Chronic treatment with exendin(9–39)amide indicates a minor role for endogenous glucagon-like peptide-1 in metabolic abnormalities of obesity-related diabetes in \textit{ob/ob} mice

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Abstract

Glucagon-like peptide-1 (GLP-1) is a potent insulino-tropic hormone proposed to play a role in both the pathophysiology and treatment of type 2 diabetes. This study has employed the GLP-1 receptor antagonist, exendin-4(9–39)amide (Ex(9–39)) to evaluate the role of endogenous GLP-1 in genetic obesity-related diabetes and related metabolic abnormalities using \textit{ob/ob} and normal mice. Acute \textit{in vivo} antagonistic potency of Ex(9–39) was confirmed in \textit{ob/ob} mice by blockade of the insulin-releasing and anti-hyperglycaemic actions of intraperitoneal GLP-1. In longer term studies, \textit{ob/ob} mice were given once daily injections of Ex(9–39) or vehicle for 11 days. Feeding activity, body weight, and both basal and glucose-stimulated insulin secretion were not significantly affected by chronic Ex(9–39) treatment. However, significantly elevated basal glucose concentrations and impaired glucose tolerance were evident at 11 days. These disturbances in glucose homeostasis were independent of changes of insulin sensitivity and reversed by discontinuation of the Ex(9–39) for 9 days. Similar treatment of normal mice did not affect any of the parameters measured. These findings illustrate the physiological extrapancreatic glucose-lowering actions of GLP-1 in \textit{ob/ob} mice and suggest that the endogenous hormone plays a minor role in the metabolic abnormalities associated with obesity-related diabetes.


Introduction

The incretin hormone glucagon-like peptide-1 (GLP-1) has potent insulino-tropic effects on pancreatic $\beta$-cells, and further promotes glucose lowering by enhancing glucose uptake and glyconeogenesis in peripheral tissues (Valverde \textit{et al.} 1994, Villaneuva-Penacarillo \textit{et al.} 1994, O’Harte \textit{et al.} 1997). In addition, GLP-1 is reported to significantly reduce food consumption through a central effect and by inhibition of gastric emptying, which ultimately generates weight loss (Turton \textit{et al.} 1996, Larsen \textit{et al.} 2001). Consequently, GLP-1 has been put forward as a potential drug candidate for type 2 diabetes mellitus, and GLP-1 analogues are currently undergoing clinical trials (Holz & Chepurny 2003).

Speculation about GLP-1 has brought renewed interest in the related peptide, exendin-4(1–39)amide (Ex(1–39)) (Eng \textit{et al.} 1992) which binds and activates the GLP-1 receptor (Göke \textit{et al.} 1993, Thorens \textit{et al.} 1993). Ex(1–39) is 39 amino acid peptide produced in the salivary glands of Helodermus suspectum (the Gila monster) and which shares similar insulino-tropic and glucose-lowering actions with GLP-1 (Greig \textit{et al.} 1999, Young \textit{et al.} 1999). Unlike GLP-1, which is rapidly degraded, Ex(1–39) is resistant to the action of dipeptidyl peptidase IV (DPP IV) and consequently has a prolonged biological action (Elahi \textit{et al.} 1999). Chronic administration of Ex(1–39) improves glucose control in diabetic \textit{db/db} mice (Greig \textit{et al.} 1999), and reduces food intake and weight gain in Zucker fatty rats (Szayna \textit{et al.} 2000). An analogue of Ex(1–39), AC2933, is currently being tested as an anti-diabetic drug (Holz & Chepurny 2003). However, the fact that Ex(1–39) is not a human physiological hormone and may trigger the immune system is something which should not be overlooked.

Interestingly, a truncated form of Ex(1–39), exendin(9–39)amide (Ex(9–39)), serves as a strong antagonist of the GLP-1 receptor, blocking the cellular and metabolic effects of GLP-1 (Göke \textit{et al.} 1993, Thorens \textit{et al.} 1993, Kolligs \textit{et al.} 1995, Green \textit{et al.} 2004). Acute studies have used Ex(9–39) to determine the contribution of GLP-1 to the incretin effect in normal animals (Kolligs \textit{et al.} 1995,
Tseng et al. 1999). These studies have revealed that GLP-1 is an important physiological insulin-releasing hormone, contributing 32–60% to the overall incretin effect.

The risk of developing type 2 diabetes, a disease clinically characterised by elevated blood glucose, impaired glucose tolerance, hyperglycaemia and insulin resistance, is greatly increased in obese individuals (Kolterman et al. 1981, Bogardus et al. 1985, Golay & Felber 1994). The role of the enteroinsular axis and GLP-1 in obesity-related diabetes remains uncertain (Ranganath et al. 1996, Flint et al. 2001, Vilsboll et al. 2003). However, recent opinion is that GLP-1 secretion may be disturbed, whereas action on pancreatic β-cells is normal (Mannucci et al. 2000, Vilsboll et al. 2003). In this investigation Ex(9–39) was employed to molecularly disrupt the action of GLP-1 in adult obese diabetic (ob/ob) mice to assess the involvement of endogenous GLP-1 in the established hyperphagia, hyperinsulinaemia, glucose intolerance and related metabolic abnormalities of this commonly employed model of type 2 diabetes (Bailey & Flatt 2003). Ex(9–39) was administered once daily for 11 days and the effects on feeding activity, body weight, glucose tolerance, pancreatic β-cell function and insulin sensitivity were recorded. These measurements were repeated after a 9-day recovery period to evaluate the effects of alleviation of GLP-1 receptor antagonism. Comparative studies were conducted in normal mice.

Materials and Methods

Reagents

Ex(9–39) was a gift from Dr Andrew Young (Amylin Corporation, San Diego, CA, USA). Na¹²⁵I for iodination of insulin was obtained from Amersham International plc (Amersham, Bucks, UK). Bovine insulin, dextran-T70 and activated charcoal were obtained from Sigma (Poole, Dorset, UK). All other chemicals used were of the utmost purity available.

Figure 1 Acute effects of Ex(9–39) on GLP-1-induced glucose lowering and insulin release in ob/ob mice. Concentrations of (A) plasma glucose and (B) insulin prior to, and 15, 30 and 60 min after intraperitoneal administration of glucose alone (18 mmol/l per kg) or in combination with 25 nmol/kg GLP-1, 25 nmol/kg GLP-1 plus equimolar GLP-1 or 25 nmol/kg GLP-1 plus equimolar Ex(9–39). The time of injection is indicated by the arrows. Plasma glucose and plasma insulin AUC values for 0–60 min after injection are shown. Values are means ± S.E.M. for eight mice. *P<0·05, **P<0·01 and ***P<0·001 compared with glucose alone. P<0·05, A*P<0·01 and A**P<0·001 compared with GLP-1 plus GLP-1.
Animals

The genetic background and characteristics of the ob/ob (Lep$^b$/Lep$^{ob}$) mouse colony and normal control homozygous (+/+) mice have been outlined elsewhere (Bailey et al. 1982). Briefly, heterozygous C57BL/6J ob+/+ breeding pairs from the Jackson Laboratory, Bar Harbor, Maine, USA were obtained by the Institute of Animal Genetics, University of Edinburgh in 1957 and out-crossed to non-inbred local strains: JH for higher litter size and CRL for higher growth rate. Heterozygous breeding pairs from this stock were obtained by Aston University in 1966 where they have been maintained in a closed non-inbred colony. The Aston colony of ob/ob mice display hyperphagia, marked hyperinsulinaemia, islet hypertrophy and moderate hyperglycaemia (Bailey & Flatt 1995).

Animals, aged 15–19 weeks, were housed individually in an air-conditioned room at 22 ± 2 °C with a 12 h light:12 h darkness cycle. Drinking water and a standard rodent maintenance diet (Trouw Nutrition Ltd, Cheshire, 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 saline or Ex(9–39).

Acute effects of Ex(9–39) on GLP-1-mediated glucose lowering and insulin release

To confirm that Ex(9–39) acted as a functional GLP-1 receptor antagonist in this group of ob/ob mice, acute experiments were performed. Fasted (18 h) ob/ob mice were administered intraperitoneally with glucose alone (18 nmol/kg body weight) or in combination with 25 nmol/kg GLP-1 or 25 nmol/kg GLP-1 plus an equivalent dose of either GLP-1 or Ex(9–39). Plasma glucose and insulin levels were measured in blood samples taken prior to and at 15, 30 and 60 min after injection.

Figure 2 Food intake, body weight and plasma glucose and insulin concentrations of ob/ob mice receiving 11 daily injections of either saline or Ex(9–39). (A) Body weight, (B) food intake, (C) plasma glucose and (D) plasma insulin concentrations were measured for 5 days prior to, for 11 days during (indicated by solid bars) and for 9 days after treatment with saline or Ex(9–39) (25 nmol/kg body weight). Values are means ± S.E.M. for eight mice. *P<0·05 compared with saline group.
Long-term effects of Ex(9–39) on metabolism

Groups of ob/ob mice and normal control mice received once daily subcutaneous injections (1700 h) of either saline (0·9% (w/v) NaCl) or Ex(9–39) (25 nmol/kg in saline) over an 11-day period. The animals were also monitored for 9 days after the cessation of treatment. Food intake and body weight were recorded daily. Blood samples were collected on days 0, 1, 3, 7, 11, 14 and 20 (0900 h) from the cut tail tips of conscious mice. Glucose tolerance (18 mmol/kg, intraperitoneally), meal tolerance (15-min refeeding after an 18-h fast) and insulin sensitivity (50 U/kg, intraperitoneally) tests were conducted on day 11 and day 20. Blood samples were collected at the times indicated in the figures into chilled fluoride/heparin-coated microcentrifuge tubes (Sarstedt, Nümbrecht, Germany) and centrifuged (30s at 13 000g) using a Beckman microcentrifuge (Beckman Instruments, Palo Alto, CA, USA). The resulting plasma was then aliquoted into fresh Eppendorf tubes and stored at −20 °C prior to analysis.

Analyses

Plasma glucose was assayed by an automated glucose oxidase procedure using a Beckman glucose analyser II (Stevens 1971). Plasma insulin was assayed by a modified dextran–charcoal radioimmunoassay (Flatt & Bailey 1981). In vivo data were compared using ANOVA, followed by the Student–Newman–Keuls post hoc test. Area under the curve (AUC) analysis employed the trapezoidal rule (Burington 1973). Groups of data from both were considered to be significantly different if P<0·05.

Results

Acute Ex(9–39) antagonism of GLP-1 action

Figure 1 shows the plasma glucose and insulin responses of ob/ob mice to glucose alone or in combination with either GLP-1 plus equipotent GLP-1 or Ex(9–39). As expected, administration of GLP-1 markedly decreased the glycaemic excursion (Fig. 1A) and enhanced insulin concentrations (Fig. 1B) compared with glucose alone.
These actions of GLP-1 were substantially blocked by the established GLP-1-receptor antagonist, Ex(9–39) (Fig. 1A and B).

Long-term effects of Ex(9–39)

Figure 2 shows the effects of long-term Ex(9–39) treatment on body weight, food intake and plasma concentrations of glucose and insulin in ob/ob mice. Ex(9–39) had no significant effect on either body weight or food intake (Fig. 2A and B). However, on day 11 of treatment basal plasma glucose levels were significantly raised in mice treated with Ex(9–39), compared with those treated with saline (P<0.05; Fig. 2C). Cessation of treatment returned glucose to pretreatment levels. No significant changes in plasma insulin levels were noted during or after the treatment period, although there appeared to be a trend towards decreased concentrations with Ex(9–39) (Fig. 2D). Treatment of normal mice with Ex(9–39) did not affect any of the parameters measured (Fig. 3).

Long-term effects of Ex(9–39) treatment on glucose tolerance

Figure 4 shows the effects of intraperitoneal glucose administration (18 mmol/kg) on glucose and insulin levels in ob/ob mice treated for 11 days with either saline or Ex(9–39). Glucose levels in Ex(9–39)-treated mice were significantly elevated 0, 15, 30 and 60 min after injection compared with mice treated with saline alone (Fig. 4A). This was confirmed by a significantly increased AUC value (P<0.05; Fig. 4A). No significant changes in glucose-mediated insulin release were noted in mice treated with Ex(9–39) compared with those treated with saline (Fig. 4B). As shown in Fig. 5, no significant changes in plasma glucose or insulin levels were evident in ob/ob mice 9 days after cessation of Ex(9–39)-treatment. Treatment of normal mice with Ex(9–39) for 11 days did not significantly affect glycaemic or insulin responses to intraperitoneal glucose (Fig. 6). Responses of normal mice were identical 9 days following cessation of treatment (data not shown).
Long-term effects of Ex(9–39) treatment on metabolic response to feeding

Figure 7 shows the glucose and insulin responses of fasted (ob/ob) mice to feeding after 11 days of treatment with Ex(9–39). An allowed period of 15 min of feeding caused significant rises in both plasma glucose (Fig. 7A) and insulin (Fig. 7B). However, responses in saline-treated mice and Ex(9–39)-treated mice were not significantly different. Food intake during the 15-min period was consistent with these data, showing no significant difference in saline-treated (0·5 ± 0·1 g/mouse per 15 min) and Ex(9–39)-treated (0·6 ± 0·1 g/mouse per 15 min) mice. Similarly 11 days of treatment with Ex(9–39) did not affect glucose or insulin responses to feeding in normal mice (Fig. 8). Food intake was 0·5 ± 0·1 g/mouse per 15 min and 0·5 ± 0·1 g/mouse per 15 min for control and Ex(9–39) respectively.

Long-term effects of Ex(9–39) treatment on insulin sensitivity

Figure 9 shows the effect of intraperitoneal administration of insulin on glucose levels in ob/ob mice (Fig. 9A) and normal mice (Fig. 9B) following 11 days of treatment with Ex(9–39). No significant changes in insulin-induced glucose lowering were observed in animals receiving treatment or 9 days following the cessation of treatment (data not shown).

Discussion

Ex(1–39) is a 39 amino acid peptide isolated from Heloderma suspectum (Gila monster) venom (Eng et al. 1992). It later transpired that Ex(1–39) was a highly potent agonist of the GLP-1 receptor (Thorens et al. 1993). Furthermore, a shortened form of this peptide, Ex(9–39), possessed significant antagonistic activity for this receptor (Göke et al. 1993, Thorens et al. 1993). These findings have been confirmed many times in acute tests involving rat GLP-1 receptors, humans (Schirra et al. 1998, Edwards et al. 1999) and various non-diabetic and diabetic animal models (Kolligs et al. 1995, Wang et al. 1995, Gault et al. 2003, Green et al. 2004).

Although many studies have demonstrated acute antagonistic actions of Ex(9–39), the chronic effects of Ex(9–39)
administration on insulin secretion and glucose homeostasis in animal models with established obesity-related diabetes such as \textit{ob/ob} mice have not been evaluated. Given the beneficial effects of chronic administration of the GLP-1 receptor agonist, Ex(1–39), in \textit{db/db} mice (Greig \textit{et al.} 1999, Szayna \textit{et al.} 2000), plus the role of GLP-1 in the enteroinsular axis, we anticipated that chronic administration of Ex(9–39) would have quite profound effects in \textit{ob/ob} mice. This animal model is characterised by a less severe form of diabetes than \textit{db/db} mice due to metabolic compensation through islet hypertrophy and marked hyperinsulinaemia (Bailey & Flatt 2003). In initial studies, we confirmed that this mutant displayed prominent insulin secretory and anti-hyperglycaemic responses to GLP-1 as described elsewhere (Green \textit{et al.} 2004). Furthermore, administration of Ex(9–39) reduced the acute glucose-lowering action of GLP-1 by 70%, while the insulin-releasing action of GLP-1 was almost completely abolished (reduced by 95%). These results corroborated the findings of earlier investigations (Kolligs \textit{et al.} 1995, Schirra \textit{et al.} 1998, Edwards \textit{et al.} 1999, Tseng \textit{et al.} 1999, Green \textit{et al.} 2004).

Administration of Ex(9–39) once daily for 11 days to \textit{ob/ob} mice had no effect on feeding activity or body weight. Since GLP-1 is believed to exert satiety effects which can be reversed by Ex(9–39) in other species (Schick \textit{et al.} 2003), this suggests that endogenous circulating GLP-1 has no such role in \textit{ob/ob} mice. However, the present study did not note an inhibitory effect of Ex(9–39) on feeding in normal mice. Consistent with a limited physiological role for circulating GLP-1 in \textit{ob/ob} mice, basal and glucose-stimulated insulin secretion were not significantly changed by chronic Ex(9–39) treatment. However, small impairments in glucose homeostasis were noted, namely elevated basal glucose and impaired glucose tolerance after 11 days. These effects were independent of changes in insulin sensitivity and reversed by discontinuation of Ex(9–39) for 9 days.

In contrast to \textit{ob/ob} mice, administration of Ex(9–39) to normal mice for 11 days was without an effect on glucose homeostasis or any of the other parameters measured. This showed that the modest effects in \textit{ob/ob} mice cannot be directly attributed to leptin deficiency. These general observations have parallels with GLP-1 receptor knockout (GLP-1R\textsuperscript{-/-}) mice which exhibit only a modest intolerance to glucose (Scrocchi \textit{et al.} 1996, Flamez \textit{et al.} 1998, Scrocchi & Drucker 1998). In these transgenic animals it appears likely that there was a compensatory increase in the glucose-dependent insulintropic polypeptide (GIP) arm of the enteroinsular axis from early life (Pederson \textit{et al.} 1998). Although a similar adaptive response might have been induced very rapidly by the present 11-day administration of Ex(9–39), there is presently no commercial assay to specifically measure active

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\caption{Long-term effects of Ex(9–39) treatment on glucose and insulin responses to intraperitoneal glucose administration in normal mice. (A) Plasma glucose and (B) plasma insulin concentrations were measured prior to and at intervals after intraperitoneal administration of glucose (18 mmol/kg body weight) after 11 days treatment with Ex(9–39). The time of injection is indicated by the arrows. Plasma glucose and plasma insulin AUC values for 0–60 min after injection are shown. Values are means ± S.E.M. for eight mice.}
\end{figure}
GIP(1–42) in small plasma volumes for mice. However, it is notable that the adverse effects on glucose homeostasis in ob/ob mice were not matched by any significant changes in insulin secretion. This supports the view that GLP-1 not only facilitates glucose lowering though glucose-dependent insulin release but also by a range of additional extrapancreatic effects (Valverde et al. 1994, Villanueva-Penacarillo et al. 1994, O’Harte et al. 1997).

Although rather little is known about concentrations of ‘active’ GLP-1 in the circulation and intestines of ob/ob mice, there are no appreciable differences compared with normal mice (Mooney et al. 2002, Anini & Brubaker 2003). Indeed, plasma concentrations of GLP-1 appear to be decreased generally in obesity due to insensitivity to the normal stimulatory effects of leptin (Ranganath et al. 1996, Anini & Brubaker 2003). Interestingly, higher levels of DPP IV activity have been reported in ob/ob mice compared with lean controls (Ruter et al. 2004), but the significance of this observation needs clarification. In sharp contrast, early studies indicate that the sister incretin hormone, GIP, is present in both the plasma and intestines of ob/ob mice at remarkably raised concentrations (Flatt et al. 1983, 1984). Taken together, these observations suggest a relatively minor role for circulating GLP-1 in the metabolic abnormalities of ob/ob mice. In contrast, GIP appears to be the major physiological component of the enteroinsular axis in these animals, as evidenced by acute blockade of GIP action with the receptor antagonist (Pro3)GIP (Gault et al. 2003). The established effects of GIP on insulin secretion and adipose tissue metabolism (Yip & Wolfe 2000) also indicate that markedly elevated GIP levels in this mutant contribute substantially to the characteristic hyperinsulinaemia, fat disposition and insulin resistance (Bailey & Flatt 2003).

In conclusion, this study has shown that prolonged antagonism of circulating GLP-1 in obese diabetic (ob/ob) mice with Ex(9–39) caused a slight impairment of glucose homeostasis without appreciable effects on food intake, pancreatic β-cell function or insulin sensitivity. No significant effects were observed in normal mice. These observations indicate a minor role for endogenous GLP-1 in the metabolic abnormalities of ob/ob mice and suggest that elevated concentrations of GIP make a major contribution to the obesity-related diabetes syndrome.
Figure 8 Long-term effects of Ex(9–39) treatment on glucose and insulin responses to feeding in normal mice. Following the 11-day treatment period mice were fasted (18 h) overnight. At 0900 h, free access to food was allowed for 15 min (indicated by the solid bars) and (A) plasma glucose and (B) plasma insulin concentrations were measured. Plasma glucose and plasma insulin AUC values for 0–105 min after feeding are shown. Values are means ± S.E.M. for eight mice.

Figure 9 Insulin sensitivity of (A) ob/ob mice and (B) normal mice chronically treated for 11 days with Ex(9–39). The time of injection is indicated by the arrows. Plasma glucose concentrations were measured prior to and at intervals after intraperitoneal administration of insulin (50U/kg body weight). Corresponding AUC values (0–60 min) for plasma glucose show overall effects to exogenous insulin administration.
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References


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