Reduction of the Incretin Effect in Rats by the Glucagon-Like Peptide 1 Receptor Antagonist Exendin (9–39) Amide

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Glucagon-like peptide 1 (7–37)/(7–36) amide (GLP-1) is derived from the intestinal proglucagon processing. It is considered an important insulin-releasing gut hormone. This study uses exendin (9–39) amide as a GLP-1 receptor antagonist to evaluate the contribution of GLP-1 to the incretin effect. Anesthetized rats were challenged by an intraduodenal glucose infusion to evaluate maximally occurring GLP-1 and gastric inhibitory polypeptide (GIP) plasma levels. Maximal immunoreactive (IR) GLP-1 plasma levels amounted to 10 pmol/l (IR-GIP 11 pmol/l). Exendin (9–39) amide abolished the insulin-stimulatory effect of 60 pmol of GLP-1 or of the GLP-1 agonist exendin-4 (0.5 nmol) injected as bolus, respectively. An intravenous bolus injection of 5.94 nmol of exendin (9–39) amide 3 min before enteral glucose infusion grossly reduced the total insulin secretory response (by 60%) and significantly increased circulating blood glucose levels ($P < 0.05$). In contrast, the GLP-1 antagonist left the insulin response after an intravenous glucose or glucose plus GIP (60 pmol) load unaltered. Our data support the concept that GLP-1 is an important incretin factor. Exendin (9–39) amide is a useful GLP-1 antagonist for in vivo studies. *Diabetes* 44:16–19, 1995

The secretory response of the endocrine pancreas is much greater (up to 50%) after oral than after intravenous glucose administration, despite identical glucose plasma levels in both incidents (1,2). This functional connection between intestine and endocrine pancreas is called the entero-insular axis, and several decades ago the term "incretin" was coined for gut-born insulin secretagogues (3,4). We have learned that peptides released after meals from the intestine are important mediators in the entero-insular axis and that intestino-pancreatic reflexes play only a minor role in this context (2). Meanwhile, several gut peptides have been proposed as incretins because of their stimulating effect on the secretion of insulin. However, most have been discounted because they exert insulinotropic effects only at supraphysiological concentrations. Their meal-related increments do not coincide with nutrient-mediated insulin secretion, or the meal-related insulin release is not altered during infusion with an antagonist against the peptide receptor of interest (5).

The first incretin was isolated more than 20 years ago and was named gastric inhibitory polypeptide (GIP) on the basis of its first observed biological activity (6). Although GIP clearly is a powerful insulin secretagogue (4,7), it turned out that it could only partly explain the incretin effect. In vivo and in vitro immunoneutralization studies using antisera against different epitopes of GIP abolished only 20–50% of the incretin effect (8,9). Furthermore, abolishment of the GIP release after an oral glucose load left the incretin effect nearly untouched (10), raising even more doubt about an exclusive role for GIP in the entero-insular axis. A wealth of recent data supports the new concept that the proglucagon-derived glucagon-like peptide 1 (GLP-1) is an important incretin factor (5,11–15). In this study we used the recently discovered GLP-1 antagonist exendin (9–39) amide (16,17) to study the contribution of GLP-1 to the incretin effect.

RESEARCH DESIGN AND METHODS

Male Wistar rats (Charles River Wiga, Sulzfeld, Germany) weighing 300–340 g were used for the experiments. The animals were fed a standard diet and tap water ad libitum. All rats were fasted for 12 h before each study. They were anesthetized by one intraperitoneal injection of pentobarbitone sodium (Nembutal, 45 mg/kg body wt). Catheters were inserted for intravenous injections into the jugular vein, extending to the right atrium, and for the withdrawal of blood samples into the inferior vena cava. Furthermore, if necessary, an additional catheter was introduced into the pylorus and fixed by a ligature (18). Experiments were performed 30 min after the placement of catheters. The body temperatures were kept constant at 38°C by means of an electric heating pad throughout the experiments.

Exendin-4 and exendin (9–39) amide were synthesized as has been detailed before (17). Briefly, the peptides were produced on solid-phase support using activated *N*-(9-fluorenyl)methoxycarbonyl amino acids on a Milligen 9050 peptide synthesizer (Burlington, MA) and were purified by preparative high-pressure liquid chromatography. Their quality was controlled by mass spectroscopy and amino acid sequencing. GLP-1 (7–36) amide and GIP were obtained from Peninsula (St. Helens, U.K.). Aprotinin (Trasylol) was from Bayer (Wuppertal, Germany). All other chemicals were from Sigma (Deisenhofen, Germany).

GLP-1 and GIP response after luminal glucose infusion. Glucose (0.5 g) was dissolved in 10 ml of 0.9% saline and was infused into the gut lumen via the pyloric catheter (5.6 mmol/kg body wt). The instilled volume was 1 ml solution/100 g body wt. Immunoreactive (IR)-GLP-1 and IR-GIP levels were determined before ($n = 6$) or 5 ($n = 8$), 10 ($n = 6$), 20 ($n = 6$), and 30 ($n = 6$) min after glucose administration in blood samples withdrawn from the inferior vena cava. Control subjects received identical amounts of saline instead of glucose solution.

Effect of exendin (9–39) amide on exendin-4–, GLP-1–, or GIP-stimulated insulin release. Previous in vitro experiments have shown that exendin (9–39) amide needs to be applied in at least a 10-fold excess to reduce GLP-1- or exendin-4–induced effects on insulin secretion (17).

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GIP, gastric inhibitory polypeptide; GLP, glucagon-like peptide; IR, immunoreactive.

To antagonize the action of GLP-1 on glucose-induced insulin secretion, we reinvestigated this relationship in vivo by injecting 5.94 nmol (20 μg) or 59.4 nmol (200 μg) of the antagonist (100-fold excess) 3 min before the application of 50 pmol (0.2 μg) or 0.5 nmol (2 μg) exendin-4 dissolved in a glucose solution (see next paragraph). Using this protocol, we also investigated the effect of truncated exendin (5.94 nmol) on the GLP-1– or GIP-stimulated (60 pmol, respectively) glucose-induced insulin secretion. These amounts of GLP-1 and GIP were chosen because injections resulted in plasma levels similar to peak plasma levels obtained after the intraduodenal administration of glucose (see later).

Injection of the GLP-1 antagonist to alter the incretin effect. A bolus of exendin (9–39) amide (5.94 nmol) was administered 3 min before glucose infusions via the jugular vein. Glucose was introduced over 1 min into the gut lumen (see above) or via infusion into the jugular vein (2.8 mmol/kg body wt; 0.17 ml/100 g body wt of 3 g glucose dissolved in 10 ml isotonic saline, pH 7.4), each glucose application achieving comparable plasma glucose levels. Blood (1 ml) from the vena cava was put on ice at 0, 5, 10, 20, and 30 min for the estimation of insulin and glucose levels. The volume loss was substituted with an injection of identical amounts of isotonic saline. The samples were kept on ice in vials containing 1.6 mg EDTA and 350 KIU of aprotinin/ml of blood and centrifuged at 3,000 U/min (4°C); then the plasma was stored at −20°C until the assays were performed.

Radioimmunoassays. Immunoreactive GLP-1 was analyzed by a competitive radioimmunoassay with the specific polyclonal antibody GA 1178 (Affinity Research, Nottingham, U.K.). It exhibits 100% reactivity with GLP-1 (1–36) amide and the truncated GLP-1 (7–36) amide. The sensitivity is 0.4 pmol/l. Further characterization of the antibody in our laboratory revealed no cross-reaction with GIP, pancreatic glucagon, glicentin, oxyntomodulin, GLP-2, and the GLP-1 agonist exendin-4. ^{125}I-labeled GLP-1 (7–36) amide (specific activity ~74 TBq/mmol) was prepared as described previously (19). The inter- and intra-assay coefficients of variation are 10.2 and 5.4%, respectively. Immunoreactivities were extracted from plasma samples (1.5 ml) using Sep-Pak C_{18} cartridges (Millipore, Eschborn, Germany), which were activated by 0.1% trifluoroacetic acid, loaded with sample, and washed with 40% acetonitrile to remove interfering plasma factors from the samples. The peptides were recovered by slowly flushing 70% acetonitrile through the column. When this technique was applied to extractions of hormone-free plasma substituted with known amounts of GLP-1, a recovery of GLP-1 between 72 and 80% was achieved. GIP plasma levels were analyzed by means of a commercially available assay system (Biermann, Bad Nauheim, Germany). For this, no extraction of samples was necessary.

Other determinations. Insulin was analyzed by radioimmunoassay using rat insulin I and II as standard (Biermann). The assay sensitivity was 30 pmol/l, the interassay variance was 11.8%, and the intra-assay variance amounted to 9.7%. Plasma glucose was determined by a glucose oxidase method (Boehringer Ingelheim, Ingelheim, Germany) using an automated glucose analyzer.

Statistical analysis. Data are means ± SE and were tested statistically by comparing the mean changes at each time point using the Student's t test for unpaired samples. Differences were considered significant at $P < 0.05$. Analysis of data was facilitated using a Statgraphics 210 computer program.

RESULTS

GLP-1 and GIP response after luminal glucose infusion. Basal IR-GLP-1 plasma levels ranged from 1.3 to 1.7 pmol/l (1.5 ± 0.1 pmol/l). In control subjects (saline infusion), these levels remained unaltered. Under luminal glucose infusion, IR-GLP-1 levels significantly increased (5 min: 5.7 ± 1.1 pmol/l, $P < 0.01$; 10 min: 3.5 ± 0.7 pmol/l, $P < 0.05$; 20 min: 3.6 ± 1.3 pmol/l, $P < 0.05$; 30 min: 1.9 ± 0.4 pmol/l, NS). Maximal single IR-GLP-1 plasma values after duodenal glucose administration amounted to 10.7 pmol/l. Bolus injection of 60 pmol of GLP-1 ($n = 4$), analyzed 10 min later, amounted to IR-GLP-1 plasma levels of 11.2 ± 2.7 pmol/l; GIP levels 5 min after injection (60 pmol) were 17.4 ± 5.2 pmol/l ($n = 3$).

Administration of luminal glucose also increased IR-GIP levels (basal: 3.0 ± 0.2 pmol/l; 5 min: 8.2 ± 1.6 pmol/l, $P < 0.01$; 10 min: 7.8 ± 0.6 pmol/l, $P < 0.05$; 20 min: 6.2 ± 1.0 pmol/l, $P < 0.05$; 30 min: 5.4 ± 1.2 pmol/l, $P < 0.05$). Maximal single IR-GIP plasma levels amounted to 11.2 pmol/l.

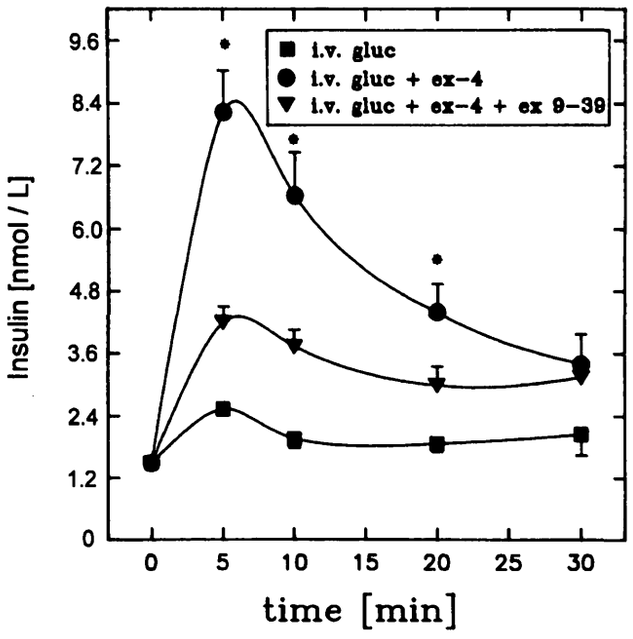

FIG. 1. Effect of exendin-4 (0.5 nmol) and exendin (9–39) amide (59.5 nmol) on plasma insulin levels after intravenous glucose administration. Values are means ± SE ($n = 6$). * $P < 0.05$, the difference between intravenous glucose plus exendin versus this combination with addition of exendin (9–39) amide.

Effect of exendin (9–39) amide on the exendin-4–, GLP-1–, and GIP-stimulated insulin release. Previous studies have demonstrated that the stimulatory effect of GLP-1 and exendin-4 on insulin secretion is glucose-dependent (17,20). Therefore, we intravenously infused exendin-4 dissolved in a glucose solution. The GLP-1 antagonist [exendin (9–39) amide] grossly reduced the exendin-4–induced enhancement of plasma insulin levels, but administered alone, exendin (9–39) amide left the intravenous glucose-induced increase of plasma insulin unaltered (data not shown). The antagonistic effect of exendin (9–39) amide became clearly evident at the higher concentration of exendin-4 (0.5 nmol) (Fig. 1) when the insulin-stimulatory action of the agonist was more prominent than at the lower amount of 50 pmol (not shown). Figure 2 demonstrates that exendin (9–39) amide (5.94 nmol) also drastically diminished the stimulatory action of GLP-1 (60 pmol) on glucose-induced insulin secretion. In control experiments, injection of exendin (9–39) amide (5.94 nmol) left the insulin-secretory response to GIP (60 pmol dissolved in glucose) unaltered (data not shown).

Effect of the GLP-1 antagonist on the incretin effect. Luminal infusion of glucose significantly increased plasma insulin levels above those observed after intravenous glucose despite even higher glucose levels (incretin effect) (Fig. 3). The GLP-1 antagonist reduced the total insulin secretory effect by 60 ± 8% ($P < 0.05$) (Fig. 4) and, consecutively, increased the blood glucose levels (areas under the curve of glucose plasma levels over 30 min; control versus exendin (9–39) amide: 9.4 ± 2.7 vs. 26.1 ± 2.2 mmol/l for 30 min; $P < 0.05$). Furthermore, the plasma insulin levels were no longer significantly above those measured under intravenous glucose alone.

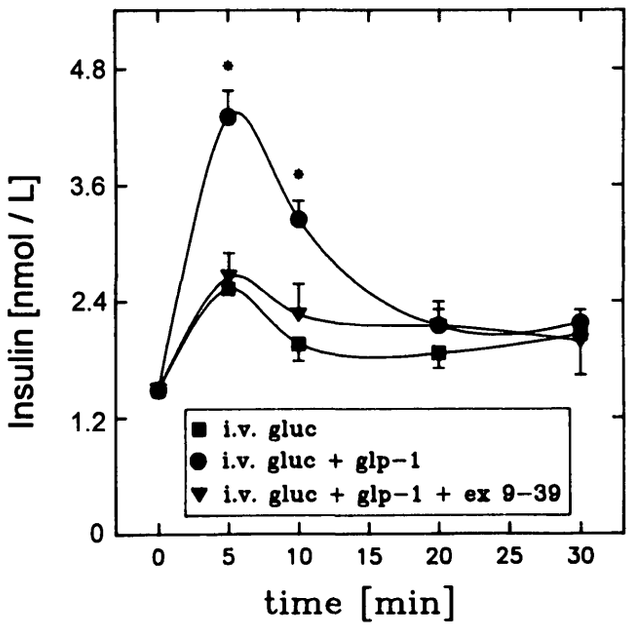

FIG. 2. Effect of GLP-1 (60 pmol) after bolus preinjection of exendin (9–39) amide (5.94 nmol) on plasma insulin levels after intravenous glucose administration. Values are means ± SE (n = 6). * $P < 0.05$, the difference between intravenous glucose plus GLP-1 versus this combination with addition of exendin (9–39) amide.

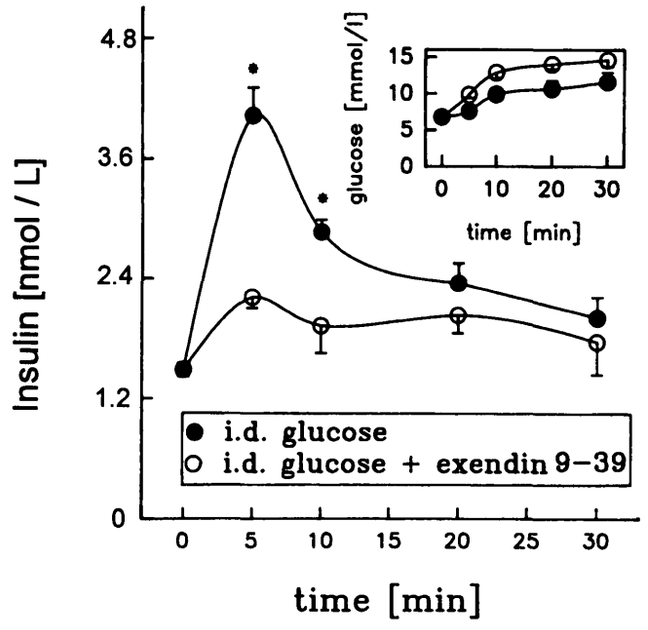

FIG. 4. Plasma insulin and glucose after the intraduodenal glucose challenge and preinjection of 5.94 nmol exendin (9–39) amide. Values are means ± SE (n = 12). * $P < 0.05$, the differences between insulin levels after intraduodenal glucose alone versus intraduodenal glucose plus preinjection of exendin (9–39) amide. Glucose levels after exendin (9–39) amide were significantly ($P < 0.05$) higher than those in control subjects at 10, 20, and 30 min.

DISCUSSION

The vast majority of studies that have investigated the effect of nutrient ingestion on enterohormonal changes and their effect on the endocrine pancreas have focused, so far, on GIP. Now evidence has accumulated proving that GIP only partly contributes to the incretin effect. It has now been realized that GLP-1 is an additional potent insulinotropic gut hormone with promising therapeutic potential in non-insulin-dependent diabetes mellitus (5,12).

Although it was possible to collect a wealth of data concerning the GLP-1 effect on the endocrine pancreas (11), the assumption of a possible importance of this gut hormone within the entero-insular axis is based mainly on indirect evidence. Direct proof would be possible if a selective blockade or induction of the GLP-1 action could be facilitated. However, the GLP-1–generating and GLP-1–releasing L-cells belong to the diffuse endocrine system of the gut (21), and this circumstance mirrors a dilemma of gut endocrinology: the classic approach of endocrinology to evaluate the significance of an endocrine organ or system simply by its removal is not possible. Therefore, it was of great interest that recently a potent antagonist against GLP-1 (exendin [9–39] amide) has become available for such studies (16,17,22).

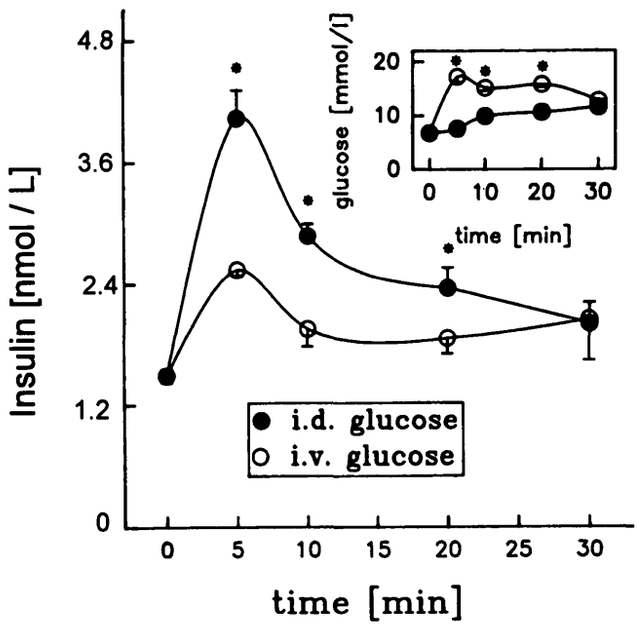

FIG. 3. Incretin effect after administration of glucose intraduodenally into the gut lumen or intravenously into the jugular vein. Values are means ± SE. * $P < 0.05$, the difference between intraduodenal and intravenous glucose administration (n = 5, respectively).

Exendin (9–39) amide is a product of exendin-4 processing, the latter purified from *Heloderma suspectum* venom. The shared biological properties of GLP-1 and exendin-4 are probably based upon their homologue primary structures. Exendin (9–39) amide has been found to interact specifically with the GLP-1 receptor on endocrine pancreatic cells in vitro (17) and in vivo when infused in dogs (23). Control experiments in our laboratory revealed that the antagonist (1 and 10 μmol/l) does not interfere with binding of labeled GIP to the GIP receptor on RINm5F cells (H. Schmidt, B.G., unpublished data). Furthermore, in this study, we report that preinjection of the GLP-1 antagonist leaves the insulin response to GIP in vivo unaltered.

Our data allow conclusions about the contribution of GLP-1 to the incretin effect, at least in rats. Furthermore, it has been shown that exendin (9–39) amide also behaves as a GLP-1 antagonist under in vivo conditions. Injection of the GLP-1 antagonist drastically reduced the incretin effect after luminal glucose.

It was surprising that antagonizing the action of GLP-1 did not result in a greater residual incretin effect, possibly induced by GIP. However, previous data support this obser-

vation: in humans, the abolishment of GIP release after an oral glucose load left the incretin effect nearly untouched (10). Recent data suggest that physiologically, GIP might exert only a permissive action on the β-cell via direct stimulation of GLP-1 secretion from intestinal L-cells (24). This is supported by our own recent data that reveal a stimulatory action of GIP on GLP-1 secretion from the vascularly perfused rat ileum (25). However, data from human studies argue against a stimulation of GLP-1 secretion by GIP (26). Furthermore, if GIP only induced a permissive effect on insulin secretion by releasing GLP-1, exendin (9–39) amide should have antagonized the GIP effect in our control experiments. This, however, was not the case, so that the whole issue is not decided yet.

Another result emerging from the presented data is that, in vivo, GLP-1 obviously has a stronger effect on insulin secretion than the agonist exendin-4, although previous in vitro data have shown that the agonist binds with higher affinity to insulinoma cells than GLP-1 itself (17). This emphasizes that, in addition to the in vitro binding data, other important factors, such as bioavailability and plasma stability, have to be considered to judge the biological effects of incretin peptides.

Taken together, our data support the concept that GLP-1 is an important hormonal mediator in the entero-insular axis and that exendin (9–39) is a useful GLP-1 antagonist for in vivo studies.

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