Comparison of sub-chronic metabolic effects of stable forms of naturally occurring GIP(1–30) and GIP(1–42) in high-fat fed mice

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Abstract

Glucose-dependent insulintropic polypeptide (GIP) is a 42 amino acid hormone secreted from intestinal K-cells, which exhibits a number of actions including stimulation of insulin release. A truncated form, GIP(1–30), has recently been demonstrated in intestine and islet α -cells. To evaluate the potential physiological significance of this naturally occurring form of GIP, the present study has examined and compared the bioactivity of enzymatically stabilised forms, [D-Ala²]-GIP(1–30) and [D-Ala²]-GIP(1–42), in high-fat fed mice. Twice-daily injection of GIP peptides for 42 days had no significant effect on food intake or body weight. However, non-fasting glucose levels were significantly lowered, and insulin levels were elevated in both treatment groups compared to saline controls. The glycaemic response to i.p. glucose was correspondingly improved ($P < 0.05$) in [D-Ala²]-GIP(1–30)- and [D-Ala²]-GIP(1–42)-treated mice.

Furthermore, glucose-stimulated plasma insulin levels were significantly elevated in both treatment groups compared to control mice. Insulin sensitivity was not significantly different between any of the groups. Similarly, plasma lipid profile, O₂ consumption, CO₂ production, respiratory exchange ratio, and energy expenditure were not altered by 42 days twice-daily treatment with [D-Ala²]-GIP(1–30) or [D-Ala²]-GIP(1–42). In contrast, ambulatory activity was significantly ($P < 0.05$) elevated during the light phase in both GIP treatment groups compared to saline controls. The results reveal that sustained GIP receptor activation exerts a spectrum of beneficial metabolic effects in high-fat fed mice. However, no differences were discernable between the biological actions of the enzyme-resistant analogues of the naturally occurring forms, GIP(1–30) and GIP(1–42).

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Introduction

Glucose-dependent insulintropic polypeptide (GIP) is a 42 amino acid gastrointestinal hormone secreted from K-cells that performs a spectrum of biological activities through activation of GIP receptors on target cells (McIntosh *et al.* 2009). Thus, in addition to classically inhibiting gastric acid secretion, GIP has been shown more recently to exert positive effects on adipocyte metabolism, remodelling of bone and cognition (Irwin *et al.* 2010). Despite these advances, the most widely recognised role of GIP is as an important glucose-dependent stimulator of insulin secretion from pancreatic β -cells (Pederson & Brown 1976). Studies indicate that conservation of the N-terminal amino acid sequence produces GIP peptides that retain insulintropic action. In agreement with this, GIP(1–30) has been shown to exhibit equal insulin-releasing potencies in the perfused rat pancreas when compared to GIP(1–42) (Pederson *et al.* 1990).

Recent studies by Fujita *et al.* (2010a) have highlighted potential differential posttranslational processing of GIP in gut K-cells. Thus, processing of the precursor protein pro-GIP by prohormone convertase 1/3 (PC1/3) is known to yield the full-length GIP(1–42) molecule (Ugleholdt *et al.* 2006).

However, PC2 immunoreactivity has been identified in a sub-population of K-cells, which appears to result in the generation of the truncated GIP isoform, GIP(1–30) (Fujita *et al.* 2010a). This distinct sub-set of cells represents ~5–15% of the total GIP-immunoreactive cells in mice (Fujita *et al.* 2010a). Furthermore, immunohistochemical studies suggest that GIP is expressed in pancreatic islet α -cells and its secretion is under the control of PC2, again yielding the GIP(1–30) isoform (Fujita *et al.* 2010b). This bioactive form of GIP differs from the PC1/3-derived form secreted from K-cells. Therefore, GIP(1–30) appears to be a naturally occurring biologically active form, as well as GIP(1–42).

However, rapid degradation of the N-terminal region of GIP poses a major obstacle in studying native peptides to delineate biological effects *in vivo*. Thus, GIP is rapidly metabolised by the ubiquitous enzyme dipeptidylpeptidase IV (DPP IV) to release the N-terminal dipeptide Tyr¹-Ala², giving rise to a biologically inactive fragment (Deacon *et al.* 2006). However, N-terminally modified analogues of GIP have profound resistance to DPP IV-mediated degradation, such as [D-Ala²]-GIP (Hinke *et al.* 2002, Widenmaier *et al.* 2010). This form, with substitution of the natural L-Ala² for the D-Ala² isomer in GIP, has been shown in several studies to

Table 1 Baseline characteristics of experimental mice. Values are means±S.E.M. for eight mice

	Saline controls	[D-Ala ²]GIP(1–30)	[D-Ala ²]GIP(1–42)
Daily food intake (g)	4.3±0.2	4.3±0.3	4.3±0.4
Body weight (g)	57.4±1.3	56.8±2.7	57.3±1.6
Non-fasting plasma glucose (mmol/l)	6.7±0.7	6.9±0.6	6.7±0.6
Non-fasting plasma insulin (ng/ml)	1.2±0.6	2.0±0.7	1.8±0.8

Parameters were measured for 5 days prior to respective treatment regimens.

enhance insulin secretion *in vitro* and *in vivo*, mediating significantly improved glucose homeostasis in diabetic animal models (Hinke *et al.* 2002, Maida *et al.* 2009, Widenmaier *et al.* 2010).

In light of this, the present study has investigated and compared the biological effects of twice-daily administration of the enzymatically stable analogues of these two naturally occurring forms of GIP, namely [D-Ala²]GIP(1–30) and [D-Ala²]GIP(1–42), in high-fat fed mice. Given GIP(1–30) shares similar insulintropic properties to the full-length molecule (Pederson *et al.* 1990), it is anticipated that the sub-chronic administration of [D-Ala²]GIP(1–30) and [D-Ala²]GIP(1–42) would recapitulate the well-established beneficial effects of prolonged GIP receptor activation on glucose homeostasis and insulin secretion in high-fat fed mice (Kerr *et al.* 2009). However, since GIP(1–30) is reportedly less potent in stimulating lipoprotein lipase activity than GIP(1–42) (Widenmaier *et al.* 2010), the GIP molecules may have diverse effects on circulating blood lipids. Finally, the present study examines the effects of 42-day administration of [D-Ala²]GIP(1–30) and [D-Ala²]GIP(1–42) on aspects of indirect calorimetry, which have never been investigated under situations of prolonged GIP receptor activation. Thus, we will firstly establish whether GIP(1–42) has effects on indirect calorimetry, and secondly if the biological effects of the truncated GIP metabolite are similar. To this end, the results of the present study demonstrate similar beneficial effects of prolonged GIP receptor activation by both GIP-based molecules in high-fat fed mice.

Materials and Methods

Peptides synthesis

[D-Ala²]GIP(1–30) and [D-Ala²]GIP(1–42) were obtained from GL Biochem Ltd (Shanghai, People’s Republic of China). Peptides were characterised using matrix-assisted laser desorption ionisation–time of flight (MALDI–TOF) mass spectrometry (Kerr *et al.* 2009). DPP IV resistance of [D-Ala²]GIP(1–30) and [D-Ala²]GIP(1–42) was confirmed as described previously (Kerr *et al.* 2009; data not shown).

Animals

NIH male Swiss TO mice (Harlan UK Ltd) were age-matched, divided into groups and housed individually in an air-conditioned room at 22±2 °C with a 12 h light:12 h darkness cycle (0800–2000 h). Experimental animals had free access to drinking water and a high-fat diet (23.6% fat, 21.3% protein and 41.2% carbohydrate; percentage of total energy of 19.46 kJ/g; Special Diets Service, Essex, UK). Prior to commencement of studies, the experimental animals were maintained on high-fat diet from 6 weeks of age for 140 days. Obesity, insulin resistance, and hyperglycaemia were clearly manifested compared to similarly aged mice maintained on normal laboratory diet as judged by body weight, plasma insulin, and glucose analyses. All animal experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986.

Experimental protocol

Mice (n=8) received twice-daily (0930 and 1630 h) s.c. injections of [D-Ala²]GIP(1–30), [D-Ala²]GIP(1–42) (each at 25 nmol/kg body weight), or saline vehicle (0.9% (w/v), NaCl) for 42 days. Food intake and body weight were recorded every 1–2 days, while plasma glucose and insulin concentrations were monitored at 1000 h twice weekly. At the end of the study, an i.p. glucose tolerance (18 mmol/kg body weight) test and insulin sensitivity (10 U/kg body weight) test were similarly performed at 1000 h in freely fed mice. The i.p. route was chosen as the method of glucose delivery, as this will bypass the stomach and intestines and so

Figure 1 Effects of twice-daily [D-Ala²]GIP(1–30) or [D-Ala²]GIP(1–42) administration on body weight, food intake, plasma glucose and insulin concentrations in high-fat fed mice. Parameters were measured for 5 days prior to and 42 days during (indicated by black bar) treatment with saline vehicle, [D-Ala²]GIP(1–30) or [D-Ala²]GIP(1–42) (both at 25 nmol/kg body weight per day). Values are means±S.E.M. for eight mice. *P<0.05, **P<0.01 and ***P<0.001 compared to saline control.

Figure 2 Effects of twice-daily [D-Ala²]GIP(1–30) or [D-Ala²]GIP(1–42) administration on glucose tolerance and plasma insulin response to glucose in high-fat fed mice. Tests were conducted after twice-daily treatment with [D-Ala²]GIP(1–30) or [D-Ala²]GIP(1–42) (both at 25 nmol/kg body weight per day) for 42 days. Glucose (18 mmol/kg body weight) was administered by i.p. injection at the time indicated by the arrow. Plasma glucose and insulin AUC values for 0–60 min post injection are also shown. Values are means $\pm$ S.E.M. for eight mice. * $P < 0.05$ and ** $P < 0.01$ compared to saline control.

does not stimulate release of the plethora of peptides and hormones involved in glucose regulation. Thus, the sole effects of [D-Ala²]GIP(1–30) and [D-Ala²]GIP(1–42) treatment on glucose uptake and transport in peripheral tissues can be more closely examined. On the days that plasma parameters were measured, the normal daily injection at 0930 h was delayed until blood sampling was complete.

Biochemical analyses

Blood samples were collected from the cut tip of the tail vein of conscious mice at the times indicated in the figures. Blood was immediately centrifuged using a Beckman microcentrifuge (Beckman Instruments, Galway, Ireland) for 30 s at 13 000 *g*. Plasma glucose was determined by an automated glucose oxidase procedure using a Beckman Glucose Analyzer II (Beckman Instruments). Plasma insulin was assayed using a modified dextran-coated charcoal RIA as described previously (Flatt & Bailey 1981). Blood triglyceride and cholesterol levels were measured using a Hitachi Automated Analyser 912 (Boehringer).

Measurement of indirect calorimetry, energy expenditure and locomotor activity

Mice were placed in Complete Laboratory Animal Monitoring System (CLAMS) metabolic chambers (Columbus Instruments, Columbus, OH, USA) following the normal 0930 h daily injection. Consumption of O₂ and production of CO₂ were measured for 30 s at 15 min intervals for a total of 22 h. Respiratory exchange ratio (RER) was calculated by

dividing VCO₂ by VO₂. Energy expenditure was calculated using RER with the following equation $(3.815 + 1.232 \times \text{RER}) \times \text{VO}_2$. Ambulatory locomotor activity of each mouse was measured simultaneously using the optical beams (Opto M3, Columbus Instruments). Consecutive photo-beam breaks were scored as an ambulatory movement. Activity counts in X- and Z-axes were recorded every minute for 22 h. All measurements were carried out on day 42. Male mice were used to avoid influence of cyclical changes in female sex hormones on glucose homeostasis, but it should be noted that female rodents display increased activity levels compared to males (van Haaren *et al.* 1990).

Statistical analyses

Results are expressed as mean $\pm$ S.E.M. Data were compared using ANOVA, followed by a Student–Newman–Keuls *post hoc* test. Area under the curve (AUC) analyses were calculated using the trapezoidal rule with baseline subtraction. $P < 0.05$ was considered to be statistically significant.

Figure 3 Effects of twice-daily [D-Ala²]GIP(1–30) or [D-Ala²]GIP(1–42) administration on insulin sensitivity in high-fat fed mice. Tests were conducted after twice-daily treatment with [D-Ala²]GIP(1–30) or [D-Ala²]GIP(1–42) (both at 25 nmol/kg body weight per day) for 42 days. Insulin (10 U/kg body weight) was administered by i.p. injection at the time indicated by the arrow. AUC values for 0–60 min post injection are also shown. Values are means $\pm$ S.E.M. for eight mice.

Figure 4 Effects of twice-daily [D-Ala²]GIP(1–30) or [D-Ala²]GIP(1–42) administration on plasma lipid profile in high-fat fed mice. Parameters were measured after twice-daily treatment with [D-Ala²]GIP(1–30) or [D-Ala²]GIP(1–42) (both at 25 nmol/kg body weight per day) for 42 days. Values are mean $\pm$ S.E.M. for eight mice.

Results

Effects of [D-Ala²]GIP(1–30) or [D-Ala²]GIP(1–42) on food intake, body weight and non-fasting plasma glucose and insulin concentrations in high-fat fed mice

Baseline characteristics of all experimental animals are shown in Table 1. Administration of [D-Ala²]GIP(1–30) or [D-Ala²]GIP(1–42) had no effect on food intake or body weight compared to saline-treated controls over the 42-day period (Fig. 1). However, non-fasted plasma glucose concentrations were significantly ($P < 0.05$ to $P < 0.001$) decreased at numerous observation points throughout the study in all GIP-treated mice (Fig. 1). In agreement with this, plasma insulin levels were significantly ($P < 0.05$ to $P < 0.01$) elevated by day 30–42 in [D-Ala²]GIP(1–30)- and [D-Ala²]GIP(1–42)-treated mice compared to controls (Fig. 1).

Effects of [D-Ala²]GIP(1–30) or [D-Ala²]GIP(1–42) on glucose tolerance and insulin sensitivity in high-fat fed mice

As shown in Fig. 2, administration of [D-Ala²]GIP(1–30) or [D-Ala²]GIP(1–42) for 42 days significantly improved individual 60 min post injection values ($P < 0.01$ and $P < 0.05$ respectively) and the 0–60 min overall glycaemic excursion ($P < 0.05$) values following an i.p. glucose load (Fig. 2). There was a strong tendency for improvements in glycaemic levels in both GIP-treated groups at 0, 15 and 30 min post injection; however, despite the lack of data variability, these responses failed to reach significance. The insulinotropic responses were significantly ($P < 0.05$ to $P < 0.01$) elevated in terms of individual and overall AUC values in both GIP-treated groups when compared to controls (Fig. 2). However, insulin sensitivity was not significantly different between any of the groups (Fig. 3).

Effects of [D-Ala²]GIP(1–30) or [D-Ala²]GIP(1–42) on plasma lipid profile in high-fat fed mice

Administration of [D-Ala²]GIP(1–30) or [D-Ala²]GIP(1–42) for 42 days in high-fat fed mice was not associated with any significant changes in plasma total cholesterol, low density lipoprotein-cholesterol, high density lipoprotein-cholesterol or triglyceride levels, despite the lack of data variability, when compared to saline-treated controls (Fig. 4).

Effects of [D-Ala²]GIP(1–30) or [D-Ala²]GIP(1–42) on locomotor activity in high-fat fed mice

Figure 5 depicts the effects of 42 days treatment with [D-Ala²]GIP(1–30) or [D-Ala²]GIP(1–42) on locomotor activity. Both peptides significantly ($P < 0.05$) increased ambulatory activity in high-fat fed mice as assessed by X beam breaks during the light phase (Fig. 5). However, there were no significant differences in ambulatory activity between control and treatment groups during the dark phase (Fig. 5). In addition, there were no significant differences in rearing or jumping episodes during the light or dark phase, as assessed by Z beam breaks, in [D-Ala²]GIP(1–30)- or [D-Ala²]GIP(1–42)-treated mice when compared to controls (Fig. 5). Notably, activity levels of high-fat control mice had a tendency to be decreased compared to lean control counterparts (data not shown).

Effects of [D-Ala²]GIP(1–30) or [D-Ala²]GIP(1–42) on indirect calorimetry and energy expenditure in high-fat fed mice

Administration of [D-Ala²]GIP(1–30) or [D-Ala²]GIP(1–42) had no significant effect on O₂ consumption or CO₂ production when compared to controls (Fig. 6). Similarly,

Figure 5 Effects of twice-daily [D-Ala²]GIP(1–30) or [D-Ala²]GIP(1–42) administration on locomotor activity in high-fat fed mice. Parameters were measured after twice-daily treatment with [D-Ala²]GIP(1–30) or [D-Ala²]GIP(1–42) (both at 25 nmol/kg body weight per day) for 42 days. Mice were placed in CLAMS metabolic chambers, and locomotor activity was measured using optical beams. Activity counts in X- and Z-axes were recorded every minute for 22 h. Values are means $\pm$ S.E.M. for six mice. * $P < 0.05$ compared to saline control.

Figure 6 Effects of twice-daily [D-Ala²]GIP(1–30) or [D-Ala²]GIP(1–42) administration on O₂ consumption, CO₂ production, respiratory exchange ratio and energy expenditure. Parameters were measured after twice-daily treatment with [D-Ala²]GIP(1–30) or [D-Ala²]GIP(1–42) (both at 25 nmol/kg body weight per day) for 42 days. Mice were placed in CLAMS metabolic chambers, and O₂ consumption or CO₂ production were measured for 30 s at 15 min intervals. RER was calculated by dividing VCO₂ by VO₂. Energy expenditure was calculated using RER with the following Default $(3 \cdot 815 + 1 \cdot 232 \times \text{RER}) \times \text{VO}_2$. The dark phase is indicated by the black horizontal bar. Insets depict the consequence of combining light and dark phase data. Values are means $\pm$ S.E.M. for six mice.

RER and energy expenditure were not significantly altered in terms of individual measurements or overall responses during the 22 h observation period in mice treated with [D-Ala²]GIP(1–30) or [D-Ala²]GIP(1–42) (Fig. 6).

Discussion

In agreement with previous studies, twice-daily injection of high-fat fed mice with long-acting GIP receptor agonists had no adverse or toxic effects (Irwin & Flatt 2009a). Food intake and body weight were similar to saline-treated control mice, consistent with other feeding studies in rodents (Irwin & Flatt 2009a, Kerr *et al.* 2009, McIntosh *et al.* 2009). As would be expected, a major benefit of twice-daily injection of GIP peptides centred on increased insulin secretion (Irwin & Flatt 2009a). Thus, both [D-Ala²]GIP(1–30)- and [D-Ala²]GIP(1–42)-treated mice exhibited significantly elevated plasma insulin concentrations compared to controls in both the non-fasted state and following administration of glucose. This was associated with decreased plasma glucose levels and improved i.p. glucose tolerance. Protective effects of GIP

receptor signalling on islet survival as well as function may also contribute, since insulin sensitivity was unaltered. For example, 4-day administration of [D-Ala²]GIP(1–30) afforded partial protection of β -cell destruction in streptozotocin-treated rats, resulting in greater glycaemic control and insulin responses (Widenmaier *et al.* 2010). This appeared to be a result of decreased apoptosis rather than enhanced β -cell proliferation. Importantly, in the present study, there were no observed differences between the beneficial effects of GIP(1–30) and GIP(1–40). Thus, the stable forms [D-Ala²]GIP(1–30) and [D-Ala²]GIP(1–42) were equally effective in restoring glycaemic control and promoting insulin secretion in high-fat fed mice. This provides conclusive evidence that the truncated GIP form, GIP(1–30), contains the essential structural motifs for full GIP receptor binding and activation. Furthermore, the relative potency of [D-Ala²]GIP(1–30) and [D-Ala²]GIP(1–42) could possibly be improved by conjugation to a fatty acid or polyethylene glycol molecule to impede renal filtration.

Although extensive studies have been conducted on β -cell secretory function and potential antidiabetic actions of long-acting forms of GIP(1–42) (Irwin & Flatt 2009a,

Irwin *et al.* 2010), there is a paucity of information on their possible effects on locomotor activity and energy expenditure. To date, a modest increase in motor activity has been reported in mice treated sub-chronically with the specific GIP receptor antagonist (Pro³)GIP (McClean *et al.* 2007, 2008) and in GIP receptor knockout mice (Hansotia *et al.* 2007). Interestingly, [D-Ala²]GIP(1–30)- and [D-Ala²]GIP(1–42)-treated mice also displayed significantly increased locomotor activity during the light phase in the current study. The reason why similar effects are observed with compromised as well as enhanced GIP receptor activation needs further study. However, the observations in the current study are interesting given that locomotor activity of mice is normally much less during light as opposed to the dark phase. Furthermore, studies in our laboratory indicate that motor activity of high-fat fed mice is somewhat reduced compared to lean counterparts. Thus, [D-Ala²]GIP(1–30) and [D-Ala²]GIP(1–42) treatment may partially reverse this consequence of high-fat feeding.

GIP receptor knockout mice exhibit a significant reduction of respiratory quotient during the light phase, but in agreement with the current study, modulation of GIP receptor signalling did not evoke changes in oxygen consumption (Miyawaki *et al.* 2002). Furthermore, since GIP has well-documented lipogenic actions that are potentially involved in the promotion of obesity (Irwin & Flatt 2009b), the lack of effect on RER could be one explanation for the weight neutral effects of the GIP peptides. Importantly, the truncated analogue [D-Ala²]GIP(1–30) had similar effects to [D-Ala²]GIP(1–42) on all parameters relating to locomotor activity and energy expenditure. Similarly, the plasma lipid profile was not different between the two groups of treated mice. Recent studies in 3T3-L1 adipocytes suggested that GIP(1–30) was significantly less potent in stimulating lipoprotein lipase activity than GIP(1–42) (Widenmaier *et al.* 2010). The present results imply that such observations are not translated functionally to the *in vivo* setting. This could be due to a high level of redundancy in GIP receptor signalling in adipocytes or other, as yet unknown, metabolic adaptations as a result of sustained GIP receptor activation. Furthermore, it appears that if GIP(1–30) is released by a sub-population of intestinal K-cells, or even pancreatic α -cells, the effects of this truncated form would be similar to GIP(1–42). Nevertheless, GIP exerts effects on brain, bone and possibly cardiac tissue (McIntosh *et al.* 2009, Irwin *et al.* 2010), and the possibility of different responses to these two naturally occurring forms of GIP in these tissues necessitates further study.

In conclusion, the present study has characterised the bioactivity of DPP IV-resistant [D-Ala²]GIP(1–30) and [D-Ala²]GIP(1–42). The results demonstrate that the truncated and full-length forms of GIP exhibit essentially similar beneficial effects on metabolic control and energy expenditure in high-fat fed mice. Whether the enhanced activity levels observed following [D-Ala²]GIP(1–30) and [D-Ala²]GIP(1–42) administration will impact on their

usefulness as therapeutic agents remains to be elucidated. In addition, further studies are needed to delineate whether subtle differences exist between their biological activities, particularly at other GIP receptor expressing sites not examined in the current study.

Declaration of interest

VAG, NI and PRF hold shares with Diabetic Ltd.

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