Intraportal GLP-1 stimulates insulin secretion predominantly through the hepatoportal-pancreatic vagal reflex pathways

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Nishizawa M, Nakabayashi H, Uehara K, Nakagawa A, Uchida K, Koya D. Intraportal GLP-1 stimulates insulin secretion predominantly through the hepatoportal-pancreatic vagal reflex pathways. Am J Physiol Endocrinol Metab 305: E376–E387, 2013. First published May 28, 2013; doi:10.1152/ajpendo.00565.2012.—We previously reported that glucagon-like peptide-1 (GLP-1) appearance in the portal vein facilitates hepatic vagal afferent activity, and this further augments reflexively the pancreatic vagal efferents in anesthetized rats, suggesting a neuroincretin effect of GLP-1. To determine whether the GLP-1-induced vagal pathways lead to a neuronal-mediated component (NMC) of insulin secretion, we infused GLP-1 at a physiological or pharmacological dose (1 or 3 pmol·kg–1·min–1, respectively) into the portal vein in conscious rats with selective hepatic vagotomy (Vagox) or sham operation (Sham). The experiments consisted of two sequential 10-min intraportal infusions (P1 and P2): glucose at a physiological rate (56 μmol·kg–1·min–1) in P1 and the glucose plus GLP-1 or vehicle in P2. Under arterial isoglycemia across the groups, the physiological GLP-1 infusion in Sham augmented promptly and markedly arterial insulin levels, approximately twofold the levels in glucose alone infusion (P < 0.005), and insulin levels in Vagox diminished apparently (P < 0.05). Almost 60% of the GLP-1-induced insulin secretion (AUC) in Sham met the NMC, i.e., difference between insulin secretion in Sham and Vagox. (AUC 976 ± 65 vs. 393 ± 94 pmol·min/l, respectively, P < 0.005). Intraportal pharmacological GLP-1 infusion further augmented insulin secretion in both groups, but the NMC remained in 46% (NS; Sham vs. Vagox). In contrast, “isoglycemic” intravenous GLP-1 infusion (3 pmol·kg–1·min–1) evoked an equal insulin secretion in both groups. Thus, the present results indicate that GLP-1 appearing in the portal vein evokes a powerful neuronal-mediated insulinoportal effect, suggesting the neuroincretin effect.

vagal hormone reception; hepatic vagotomy; glucose-induced early-phase insulin secretion; unrestrained conscious rat

THE CLEAR DIFFERENCE between insulin secretion(s) to an oral, and that to intravenous, glucose load under isoglycemia was first coined in 1964 (13, 29). As to the concept of the gut-to-islet connections, the term “enteroinsular axis” was proposed by Unger and Eisenraut (48), referring particularly to the role of peptides of the gastrointestinal tract in the axis. Later, an augmentation of insulin secretion upon glucose ingestion by still-undefined alimentary factors in those times, in the term “incretin”, was conceptualized under the term “incretin effect”, postulating complex humoral and neural mechanisms in the effect (10). Since in vitro and in vivo glucose-dependent insulinoportal actions by two of the intestinal hormones, gastric inhibitory polypeptide (also called glucose-dependent insulinoportal polypeptide, GIP) and glucagon-like peptide-1 (GLP-1) have been recognized (see reviews, Refs. 4, 16), they have been listed as incretin hormones operating in physiological situations. These peptides have been assumed to act directly on the respective receptor on β-cells of the pancreatic islets as circulating hormones, particularly concomitant with glucose absorbed through the gut (18, 25). Thus, their roles in diabetes mellitus have also been implicated (19).

The central role of the enteroinsular axis in meal-induced insulin secretion has attracted notice since it was known that at least more than 50% of the insulin released upon glucose ingestion was derived from gastrointestinal factors (34, 44). However, the relative importance of each of the humoral and neural factors in the enteroinsular axis still remains to date. In an attempt to delineate the role(s) of the factors in the axis, we performed a series of studies on GLP-1-(7–36) amide (GLP-1). The results were the following. First, GLP-1 infusion into the portal vein at a physiological dose as well as a pharmacological one facilitated dose-dependently the hepatic vagal afferent activities measured electrophysiologically, in contrast to no facilitating effect of noninsulinoportal full-length GLP-1-(1–37) or GIP in anesthetized rats (32, 42). The thus-facilitated afferent activities led further to an augmentation of the pancreatic vagal efferent activities in normal, but not in hepatic vagotomized, rats. This indicated the existence of the vagovagal reflex pathways induced by an appearance of GLP-1 into the portal circulation, suggesting another nature of GLP-1 as a neuroincretin (32). Second, gene expression of GLP-1 receptor (GLP-1R) in the primary afferent ganglion of the vagus, the nodose ganglion, was evidenced by methods of RT-PCR, Northern blotting, and in situ hybridization (33). Third, when neuronal cells isolated from the ganglion were exposed to GLP-1, an appearance of action potential and its burst was evoked and an increase of intracellular free Ca2+ levels was observed, even at as low a concentration as picomolar order (23). Together, our results have revealed that the hepatic vagal chemoreception to GLP-1 in the hepatoportal area plays a pivotal role in establishing the hepatoportal-pancreatic vagal reflex pathways and that the reception is exclusively sensitive to intraportal GLP-1 as observed at a much lower concentration than that reported in in vitro and in vivo humoral insulinoportal action (32, 42).

The results in our previous studies thus favored the idea that GLP-1 augments insulin secretion as a neuroincretin rather than as a circulating incretin hormone. Furthermore, insulin secretion has been known to be under the strong influence of autonomic innervation, particularly of vagal stimulatory control (2, 54). The hypothesis was subsequently reinforced when a unique tissue distribution of dipeptidyl peptidase-4 (DPP-4)

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that powerfully and rapidly degrades GLP-1 and its tissue specificities of GLP-1 degradation had been revealed (12, 15). Namely, there exists a juxtapositioning of L cells containing GLP-1, and the capillaries consist of DPP-4-positive endothelia, and only 25% of GLP-1 secreted from L cells appears into the portal circulation. Following a large proportion of GLP-1 having already been degraded by DPP-4, 40% of the remainder is then metabolized by the liver, and finally, only 10–15% of GLP-1 released from L cells enters the systemic circulation (15, 16). Consequently, its short half-life of only 1–1.5 min and a low concentration in plasma were reported (12, 49). Considering the half-life and the concentration of circulating GLP-1, there has been debate whether an endocrine (hormonal direct) effect of the peptide on β-cells operates in physiological circumstances (17, 35), leading to attract much attention to our hypothesis (32).

The aim of our present research was thus to determine whether or to what extent the electrophysiological vagal pathways triggered by chemoreception of GLP-1 in the hepatoporal area play actual role(s) in GLP-1-induced insulin secretion. To this end, a physiological dose for GLP-1 or glucose infusion into the portal vein, which achieves arterial GLP-1 and glucose levels comparable to those in meal ingestion, was first determined in conscious and unrestrained rats, expecting of the undisturbed pertinent neuronal pathways and physiological circulating route of the hormone released. Then, we infused GLP-1 concurrent with glucose at the physiological doses and evaluated arterial insulin levels in rats with a selective hepatic vagotomy (Vagox) or its sham operation (Sham). The results indicated an important role of the vagal pathways triggered by chemoreception of GLP-1 in the hepatoporal region and secured in place, through which GLP-1 [GLP-1-(7–36) amide, Bachem, Bubendorf, Switzerland] dissolved in physiological saline solution containing 0.2% bovine serum albumin or vehicle was infused into the portal vein. After closure of the abdominal walls, two silicon catheters (1.0 mm OD) were placed, one into the left common carotid artery for blood sampling and the other into the right jugular vein for systemic infusion (in some groups). The three catheters were exteriorized at the nape of the neck via subcutaneous tunnels and filled with heparinized saline solution (10 U/ml) using a stopper of metal wire until experiments. The animals, which were confirmed to have regained their preoperative weight and stayed in a good healthy condition within 6–8 days postoperatively, were subjected to the study, and body weights of Vagox and Sham groups were matched. After a 14-h overnight fast with free access to water, experiments were carried out in the morning (0930–1100) following a composure period of 30 min under conscious and unrestrained conditions in metabolic cages. Blood loss (0.4 ml) at each sampling time was simultaneously replaced with the same amount of a 50% suspension of red blood cells, which was obtained from a donor rat fasted similarly, and rinsed with 0.9% saline. Completeness of the hepatic vagotomy was microscopically substantiated at autopsy after the experiment, confirming the separating condition of the proximal and distal cut ends of the nerve and also lack of the adjunctive hepatic branch(es) from the ventral vagal trunk. Patency of the implanted catheters and the portal-to-portal circulation were also verified by injecting ink via the catheters after the experiment. The animals found to have incompleteness of the vagotomy and any troubles in the catheters and the portal circulation were excluded from the study. The protocols and procedures of the present study were approved by the Ethics Committee for Animal Experiments of Kanazawa Medical University.

**Determination of rates of glucose and GLP-1 infusion into the portal or jugular vein to achieve arterial plasma glucose and GLP-1 levels comparable to postprandial concentrations observed in conscious unrestrained rats.** To evaluate changes of postprandial glucose and GLP-1 levels in arterial plasma upon meal ingestion in conscious and freely moving conditions, rats were allowed to take standard chow and water for 60 min after an overnight fast (with free access to water) for 14 h (1900–0900). They consumed an average of 5.5 ± 0.7 g of the chow during the period of time, corresponding to ~23 kcal. And the samples were taken from the carotid artery before and at 15, 30, 45, and 60 min after starting the ingestion. Next, through our preliminary experiments in conscious rats, it was confirmed that intraportal infusion of GLP-1 at a rate of 1 pmol·kg⁻¹·min⁻¹ reproduced arterial plasma GLP-1 levels comparable to those observed upon the above-mentioned meal ingestion. On the basis of the rates obtained in the preliminary experiments, we thus employed intraportal GLP-1 infusion at a rate of 1 pmol·kg⁻¹·min⁻¹ as a physiological dose infusion in the following experiments. Furthermore, regarding the rate of glucose infusion into the portal vein to achieve the postprandial arterial glucose levels observed upon the meal ingestion, the infusion using a rate of 56 μmol·kg⁻¹·min⁻¹ was also employed as a physiological dose infusion. And the rate of systemic glucose infusion was reduced to 44 μmol·kg⁻¹·min⁻¹ so that arterial glucose levels matched those observed in the intraportal glucose infusion at 56 μmol·kg⁻¹·min⁻¹ were obtained, again through the results of our preliminary experiments (see RESULTS and DISCUSSION).

**Experimental protocol for intraportal glucose or glucose plus GLP-1 infusion studies in conscious rats.** Since our preliminary studies in anesthetized rats showed that intraportal GLP-1 infusion quickly stimulated insulin secretion and the secretion was more evident in an early phase, the framework of the present experimental protocol for intraportal infusion studies was set as follows (Fig. 1). Each experiment consisted of two periods of 10-min intraportal infusion (P1 and P2) following a composure period of 30 min. In P1, infusion of a mixture of glucose solution (at rate of 56 μmol/50 μl·kg⁻¹·min⁻¹) and vehicle (50 μl·kg⁻¹·min⁻¹) into the portal vein was continued for 10 min (from t = 0 to t = 10 min) using a two-barreled infusion pump.
humoral-mediated effect of GLP-1 represented by iAUC upon GLP-1 infusion in the Vagox group (iAUC\textsubscript{vagox}).

Statistical comparisons were performed using one-way repeated-measures analysis of variance (ANOVA) followed by Scheffe’s test as post hoc analysis for data in a group and two-way repeated-measures ANOVA for data among groups. To analyze data at each time point or for time period among the groups, one-way ANOVA followed by Scheffe’s test or unpaired t-test was used, when appropriate. All statistical tests were performed using Statview version 5.0 software (SAS Institute, Cary, NC). Statistical significance was accepted at $P < 0.05$.

RESULTS

Arterial plasma glucose, insulin, and GLP-1 levels during meal ingestion and arterial plasma glucose and GLP-1 levels upon intraportal glucose alone or GLP-1 infusion in rats. As mentioned in MATERIALS AND METHODS, rats were allowed to take standard chow for 60 min after 14-h fasting in conscious and freely moving condition. Upon this meal ingestion, arterial plasma glucose level averaged $5.7 \pm 0.2$ mmol/l before the feeding, increased significantly to $9.3 \pm 0.2$ mmol/l at 15 min after starting the feeding, and remained thereafter significantly elevated in the face of ongoing eating for the ensuing 45 min ($n = 6$, $P < 0.0001$ at 15, 30, and 60 min vs. the value at 0 min; Fig. 2A). Arterial plasma insulin level also increased significantly from $51 \pm 5$ pmol/l before the meal to $376 \pm 77$ pmol/l at 15 min and stayed at significantly higher levels until 60 min ($P < 0.01$ at 15 min and $P < 0.05$ at 30 and 60 min vs. the value at 0 min; Fig. 2B). Arterial plasma GLP-1 concentration increased from a premeal level of $8.9 \pm 3.0$ to $17.6 \pm 4.2$ pmol/l at 15 min, declining thereafter slightly to $16.0 \pm 3.8$ and $14.7 \pm 2.7$ pmol/l at 30 and 60 min, respectively ($P < 0.01$ at 15 min, $P < 0.05$ at 30 min, $P = 0.054$ at 60 min vs. the value at 0 min; Fig. 2C).

GLP-1 infusion into the portal vein at a rate of $1 \text{pmol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ for 10 min in 14-h fasted conscious rats induced a significant rise in arterial plasma GLP-1 level from $9.3 \pm 0.3$ pmol/l at the basal to $14.2 \pm 1.9$ and $17.0 \pm 2.7$ at 5 and 10 min, respectively ($n = 5$, $P < 0.05$ at 5 min, $P < 0.005$ at 10 min vs. the value at 0 min; Fig. 3). This 10-min infusion induced an increment of plasma GLP-1 concentration from the baseline by $7.6 \pm 2.4$ pmol/l, which approximated a peak incremental value of $8.7 \pm 2.4$ pmol/l at the first 15 min upon the meal ingestion mentioned above.

Likewise, a rise in arterial plasma glucose concentration matching that upon the meal ingestion was induced by intraportal infusion of $56 \text{pmol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ glucose in conscious rats ($n = 5$, data not shown; see also Fig. 4). Thus, as targeted, the intraportal infusion of GLP-1 at a rate of $1 \text{pmol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ and glucose at a rate of $56 \text{pmol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ was verified to achieve arterial plasma GLP-1 and glucose levels expected as a result of meal ingestion in conscious and unrestrained rats.

Intraportal infusion of glucose or glucose plus 1 pmol·kg\textsuperscript{-1}·min\textsuperscript{-1} GLP-1 in sham and vagox rats. Based on the results mentioned above, intraportal glucose ($56 \text{pmol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) plus GLP-1 (1 pmol·kg\textsuperscript{-1}·min\textsuperscript{-1}) or vehicle infusions were selected to simulate those variables upon the meal ingestion, and arterial insulin levels in Sham and Vagox rats were compared.

Upon intraportal glucose-alone infusion (P1 and P2), arterial plasma glucose levels increased significantly from the basal levels but remained within the postprandial range observed in normal rats ($n = 5$, each, $P < 0.0001$ at all time points in both
similarly in both Sham and Vagox groups, showing no differences from those in glucose-alone infusions (Fig. 4A). This glucose plus GLP-1 infusion in Sham evoked a prompt and robust rise in arterial plasma insulin levels from 106 ± 13 pmol/l at 10 min to 200 ± 21 pmol/l at 13 min, 209 ± 16 pmol/l at 15 min, and further to a mean peak of 258 ± 23 pmol/l at 20 min (P < 0.0001 at 13, 15, and 20 min vs. the value at 10 min; Fig. 4B), showing almost twofold the level of 135 ± 7 pmol/l at 20 min in Sham infused glucose alone. Contrastingly, despite the similar glucose changes in P2 in Sham and Vagox, insulin levels in P2 in Vagox were greatly attenuated: the levels did not significantly increase at 13 and 15 min from the value of 108 ± 17 pmol/l at 10 min and finally reached a significant level of 193 ± 21 pmol/l at 20 min (P < 0.01 at 20 min, NS at 13 and 15 min vs. the value at 10 min; Fig. 4B), showing a value of as small as 75% of that at 20 min in Sham. Additionally, after cessation of the glucose plus GLP-1 infusion, insulin levels returned promptly to the basal levels, whereas glucose levels declined rather slowly toward the basal levels in both groups.

The amount of insulin secreted by intraportal glucose or glucose plus 1 pmol·kg⁻¹·min⁻¹ GLP-1 infusion in P2 was further evaluated using iAUC, as shown in Fig. 6A. In Sham, the glucose plus GLP-1 infusion evoked an iAUC of 976 ± 65 pmol-min/l in contrast to 181 ± 90 pmol-min/l in the glucose-alone infusion (P < 0.001), showing a marked, 5.4-fold augmentation of insulin secretion by the physiological dose of GLP-1. However, in Vagox, augmentation of insulin secretion by glucose plus GLP-1 was strikingly diminished, where iAUC reached a level of only 393 ± 94 pmol-min/l (P < 0.005 vs. Sham), exhibiting a reduction by 60% compared with that of Sham. The diminished iAUC upon glucose plus GLP-1 in Vagox showed a significant attenuation in both groups (P < 0.001 vs. value before start of ingestion (0 min).

Next, when GLP-1 infusion at the rate in parallel with the glucose was performed in P2 in Sham and Vagox rats (n = 6 each), arterial plasma glucose levels in P2 again changed
Intraportal infusion of glucose or glucose plus 3 pmol·kg⁻¹·min⁻¹ GLP-1 in sham and vagox rats. To examine a dose-effect relationship between the GLP-1 doses in intraportal infusion and the augmentation of insulin secretion, the infusion was performed by stepping up the rate to 3 pmol·kg⁻¹·min⁻¹ to achieve arterial GLP-1 levels beyond a physiological range, adopting otherwise the same framework of protocol as shown in Fig. 1.

When glucose alone was infused intraportally at a rate of 56 μmol·kg⁻¹·min⁻¹ in P1 and P2, arterial plasma glucose levels before, during, and after the infusion were similar in Sham and Vagox and stayed again within a physiological postprandial range (n = 6, each; Fig. 4C). As shown in Fig. 4D, arterial insulin levels in Sham and Vagox were not significantly different at baseline and increased significantly upon the glucose-alone infusion through P1 to P2 in both groups (Fig. 4D; less than P < 0.05 at 5–20 min vs. the value at 0 min in both groups). But insulin levels in Sham tended to be slightly higher than those in Vagox (in P1) in this subset of experiments. Additionally, since glucose-alone infusion (in P1 and P2) in the
Intrajugular infusion of glucose or glucose plus 3 pmol·kg⁻¹·min⁻¹ GLP-1 in sham and vagox rats. The characteristic difference between insulin secretions in response to intraportal glucose plus GLP-1 in Sham and Vagox prompted us to examine how systemic infusion of glucose plus GLP-1 via the jugular vein acts on insulin secretion with respect to the vagal pathways. Since glucose and the hormone infused systemically were supposed to directly stimulate β-cells of the islet, systemic infusion of glucose at a rate of 44 µmol·kg⁻¹·min⁻¹ and GLP-1 at 3 pmol·kg⁻¹·min⁻¹ or vehicle was employed in Sham or Vagox rats following the same time framework of the protocol as used in intraportal infusion in Fig. 1 (see MATERIALS AND METHODS).

The systemic glucose-alone infusions achieved similar arterial glucose levels in Sham and Vagox, and, as expected, the levels matched those upon the intraportal infusions using a glucose dose of 56 µmol·kg⁻¹·min⁻¹ in all subset groups (Fig. 4E). Arterial plasma insulin levels in Sham and Vagox infused with glucose alone in P1 and P2 increased similarly and significantly, reaching ~240% of the baseline at 20 min (n = 5, each, less than P < 0.05 at 10–20 min vs. the value at 0 min in both groups; Fig. 4F). This glucose-alone infusion induced an insignificant difference of iAUC in P1 and P2 in both groups, although the iAUC in Vagox tended to be slightly smaller than that in Sham (P = 0.346; Fig. 5D).

Upon systemic glucose plus GLP-1 in P2 in Sham, insulin levels increased promptly within 3 min and reached a level of five times the preinfusion value at 20 min (n = 5; Fig. 4F). Moreover, the infusion in Vagox elicited a rapid and obvious insulin secretion almost comparable to those in Sham (n = 6; Figs. 4F and 6C). The systemic GLP-1 infusions under the isoglycemic condition revealed a quite contrasting feature of insulin secretions not involving the neuronal-mediated mechanism (Figs. 4 and 6).

Insulin response expressed as iAUC to systemic glucose plus GLP-1 in Sham was 27 times that caused by glucose alone (3,494 ± 667 vs. 129 ± 152 pmol·min⁻¹, P < 0.005; Fig. 6C).

Note, the iAUC in Sham was not significantly different from that in Vagox (3,494 ± 667 vs. 3,094 ± 737 pmol·min⁻¹, respectively; P = 0.703). The results again demonstrated a unique difference in insulinotropic action between intraportal and systemic GLP-1 infusion (Figs. 4 and 6), implying little contribution of the vagal pathways in stimulating insulin secretion by systemic GLP-1. In addition, iAUCs to intrajugular glucose alone (in P2 as well as in P1) were also similar in both Sham and Vagox groups, also suggesting little contribution of the vagal pathways in insulin secretion by such a physiological change of glucose levels in the artery as well as by a rather large change of glucose levels in the portal vein in the present series of the studies (Figs. 4 and 5).

Neuronal-mediated component of insulin secretion stimulated by intraportal or intrajugular GLP-1 infusion in sham and vagox rats. To delineate further characteristics of the neuronal-mediated component and humoral-mediated one in insulin secretions induced by intraportal and intrajugular GLP-1 infusions, incremental insulin levels above the preinfusion value (at 10 min) in Sham and Vagox were compared as shown in Fig. 7.

Upon intraportal infusion of a physiological dose of 1 pmol·kg⁻¹·min⁻¹ GLP-1, the incremental insulin levels in Sham were apparently greater than those in Vagox throughout P2 (P < 0.01; Fig. 7A). The difference in incremental insulin levels between both groups, namely corresponding to the NMC, was
prominent at 13 min and thereafter remained also in a quite
large range (P/H110210.0005, 0.005, and 0.05 at 13, 15, and 20 min,
respectively; Fig. 7A). The results clearly indicated that, par-
ticularly in a physiological situation, the NMC occurs promptly
and substantially. Intraportal infusion of pharmacological
GLP-1 (3 pmol·kg⁻¹·min⁻¹) also seemed to induce a consid-
erable difference in the incremental insulin levels between the
two groups, but the overall difference between groups was not

Fig. 6. Insulin-secretory responses to intraportal or intrajugular GLP-1 infusion under arterial isoglycemia in Sham and Vagox. iAUC of arterial plasma insulin changes
to intraportal infusions of glucose (56 μmol·kg⁻¹·min⁻¹) + a physiological GLP-1 dose (1 pmol·kg⁻¹·min⁻¹) or vehicle (A) to the intraportal glucose + a
pharmacological GLP-1 (3 pmol·kg⁻¹·min⁻¹) or vehicle (B), and to intrajugular infusion of glucose (44 μmol·kg⁻¹·min⁻¹) + GLP-1 (3 pmol·kg⁻¹·min⁻¹) or vehicle
(C) from start of GLP-1 infusion in P2 (at 10 min). Data are means ± SE. P values indicate significance between iAUCs in Sham and Vagox infused with the same
solution. **P < 0.01, ***P < 0.005, ****P < 0.0001 vs. glucose-alone infusion in Sham; †P < 0.05 vs. glucose-alone infusion in Vagox. NS, not significant.

Fig. 7. Time course of incremental changes of insulin-secretory response to intraportal or intrajugular GLP-1 infusion under arterial isoglycemia in Sham and Vagox.
Ratio of neuronal-mediated insulin component (NMC) to total GLP-1-induced insulin secretion is calculated according to the formula indicated. IAUCinsulin to intraportal
infusion of glucose (56 μmol·kg⁻¹·min⁻¹) + GLP-1 (1 pmol·kg⁻¹·min⁻¹) in P2 in Sham and Vagox (n = 6, each; A), intraportal infusion of the glucose + GLP-1
(3 pmol·kg⁻¹·min⁻¹) in Sham and Vagox (n = 5 and 6, respectively; B), and intrajugular infusion of glucose (44 μmol·kg⁻¹·min⁻¹) + GLP-1 (3 pmol·kg⁻¹·min⁻¹)
in Sham and Vagox (n = 5 and 6, respectively; C) from start of GLP-1 infusion in P2 (at 10 min). IAUCinsulin in Sham, stippled areas, in Vagox, hatched areas. Data
are means ± SE. *P < 0.05, **P < 0.005, ***P < 0.0001 vs. value at corresponding time in Vagox. Note that scales of the ordinates are different to depict relative
ratio of NMC.
statistical significant ($P = 0.342$; Fig. 7B). Intrajugular GLP-1 infusion (3 pmol·kg$^{-1}·$min$^{-1}$) induced no significant difference between the groups ($P = 0.873$; Fig. 7C).

When the ratio of the neuronal-mediated insulin component to the total insulin secretion stimulated by GLP-1 was calculated, using iAUC$_{\text{sham}}$ and iAUC$_{\text{vagox}}$, as mentioned in MATERIALS AND METHODS, the ratio showed values of 59.7, 46.2, and 11.4% in intraportal physiological or pharmacological and in a systemic pharmacological GLP-1 infusion, respectively (Fig. 7). Those results together indicated that the neuronal-mediated insulin component dominates the direct (hormonal-mediated) component in a physiological situation. The present results further showed that the non-neuronal-mediated insulin component increases in accordance with an addition of arterial GLP-1 levels beyond the physiological ranges.

Moreover, there was still observed a dose-relationship between the neuronal-mediated components induced by the two intraportal GLP-1 infusions in Sham: when the components were calculated according to the value of the ratio (59.7%) in the physiological GLP-1-induced insulin amount (iAUC 976 pmol-min/l) and to 46.2% in the pharmacological one (1,968 pmol-min/l), the neuronal-mediated insulin secretions to those GLP-1 doses showed a ratio of 1:1.56, respectively (Figs. 6 and 7). Thus, it was revealed that the neuronal-mediated component of insulin secretion evoked by intraportal GLP-1 behaves in a dose-dependent, but not all-or-none, fashion.

**DISCUSSION**

As mentioned in the introduction, our previous electrophysiological studies in anesthetized rats revealed that the vagal chemoreception of intraportal GLP-1 establishes the hepatopancreatic vagovagal reflex pathways, suggesting a neuroincretin’s role of GLP-1 (32, 42). The present studies have shown that the vagovagal pathways triggered by chemoreception actually lead to GLP-1-induced neuronal insulin secretion in conscious and unrestrained rats. The results were the following. 1) Under an intraportal glucose infusion achieving postprandial glycemia, concurrent intraportal GLP-1 infusion at a physiological dose (1 pmol·kg$^{-1}·$min$^{-1}$ for 10 min) promptly and markedly augmented insulin secretion in Sham, whereas the GLP-1-induced insulin secretion in Vagox greatly reduced by 60% despite isoglycemia. 2) Intraportal GLP-1 infusion at a pharmacological dose (3 pmol·kg$^{-1}·$min$^{-1}$ for 10 min) plus glucose induced a larger insulin secretion in Sham, but again a 46% reduction of the secretion in Vagox occurred. 3) Glucose plus GLP-1 infusion at a pharmacological dose (3 pmol·kg$^{-1}·$min$^{-1}$ for 10 min) into the jugular vein, under isoglycemia, induced approximately equal insulin secretions in Sham and Vagox. Taken together, the present observations clearly showed that intraportal appearance of GLP-1, particularly in the physiological amount, evokes a prompt and strong insulinotropic effect predominantly through the vagal neuronal-mediated pathways. This suggests that GLP-1 behaves as a neuroincretin in the enteroinsular axis under physiological circumstances.

The insulin secretion to a physiological arterial glucose excursion induced by intraportal or systemic glucose administration in Sham and Vagox showed rather unexpected results in aspects of the hepatic vagal innervation. The endocrine pancreas is known to be under strong influence of pancreatic, particularly the vagal efferent, innervation [see reviews (31, 54)]. In addition, the hepatic vagal branch, about three-fourths of the fibers of which are of afferent origin (1, 46), is known to be receptive to intraportal glucose levels, showing different response patterns of the afferents according to various intraportal glucose changes (37, 39). This portal vagal glucose sensing (37, 40), as well as the brain glucose sensing via the systemic circulation, is also known to send the vagal efferent signals to the pancreas (38) and thus evokes insulin secretion (26). It was also reported that the hepatic vagotomy modifies insulin secretion to glucose load (27, 55). In the present study, however, arterial insulin levels were not statistically different among Vagox and Sham subgroups, in the condition that arterial glucose levels in Sham and Vagox were “isoglycemic” across all subgroups in either intraportal or intrajugular glucose-alone infusions (Figs. 4 and 5). Those results strongly support the notion that the hepatic vagotomy in itself hardly influences fasting insulin levels and insulin responses to either intraportal or systemic glucose, as far as physiological glucose administrations of a short period of 20 min were applied (anatomic consideration on the hepatopancreatic vagal reflex pathways will be discussed later).

A clear and unique difference of insulin responses to intraportal GLP-1 between in Sham and Vagox rats was shown in the present study. A physiological dose of GLP-1 in parallel with glucose in P2 induced a peak insulin level of almost twofold the level in the glucose-alone infusion in Sham, indicating a clear glucose-potentiated insulinotropic action of intraportal GLP-1. Moreover, the amount of insulin secreted by the GLP-1 infusion in Sham, when expressed as iAUC, was drastically attenuated by 60% compared with that in Sham. Thus, it is quite conceivable to assume that a physiological amount of GLP-1 brought about by meal ingestion evokes insulin secretion predominantly through the neuronal-mediated mechanism rather than through the hormonal-mediated one in conscious rats. However, there are some discrepancies between the results in our previous electrophysiological studies using anesthetized rats (32) and those in the present study. First, the hepatic afferents increased dose dependently in response to a 1-min bolus intraportal GLP-1 administration in the range from a physiological dose of as low as 0.05 pmol to an apparent pharmacological dose of 4.0 pmol and showed no further increase to a dose above 0.2 pmol in the previous study, implying a ceiling effect in the reception. And the pancreatic vagal efferents to intraportal GLP-1 doses behaved in the same way. However, the present physiological dose (1 pmol·kg$^{-1}·$min$^{-1}$ for 10 min) had already exceeded the ceiling effect dose of 0.2 pmol/min. Nevertheless, in the present study we still observed a dose relationship in the neuronal-mediated insulin components induced by a physiological and a pharmacological in Sham (Figs. 6 and 7). Second, the pancreatic vagal efferents to the various doses of intraportal GLP-1 behaved as if in an on-and-off fashion upon the presence or absence of the hepatic vagus in the previous study (under rather less neuronal influences by anesthesia and constant fasting glucose level) (32). But again, the dose-related responses of the neuronal insulin components were observed in the present study, reflecting the pertinent neuronal pathways and ambient intraportal glucose levels. Those discrepancies strongly suggest that the neuronal-mediated insulin component in itself pertains to the inherent nature of glucose dependence. In this context, it is interesting whether the glucose dependence resides in the step of the
hepatoportal GLP-1 reception (7) and/or in the step of the pancreatic β-cells. In the latter step, the activities of the pancreatic efferent limb (probably via acetylcholine receptor and/or other neurotransmitters) in conjunction with the activities of intracellular ATP-sensitive K⁺ and Ca²⁺ channel induced by glucose in the β-cell might operate concomitantly (2, 14). Future investigations are really needed. Whatever the mechanistic process(es) may be, the present observations stress an important role of the intraportal GLP-1-induced neuronal events (together with glucose) in the incretin effect by GLP-1 in physiological circumstances.

An early insulin response to intraportal GLP-1 mediated through the neuronal mechanism(s), particularly at a physiological dose, was of note. Arterial insulin levels upon the intraportal infusion increased by 89, 97, and 144% at 13, 15, and 20 min from the preinfusion level in Sham, respectively, whereas only by 19, 36, and 79% in Vagox, respectively (P < 0.01; Figs. 4 and 7). A similar augmentation in early insulin secretions, but to a lesser extent, was also observed upon the pharmacological dose infusion. This early and dominant increase of the neuronal-mediated insulin component to intraportal GLP-1 might be of physiological and pathophysiological significance, since early secretion has long been implicated as a significant causative factor in glucose tolerance in normal subjects and also in those with type 2 diabetes mellitus (8, 45). Thus, the prompt insulinotropic effect induced by intraportal GLP-1 seems also to be of clinical relevance in GLP-1-related therapy, such as DPP-4 inhibitor application on type 2 diabetic patients. Taken together, GLP-1-induced insulin secretion at the very beginning of the intestinal phase of meal ingestion awaits further studies from the standpoint of the vagal chemo-reception to the circulating hormone in the hepatoportal area as well as in view of the local reception to the peptide near L cells in the intestine.

The insulin secretion by the present systemic GLP-1 administration raised an intriguing question regarding the role of GLP-1 receptor in the hepatoportal area. It is known that systemic GLP-1 plus glucose infusion, which achieve their physiological postprandial levels, induces far more insulin secretion than that by glucose alone in humans (50). In fact, the present systemic pharmacological GLP-1 infusion under physiological isoglycemia induced glucose-potentiated and equal insulin secretion in Sham and Vagox (Figs. 4, E and F, 5, C and D, 6C, and 7C). The results will be interpreted as that circulating GLP-1 directly stimulated insulin secretion mainly through direct hormonal-mediated action in both groups. However, it is interesting to speculate that pharmacological GLP-1 infusion should increase to some extent GLP-1 levels in the portal circulation (as mentioned in Materials and Methods), and this may in turn evoke the vagal chemo-reception of the hormone in the hepatoportal area and the putative neuronal-mediated action. And the neuronal-mediated insulin component of 11% was calculatedly obtained upon the systemic GLP-1 infusion. This notion, however, seems to be weakened, since the insulin levels were statistically not different between Sham and Vagox. At the same time, the observations might also raise the interesting question whether a negative portal-to-arterial gradient of GLP-1 concentrations is relevant, if any, to the neuroincretin effect seen in intraportal GLP-1 infusion. The issue remains for future study. In any event, the distinction between underlying mechanisms in insulinotropic action of intraportal or systemic GLP-1 administration highlighted the discrete action of endogenous or exogenous GLP-1. (As to systemic circulating GLP-1 and the CNS, see below).

The essential linkage between the vagal reflex pathways (32) and an augmentation of insulin secretion by intraportal GLP-1 was clearly shown in the present study. However, there are several points to be discussed. First, as to the pancreatic efferent limb of the reflex pathways, it is known that the pancreatic vagal efferent innervation comes from the hepatic branch and the ventral gastric branch of the ventral trunk (the left vagus), and from the dorsal gastric branch and the celiac branch of the dorsal trunk (the right vagus) (6, 46). When a minute amount of the efferent fibers in the hepatic branch was sectioned, although the afferent fibers outnumber the efferents (1, 46), the reduction of insulin secretion to intraportal GLP-1 might be caused, raising the question in interpreting comprehensively the present results. In this context, the reliable sectioning of the proper afferent filaments distributing only to the hepatoportal area in the hepatic branch has not been technically feasible to us. Moreover, we have unfortunately not been successful in the electrophysiological recording of the pancreatic efferent activities in the filaments derived from other pancreatic filaments (of the gastric branch) of the ventral trunk ipsilateral to the hepatic vagotomy. In addition, our previous electrophysiological study (32), which measured the pancreatic vagal efferents in the celiac division of the dorsal trunk contralateral to the vagotomy, revealed an apparent augmentation of the pancreatic efferents by intraportal GLP-1 administration in Sham and further disclosed that the hepatic vagotomy completely abolished the augmentation at either a physiological or even a pharmacological dose. Furthermore, it was reported that the vagal postganglionic neurons in the pancreas were almost equally innervated by both the ventral and the dorsal trunks (52). Additionally, crossing as many as 20% of the afferent, but not the efferent, axons between the left and right vagus at the thoracic level is known to exist in rats (47). And, we have recently observed that the gastric vagal efferents in both the ventral and dorsal gastric branches do change equally upon intraportal GLP-1 infusion at a physiological as well as a pharmacological dose, and the changes in both of the gastric efferents were completely abolished by the hepatic vagotomy (unpublished observation), suggesting an extrapolation to the present results. Along these lines, it is now reasonable to propose that the pancreas of rats with the selective hepatic vagotomy is functionally ousted from the efferent vagus ipsilateral and contralateral to the hepatic vagotomy, as far as the intraportal GLP-1-induced reflex pathways are concerned, making the pancreas vagally deefferentated not to respond reflexively to intraportal GLP-1. All together, it is quite conceivable that the appearance of GLP-1 in the portal vein induces such a dominant neuronal-mediated insulin secretion, particularly in physiological circumstances.

The second point to be discussed is that, in Vagox, both the electrophysiological response of the pancreatic efferents in our previous study and the present neuronal-mediated component of insulin secretion to intraportal GLP-1 were no longer noticed even at a pharmacological dose. It is, however, known that some parts of the brain, including the medulla oblongata, remain outside the blood-brain-barrier (BBB) (30), that there are some sites binding to circulating GLP-1 and expressing GLP-1R in the CNS (43), and that circulating GLP-1 penen-
trates the BBB to some extent (16, 24). Thus, it is of particular interest that the previous electrophysiological events and the present neuronal-mediated insulin secretion to intraportal GLP-1 were exclusively dependent on the pivotal role of the GLP-1 receptor by the hepatic afferent vagus. Our results strongly suggest that the penetration of circulating GLP-1 into the brain and consequent direct GLP-1R activation in the CNS (51), if it occurs at all, exert hardly the effect(s) on this type of the vagovagal reflex pathways and on the polysynaptic CNS activation related to the efferent limb of the vagal pathways triggered by intraportal GLP-1. In addition, the present systemic GLP-1 administration at a pharmacological dose also exhibited almost no neuronal effects on the insulin secretion, being partly in concord with the results reported previously by others (22). This concept, together with the pertinent questions, remains in the subject of further study.

The third, interesting but rather unexpected, point to be discussed is that the glucose levels before, during, and after the GLP-1 infusions remained quite similar in Sham and Vagox, despite large differences in insulin levels between the two groups. Several contributing factors to the observation might be listed. One is the short period of time (10 min) in GLP-1 infusion that was chosen on the basis of data from our preliminary studies, wherein intraportal GLP-1 infusion augmented clearly early insulin secretion in anesthetized rats. In fact, the present GLP-1 infusion evoked an early increase of insulin particularly from 15 min to a peak at 20 min, the latter part in P2. The other is the concurrent intraportal infusion of glucose that might prevent arterial glucose levels from declining within the short period of time. Consequently, it is quite plausible to explain that the GLP-1-induced insulin secretion had ceased before insulin fully exerted a glucose-lowering effect in the systemic circulation, which takes some time, particularly under the condition of concomitant intraportal glucose infusion (appearance). An additional possibility is that the presence or absence of the intraportal GLP-1-induced neuronal efferent signals to insulin-sensitive organs might also have been reflected in the arterial glucose levels (7, 9, 28). However, our detailed analysis on the disappearance of arterial glucose and insulin levels within 10 min after cessation of the glucose plus GLP-1 infusion failed to reveal significant relationships across the groups. Since the present experimental design was aimed to substantiate the presence of an important neuronal-mediated insulin secretion itself, another type of experimental design might be needed for clarification of the relationship between intraportal GLP-1-induced insulin and the resultant glucose handling in various tissues.

As for the role of the neural sensory system in GLP-1-induced insulin secretion, a few sets of interesting observations, to our knowledge, are worth noting. First, Balkan and Li (5) showed that insulin secretion augmented by intraportal administration of glucose and GLP-1 was inhibited by gangliionic blockade, but not muscarinic blockade, in rats. In addition, when GLP-1 was given into the systemic circulation, gangliionic blockade no longer inhibited the augmented insulin secretion. The authors claimed that stimulation of β-cells of pancreatic islets by GLP-1 was evoked through a neural reflex triggered in the hepatoportal system, introducing our notion that the vagal reflex pathways induced by the intraportal GLP-1 might exert the neuroincretin effect (32). However, usage of an apparently pharmacological dose of GLP-1 (10 pmol/kg as a bolus) and application of a ganglionic blocker could give a limitation in interpreting the results, because the blocker may perturb systemic and pancreatic local circulatory state through the effects on the sympathetic and nonmuscarinic parasympathetic activities. Second, Ahren (3) administered intravenously a low and a high dose of GLP-1 with glucose, using anesthetized adult mice that had been deafferentated by neonatal capsicain treatment, which might affect the vagal and nonvagal afferent systems. That stimulating study showed the results that insulin secretion induced by a bolus administration of supra-physiological glucose plus GLP-1 at a pharmacological dose of 0.1 nmol/kg, but not 10 nmol/kg, was reduced in the capsicain-treated group compared with vehicle-treated one, suggesting that GLP-1 augmented directly and/or indirectly (neurally) glucose-induced insulin secretion. In this line, it has recently been reported that neonatal capsicain treatment causes a reduction of GLP-1R density in the nodose ganglion (56). Our present results, thus, for the first time provide a neuronal background in the incretin effect exerted by GLP-1 that appears postprandially into the portal circulation, stressing an important role of hormone sensing in the hepatoporal region for the physiological neuroincretin effect. Additionally, as to another aspect of the sensing system relevant to intraportal GLP-1 and glucose, it was reported that infusion of glucose and GLP-1 into the portal vein produces lower peripheral glycemia than that of intraportal glucose alone (7, 21), although the precise mechanisms are still unknown. Thus, the potential role of vagal GLP-1 reception in the hepatoporal area and/or outside the area, which leads to induction of multiple effects in addition to the present neuroincretin effect, such as gastric emptying (20, 36, 53) and glucose metabolism probably accompanied by stimulation of glucose clearance in nonhepatic tissues (11, 21, 22, 41), will be the subject of further studies on nutrient homeostasis after meal ingestion.

Finally, the unique, but hitherto only electrophysiologically proven, neuroincretin effect through the GLP-1-induced vagovagal reflex pathways in the enteroinsular axis was clearly shown in the present study. Among a variety of the gut-liver-brain-pancreas axes that comprise humoral and/or neural signaling upon meal ingestion, the concept that GLP-1 (hormone)-translating neuronal pathways play important role(s) in postprandial nutrient homeostasis has gained some evidences. Such neuronal monitoring systems for ongoing changes of gut hormones and nutrients appearing into the portal circulation following meal ingestion will effectively contribute to nutrient homeostasis. Any pharmacological mean(s) that potentiates the potential role of vagal GLP-1 reception in the hepatoportal area might prevent arterial glucose levels from declining within the short period of time. Consequently, it is quite plausible to explain that the GLP-1-induced insulin secretion had ceased before insulin fully exerted a glucose-lowering effect in the systemic circulation, which takes some time, particularly under the condition of concomitant intraportal glucose infusion (appearance). An additional possibility is that the presence or absence of the intraportal GLP-1-induced neuronal efferent signals to insulin-sensitive organs might also have been reflected in the arterial glucose levels (7, 9, 28). However, our detailed analysis on the disappearance of arterial glucose and insulin levels within 10 min after cessation of the glucose plus GLP-1 infusion failed to reveal significant relationships across the groups. Since the present experimental design was aimed to substantiate the presence of an important neuronal-mediated insulin secretion itself, another type of experimental design might be needed for clarification of the relationship between intraportal GLP-1-induced insulin and the resultant glucose handling in various tissues.

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