The mediation by GLP-1 receptors of glucagon-induced insulin secretion revisited in GLP-1 receptor knockout mice

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ABSTRACT

To study whether activation of GLP-1 receptors importantly contributes to the insulinotropic action of exogenously administered glucagon, we have performed whole animal experiments in normal mice and in mice with GLP-1 receptor knockout. Glucagon (1, 3 or 10 μg/kg), the GLP-1 receptor antagonist exendin 9-39 (30 nmol/kg), glucose (0.35 g/kg) or the incretin hormone glucose-dependent insulinotropic polypeptide (GIP; 3 nmol/kg) was injected intravenously or glucose (75 mg) was given orally through gavage. Furthermore, islets were isolated and incubated in the presence of glucose with or without glucagon. It was found that the insulin response to intravenous glucagon was preserved in GLP-1 receptor knockout mice but that glucagon-induced insulin secretion was markedly suppressed in islets from GLP-1 receptor knockout mice. Similarly, the GLP-1 receptor antagonist markedly suppressed glucagon-induced insulin secretion in wildtype mice. These data suggest that GLP-1 receptors contribute to the insulinotropic action of glucagon and that there is a compensatory mechanism in GLP-1 receptor knockout mice that counteracts a reduced effect of glucagon. Two potential compensatory mechanisms (glucose and GIP) were explored. However, neither of these seemed to explain why the insulin response to glucagon is not suppressed in GLP-1 receptor knockout mice. Based on these data we confirm the hypothesis that glucagon-induced insulin secretion is partially mediated by GLP-1 receptors on the beta cells and we propose that a compensatory mechanism, the nature of which remains to be established, is induced in GLP-1 receptor knockout mice to counteract the expected impaired insulin response to glucagon in these mice.

1. Introduction

It is well known that glucagon is involved in the regulation of circulating glucose and contributes to the development of type 2 diabetes [1,2]. It is also established that a primary effect of the hormone is to stimulate glycogenolysis and gluconeogenesis in the liver, which increases hepatic glucose production and circulating glucose [3].

What is perhaps less well known is that glucagon also stimulates insulin secretion, although this was demonstrated several decades ago in studies both in animals [4,5] and in humans [6,7]. The insulinotropic effect of glucagon has been thought to be mediated by glucagon receptors, which are expressed in beta cells [8-10]. Glucagon therefore also stimulates insulin secretion in vitro in islet studies [11-13]. This direct beta cell effect of glucagon has been shown to be of critical importance for beta cell function beyond the insulinotropic effect of exogenously added glucagon. Thus, the insulin response to glucose from isolated islets is reduced in mice with genetic deletion of glucagon receptors [14] and, conversely, the insulin response to glucose is enhanced after beta cell overexpression of glucagon receptors [15,16]. Similarly, mice with selective inhibition of glucagon secretion have impaired insulin secretion as evident both under in vivo conditions and in perfused islets [17,18]. Effects of glucagon on beta cell function are thus both pharmacologically important and of critical physiological relevance.

A novel concept has emerged that the stimulation by glucagon of insulin secretion is not only mediated by activation of glucagon receptors but also dependent on activation of glucagon-like peptide-1 (GLP-1) receptors, although the relative contribution of these two receptor types in this respect remains to be established. It is well known

Abbreviations: AUC, area under the curve; GIP, glucose-dependent insulinotropic polypeptide; GLP-1, glucagon-like peptide-1; DIRKO, double incretin hormone receptor knockout.

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The resulting offspring was used to establish breeding pairs, whose receptor antagonist exendin 9-39 displaces glucagon and inhibits temperature-controlled room (22°C). Heterozygotes were mated to yield GLP-1 receptor knockout deletion mutant mice by rederivation at Taconic Europe (Silkeborg, Denmark). We used a C57BL6J background being heterozygous for the deletion of both glucagon and GLP-1 receptors. The generation of GLP-1 receptor knockout mice, GIP receptor knockout mice and mice with double knockout of both GLP-1 receptors and GIP receptors (DIRKO) has been described previously [28]. Briefly, mice on a C57BL6J background being heterozygous for the deletion of both the Glp1r and Gipr genes were generated from double homozygous deletion mutant mice by rederivation at Taconic Europe (Silkeborg, Denmark). Heterozygotes were mated to yield GLP-1 receptor knockout mice, GIP receptor knockout mice, DIRKO mice and wildtype (wt) mice. The resulting offspring was used to establish breeding pairs, whose offspring was used in the experiments. All experiments were undertaken in mice of 4–6 months of age. The animals were maintained in a temperature-controlled room (22°C) on a 12:12 h light-dark cycle (light on at 7:00 AM). Mice were fed a standard pellet diet (total energy 14.1 MJ/kg with 14% from fat, 60% from carbohydrate and 26% from protein; SAFE, Augy, France) and tap water ad libitum. During experimental days, food was removed from the cages at 7:30 AM and the actual experiments started at 12:30, i.e., during the light cycle. For in vivo studies, we used female mice only to avoid the stress of single housing, which is used in male mice, and to be in line with the previous study in GLP-1 receptor knockout mice [29]. We used the mice randomly during the estrous cycle. The study was approved by the Lund/Malmö Animal Ethics Committee (Approval No. 5.8.18-06417/2020) and performed according to Good Laboratory Practice.

2.2. Animal disposition

A total of 255 animals were allocated for the experiments (139 wildtype mice, 89 GLP-1 receptor knockout mice, 12 GIP receptor knockout mice and 15 DIRKO mice). In vivo studies were undertaken in batches of 6–8 mice on each experimental day. All experiments were undertaken by one experienced technician. All individual results from the complete population were included in the final analysis and statistics.

2.3. Experiments

2.3.1. Intravenous test

After 5-h fast, mice were anesthetized with an intraperitoneal injection of a fixed-dose combination of fentanyl (0.02 mg/mouse) – fluanisone (0.5 mg/mouse; Hypnorm®; Vetpharma, Leeds, UK), and midozalam (0.125 mg/mouse; Roche, Basel, Switzerland). Twenty minutes later, a first blood sample was taken and an intravenous bolus dose over 3 s was given in a tail vein. The bolus consisted of glucagon (1, 3 or 10 μg/kg), glucagon at 3 μg/kg in combination with exendin 9-39 (Sigma-Aldrich, St Louis, MO; 30 nmol/kg; dose selection from Ref. [30]) or β-glucagon (Sigma; 0.35 g/kg) with or without combination with glucose-dependent insulinotropic polypeptide (GIP; Sigma; 3 nmol/kg; dose selection from Ref. [31]). Whole blood was sampled in heparinized pipettes from the intraorbital retrobulbar sinus plexus (40 μl) immediately before the intravenous injection (time t = 0) and specified time points up to 50 min after injection. Plasma was separated by centrifugation and stored at −20°C until analysis of insulin and C-peptide.

2.3.2. Oral test

After a 5-h fast, wildtype and GLP-1 receptor knockout mice were anesthetized and a blood sample was taken as above. Then, glucose (75 mg in volume of 0.3 ml) was administered through a gastric tube (outer diameter 1.2 mm) and additional blood samples were taken at 5, 10 and 30 min. Plasma was separated by centrifugation and stored at −20°C until analysis of glucose and GIP.

2.3.3. In vitro studies

Mouse islets were isolated by collagenase digestion and hand-picked in an inverted microscope. Batches of freshly isolated islets were pre-incubated in HEPES balanced salt solution containing 125 mmol/l MgCl2, 25 mmol/l HEPES (pH 7.4), 5.6 mmol/l glucose and 0.1% fatty acid free BSA (Boehringer Mannheim, Germany) for 60 min. Thereafter, islets in groups of three were incubated for 60 min in 200 μl of the buffer described above with 2.8, 11.1 or 16.7 mmol/l glucose or with 11.1 mmol/l glucose in the presence of 100 nmol/l glucagon.

2.4. Assays

Plasma was immediately separated after collection and stored at −20°C until analysis. Glucose was measured using an Accu-Chek Aviva (Hoffman-La Roche, Basel, Switzerland). Insulin concentration was determined by ELISA (Merrick, Uppsala, Sweden). The intra-assay coefficient of variation (CV) of the method is 4% at both low and high levels, and the interassay CV is 5% at both low and high levels. The lower limit of the assay is 6 pmol/l. Mouse C-peptide was determined by ELISA (Crystal Chem, Brook Drive, IL). The intra- and interassay CVs of the method are <10% at both low and high levels. The lower limit of the assay is 25 pmol/l. Total GIP was determined by ELISA (Crystal Chem, catalog #81527). The interassay CV is <10% at low and high values and the detection limit is 2.5 pmol/l. The assay is specific for GIP and show no cross-reactivity to glucagon, GLP-1 or GIP-2.

2.5. Data analysis and statistics

Data are presented as means ± SE. Areas under the curves (AUCs) were calculated with the trapezoid rule for incremental values above baseline (suprabasal AUC). The acute insulin response (AIR) was estimated as the increase in mean of 1 and 5 min insulin levels and insulinoergic index was estimated by dividing AIR by mean glucose levels at 1 and 5 min. Differences between experimental groups were determined using Student’s unpaired t-test. Statistics on insulin and C-peptide were performed after logarithmic transformation since they do not display normal distribution in mice. For all analyses, statistical significance was defined as P < 0.05. Analyses were carried out using SPSS, version 25.
3. Results

3.1. Effect of iv glucagon in wildtype and GLP-1 receptor knockout mice

Glucagon was administered intravenously at three different dose levels to wildtype and GLP-1 receptor knockout mice. As seen in Fig. 1, glucose levels increased at 5 min after glucagon administration at all doses in both groups with the maximal glucose levels seen at 10–20 min after administration. Also insulin and C-peptide levels increased after all three dose levels of glucagon in both wildtype and GLP-1 receptor knockout mice. At 1 and 3 μg/kg of glucagon, peak insulin and C-peptide levels were seen at 5 min after administration, whereas after the highest dose of glucagon (10 μg/kg), the peak was evident already at 1 min. Insulin and C-peptide levels increased dose-dependently by increasing dose of glucagon with no significant difference between wildtype and GLP-1 receptor knockout mice, as evident from plotting the AIR versus dose of glucagon (Fig. 2 left panel). Fig. 2 (right panel) shows the insulinogenic index after glucagon administration (estimated by dividing AIR by glucose levels), which was assessed to normalize for the different glucose between the two groups. Again, no difference between wildtype and GLP-1 receptor knockout mice were seen. These studies therefore show that the insulin response to intravenous glucagon is not reduced in GLP-1 receptor knockout mice.

3.2. Effects of exendin 9-39 in wildtype mice

We next determined the influence of the GLP-1 receptor antagonist exendin 9-39 on glucagon-stimulated insulin secretion in wildtype mice. Exendin 9-39 was given together with the intravenous administration of glucagon (3 μg/kg). Fig. 3 shows the glucose, insulin and C-peptide data. It is seen that glucose levels after glucagon were augmented by exendin 9-39, whereas insulin and C-peptide levels were reduced. Fig. 3 also shows that the 5 min increase in insulin levels after glucagon was significantly reduced by approximately 85% by exendin 9-39 (P = 0.042). Similarly, the 5 min increase in glucagon was reduced from 43 ± 18 to -21 ± 17 pmol/l by exendin 9-39 (P = 0.018). Therefore, exendin 9-39 efficiently reduces glucagon-induced insulin secretion in normal mice.

3.3. Effects in islets from GLP-1 receptor knockout mice

The combined findings that exendin 9-39 impaired glucagon-induced insulin secretion in normal mice yet the insulin response to glucagon was not impaired in GLP-1 receptor knockout mice, suggests that a compensatory mechanism exists in GLP-1 receptor knockout mice. If so, insulin secretion in response to glucagon should be impaired in isolated islets, when such a compensatory does not exist. We therefore proceeded to examine effects of glucose and glucagon on insulin secretion in islets from mice with GLP-1 receptor knockout. We found that glucose-stimulated insulin secretion was suppressed in GLP-1 receptor knockout mice compared to wildtype mice. Furthermore, we found that at 11.1 mmol/l glucose (i.e., a stimulatory dose level), glucagon failed to stimulate insulin secretion in female GLP-1 receptor knockout mice but had a clear effect in female wildtype mice (Fig. 4). To examine whether there could be a gender difference in the effect of glucagon, we also incubated islets from male wildtype and male GLP-1 receptor knockout mice. We found that during incubation at 100 nmol/l glucagon in the presence of 11.1 mmol/l glucose, insulin secretion was significantly lower in islets from GLP-1 receptor knockout mice (415 ± 53 pg/islet/h) compared to islets from wildtype mice (547 ± 55 pg/islet/h; P = 0.037).

Fig. 1. Glucose and insulin levels before and after intravenous injection of glucagon (1, 3 and 10 μg/kg) in wildtype and GLP-1 receptor knockout mice. Means ± SEMs are shown. n indicates number of animals.
(studies performed in 16 incubations with three islets in each incubation performed as 8 incubations in two different independent experiments; 8 wildtype mice and 8 GLP-1 receptor knockout mice). In contrast, there was no difference in the insulin response to glucagon at 1 or 10 nmol/l in between islets from male wildtype and GLP-1 receptor knockout mice. These results show that GLP-1 receptors indeed contribute to the insulinotropic action of glucagon, although the sensitivity of glucagon for this action was lower in islets from male than in female mice. Overall, however, these data support the hypothesis that a compensatory mechanism has evolved in vivo in GLP-1 receptor knockout mice and this mechanism has masked the blunting of the insulin response to glucagon in these mice.

3.4. Potential compensatory mechanisms in vivo

Two potential compensatory mechanisms were explored. A first possibility is glucose, the level of which is elevated in GLP-1 receptor knockout mice which may augment the action of glucagon to stimulate insulin secretion and therefore mask the impaired action due to the lack of GLP-1 receptors. This hypothesis was explored by comparing the glucose and insulin responses to glucagon at 3 μg/kg (Fig. 5). After glucagon administration at this dose level, glucose levels were similarly raised in wildtype and GLP-1 receptor knockout mice and also insulin levels were similar. Therefore, when matching for glucagon levels after glucagon administration, the insulin levels were similarly raised by
Fig. 4. Insulin secretion from isolated mouse islets from wildtype or GLP-1 receptor knockout mice. Islets were incubated for 60 min in the presence of 2.8, 11.1 or 16.7 mmol/l glucose (panel A) or 11.1 mmol/l glucose in the presence of 0, 1, 10 or 100 nmol/l of glucagon (panel B). The results are presented as mean ± SEM from 32 incubations with three islets in each incubation performed as 8 incubations in four different independent experiments (16 wildtype mice and 16 GLP-1 receptor knockout mice). Asterisks indicate probability level of random difference between the groups; **p < 0.01; ***p < 0.001.

Fig. 5. Glucose and insulin levels before and after intravenous injection of glucagon (3 μg/kg) in wildtype and GLP-1 receptor knockout mice. Means ± SEMs are shown. n indicates number of animals.

Fig. 6. Panel A shows plasma GIP levels before and after oral administration of glucose (75 mg) in wildtype and GLP-1 receptor knockout mice, and panel B shows the 30 min AUC_{GIP}. Means ± SEMs are shown. P-value indicates probability level of random difference between the groups. n indicates number of animals.
glucagon in wildtype and GLP-1 receptor knockout mice, suggesting that glucose is not a compensatory mechanism in vivo in GLP-1 receptor knockout mice.

We next examined a potential compensatory mechanism through the other incretin hormone, GIP. It has previously been reported that the secretion and effect of GIP are enhanced in GLP-1 receptor knockout mice [17,32], and such a mechanism could enhance the beta cell sensitivity to glucagon which could counteract the impaired response to glucagon due to lack of GLP-1 receptors. To explore such a compensation we studied both the release and effect of GIP in GLP-1 receptor knockout mice. We found that baseline GIP levels were not different between wildtype and GLP-1 receptor knockout mice. Furthermore, after oral administration of 75 mg glucose, the raise in GIP levels was significantly augmented in GLP-1 receptor knockout mice (Fig. 6). However, there was no difference in GIP levels after intravenous administration of glucagon at 3 μg/kg in GLP-1 receptor knockout mice (Fig. 7).

We also examined the effect of GIP on insulin secretion in wildtype and GLP-1 receptor knockout mice. Glucose (0.35 g/kg) was injected intravenously alone or together with GIP (3 nmol/kg). It was found, as seen in Fig. 8, that GIP augmented the glucose-induced increase in insulin and C-peptide levels and also increased glucose disposal. The augmentation of glucose-induced insulin secretion by GIP was higher in GLP-1 receptor knockout mice than in wildtype mice, as evident both by higher AUC_{insulin} (58.8 ± 8.3 versus 29.3 ± 1.8 nmol/l min, P = 0.004) and AIR (3.5 ± 0.4 versus 2.3 ± 0.2 nmol/l/min = 0.026). Hence, the insulinoceptive effect of GIP is augmented in GLP-1 receptor knockout mice.

We also examined the effect of glucagon in GIP receptor knockout mice to examine whether the GIP receptor might contribute to the action of glucagon. We found that glucose-stimulated insulin secretion was suppressed in GIP receptor knockout mice compared to wildtype mice. Furthermore, we found that at 11.1 mmol/l glucose (i.e., a stimulatory dose level), the insulinoceptive action of glucagon was the same in GIP receptor knockout mice as in wildtype mice, although the resulting insulin concentrations were significantly reduced due to the lower action of glucose (Fig. 9). This suggests that GIP receptors do not contribute to the insulinoceptive action of glucagon.

Finally, we examined the insulin response to intravenous glucagon in DIRKO mice, i.e., mice with deletion of both GLP-1 receptors and GIP receptors. The rationale for such an experiment was that if it is GIP that restores the reduced insulinoceptive effect of glucagon in GLP-1 receptor knockout mice, such a compensation would be absent in DIRKO mice. The results show, however, that in DIRKO mice, the insulin response to glucagon was not suppressed when compared to wildtype mice (Fig. 10). On the contrary, the 1 min insulin response to glucagon was augmented in these mice (P = 0.04). This further enforces that GIP is not the compensatory mechanism saving the insulinoceptive action of glucagon in GLP-1 receptor knockout mice.

4. Discussion

We have demonstrated in several studies that administration of glucagon increases insulin secretion in mice [2,4,5,33] and we have shown that this is elicited without changing insulin clearance [34]. This is in line with several other reports [2,25,35] and also studies in humans [6,7]. This effect is partially dependent on glucose, the level of which is increased by glucagon through stimulated hepatic glucose production [2], and a direct action by glucagon on the insulin producing beta cells [13–15]. Glucagon-stimulated insulin secretion is regulated by several factors and it is known that the insulin response to glucagon is inhibited by GLP-1, beta adrenergic receptor blockade (propranolol) and somatostatin and also reduced by fasting [25,36–39].

In this study we explored whether glucagon-induced insulin secretion in mice is dependent on GLP-1 receptors. Such a mechanism was proposed more than 20 years ago based on studies with the GLP-1 receptor antagonist exendin 9-39 [24]. Similar results have been presented more recently [17,25,26] although other results have challenged this view [28,29]. We explored the hypothesis in GLP-1 receptor knockout, which we previously have characterized in regard to insulin and glucagon secretion [40]. We expected that if glucagon-induced insulin secretion is mediated by GLP-1 receptors, the insulin response to glucagon would be impaired in these mice. We indeed found this to be the case in islets isolated from these mice, and therefore we confirm the previous data of deficient glucagon-induced insulin secretion in islets with GLP-1 receptor deficiency [24,25] and which support that GLP-1 receptors contribute to the insulinoceptive action of glucagon. This was also supported by our data that the GLP-1 receptor antagonist exendin 9-39 inhibited glucagon-induced insulin secretion in normal mice, which thus also confirms previous data [24]. The latter result would be the case also if exendin 9-39 inhibits glucagon receptor activity, which, however, is less likely considering that glucagon does not displace exendin-derived peptides in other cell systems [41]. The conclusion is therefore that GLP-1 receptors contribute to the insulinoceptive action of glucagon. The relative contribution of this mechanism versus a
mechanism mediated by the glucagon receptors are difficult to establish, since receptor activity is dependent on glucose levels, baseline insulin secretion and receptor interactions. An estimation from the exendin 9-39 experiments reveal that glucagon induced insulin secretion is reduced by more than 85% by exendin 9-39, and in islets from GLP-1 receptor knockout mice, glucagon-induced insulin secretion is similarly reduced by approximately 85%. This would suggest that GLP-1 receptors are of large importance for the beta cell actions of glucagon in these systems. Also somatostatin secretion is stimulated by glucagon [42] and the so- matostatin producing delta cells express GLP-1 receptors [43]. It would therefore also be of interest to examine whether GLP-1 receptors contribute also to glucagon-induced somatostatin secretion.

In contrast to the clear cut results with exendin 9-39 in normal mice and effects of glucagon in islets from GLP-1 receptor knockout mice, the insulin and C-peptide responses to glucagon were not reduced under in vivo conditions in GLP-1 receptor knockout mice. This would be explained if a compensatory mechanism has evolved in these mice, a mechanism which would augment glucagon-induced insulin secretion beyond GLP-1 receptors and therefore would mask the impaired insulin response that is expected. We examined two potential compensatory mechanisms, glucose and GIP. In regard to glucose, we found, however, that there was a similar increase in glucose and insulin levels after glucagon at 3 \( \mu \text{g/kg} \). This does not support that glucose is a compensatory mechanism explaining the expected failure of glucagon to increase insulin in GLP-1 receptor knockout mice. Another potential compensatory mechanism is GIP if its tonus on beta cells would augment the insulin response to glucagon in GLP-1 receptor knockout mice. It has previously been observed that GIP secretion is enhanced in GLP-1 receptor knockout mice and also that the sensitivity to administered GIP is increased [24,32], which is in analogy with the previously reported

![Graphs showing glucose, insulin, and C-peptide levels](image-url)

**Fig. 8.** Glucose, insulin and C-peptide levels before and after intravenous injection of glucose (0.35 g/kg) or glucose + GIP (3 nmol/kg) in wildtype mice or in GLP-1 receptor knockout mice. Means ± SEMs are shown. n indicates number of animals.
enhanced beta cell effect of GLP-1 levels in GIP receptor knockout mice [44]. We confirmed these findings since the increase in GIP levels after oral glucose and the insulinotropic effect of exogenously administered GIP were significantly higher in GLP-1 receptor knockout mice than in wildtype mice. However, we did not detect higher GIP levels after intravenous glucagon in GLP-1 receptor knockout mice and it is therefore unlikely that circulating GIP may contribute to the compensatory mechanism after glucagon administration in vivo in GLP-1 receptor knockout mice. This conclusion is also supported by the results in DIRKO mice, in which the insulinotropic action of glucagon was not suppressed when compared to wildtype mice. If GIP would have been the compensatory mechanism in GLP-1 receptor knockout mice, such a compensation would be absent in DIRKO mice, which thus did not seem to be the case. There was on the contrary an augmented 1 min insulin response to glucagon in DIRKO mice compared to wildtype mice. Interactions between glucagon and GIP may therefore exist, but not in terms of GIP being a compensatory mechanism to restore the insulin response to glucagon in GLP-1 receptor knockout mice. Interaction through GIP receptors is, however, unlikely considering that we found that glucagon-induced insulin secretion was preserved in islets from GIP receptor knockout mice. It should also be emphasized that the effect of GIP on glucagon secretion vanishes by increasing glucose [45] and that islets have been shown to express GIP [46], which mainly is the truncated GIP 1–30 form [47], which, however, exhibits the same effects as the longer GIP (1–42) form [48]. Other mechanisms, such as dependent on islet forms of GIP, may, however, not be excluded.

Therefore, based on our data we support the hypothesis that glucagon-induced insulin secretion is partially mediated by GLP-1 receptors on the beta cells and we propose that a compensatory mechanism is induced in GLP-1 receptor knockout mice to counteract the expected impaired insulin response to glucagon in these mice.

CRediT authorship contribution statement

Bo Ahrén: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Writing - original draft, Writing - review & editing. Yuichiro Yamada: Writing - review & editing. Yutaka Seino: Writing - review & editing.

Declaration of Competing Interest

The authors reported no declarations of interest.
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