ORIGINAL

Dynamics of plasma active GLP-1 *versus* insulin and glucose concentrations during GLP-1 infusion in rat model of postprandial hyperglycemia

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Abstract. *In vitro* studies in isolated pancreas and islets have shown that glucagon-like peptide-1 (GLP-1) promotes insulin release in a typical concentration-dependent manner. In contrast, the relationship between plasma GLP-1 and insulin concentrations *in vivo* is complicated, because GLP-1-promoted insulin release lowers blood glucose, which influences glucose-dependent insulinotropic ability of GLP-1. GLP-1 also stimulates insulin release *via* hepatoportal neuronal mechanism. Hence, the dynamic relationship between plasma active GLP-1 *vs.* insulin and glucose concentrations is obscure. In this study, we aimed to determine *in vivo* relationships between these parameters in rats. To mimic postprandial state, intraduodenal glucose challenge in anesthetized rats was performed, which can minimize the release of endogenous GLP-1. The glucose challenge induced the 1st phase and 2nd phase insulin release. GLP-1 infusion from jugular vein significantly and concentration-dependently enhanced area under the curve (AUC) of the 1st phase insulin, in which the minimum effective active GLP-1 concentration was 6.6 pmol/L. In contrast, bell-shaped dose responses were observed for both the 2nd phase and total insulin AUCs, in which a significant increase was obtained only with 11 pmol/L of active GLP-1 for total insulin AUC. A statistically significant reduction in the plasma glucose AUC was observed when active GLP-1 concentration was 11 pmol/L and 21 pmol/L. These results indicate that GLP-1 markedly enhances the 1st phase insulin release while less potently the 2nd phase insulin release, possibly due to a negative feedback regulation of β-cells *via* reduced plasma glucose levels by the enhanced 1st phase insulin release.

Key words: GLP-1, Insulin, Glucose, Rat

GLUCAGON-LIKE PEPTIDE-1 (GLP-1) is an incretin hormone secreted from enteroendocrine L-cells located on the proximal through distal gut. GLP-1 augments insulin release in a glucose concentration-dependent manner, thereby minimizing the risk of hypoglycemia [1]. It has been shown that GLP-1 interacts with the GLP-1 receptor in the pancreatic β-cells to elicit cAMP - protein kinase A (PKA) signaling in the presence of stimulatory glucose concentrations. The stimu-

lation of cAMP - PKA signaling enhances the activity of L-type Ca^{2+} channels and increases cytosolic Ca^{2+} concentration ($[Ca^{2+}]i$) in β-cells in a glucose-dependent manner [2-4]. The stimulated cAMP and $[Ca^{2+}]i$ systems, which could further induce a series of amplifying signals in β-cells, are thought to play key roles in the insulinotropic action on GLP-1 [4].

The active GLP-1 is cleaved into inactive form with the life time around 1-2 min by the action of dipeptidylpeptidase-4 (DPP4 or DPP-IV). Both the selective DPP4 inhibitors and the stable analogs of active GLP-1 have recently been introduced to the treatment of type 2 diabetic patients, providing remarkable advantages of potent insulinotropic ability and minimized risk of hypoglycemia [5]. As the mechanistic basis for the new medication with these drugs, the understanding of precise relationship between GLP-1, insulin and glu-

cose concentrations is essential.

The *in vitro* concentration relationship between GLP-1 and insulin release has been well studied [6-11]. In perfused rat pancreas, GLP-1 infusion enhances both 1st and 2nd phases of insulin releases in a GLP-1 concentration-dependent manner under glucose clamp conditions [6, 7, 10]. The *in vivo* effect of infused GLP-1 on insulin release has also been studied using glucose clamp method in animals and humans. The method of clamping glucose is useful for accurately assessing the concentration relationship between GLP-1 and insulin release both *in vitro* and *in vivo*. In contrast, *in vivo* concentration relationship between plasma active GLP-1 and dynamics of plasma insulin and glucose is complex, because both glucose and insulin levels are fluctuating at physiological states, especially in prandial period. Under *in vivo* postprandial situation, plasma GLP-1, insulin and glucose concentrations are related to each other: GLP-1-promoted insulin release subsequently accelerates the lowering of blood glucose levels and consequently attenuates glucose-dependent insulinotropic action of GLP-1. This feedback regulation to suppress excessive insulin release may occur in the later postprandial period, making it difficult to accurately evaluate *in vivo* dynamic relationship between plasma active GLP-1 and insulin concentrations.

Accumulating evidences suggest that GLP-1 also acts through neuronal pathway. GLP-1 infusion from portal vein, which is the physiological route for GLP-1 released from the intestine, affects impulse discharge rate of hepatic and pancreatic vagal nerves in rats [12, 13]. GLP-1 receptor is expressed in the nodose ganglion neurons that compose afferent vagus nerve [14], and GLP-1 evokes action potentials and increases $[Ca^{2+}]i$ in nodose ganglion neurons [15]. The increase in plasma insulin evoked by co-administration of GLP-1 and glucose into portal vein was blocked by ganglionic blocker [16]. These data suggest that the GLP-1-induced prandial augmentation of insulin release from pancreatic β-cells is partly mediated by the hepato-portal vagal pathway, and that the time course could be different for the vagus-mediated effect and direct effect of GLP-1 on β-cells. GLP-1 also inhibits gastric emptying and slows glucose absorption [17] through the neural mechanism and/or direct effect on stomach. Taken together, GLP-1 regulates *in vivo* insulin release and blood glucose through various mechanisms in postprandial states. Thus, it is of importance to evaluate the concentration-dependent effect of GLP-1 on the

plasma glucose and insulin dynamics through its direct action on pancreatic β-cells in the postprandial states.

In order to address this unanswered question, we developed an experimental condition in which GLP-1 concentrations were clamped in a physiological range, while glucose and insulin were variables. Furthermore, we employed intraduodenal, but not oral, glucose challenge under anesthesia to minimize endogenous GLP-1 release, and GLP-1 infusion from jugular vein to minimize the effects of GLP-1 on the vagal afferent nerve and stomach, allowing us to observe predominantly the direct effect of exogenously administered GLP-1 on pancreatic β-cells *in vivo*. By using this model, the present study aimed to clarify the *in vivo* relationship between plasma concentrations of active GLP-1 in a physiological range and postprandial dynamics of plasma insulin and glucose concentrations as determined primarily by the direct effect of GLP-1 on β-cells. We also aimed to determine minimum effective concentration of plasma active GLP-1 that affects pharmacodynamics of postprandial plasma insulin and glucose.

Materials and Methods

Chemicals

All chemicals were purchased from Wako Pure Chemical (Osaka, Japan) or Sigma (St Luis, MO, USA) unless specifically described. Thiobutabarbital solution was prepared by dissolving thiobutabarbital sodium (inactin) into propylene glycol at a concentration of 200 mg/mL, then, diluted into 100 mg/mL with saline. GLP-1 (7-36) amide was purchased from Peptide Institute, Osaka, Japan.

Animal surgery

Male Wistar rats aged 9 weeks (280-320g, Charles River Japan, Yokohama, Japan) were used in this study. The animals were fasted overnight. On the day of the experiment, animals were anesthetized by intraperitoneal (i.p.) injection of thiobutabarbital (100 mg/kg) at 13:00. Then, catheters were placed onto the left carotid artery and right jugular vein for blood sampling and GLP-1 infusion, respectively. Another catheter was placed onto the duodenum according to the method described previously [27] as the route for glucose load. The animals were placed in the hot plate and maintained at 37°C during the experiment. All animal experimental protocols were approved by the Institutional Animal

Care and Use Committee.

GLP-1 infusion and glucose tolerance test

Thirty minutes after completion of the surgery, either vehicle (0.1% w/v bovine serum albumin in saline) or GLP-1(7-36) amide was infused from right jugular vein at a rate of 0.01 mL/min. Five minutes later, glucose at 0.5 g/kg was infused into the duodenum *via* catheter at a rate of 30 mL/kg/min for 1 min. Blood was sampled from catheter placed on the left carotid artery by a syringe chilled with EDTA 2Na (1 mg/mL, DOJINDO, Osaka, Japan), aprotinin (500 U/mL, Boelinger Manheim, Germany) and DPP-IV inhibitor (10μL/mL, Linco, St. Charles, MO, USA). Plasma was collected from the blood by centrifugation, and subjected to chemical analysis.

Chemical analysis

Plasma glucose was measured by glucose kit (Determinar GL-E, Kyowa Medex, Japan). Plasma insulin was determined by use of sandwich ELISA kit (Morinaga Seikagaku, Yokohama, Japan). Plasma active GLP-1 was determined by the active GLP-1 ELISA kit (Linco) by using synthetic GLP-1 (7-36) amide (Peptide Institute, Osaka, Japan) as a standard. Lower limit of quantification (LLQ) of active GLP-1 was 0.78 pmol/L. Values that show below LLQ were treated as 0.78 pmol/L.

Data analysis

Data were presented as mean ± SE unless specified. Area under the curve (AUC) of plasma insulin and glucose were calculated using the trapezoidal rule. Temporal profiles of plasma active GLP-1, insulin and glucose were analyzed by two-way ANOVA with the Bonferroni post hoc test. AUC of insulin and glucose excursion were analyzed by one-way ANOVA, followed by Dunnett's Multiple Comparison Test. All statistical analyses were performed by using Graphpad Prism ver. 4.03 (Graphpad Software Inc., CA, USA). A *p* value of <0.05 was considered statistically significant.

Result

Plasma active GLP-1 levels during GLP-1 infusion

The animals were given either vehicle or GLP-1 (7-36)amide (GLP-1) at 5 min prior to intraduodenal glucose load (0.5 g/kg) under anesthetic condition, with the GLP-1 infusion rate of 0.42, 0.83, 1.7 and

3.3 pmol/kg/min, respectively. Basal plasma active GLP-1 level was mostly below limit of quantification (LLQ; 0.78 pmol/L). Intraduodenal glucose did not significantly increase plasma active GLP-1 (1.3 ± 0.17 pmol/L including samples below LLQ). GLP-1 infusion elevated plasma active GLP-1 concentrations to the plateau levels of 3.5 ± 0.33, 6.6 ± 0.70, 11 ± 0.92 and 21 ± 0.90 pmol/L, respectively, within 10 min after initiation of infusion (5 min after intraduodenal glucose load) (Table and Fig. 1A).
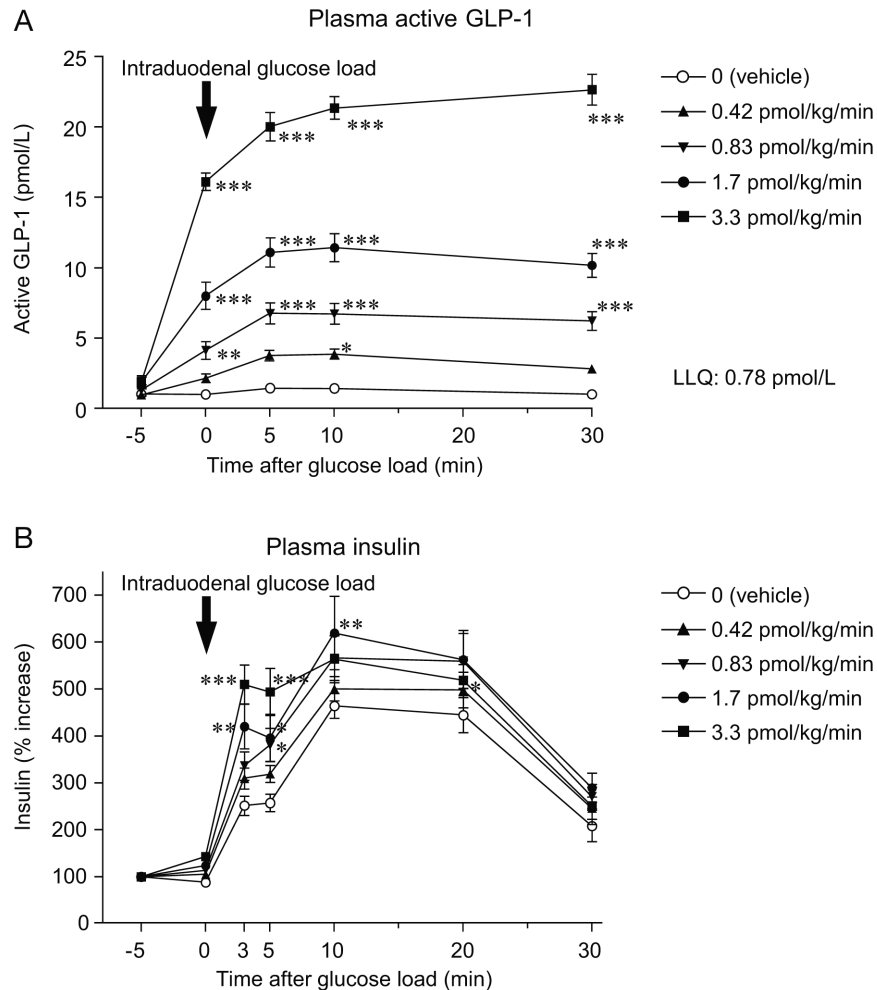
Effect of GLP-1 infusion on insulin dynamics

Glucose load at 0.5 g/kg was performed intraduodenally at 5 min after initiation of either vehicle or GLP-1 infusion. The glucose load induced biphasic rise in the plasma insulin, the 1st phase between 0 and 5 min followed by the 2nd phase between 5 and 30 min (Fig. 1B). The glucose-induced 1st phase insulin release was enhanced by GLP-1 infusion in a concentration-dependent manner; Statistical significance was observed at 3 and/or 5 min after glucose load in the groups infused with GLP-1 at 0.83, 1.7 and 3.3 pmol/kg/min (Fig. 1B) with average peripheral plasma active GLP-1 concentrations of 6.6, 11 and 21 pmol/L, respectively (Table and Fig. 1A). Although the 2nd phase insulin tended to increase in GLP-1-treated groups, the statistical significance was not observed except for the time points with GLP-1 at 1.7 and 3.3 pmol/kg/min.

The AUC for the 1st phase insulin ($AUC_{(0-5\ min)}$) showed a linear concentration response relationship with statistically significant increases in groups with GLP-1 at 0.83 pmol/kg/min and higher corresponding to average plasma active GLP-1 concentrations of 6.6 pmol/L and higher (Fig. 2A). In contrast, both the 2nd phase insulin AUC ($AUC_{(5-30\ min)}$) and total insulin AUC ($AUC_{(0-30\ min)}$) exhibited bell-shaped dose responses, in which the highest values were observed at plasma active GLP-1 of 11 pmol/L and a statistically significant increase was obtained only for total insulin AUC. (Fig. 2B and 2C). The data suggested that GLP-1 predominantly affects to 1st phase insulin release during intraduodenal glucose-induced hyperglycemia, with a minimum effective plasma active GLP-1 concentration of 6.6 pmol/L.

Effect of GLP-1 infusion on glucose excursion

At 5 min after initiation of either vehicle or GLP-1 infusion, glucose at 0.5 g/kg was challenged intraduodenally. Plasma glucose excursion achieved at a peak

A

Plasma active GLP-1

Intraduodenal glucose load

- ○ — 0 (vehicle)
- ▲ — 0.42 pmol/kg/min
- ▼ — 0.83 pmol/kg/min
- ● — 1.7 pmol/kg/min
- ■ — 3.3 pmol/kg/min

LLQ: 0.78 pmol/L

Active GLP-1 (pmol/L) vs *Time after glucose load (min)*

B

Plasma insulin

Intraduodenal glucose load

- ○ — 0 (vehicle)
- ▲ — 0.42 pmol/kg/min
- ▼ — 0.83 pmol/kg/min
- ● — 1.7 pmol/kg/min
- ■ — 3.3 pmol/kg/min

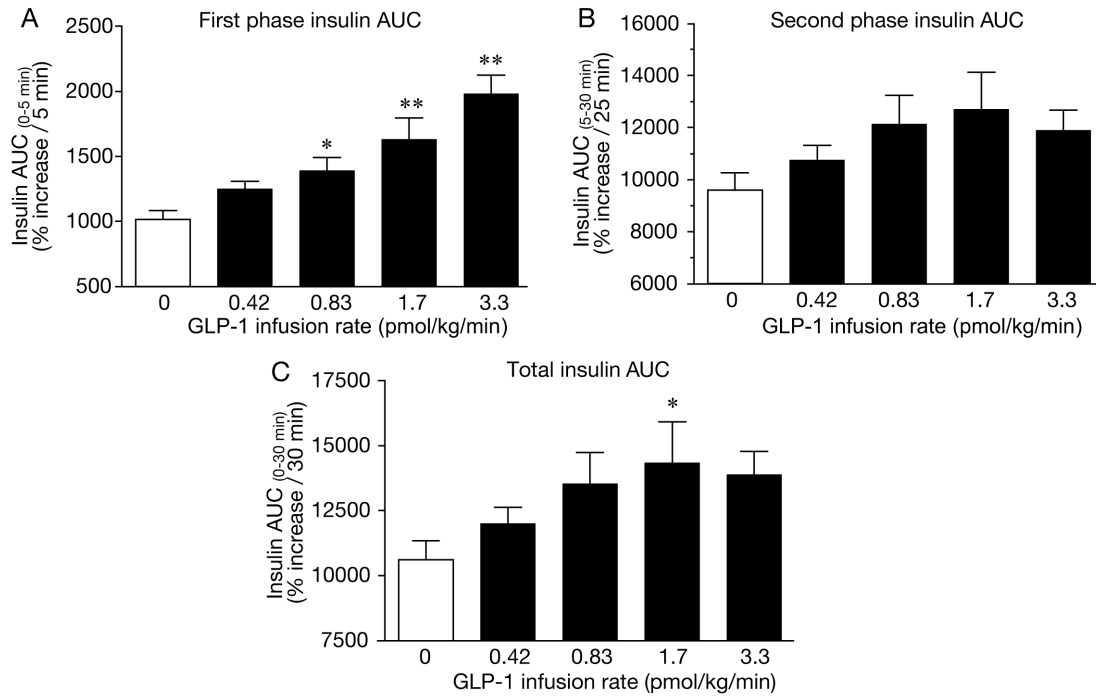*Insulin (% increase)* vs *Time after glucose load (min)*

Fig. 1 Plasma active GLP-1 (A) and plasma insulin concentrations (B) during GLP-1 infusion and in response to intraduodenal glucose load. GLP-1 infusion was initiated at 5 min prior to glucose load and continued throughout the measurements. Glucose at 0.5 g/kg was loaded intraduodenally at 0 min (arrow). Data represent means ± SE, n=7-16. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ compared with vehicle-treated control group. LLQ; lower limit of quantification.

Table

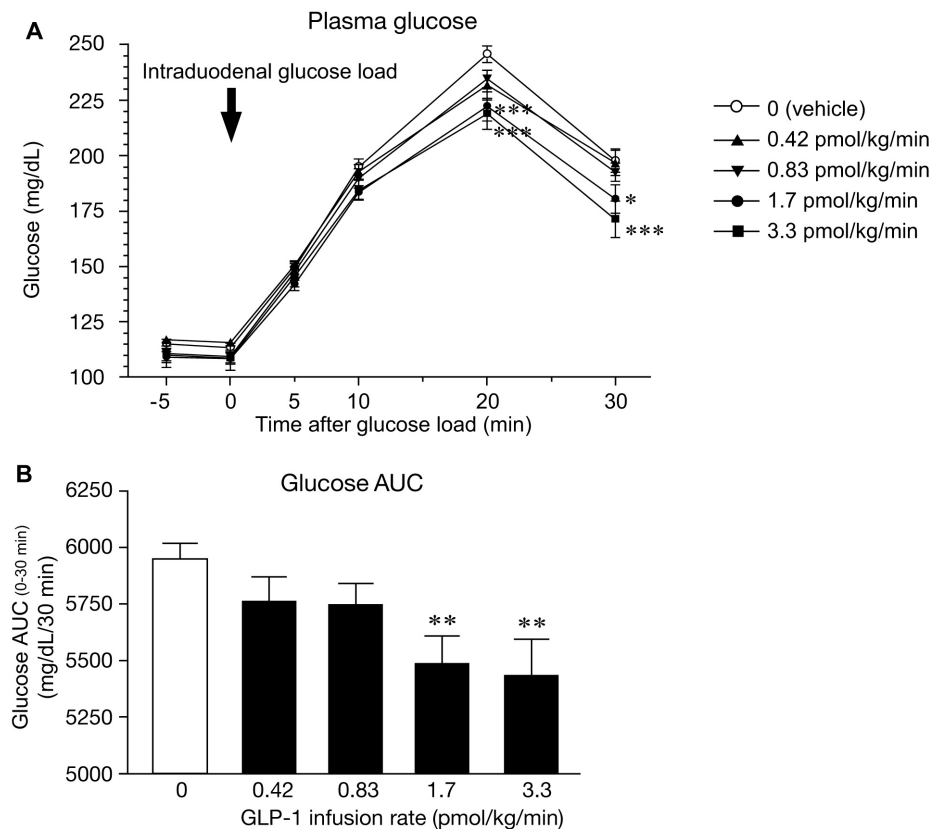| Treatment group	GLP-1 infusion rate (pmol/kg/min)	Average concentration of plasma active GLP-1 (pmol/L)
Vehicle control	-	1.3 ± 0.17
GLP-1 infusion	0.42	3.5 ± 0.33
	0.83	6.6 ± 0.70
	1.7	11 ± 0.92
	3.3	21 ± 0.90

GLP-1 infusion rates and average active GLP-1 concentrations between 5 and 30 min after intraduodenal glucose load. Data represent means ± SE, n=7-16.

concentration at 20 min after glucose load, and then, partially declined in all groups (Fig. 3A). Presence of GLP-1 infusion improved glucose tolerance in a GLP-1 concentration-dependent manner. Minimum effective concentration of plasma active GLP-1 for statistical significance was 11 pmol/L in both plasma glucose excursion curve and glucose AUC ($AUC_{(0-30\ min)}$) (Fig. 3A and 3B). No further decrease in the glucose AUC was observed even with 21 pmol/L of plasma active GLP-1, the highest concentration used in the present study, suggesting that an average plasma active GLP-1 concentration of 11 pmol/L gives suboptimal effect of improving glucose excursion curve as well as glucose AUC in the present experimental condition.

Fig. 2 AUC of 1st phase (0-5 min; A), 2nd phase (5-30 min; B) and total (0-30 min, C) plasma insulin after intraduodenal glucose load during GLP-1 infusion in rats. Data represent means ± SE, n=7-16.   * $p<0.05$ and ** $p<0.01$ compared with vehicle-treated control group.

Fig. 3 Time profile (A) and $AUC_{(0\text{-}30\,min)}$ (B) of plasma glucose after intraduodenal glucose load during GLP-1 infusion in rats. Data represent means ± SE, n=7-16.   * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ compared with vehicle-treated control group.

Discussion

In the present study, we aimed to assess *in vivo* relationship between plasma active GLP-1 concentration and dynamics of plasma insulin and glucose that is determined primarily by the direct effect of GLP-1 on pancreatic β-cells during postprandial hyperglycemia. GLP-1's action to enhance insulin release includes both direct effect on pancreatic β-cells and indirect mechanisms through neuronal regulation [12-16]. We infused GLP-1 from the jugular vein instead of the portal vein in this study. Although the portal vein is the physiological route for the GLP-1 released from the intestinal endocrine L-cells, the jugular vein route has several advantages. First, this route is able to deliver GLP-1 directly to the islets of Langerhans and therefore suitable for assessing the direct immediate effect of GLP-1 on islets. Furthermore, this method can exclude the action of GLP-1 that may secondarily affect insulin release and/or glucose metabolism: GLP-1 of the intestinal/portal origin inhibits gastric emptying *via* neural mechanisms and/or direct effect on stomach [17], which could influence glucose absorption by the intestine. Secondly, we used intraduodenal, instead of oral, glucose load in this study. The glucose absorption rate after intraduodenal glucose challenge under anesthetic condition was comparable to that after oral glucose challenge in conscious animals (*J.E. T.Y.*, unpublished data). The advantage of intraduodenal glucose challenge is to mitigate the release of endogenous GLP-1 that is known to be stimulated by oral glucose administration possibly *via* neural mechanisms [18]. Intraduodenal glucose load can also diminish the effect of GLP-1 on gastric emptying *via* neural mechanisms and/or direct effect on stomach. Our data that intraduodenal glucose load increased plasma glucose without elevating plasma active GLP-1 concentration under anesthetic condition suggests that the present experimental condition succeeded in minimizing the induction of endogenous GLP-1 release, allowing the accurate assessment of active GLP-1 concentration-insulin release relationship under the conditions reflecting postprandial hyperglycemia.

Our study revealed that GLP-1 enhanced insulin release at the 1st phase to a greater extent than at the 2nd phase. Minimum effective peripheral plasma active GLP-1 concentration to increase 1st phase insulin release and glucose tolerance was 6.6 and 11 pmol/L, respectively. The apparent discrepancy in the minimum effective concentrations to affect plasma insulin and glucose levels might be due to the following mechanism. The GLP-1-induced 1st phase insulin release attenuates the elevation of blood glucose, which in turn has negative direct influence on pancreatic β-cells and reduces glucose-dependent insulinotropic ability of GLP-1, thereby attenuating the 2nd phase insulin release. In fact, when the plasma active GLP-1 concentration was elevated form 11 pmol/L to the highest (21 pmol/L), the 1st phase insulin release was much enhanced but 2nd phase insulin was suppressed (Fig. 2A and 2B).

Previous studies in healthy subject and patients with type 2 diabetes reported that GLP-1 infusion enhanced both 1st phase and 2nd phase insulin release [19, 20]. In these studies, plasma active GLP-1 reached at high levels of 40-150 pmol/L, the supraphysiological to pharmacological concentrations. By contrast, after oral glucose or meal challenge, plasma active GLP-1 concentration increases to peak levels of 9-13 pmol/L in human subjects [21, 22], and the treatment with selective DPP4 inhibitors further elevates peak plasma active GLP-1 concentrations to 15-20 pmol/L [21, 22]. In rats, oral glucose or meal loading elevates peak active GLP-1 concentrations to 13-22 pmol/L [23, 24]. Taken together, the active plasma GLP-1 concentrations used in the present study (3.5-21 pmol/L) are thought to be within a physiological range. Thus, it is suggested that the peripheral plasma active GLP-1 physiologically elevated by glucose and/or meals and that therapeutically elevated with DPP4 inhibitor promote insulin release *via* the kinetics shown in the present study. However, extrapolation of our data to *in vivo* physiological situation should be carefully performed by taking the following *in vivo* dynamics of GLP-1 into account. GLP-1 is rapidly degraded by DPP4 located on the intestinal capillary endothelium right after its release from L-cells [25], and only 25% of GLP-1 arrives as an active form in liver [25], where it is further decreased by hepatic clearance. As a result, only 10–15% of newly secreted GLP-1 reaches the systemic circulation as an active form [26]. These reports indicate that active GLP-1 concentration in portal vein is much higher than that in systemic circulation to islets and suggest that the portal sensing of GLP-1 and resultant activation of neural circuit could also contribute to the potentiation of insulin release [13, 16]. However, both the mechanisms and physiological role for the insulinotropic action of GLP-1 through neural circuit

remain to be further clarified.

In conclusion, the present data demonstrate that physiological level of elevated active GLP-1 in peripheral blood can primarily promote 1st phase insulin release at least partly *via* its direct action on pancreatic β-cells.

Acknowledgement

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