Reduced GLP-1 and insulin responses and glucose intolerance after gastric glucose in GRP receptor-deleted mice

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Persson, Kristin, Ronald L. Gingerich, Sonali Nayak, Keiji Wada, Etsuko Wada, and Bo Ahren. Reduced GLP-1 and insulin responses and glucose intolerance after gastric glucose in GRP receptor-deleted mice. Am J Physiol Endocrinol Metab 279: E956–E962, 2000.—By applying a newly developed ELISA technique for determining biologically active intact glucagon-like peptide [GLP-1, GLP-1-(7–36)amide] in mouse, plasma baseline GLP-1 in normal NMRI mice was found to be normally distributed (4.5 ± 0.3 pmol/l; n = 72). In anesthetized mice, gastric glucose (50 or 150 mg) increased plasma GLP-1 levels two- to threefold (P < 0.01). The simultaneous increase in plasma insulin correlated to the 10-min GLP-1 levels (r = 0.36, P < 0.001; n = 12). C57BL/6J mice deleted of the gastrin-releasing peptide (GRP) receptor by genetic targeting had impaired glucose tolerance (P = 0.030) and reduced early (10 min) insulin response (P = 0.044) to gastric glucose compared with wild-type controls. Also, the GLP-1 response to gastric glucose was significantly lower in the GRP receptor-deleted mice than in the controls (P = 0.045). In conclusion, this study has shown that 1) plasma levels of intact GLP-1 increase dose dependently on gastric glucose challenge in correlation with increased insulin levels in mice, and 2) intact GRP receptors are required for normal GLP-1 and insulin responses and glucose tolerance after gastric glucose in mice.

INSULIN SECRETION IS REGULATED by circulating nutrients, such as glucose and amino acids, by paracrine factors produced by islet endocrine cells, by autonomic nerves innervating the islets, and by gut hormones released during food intake (2). The integrative action of these factors enables the $\beta$-cells to secrete insulin in an optimal fashion, thereby preventing glucose from deviating to hyper- or hypoglycemic levels. This interplay between several factors is particularly important during food intake, when, besides the autonomic (parasympathetic) nerves and ingested and absorbed nutrients, gut hormones, the so-called incretin factors, are relevant (13). One important gut hormone in this respect is glucagon-like peptide 1 (GLP-1), which is released from the intestinal L cells during food intake and which augments glucose-stimulated insulin secretion (1).

GLP-1 is stored in the secretory granules of the L cells, which are located mainly in the distal portion of the small intestine and in the colon (9). The peptide is released into the bloodstream when the cells are activated. The most important stimulus for secretion of GLP-1 is ingestion of a mixed meal or oral ingestion of glucose (3, 24, 28). The main activator of the L cells is enteral glucose reaching the small intestine, because the secretion of GLP-1 correlates with the gastric emptying rate (28), and enteral glucose stimulates GLP-1 secretion in the isolated perfused canine ileum (36). However, intestinal hormones and neurotransmitters also affect GLP-1 secretion from the intestine. Thus it has been shown in rat L cells that GLP-1 secretion is stimulated by gastric inhibitory polypeptide (GIP), gastrin-releasing peptide (GRP), and the muscarinic agonist bethanechol and is inhibited by somatostatin (6, 16, 31). Furthermore, in the pig ileum, GLP-1 secretion is stimulated by GRP, substance P, and neurokinin A, whereas the secretion is inhibited by adrenergic nerve activation (17). The regulation of GLP-1 secretion is important to establish, because GLP-1 release seems to be impaired in fully developed type 2 diabetes (37). Furthermore, GLP-1 is required for normal glucose tolerance, because GLP-1 receptor antagonism by exendin results in glucose intolerance after oral glucose in rats and humans (8, 23, 38) and because mice lacking the GLP-1 receptor display glucose intolerance after oral glucose (32). Improvement of GLP-1 release, therefore, might be a feasible mode for the treatment of diabetes in conjunction with the use of exogenous administration of the peptide (1). To enforce such a strategy, however, a prerequisite is more detailed knowledge of the regulation of endogenous GLP-1 levels.
In this study, we examined whether the neuropeptide GRP is important for the circulating levels of GLP-1. GRP is the mammalian homolog to the amphibian peptide bombesin and consists of a 27-amino acid residue that was initially isolated from the porcine gastrointestinal tract (26). The peptide has been shown to be a neuropeptide in the gastrointestinal tract and thereby is located in intrinsic neurons distributed from the fundus to the distal colon (29). In the gastrointestinal tract, a variety of actions of GRP have been documented, like stimulation of gastrin and pepsinogen secretion, and actions on motility (26, 33, 35). GRP seems also to be involved in the local regulation of GLP-1 secretion, because, in rat L cells and pig ileum preparations, the peptide stimulates the secretion of GLP-1 (6, 16, 17), and, in the anesthetized rat, GRP potentiates GLP-1 release induced by intraduodenal fat, and a GRP antagonist abolishes the GLP-1 response to duodenal fat (31). GRP is, however, also of relevance for insulin secretion and glucose tolerance as an islet neuropeptide, because it has been localized at islet nerve terminals to be released from the pancreas during vagal nerve activation and to stimulate insulin secretion both in vivo and in vitro through a direct action on the islet β-cells (2, 11, 19, 20, 22, 30). Therefore, the insulinotropic action of GRP might be due to both a direct islet action of the peptide and an indirect action through the release of GLP-1.

The bombesin-like peptides, like GRP, bind to G protein-coupled receptors on the cell surface, of which three have been cloned: the GRP receptor, the neuromedin B receptor, and the bombesin receptor subtype 3 (4, 10, 40). Recently, GRP receptor-deleted mutant mice were generated by gene targeting (14, 39). These mice were shown to display increased locomotor activity during the dark period and increased social responses against an intruder but otherwise normal development (39). Furthermore, the mice exhibited loss of bombesin-induced suppression of feeding (14). Moreover, these mice displayed normal baseline values of glucose and insulin (39). This does not exclude, however, involvement of GRP and the GRP receptor in the homeostasis of insulin secretion and glucose tolerance after food intake. In this study, therefore, we have examined the relevance of the GRP receptor for GLP-1 and insulin responses to enteral administration of glucose by giving gastric glucose gavage to mice lacking the GRP receptor.

For these studies, we applied an ELISA technique for the nonradioactive quantification of the biologically active GLP-1 to studies in mouse plasma. It is known that active GLP-1 [i.e., GLP-1(7–36)amide] is rapidly inactivated after its release from the intestinal L cells by the enzyme dipeptidyl peptidase IV (DPP IV) (15, 27). This enzyme truncates GLP-1 by liberating the two NH₂-terminal amino acids (histidine-alanine), thereby yielding GLP-1(9–36)amide, which is not only an inactive metabolite but may also function as an inhibitor of GLP-1 (15, 17, 21, 41). Due to this rapid inactivation, the half-life of active GLP-1(9–36)amide is only 1–1.5 min (7), and only ~20% of total GLP-1 immunoreactivity consists of the active GLP-1 (12). Assays using an antibody directed to the NH₂-terminal end of GLP-1, however, allow direct conclusions concerning the active form of GLP-1.

The aims of the study were, therefore, 1) to characterize the ELISA technique for measuring biologically active GLP-1 in mice and 2) to explore the relevance of the GRP receptor to GLP-1 and insulin responses and glucose tolerance after gastric glucose gavage in mice.

MATERIALS AND METHODS

Animals. For the characterization of the GLP-1 assay in vivo and the study on the dose-response relationship between gastric glucose and plasma GLP-1, normal female NMRI mice weighing 20–25 g were used. These mice were obtained from Bomholtgaard Breeding and Research Center, Ry, Denmark. For the studies on the involvement of the GRP receptor in the regulation of plasma GLP-1, male GRP receptor-deleted mutant mice and their wild-type littermates were used. The GRP receptor gene is located on chromosome X in both mice and humans (25). Male mice of the C57BL/6J background hemizygous for deletion of the GRP receptor gene were generated by homologous recombination in embryonic stem cells as previously described (39). The disruption of the GRP receptor gene in mutant was confirmed by Southern hybridization with the use of tail DNA. As previously demonstrated, mutant (+/Y) and wild-type (+/+Y) offspring were born at the same ratio, and GRP receptor-deficient mice were viable and fertile, with no abnormality observed on gross and routine histological analysis in the brain, lung, and gastrointestinal tract (39). All animals in the study were fed a standard pellet diet and tap water ad libitum. The study was approved by the Ethics Committee of Lund University.

Gastric glucose tolerance test. The mice were fasted for 2 h and then anesthetized with an intraperitoneal injection of midazolam (Dormicum, Hoffman-LaRoche, Basel, Switzerland, 0.4 mg/mouse) and a combination of fluanison (0.9 mg/mouse; Hyp Nora, Janssen, Beerse, Belgium). At 30 min after induction of anesthesia, a blood sample (75 or 150 μl) was taken from the retrobulbar intraorbital capillary plexus, whereafter D-glucose (17, 50, or 150 mg/mouse dissolved in 0.5 ml saline) or saline was administered through a gavage tube (OD 1.2 mm) placed in the stomach. New blood samples were taken after 10, 30, and 60 min. The samples were taken in heparinized tubes and stored on ice. After centrifugation, plasma was separated and stored at −20°C until analysis.

Assay of GLP-1. An ELISA was recently developed to measure biologically active GLP-1 (GLP-1(7–36)amide) in plasma (Linco Research, St. Charles, MO). The assay uses monoclonal guinea pig antibodies specific for the NH₂- and COOH-terminal ends of GLP-1. The assay, therefore, does not react with its inactivation product, GLP-1(9–36)amide. Synthetic GLP-1 (Peninsula Labs, Merseyside, UK) was used as a calibrator (2, 5, 10, 20, 50, and 100 pmol/l). The assay was performed as described in the kit protocol (Linco). Recovery of different amounts of synthetic GLP-1 (Peninsula; 2–100 pmol/l) added to a mouse plasma pool (with baseline GLP-1 concentration of 4 pmol/l) is shown in Table 1. Recovery ranged from 85 to 103% over a range of 4 to 108 pmol/l. Furthermore, by use of 100, 50, and 25 pmol/l of plasma in the assay, measures were revealed in the range from 67 to 120% of expected values (Table 2). Intra- and interassay variations were assessed by repeated analysis of four plasma samples containing from 4.8 to 72.3 pmol/l GLP-1. Coefficients of
Table 1. Recovery of GLP-1 in mouse plasma

<table>
<thead>
<tr>
<th>Added GLP-1, pmol/l</th>
<th>Recovery, %</th>
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<tr>
<td>0</td>
<td>100 ± 3</td>
<td>7</td>
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<tr>
<td>2</td>
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<td>8</td>
</tr>
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<tr>
<td>100</td>
<td>103 ± 2</td>
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Values are means ± SE; n = number of samples. Glucagon-like peptide 1 (GLP-1) at different concentrations was added to a mouse plasma pool with a plasma concentration of 4 pmol/l. Plasma was then determined for GLP-1 concentrations, and recovery was calculated.

Table 2. Dilution of mouse plasma and determination of GLP-1

<table>
<thead>
<tr>
<th>Added Volume</th>
<th>Observed % of Expected Concentration</th>
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<tbody>
<tr>
<td>100 µl</td>
<td>100 ± 1</td>
<td>4</td>
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<tr>
<td>50 µl</td>
<td>99 ± 4</td>
<td>4</td>
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<tr>
<td>25 µl</td>
<td>86 ± 4</td>
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Values are means ± SE; n = number of samples. Four plasma samples with known concentrations of GLP-1 were diluted, and GLP-1 concentrations of the diluted samples were determined.

RESULTS

Baseline GLP-1, glucose, and insulin in mice. Figure 1 shows the pattern of distribution of baseline GLP-1 concentration in 72 female NMRI mice subjected to a standardized 2-h fasting period. It is seen that fasting GLP-1 displayed a normal distribution pattern with a tendency toward a distribution skewed to the right (P = 0.070 according to the Kolmogorov-Smirnov goodness-of-fit test). The mean of the distribution of plasma GLP-1 was 4.5 pmol/l (SD 2.1 pmol/l, SE 0.3 pmol/l). In 42 of the mice, glucose and insulin were also determined in the same samples. In these 42 mice, plasma GLP-1 was 4.5 ± 0.2 pmol/l, plasma glucose was 9.2 ± 0.2 mmol/l, and plasma insulin was 293 ± 25 pmol/l.

Fig. 1. Distribution of baseline plasma glucagon-like peptide 1 (GLP-1) in 2-h-fasted female NMRI mice (n = 72).

Statistics. Values shown are means ± SE unless otherwise stated. Statistical analyses were performed with the SPSS for Windows system. Statistical comparisons between groups were performed with Student's unpaired t-test with Bonferroni post hoc correction for multiple comparisons and with ANOVA. Pearson's product-moment correlation coefficients were obtained to estimate linear correlation between variables, and forward stepwise multiple linear regression was applied for determination of dependency of influences between significantly related variables. Normality of distribution was tested with the Kolmogorov-Smirnov goodness-of-fit test.

Comparison of GLP-1, glucose, and insulin responses to gastric glucose in mice. Figure 2 shows the GLP-1, glucose, and insulin responses to a gastric challenge of various amounts of glucose or saline in 2-h-fasted female NMRI mice. It is seen that, after gastric administration of glucose at 150 mg, plasma GLP-1 levels had significantly increased to 11 ± 0.8 pmol/l (n = 31, P < 0.001) after 10 min, i.e., approximately threefold (P = 0.009). Plasma GLP-1 levels were also significantly elevated at 30 min (P = 0.018) but had returned to baseline levels at 60 min. Like baseline GLP-1, stimulated GLP-1 levels also displayed a normal distribution pattern (P = 0.105 according to the Kolmogorov-Smirnov goodness-of-fit test). After the challenge by 50 mg of glucose, only the 10-min GLP-1 levels were significantly elevated above baseline (P = 0.002), and after the challenge by 17 mg of glucose, no significant changes in plasma GLP-1 levels were seen. Glucose levels were markedly increased after the challenge of 150 and 50 mg glucose (P < 0.001 for both) but were not significantly elevated after the 17-mg glucose challenge. Plasma insulin levels were markedly increased after the challenge of 150 and 50 mg of glucose and also increased at 10 min (640 ± 82 pmol/l vs. 282 ± 29 pmol/l at baseline, P = 0.003) after the challenge by 17 mg of glucose. GLP-1, glucose, or insulin levels were not significantly altered after administration of saline.

Comparison of GLP-1, glucose, and insulin responses to gastric glucose. Figure 3 compares the increase in the three variables at 10 min after administration of glucose at the three doses or saline in percentage of the maximal increase. It is seen that, except for the ab-
sence of any effect on plasma GLP-1 and glucose after administration of 17 mg of glucose, the three variables increased in parallel and in linearity to the logarithmically transformed glucose dose. To compare the relative importance of the increase in GLP-1 vs. glucose for the increase in insulin after gastric glucose, univariate and multiple correlations between these variables were performed. It was found that the increase in plasma insulin at 10 min (i.e., the Δ value obtained by subtracting the baseline insulin value from the 10-min value) across all animals (n = 58) subjected to the gastric gavage (regardless of glucose dose or saline given) correlated significantly with the 10-min values of both glucose (r = 0.36, P = 0.011) and GLP-1 (r = 0.36; P = 0.017). This suggests that both glucose and GLP-1 are determinators of the increase in insulin after gastric glucose. To determine whether glucose and GLP-1 are independent predictors of the rise in plasma insulin after gastric glucose, a forward stepwise multiple linear regression model was performed, with the increase in plasma insulin at 10 min as the dependent variable and the 10-min values of plasma glucose and GLP-1 as independent variables. It was found that only the GLP-1 level was an independent predictor of insulin (r = 0.36; P = 0.017), whereas in this model, the 10-min glucose value was not significantly independently related to insulin (r = 0.22, NS).

GLP-1, glucose, and insulin responses to gastric glucose in GRP receptor deleted mice. To study whether intact GRP receptors are required for the GLP-1, glucose, and insulin responses to enteral administration of glucose, glucose (150 mg) was administered gastrically to GRP receptor-deleted C57BL/6J mice and their wild-type controls. It was found (Fig. 4) that the GLP-1 response to gastric glucose administration was reduced compared with the controls, because the 10-min GLP-1 value was significantly lower (P = 0.045). Glucose levels were higher in GRP receptor-deleted mice than in controls at 30 min after glucose administration (P = 0.030), whereas plasma insulin levels were significantly lower at 10 min (P = 0.044). Thus GRP receptor-deficient mice displayed inhibited GLP-1 response to gastric glucose in conjunction with impaired insulin secretion and glucose intolerance. Analyzing the entire data pool with ANOVA revealed significantly lower GLP-1 (P = 0.036) and insulin (P = 0.022) responses to gastric glucose in the GRP receptor-deleted mice than in the controls.

DISCUSSION

In this study, we have applied a newly developed ELISA technique for measurement of biologically active GLP-1 in mice. This assay will allow investigations of factors involved in the regulation of this important incretin hormone in experimental mouse models. A monoclonal antibody used in the assay is reacting specifically with the NH₂-terminal portion of GLP-1, and
We also found that plasma GLP-1 was increased within the same range as that previously determined in humans. The plasma concentration of active GLP-1 in the basal state in the mice was 4.5 pmol/l, which is within the same range as that previously determined in humans (3). We also found that plasma GLP-1 was increased when glucose was administered through a gastric tube. A twofold increase was observed after administration of 50 mg of glucose and a threefold increase when 150 mg of glucose were administered. The maximal increase was observed at 10 min after gastric glucose administration, illustrating the rapid secretory response in the L-cells to ingested nutrients. The glucose levels at 10 min after glucose administration did not differ in mice given 50 vs. 150 mg of glucose, yet the GLP-1 levels were more markedly elevated after 150 mg of glucose. This shows that plasma glucose levels do not contribute to the degree of the GLP-1 response, which confirms previous results that the main stimulus for GLP-1 secretion is the enteric presentation of glucose (1, 28, 36). The rapid GLP-1 response is, however, not due to the presence of nutrients close to the L cells, because passage of nutrients to distally located cells requires a longer period of time. Rather, the rapid GLP-1 response seems mediated by nerves that are activated by nutrient ingestion, which innervate the L cells (1). The impairment of the GLP-1 response to gastric glucose in the GRP receptor-deleted mice indicates, furthermore, that GRP nerves are involved in this action. In fact, previous studies in rat L cells and in the perfused pig ileum have shown that exogenous administration of GRP stimulates the secretion of GLP-1 (6, 16, 17), and, in the anesthetized rat, a GRP antagonist abolishes the GLP-1 response to duodenally introduced fat (31). Thus, because GRP is a gut neurotransmitter (29), a neural GRP-ergic action seems to be responsible for at least part of the rapid GLP-1 response to food intake, and our study also shows that this is executed through the GRP receptors.

The increase in plasma insulin after gastric glucose administration was directly proportional to the glucose load, displaying a straight linear relation with the logarithmic transformation of the glucose dose. The 10-min increase in plasma insulin correlated with the 10-min values in both GLP-1 and glucose, showing that both of these variables contribute to the insulin response. Interestingly, however, a forward stepwise multiple regression model including both GLP-1 and glucose levels as independent variables showed that the increase in insulin was significantly dependent only on the GLP-1 levels and not on the glucose levels. This shows that, under these conditions, GLP-1 is of greater importance than glucose for eliciting a graded insulin response. This is also evident considering that the increase in plasma glucose at 10 min after glucose administration was the same regardless of administration of 50 or 150 mg of glucose, yet the insulin response was augmented at 150 vs. the response at 50 mg of glucose (as was the GLP-1 response), illustrating that GLP-1 is an important incretin factor in mice. It may be hypothesized that glucose initiates secretion of insulin by the β-cells but that the fine tuning of the degree of stimulation is governed by the increase in GLP-1 levels. Previously, an important incretin action of GLP-1 was suggested in rats and humans, because exendin, a specific GLP-1 receptor antagonist, prevents the insulin response to oral glucose (8, 23, 38) and in

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**Fig. 4.** Plasma levels of GLP-1 (top), glucose (middle), and insulin (bottom) immediately before and at 10, 30, and 60 min after gastric administration of glucose (150 mg/mouse) in 2-h-fasted anesthetized male GRP receptor-deleted C57BL/6J mice or their wild-type controls. Values are means ± SE. Asterisks indicate the probability level of random difference between the groups: *P < 0.05; n = 10 GRP receptor-deleted animals, n = 9 controls.
GLP-1 receptor-deleted mice, which exhibit impaired insulin response to oral glucose (32).

In this study, we also found that glucose tolerance after gastric glucose administration was slightly impaired in GRP receptor-deleted mice, accompanied by an impaired early (10 min) insulin response. This shows that intact GRP receptors are required for a normal insulin response and glucose tolerance after enteral glucose, supporting a role for GRP in the prandial insulin response. Previously, GRP was shown to be localized at nerve terminals in the islets (29) and to be released from the pancreas by vagal nerve activation (22). GRP has also been shown to stimulate insulin secretion in mice (19, 30). This shows that GRP is a parasympathetic islet neurotransmitter, and because the parasympathetic nerves have been shown to be important for the normal insulin response to food intake, i.e., the so-called cephalic phase of insulin secretion (5, 34), the impaired insulin response to gastric glucose in our present study would at first impression indicate that GRP participates in this process by being released from islet nerve terminals and then directly stimulating insulin secretion from the islet β-cells. However, the present study offers an alternative suggestion, because the GRP receptor-deleted mice were found to exhibit impaired GLP-1 response to the gastric glucose administration. Hence, the impairment of the GLP-1 response to gastric glucose in the GRP receptor-deleted mice may explain the impaired insulin response. The relative contribution of this effect vs. impairment of local islet neurotransmitter action for the impaired insulin response to gastric glucose in GRP receptor-deleted mice remains to be established.

Previously, the GRP receptor was suggested to be involved in behavioral physiology, because GRP receptor-deleted mice were shown to display increased locomotor activity during the dark period and increased social responses against an intruder but showed otherwise normal development (39). Furthermore, GRP receptor-deleted mice have also been shown to exhibit abnormal feeding behavior, because the bombesin-induced suppression of feeding was absent (14). The present study suggests that another function of the GRP receptor is its involvement in the regulation of the GLP-1 and insulin responses to enteral administration of glucose. In fact, based on the results of this study of lowered plasma GLP-1 and insulin levels and glucose intolerance after gastric glucose in the GRP receptor-deleted mice, we suggest that GRP is involved in the regulation of glucose intolerance. This may be caused both by a local islet neural effect and by regulation of GLP-1 secretion. The relative contribution of the impaired GLP-1 response vs. impairment by intra-islet neural action of GRP in the GRP receptor-deficient mice remains to be established.

In conclusion, besides showing that the ELISA GLP-1 assay is possible to use in studies on the regulation of circulating GLP-1 in mice, this study shows that 1) enteral presentation of glucose increases plasma levels of intact GLP-1 in mice; 2) a normal GLP-1 response to enteral glucose requires intact GRP receptors; and 3) a normal early insulin response to enteral glucose requires intact GRP receptors in mice.

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