

Effects of short-term chemical ablation of the GIP receptor on insulin secretion, islet morphology and glucose homeostasis in mice

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Abstract

Glucose-dependent insulintropic polypeptide (GIP) is an incretin hormone secreted by endocrine K-cells in response to nutrient absorption. In this study we have utilized a specific and enzymatically stable GIP receptor antagonist, (Pro³)GIP, to evaluate the contribution of endogenous GIP to insulin secretion and glucose homeostasis in mice. Daily injection of (Pro³)GIP (25 nmol/kg body weight) for 11 days had no effect on food intake or body weight. Non-fasting plasma glucose concentrations were significantly raised ($p < 0.05$) by day 11, while plasma insulin concentrations were not significantly different from saline treated controls. After 11 days, intraperitoneal glucose tolerance was significantly impaired in the (Pro³)GIP treated mice compared to control ($p < 0.01$). Glucose-mediated insulin secretion was not significantly different between the two groups. Insulin sensitivity of 11-day (Pro³)GIP treated mice was slightly impaired 60 min post injection compared with controls. Following a 15 min refeeding period in 18 h fasted mice, food intake was not significantly different in (Pro³)GIP treated mice and controls. However, (Pro³)GIP treated mice displayed significantly elevated plasma glucose levels 30 and 60 min post feeding ($p < 0.05$, in both cases). Postprandial insulin secretion was not significantly different and no changes in pancreatic insulin content or islet morphology were observed in (Pro³)GIP treated mice. The observed biological effects of (Pro³)GIP were reversed following cessation of treatment for 9 days. These data indicate that ablation of GIP signaling causes a readily reversible glucose intolerance without appreciable change of insulin secretion.

Keywords: enteroinsular axis; GIP receptor antagonist; (Pro³)GIP.

Introduction

Glucose-dependent insulintropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are considered to be important incretin hormones of the enteroinsular axis (Dupré et al., 1973; Brown, 1994; Holst, 1994). GIP was first isolated on the basis of its inhibitory effects upon gastric acid secretion (Kosaka and Lim, 1930), but it is now recognized mainly through its ability to stimulate insulin secretion exerting only weak effects on other islet cell types (Fehmann et al., 1995). GIP and GLP-1 both stimulate insulin secretion in a glucose-dependent manner (Creutzfeldt, 2001), but their relative contribution to the enteroinsular axis remains unresolved. In spite of this, acute studies with GIP antagonists (Tseng et al., 1996; Gault et al., 2003a) have demonstrated that GIP is a particularly important physiological incretin. Other studies suggest that elevation of circulating GIP can almost completely account for the insulin secretory response to oral glucose (Nauck et al., 1989). In addition, GLP-1 receptor-deficient mice appear to display important compensatory changes through enhanced secretion of GIP (Pederson et al., 1998), whereas GIP receptor-deficient mice display a more severe form of glucose intolerance (Miyawaki et al., 1999). Interestingly, recent studies in double incretin receptor knockout mice demonstrated that glucose homeostasis and insulin secretion were reasonably well maintained despite the absence of both GIP and GLP-1 receptors (Hansotia et al., 2004; Preitner et al., 2004). However, an inherent problem with this approach is the lifetime opportunity for compensatory metabolic adaptation.

GIP stimulates proinsulin gene transcription and translation (Fehmann and Goke, 1995; Wang et al., 1996) and also acts synergistically as both a growth and anti-apoptotic factor for pancreatic β -cells (Trumper et al., 2001, 2002; Ehses et al., 2003). GIP's contribution to glucose homeostasis is not restricted to its insulintropic effects as also other facets of its biological activity are physiologically relevant (Meier et al., 2002; Gault et al., 2003b). In addition to this, GIP exerts various extrapancreatic effects that further enhance its glucose-lowering potency (Gault et al., 2003c). For example, GIP has been shown to inhibit hepatic glucose production (Elahi et al., 1986) and to promote glucose uptake in isolated mouse diaphragm muscle (O'Harte et al., 1998a). Functional GIP receptors have also been identified on adipocytes (Yip et al., 1998), where GIP has been shown to stimulate glucose transport (Eckel et al., 1979), increase fatty acid synthesis (Oben et al., 1991) and stimulate lipoprotein lipase activity (Knapper et al., 1995). In view of these

attributes, increasing attention has focused recently on GIP and its various 'super-agonist' analogs as a possible treatment option for type 2 diabetes (Gault et al., 2003b,c; Hinke et al., 2003). A recent study in GIP receptor-deficient mice has also linked GIP to the development of obesity through an effect on adipose tissue triggered by overnutrition (Miyawaki et al., 2002). This suggests that GIP receptor antagonists as well as agonists may be useful as therapeutic drugs (Holst 2002; Gault et al., 2003c; Kieffer, 2003).

In the present study, the recently developed specific GIP receptor antagonist (Pro³)GIP (Gault et al., 2002a), has been utilized to evaluate the effects of short-term functional ablation of the GIP receptor on insulin secretion, islet morphology and glucose homeostasis in normal mice. In view of the possible therapeutic application, evaluation of the effects of extended chemical GIP receptor antagonism is also of value. Recent studies in our laboratory using (Pro³)GIP have established its specificity and key involvement in the enteroinsular axis of obese hyperglycaemic *ob/ob* mice (Gault et al., 2003a). The present study indicates that GIP is an important contributor to the maintenance of effective glucose homeostasis and that longer-term extrapancreatic actions of GIP may be especially relevant for efficient blood glucose control (Meier et al., 2002; Gault et al., 2003c).

Results

Effects of (Pro³)GIP on food intake, body weight, glycated hemoglobin and non-fasting plasma glucose and insulin concentrations

Administration of (Pro³)GIP had no effect on food intake and body weight (Figure 1A,B). A significant increase in plasma glucose concentrations ($p < 0.05$) was observed on day 11 of the study, but plasma insulin concentrations were similar in treated and control mice (Figure 1C,D). Glycated hemoglobin concentrations were also similar in the two groups on days 11 and 20 of the study, ranging from $1.8 \pm 0.1\%$ to $1.9 \pm 0.1\%$ (mean \pm SEM, $n=8$; data not shown).

Effects of (Pro³)GIP on glucose tolerance

As shown in Figure 2 (Pro³)GIP administration for 11 days resulted in impaired glucose tolerance, with elevated glucose concentrations at 15, 30 and 60 min after intraperitoneal glucose injection ($p < 0.01$, $p < 0.05$ and $p < 0.05$, respectively). The detrimental effect of (Pro³)GIP was also clearly evident from the 0–60 min AUC values (Figure 2), which were 1.5-fold elevated ($p < 0.01$) compared to controls. Somewhat surprisingly, plasma insulin concentra-

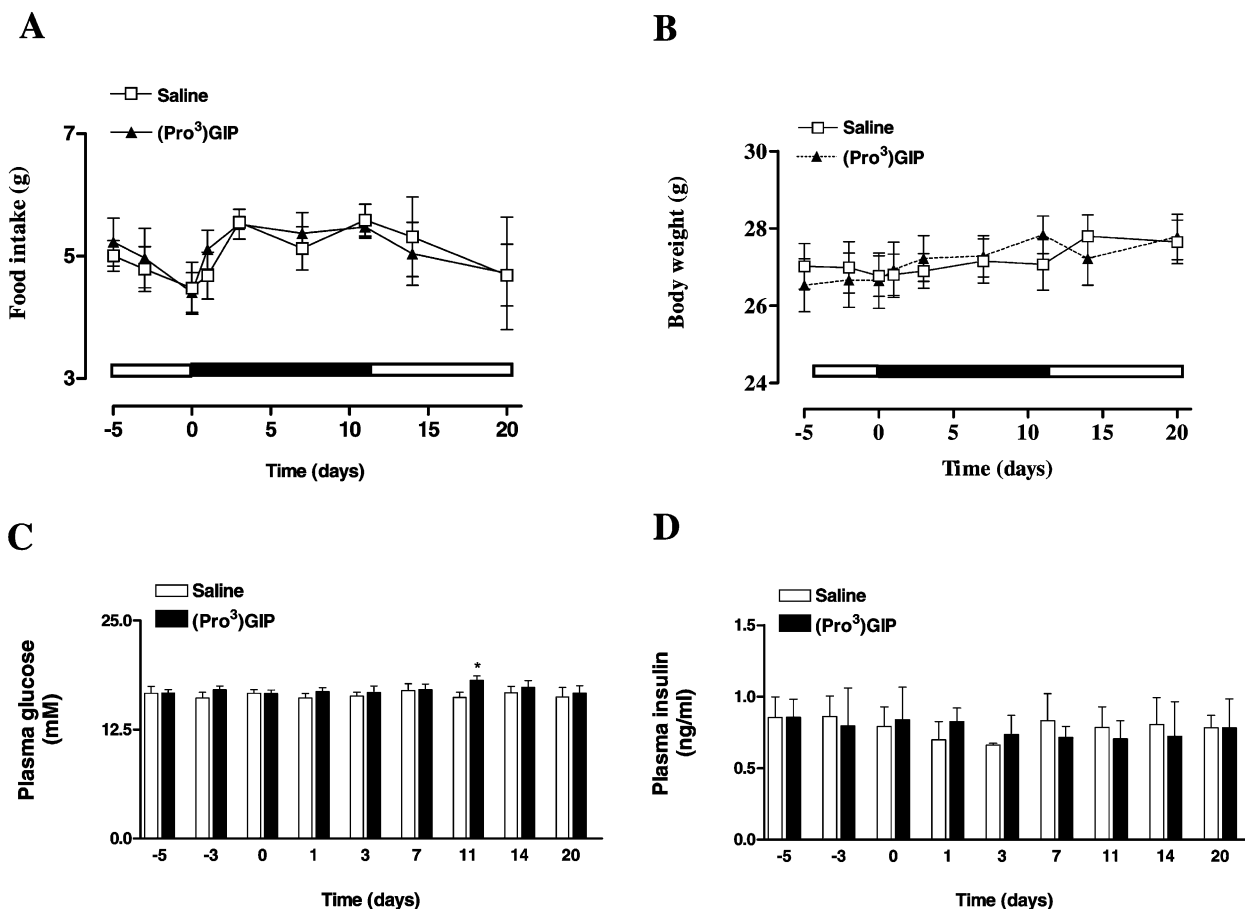


Figure 1 Effects of (Pro³)GIP treatment food intake (A), body weight (B), plasma glucose (C) and insulin (D) concentrations. Parameters were measured 5 days prior to, 11 days during (indicated by horizontal black bar) and 9 days after cessation of treatment with saline or (Pro³)GIP (25 nmol/kg body weight). Values are mean \pm SEM for eight mice. * $p < 0.05$ compared with the saline treated group.

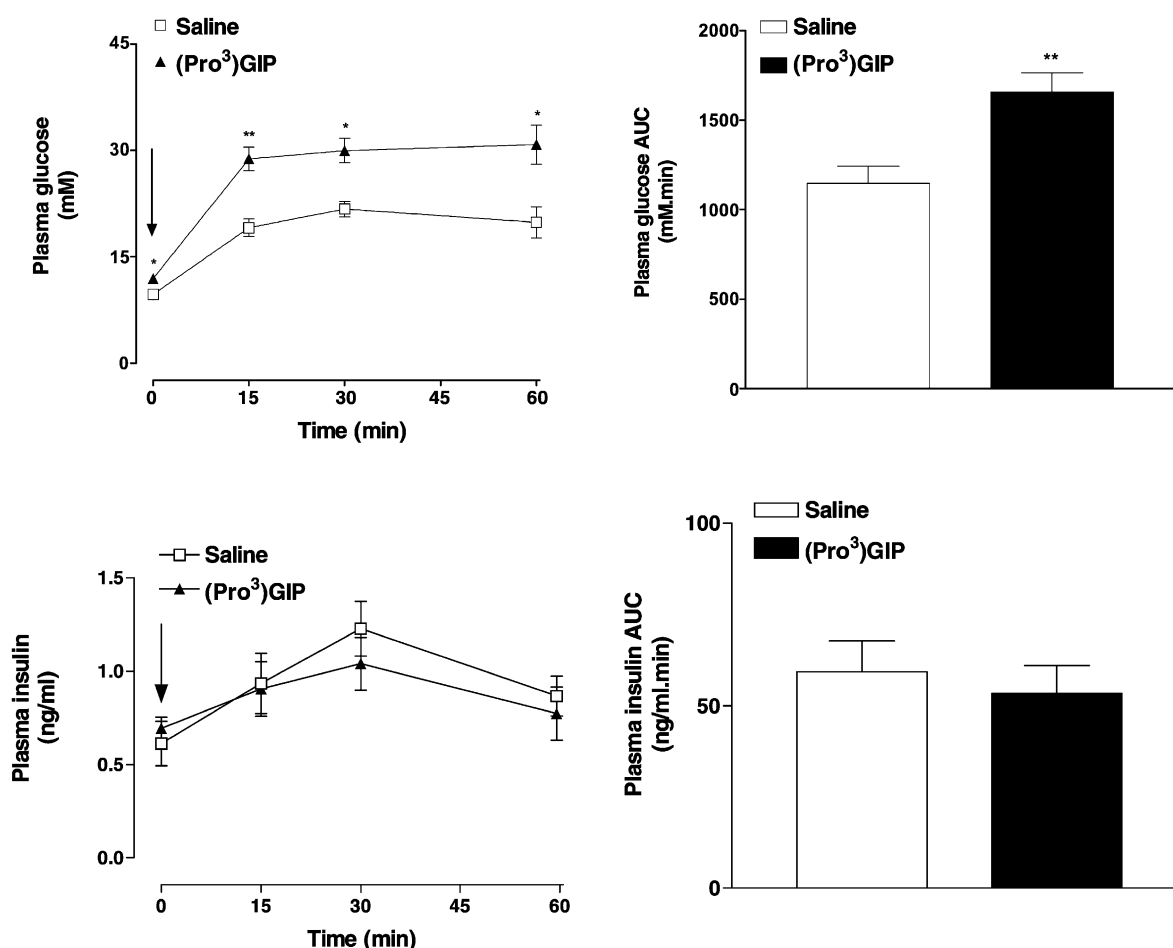


Figure 2 Effects of (Pro³)GIP treatment on glucose tolerance and plasma insulin response to glucose.

Tests were conducted after daily treatment with (Pro³)GIP (25 nmol/kg body weight) or saline for 11 days. Glucose (18 mmol/kg body weight) was administered by intraperitoneal injection at the time indicated by the arrow. Plasma glucose and insulin AUC values for 0–60 min post injection are shown in the insets. Values are mean±SEM for eight mice. * $p<0.05$ and ** $p<0.01$ compared with the saline group.

tions were similar in the two groups of mice after 11 days of (Pro³)GIP treatment, with identical AUC responses (Figure 2). Discontinuation of (Pro³)GIP treatment for 9 days (20th day of study) resulted in identical plasma glucose and insulin responses (Figure 3), indicating the reversibility of the actions of (Pro³)GIP in these animals.

Effects of (Pro³)GIP on metabolic response to feeding and insulin sensitivity

Plasma glucose concentrations were significantly raised ($p<0.05$) at 30 and 60 min after 15 min feeding in mice treated with (Pro³)GIP for 11 days (Figure 4). Food intake (0.3 g/mouse/15 min), AUC glucose and insulin were not significantly different. Similarly the hypoglycaemic actions of insulin were almost identical in (Pro³)GIP and control groups after 11 days treatment with (Pro³)GIP (Figure 5). Glucose concentrations were significantly raised ($p<0.05$) at 60 min in the (Pro³)GIP group after administration of insulin, but this was not translated into a significant difference in the overall AUC values. The responses following discontinuation of (Pro³)GIP treatment were also similar to saline-treated control mice on day 20 (data not shown).

Effects of (Pro³)GIP on pancreatic insulin and islet morphology

The pancreatic weights and insulin content were similar in the control and (Pro³)GIP mice after 11 days of treatment (Figure 6A,B). Similarly, no significant differences were observed in islet number per pancreatic section or average islet diameter (Figure 6C,D). Furthermore, the percentage of large diameter (>0.15 mm), medium diameter (0.1–0.15 mm) and small diameter (<0.1 mm) islets were not significantly different between the two groups after 11 days of treatment (Figure 7A). Representative images of pancreata immunohistologically stained for insulin from 11-day (Pro³)GIP treated (Figure 7B) and saline treated (Figure 7C) mice revealed no apparent disturbances in islet morphology (arrows indicate islets).

Discussion

Original studies showed that knockout of the GIP receptor in mice resulted in significant impairment of oral glucose tolerance and meal-induced insulin secretion without appreciable effects on body weight, food intake

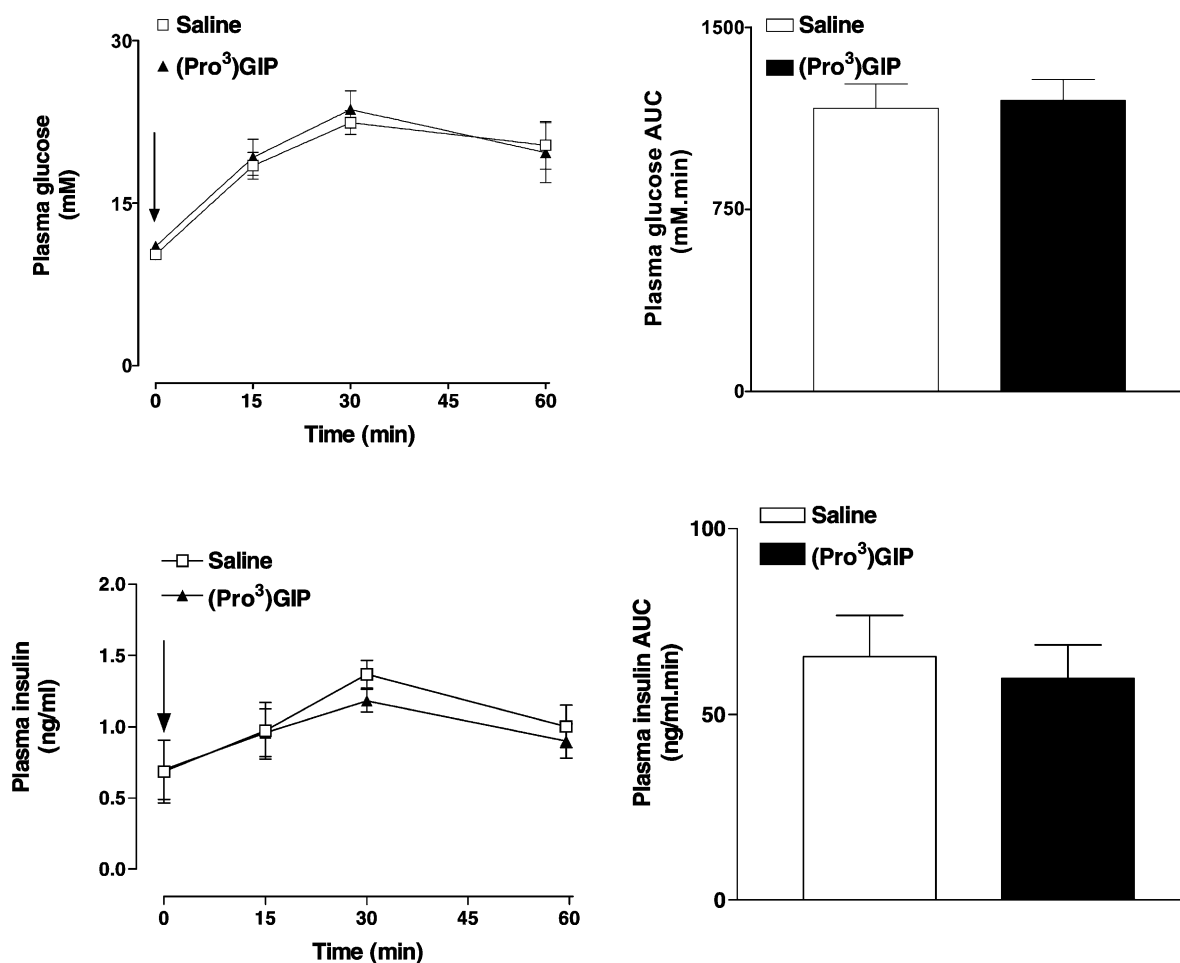


Figure 3 Reversal of glucose intolerance induced by (Pro³)GIP treatment.

Tests were conducted 9 days after cessation of daily injection of (Pro³)GIP (25 nmol/kg body weight for 11 days). Glucose (18 mmol/kg body weight) was administered by intraperitoneal injection at the time indicated by the arrow. Plasma glucose and insulin AUC values for 0–60 min post injection are shown in the insets. Values are mean \pm SEM for eight mice.

or basal glucose and insulin concentrations (Miyawaki et al., 1999). These results have largely been confirmed by more recent studies that together indicate significant involvement of GIP in the enteroinsular axis (Hansotia et al., 2004; Preitner et al., 2004). Nevertheless, the metabolic effects of single or double knockout of GIP with GLP-1 are relatively modest (Hansotia et al., 2004; Preitner et al., 2004), indicating the up-regulation of compensatory mechanisms, possibly involving other enteroendocrine factors (Pederson et al., 1998; Pamir et al., 2003).

Our previous *in vitro* and *in vivo* studies in *ob/ob* mice have demonstrated that (Pro³)GIP is a potent, specific and enzyme resistant antagonist of the GIP receptor (Gault et al., 2002a, 2003a). In the present study, daily injection of normal mice with (Pro³)GIP for 11 days had no adverse or toxic effects. In fact, food intake, body weight, islet structure and pancreatic morphology were identical to saline treated control mice. These observations are in accord with the basic features of GIP receptor knockout mice (Miyawaki et al., 1999) and indicate that functional ablation of GIP receptors does not have obvious and unexpected detrimental effects. The finding in one study (Pamir et al., 2003) of increased β -cell mass, altered islet architecture and pancreatic insulin content in GIP receptor knockout mice could not be replicated in

the present or previous investigations (Miyawaki et al., 1999; Preitner et al., 2004).

Consistent with a physiological role in glucose homeostasis, mice receiving daily (Pro³)GIP injections developed modest hyperglycaemia by day 11. When challenged with a test meal, glucose concentrations were also raised compared with those of saline treated controls. These observations have their counterparts in the results of oral glucose tolerance tests in GIP receptor knockout mice (Miyawaki et al., 1999; Preitner et al., 2004; Hansotia et al., 2004). However, chemical ablation of the GIP receptor appeared to have more profound consequences. Firstly, the animals exhibited basal hyperglycaemia, but more importantly intraperitoneal glucose tolerance was quite substantially impaired by prolonged treatment with (Pro³)GIP. Thus all transgenic animal studies to date exhibited normal disposal of an intraperitoneal glucose load. This may reflect an adaptive response enabled by lifelong, as opposed to 11 day, deficit in GIP action, possibly through secretion of GLP-1 (Pamir et al., 2003). However, this observation in (Pro³)GIP treated mice reveals an important glucose lowering action of endogenous GIP independent of enteroendocrine K-cell secretion.

A significant observation in the present study was the apparent lack of effect of (Pro³)GIP on basal plasma insu-

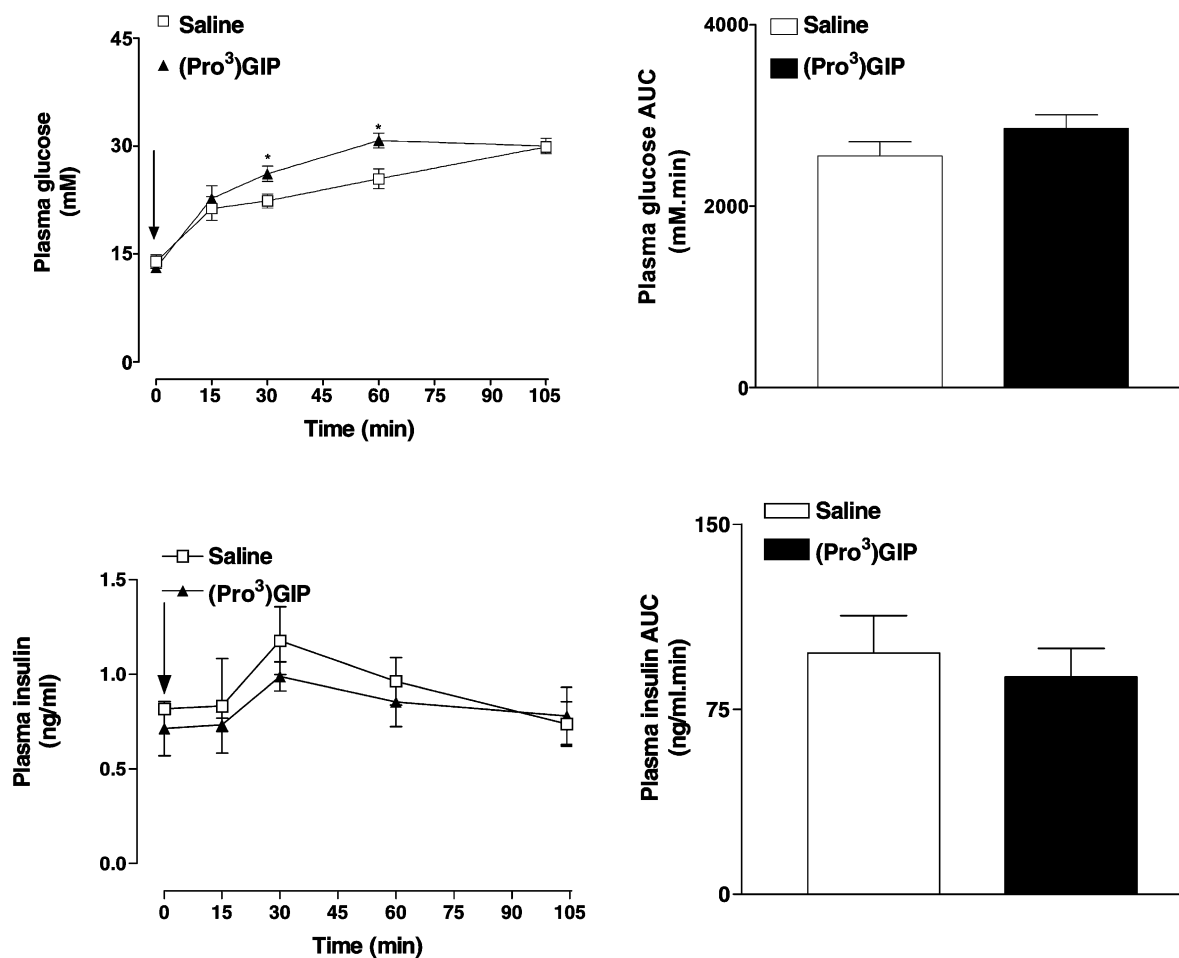


Figure 4 Effects of (Pro³)GIP treatment on glucose and insulin responses to feeding in 18 h fasted mice. Tests were conducted after daily treatment with (Pro³)GIP (25 nmol/kg body weight) or saline for 11 days. The arrow indicates the time of feeding (15 min). AUC values for 0–60 min post-feeding are also shown. Values are mean±SEM for eight mice. **p*<0.05 compared with the saline group.

lin concentrations and pancreatic β -cell responses to intraperitoneal glucose or a test meal. Small, but significant, deficiencies of insulin secretion were previously observed in GIP receptor knockout mice (Miyawaki et al., 1999). However, more recently it has been shown that direct, as opposed to alimentary derived, effects of nutrient stimuli on pancreatic β -cells are intact in GIP receptor knockout mice (Pamir et al., 2003; Preitner et al., 2004). Nevertheless, it is clear in the present study that the impairment of glucose homeostasis induced by (Pro³)GIP is dependent on mechanisms other than changes of pancreatic β -cell function.

Assessment of insulin sensitivity in (Pro³)GIP treated mice indicated a very minor deterioration of glucose lowering ability. Although a possible contributing factor, it seems unlikely that this small change of insulin sensitivity alone could be the sole explanation for the observed glucose intolerance. Thus, it appears highly likely that ablation of other glucose-lowering actions of GIP participate in the impaired glucose homeostasis of (Pro³)GIP treated mice. These pathways clearly merit further investigation but may include inhibition of glucagon secretion and effects on glucose transfer in the gastrointestinal tract, muscle or adipose tissue (Meier et al., 2002). For example, GIP is known to promote glucose uptake, glucose oxidation and glycogenesis in muscle tissue (O'Harte et

al., 1998) and to stimulate glucose transport and increase fatty acid synthesis in adipocytes (Eckel et al., 1979; Oben et al., 1991) at physiological concentrations.

In conclusion, the present study has demonstrated that short-term chemical ablation of the GIP receptor using (Pro³)GIP results in reversible indicators of glucose intolerance in normal mice. Observation of a very modest decrement in insulin sensitivity without change of pancreatic β -cell function indicates important glucose lowering actions other than the stimulation of insulin secretion to be involved in the observed glucose intolerance. Overall GIP receptor antagonism appears to be a safe and effective means of preventing the biological actions of endogenous GIP. The phenotype induced appears to be more severe than that generated in GIP receptor knockout mice probably due to limited opportunity for lifelong compensatory adaptations.

Materials and methods

Synthesis, purification and characterization of (Pro³)GIP

(Pro³)GIP was sequentially synthesized on an Applied Biosystems automated peptide synthesizer (Model 432 A) using stan-

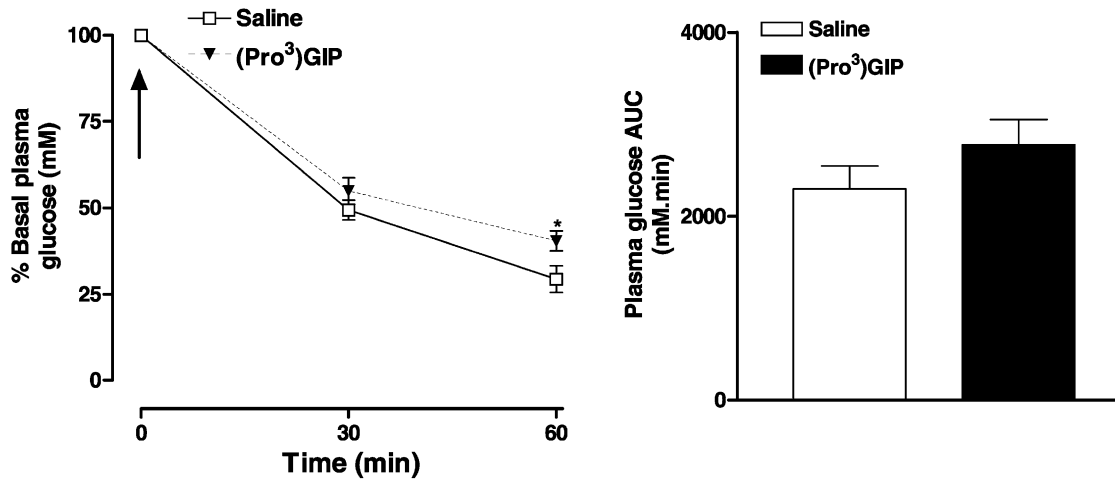


Figure 5 Effects of (Pro³)GIP treatment on insulin sensitivity.

Tests were conducted after daily treatment with (Pro³)GIP (25 nmol/kg body weight) or saline for 11 days. Insulin (50 U/kg body weight) was administered by intraperitoneal injection at the time indicated by the arrow. AUC values for 0–60 min post-injection are also shown. Values are mean±SEM for eight mice. * $p < 0.05$ compared with the saline group.

standard solid-phase Fmoc peptide chemistry as described previously (Gault et al., 2002b). The synthetic peptides were judged pure by reversed-phase HPLC on a Waters Millennium 2010 chromatography system (Software version 2.1.5) and subsequently characterized using electrospray ionization mass spectrometry (ESI-MS) (Gault et al., 2002b).

Animals

Normal mice derived from the colony maintained at Aston University, UK (Flatt and Bailey, 1981) were used at 12–16 weeks of age. Animals were housed individually in an air-conditioned room at 22±2°C with a 12 h light/12 h dark cycle. Drinking water and standard rodent maintenance diet (Trouw Nutrition, Che-

shire, UK) were freely available. All animal experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986. No adverse effects were observed following administration of (Pro³)GIP.

Experimental procedures

Mice received, over an 11-day period, once daily intraperitoneal (i.p.) injections (17.00 h) of either (Pro³)GIP (25 nmol/kg body weight) or saline vehicle (0.9%, w/v, NaCl). In the final 9 days of the study, observations were continued following discontinuation of (Pro³)GIP. Food intake and body weight were recorded daily while plasma insulin and glucose concentrations were assessed at 2–6 day intervals. Whole blood for the measure-

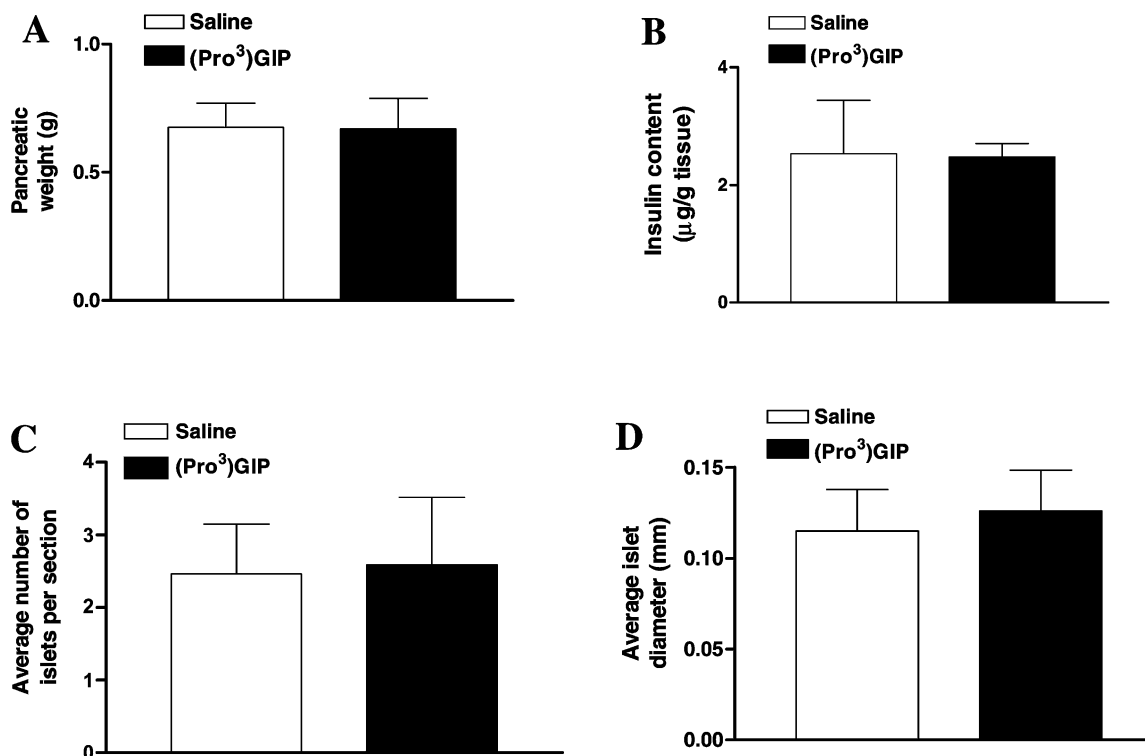


Figure 6 Effects of (Pro³)GIP treatment on (A) pancreatic weight, (B) insulin content, (C) islet number and (D) islet diameter.

Parameters were measured after daily treatment with (Pro³)GIP (25 nmol/kg body weight) or saline for 11 days. Values are mean±SEM for eight mice.

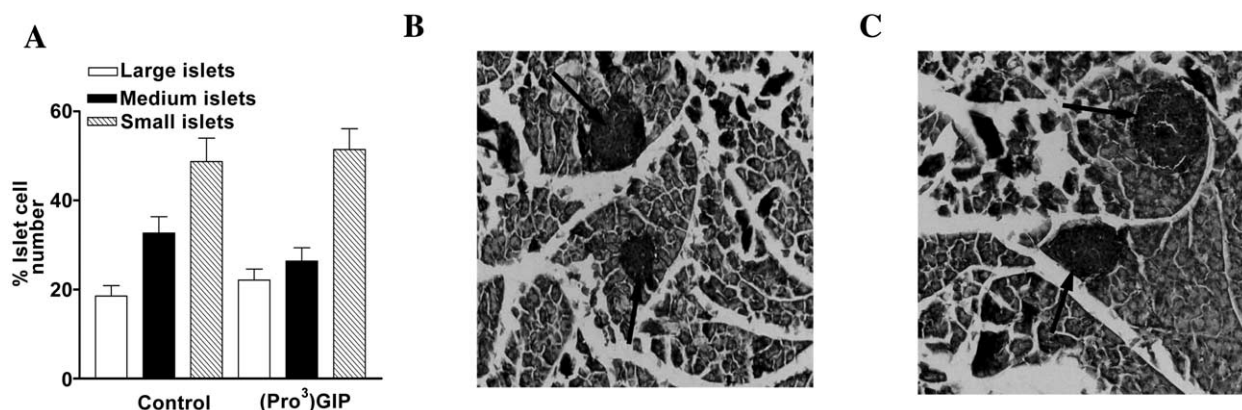


Figure 7 Effects of (Pro³)GIP treatment on islet size and morphology.

Parameters were measured after daily treatment with (Pro³)GIP (25 nmol/kg body weight) or saline for 11 days. (A) Proportion of islets classified as large (>0.15 mm) diameter, medium (0.1–0.15 mm) diameter and small (<0.1 mm) diameter are shown. Values are mean \pm SEM for eight mice. (B,C) Representative images ($\times 40$ magnification) of pancreata stained for insulin following treatment with (Pro³)GIP or saline, respectively; arrows indicate islets.

ment of glycated hemoglobin was taken on days 11 and 20. Intraperitoneal glucose tolerance (18 mmol/kg body weight) and insulin sensitivity (50 U/kg body weight) tests were performed on days 11 and 20. In a separate series, the metabolic response to 15 min feeding was examined in 18 h fasted mice. At the end of the 11 day treatment period, pancreatic tissues were excised and processed for immunohistochemistry or measurement of insulin following extraction with 5 ml/g of ice-cold acidic ethanol (750 ml ethanol, 235 ml water, 15 ml concentrated HCl). Blood samples, taken from the cut tip of the tail vein of conscious mice at times indicated in the Figures, were immediately centrifuged using a Beckman microcentrifuge (Beckman Instruments, High Wycombe, UK) for 30 s at 13 000 *g*. The resulting plasma was then aliquoted into fresh Eppendorf tubes and stored at -20°C prior to glucose and insulin determinations.

Immunohistochemistry and other analyses

For immunohistochemistry, tissue fixed in 4% paraformaldehyde/PBS and embedded in paraffin was sectioned at 8 μm . After de-waxing, sections were incubated with blocking serum (Vector Laboratories, Burlingame, USA) prior to exposure to insulin antibody. Tissue samples were then incubated consecutively with secondary biotinylated universal, pan-specific antibody (Vector Laboratories) and streptavidin/peroxidase preformed complex (Vector Laboratories). Following washing, the stained pancreatic tissue was counterstained with haematoxylin (BDH Chemicals, Dorset, UK) and then plunged into acidic methanol (500 ml methanol, 500 ml H₂O and 2.5 ml concentrated HCl) prior to dehydration and mounting in Depex (BDH Chemicals). The stained slides were then viewed under a microscope (Nikon Eclipse E2000, Diagnostic Instruments Inc., ●●●city?●●●, USA) attached to a JVC camera Model KY-F55B (JVC, London, UK) and analyzed using Kromoscan imaging software (Kinetic Imaging Limited, Faversham, Kent, UK). The average number and diameter of every islet in each section was estimated in a blinded manner using an eyepiece graticule (Graticules Limited, Tonbridge, Kent, UK) calibrated with a stage micrometer (Graticules Limited). The longest and shortest diameters of each islet were determined with the graticule. Half of the sum of these to values was then considered to be the average islet diameter. Approximately 60–70 random sections were examined from the pancreas of each mouse.

Plasma glucose was assayed by an automated glucose oxidase procedure using a Beckman Glucose Analyser II (Stevens, 1971). Plasma and pancreatic insulin was assayed by a modified dextran charcoal radioimmunoassay (Flatt and Bailey,

1981). Glycated hemoglobin (Bunn et al., 1975) was determined using cation exchange columns (Sigma, Poole, Dorset, UK) with measurement of absorbance (415 nm) in wash and eluting buffer using a VersaMax microplate spectrophotometer (Molecular Devices, Wokingham, Berkshire, UK).

In vivo data were compared using ANOVA, followed by a Student-Newman-Keuls *post hoc* test. Area under the curve (AUC) analysis performed employed the trapezoidal rule (Burlington, 1973). Groups were considered to be significantly different if $p < 0.05$.

Acknowledgments

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