8 ± 0 .5	8.6 ± 0.7	1.5 ± 0.1	236 ± 69	354 ± 153

[a] Values are mean ± SEM, $n = 6–8$. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared to lean mice.

normal and diabetic *ob/ob* mice. The levels of GIP present within the intestines were determined and, through affinity chromatographic separation, the fraction occurring in the glycated form was quantified.

Results

General Characteristics and Intestinal GIP Content

As shown in Table 1, obese mice had significantly higher ($p < 0.001$) plasma glucose concentrations compared to lean control mice. The body weight and mass of excised intestinal tissue were also significantly higher ($p < 0.001$) in the obese group. Substantial amounts of GIP were detected within the intestines of both groups of animals with obese mice demonstrating significantly higher levels compared to lean controls when expressed as pmol per g of tissue ($p < 0.05$) or as pmol per intestine ($p < 0.01$).

Non-glycated and Glycated GIP in Intestinal Tissue

The side-viewing GIP antiserum used for radioimmunoassay of both GIP and glycated GIP illustrated a high degree of cross-reactivity (Fig. 1). Comparison of best-fit values obtained following nonlinear regression analysis revealed no significant difference between both sets of binding data. Figure 2 illustrates the tissue concentrations of GIP in non-glycated and glycated fractions following extraction of lean and obese mouse intestines, affinity chromatographic separation, and radioimmunoassay. Despite measurement of high levels of non-glycated GIP, less than 2% of GIP extracted from lean mouse intestines was in glycated form. In contrast, approx 20% (90 ± 19 pmol/g) of the total GIP extracted from obese mice intestines was found to be in the glycated form. The percentage recovery of GIP and glycated GIP obtained following affinity separation of intestinal extracts was over 90%.

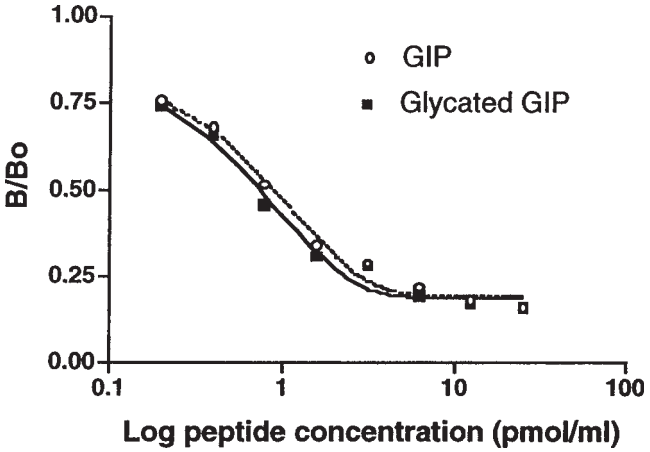

Fig. 1. Cross-reactivity of antiserum with GIP and glycated GIP. Values are bound/bound with no antigen (B/Bo, $n = 3$) versus $\log_{10}$ peptide concentration.

Discussion

The peptide hormone GIP produces a glucose-dependent stimulation of insulin release from pancreatic β-cells following meal-induced secretion into the circulation from endocrine cells of the small intestine *(32)*. The present study has found that the tissue concentration and total immunoreactive GIP levels within the small intestines of obese (*ob/ob*) mice were increased compared to age-matched lean control animals. These findings concur with previous reports indicating that GIP concentrations are substantially raised in the intestines and plasma of the obese mutant *(33–35)*. Hyperplasia and hyperactivity of GIP-secreting cells resulting from increased feeding appear as major stimuli to the hyperinsulinemia, insulin resistance, and associated metabolic derangements of the syndrome *(33–35)*.

Affinity separation of intestinal extracts and subsequent radioimmunoassay using a fully cross-reacting side-viewing antiserum for GIP demonstrated very low levels of glycated GIP in lean mouse intestinal extracts (less than 2%). The levels of GIP detected in the corresponding non-glycated fraction represented >98% of the total GIP concentration in lean mice. In contrast, in obese mice approximately 20% of the total GIP was found to occur in the glycated fraction. These observations parallel previous investigations which have demonstrated that both insulin and proinsulin are glycated within the pancreatic β-cells of diabetic

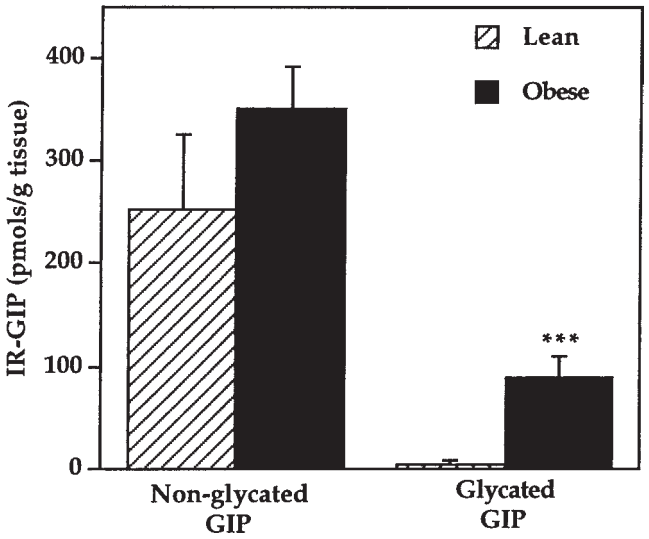

Fig. 2. Levels of GIP present in non-glycated and glycated fractions obtained following affinity separation of intestinal extracts from lean and obese (*ob/ob*) mice. Values are mean ± SEM, $n = 6–8$. ***$p < 0.001$ compared with lean mice.

animals *(36)*. The higher levels of glycated GIP in obese as compared to lean mice might reflect the marked hyperphagia as well as the hyperglycemia of the mutant *(29)*.

GIP has previously been shown to undergo glycation at the Tyr^1 residue under in vitro hyperglycemic conditions *(28)*. This study has demonstrated that such glycation may occur naturally in vivo within the endocrine cells of the glucose-rich and highly vascularized environment of the gastrointestinal tract. The realization that important endocrine peptides such as GIP are prone to glycation in vivo raises important questions concerning peptide physiology and the potential roles that these modified peptides may play in disease pathophysiology. The glycation of GIP has been shown to result in a reduced ability of this peptide to stimulate glucose uptake and metabolism in isolated mouse diaphragm muscle tissue *(5)*, while at the same time significantly enhancing its insulinotropic activity *(28)*. Recent studies *(37,38)* have also established that glycation confers GIP with resistance to enzymatic hydrolysis by the exopeptidase dipeptidylpeptidase IV, which has been identified as the enzyme primarily responsible for GIP degradation and inactivation in vivo *(39,40)*. Thus, glycated GIP has an extended half-life compared with the native hormone in the plasma of both normal and obese-diabetic rodents *(37,38)*. It is therefore clear that the glycation of GIP within the endocrine cells of the intestinal tract may have significant consequences on the subsequent half-life and activity of the circulating peptide. In this context it is noteworthy that glycated GIP has been shown to exhibit increased insulin-releasing and antihyperglycemic activity in rats and *ob/ob* mice in vivo *(37,38)*.

In conclusion, this study has demonstrated that GIP is present in a glycated form within the enteroendocrine cells from which it is secreted in vivo. These findings suggest that glycated circulating peptides warrant further investigation to determine what consequences this modification may have on normal body physiology particularly in the diabetic state.

Materials and Methods

Materials

Dextran T-70, activated charcoal, aprotinin, sodium cyanoborohydride, chloramine-T, and bovine serum albumin fraction V (BSA) were purchased from Sigma (Poole, Dorset, UK). C-18 reverse-phase Sep-Pak cartridges were purchased from Millipore-Waters, and all water used in these experiments was purified using a Milli-Q Water Purification System (Millipore Corporation, Milford, MA, USA). Human GIP was purchased from the American Peptide Company (Sunnyvale, CA, USA). Sac-Cel (anti-rabbit IgG) was purchased from Immunodiagnostic Systems (IDS) Ltd. (Boldon, Tyne and Wear, UK). GlycoGel B was obtained from Pierce and Warriner Ltd. (Chester, UK). Glucose oxidase reagent was obtained from Beckman Instruments Ltd. (Galway, Ireland). $Na^{125}I$ was obtained from Amersham International (Aylesbury, UK).

Animals

Adult (14–16 wk old) diabetic obese (*ob/ob*) mice and lean littermates were used in this study. The origin, genetic background, and characteristics of this colony have been described in detail elsewhere *(41,42)*. Mice were housed in an air-conditioned room at 22 ± 2ºC with a 12 h light/12 h dark lighting cycle. Mice were allowed *ad libitum* access to tap water and rodent pelleted-chow (FDS rodent maintenance diet, Trouw Nutrition, Cheshire, UK). The glycemic status of the mice was monitored by obtaining blood samples from the cut tip of the tail of conscious animals into chilled fluoride/heparin microcentrifuge tubes (Sarstedt, Nümbrecht, Germany). Samples were centrifuged immediately using a Beckman microcentrifuge (13,000*g*, 30 s) and plasma was aliquoted and stored at −20ºC prior to glucose determinations. Plasma glucose was assayed by an automated glucose oxidase procedure *(43)* using a Beckman Glucose Analyzer II (Beckman Instruments Inc., UK). All animal studies were carried out in accordance with the Animals (Scientific Procedures) Act 1986.

Tissue Excision and Peptide Extraction

Non-fasted animals were killed by cervical dislocation, and the small intestine was excised from the base of the stomach to the start of the large intestine. Once removed, the tissue was gently washed in ice cold saline (0.9% w/v NaCl) to remove any extraneous material, while the contents of the intestinal lumen were also gently purged. Excised tissue samples were then individually wrapped in aluminum foil, snap frozen in liquid nitrogen, and stored at −70ºC.

Individual excised intestinal samples were finely cut from frozen using a scalpel and extracted using 5 mL/g ice-cold acid-ethanol (75% ethanol, 23.5% H_2O, 1.5% conc HCl containing 1 mg/mL sodium cyanoborohydride added immediately before use). The tissue extract was subjected to a series of intermittent 30 s bursts of sonication at 4ºC over a 3 min period and then allowed to stir on ice for 3 h. After centrifugation for 20 min (3000*g*, 4ºC) the resulting supernatant was aliquoted, dried under vacuum using a Speed-Vac concentrator (AES 1000 Savant, Life Sciences International), and stored at –20ºC until required.

Separation of Non-glycated and Glycated Peptide Fractions

Separation of non-glycated and glycated peptides in tissue extracts was achieved by affinity chromatography essentially as described elsewhere *(36)*. In brief, columns containing 1 mL of a *m*-aminophenylboronate agarose gel (GlycoGel B) were equilibrated with 5 mL wash buffer (250 m*M* ammonium acetate, 50 m*M* magnesium chloride, and 3 m*M* sodium azide, pH 8.5). Tissue extract aliquots reconstituted in 1 mL wash buffer were applied to pre-equilibrated GlycoGel B columns. After the extract had been allowed to soak into the column, the unbound non-glycated peptide fraction was eluted by passing a further 4 mL of wash buffer through the column. Eluting buffer (3 mL, 200 m*M* sorbitol, 50 m*M* EDTA, 3 m*M* sodium azide, and 100 m*M* Tris buffer, pH 8.5) was then passed through the column to remove the bound glycated fraction. Both non-glycated and glycated fractions were aliquoted, dried under vacuum and stored at –20ºC until required.

Tissue Extract GIP Determination

Concentrations of immunoreactive GIP in the various intestinal extracts were determined by radioimmunoassay using rabbit anti-porcine GIP antiserum RIC34/11J, ^{125}I-human GIP, and human GIP standard. Bound and free hormone moieties were separated by addition of anti-rabbit IgG (Sac-Cel). The properties of this antiserum have been described previously *(44)*. Human GIP was iodinated using the chloramine-T (*N*-chloro *p*-methyl benzenesulfonamide) method as described previously *(45)* and iodination reaction products separated by HPLC on a Vydac C-18 analytical column (4.6 × 250 mm). Using a 1:9000 antibody dilution, a GIP standard curve was prepared over a concentration range from 0.39 to 25 pmol/mL through serial dilution of a frozen stock. Cross-reaction was checked with glycated GIP, prepared, and purified by methods as described elsewhere *(5,28)*. No loss of GIP immunoreactivity was observed following extraction procedures.

Analysis of Data

Groups of data are presented as mean ± SEM. Binding data were analyzed with the nonlinear regression program PRISM (Graphpad, San Diego, CA, USA). Statistical evaluation was performed using Student's unpaired *t* test and differences were considered to be significant if $p < 0.05$.

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