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Reproducible insulin secretion from isolated rat pancreas preparations using an organ bath

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Abstract: Diabetes mellitus is a lifestyle-related disease that is characterized by inappropriate or diminished insulin secretion. *Ex vivo* pharmacological studies of hypoglycemic agents are often conducted using perfused pancreatic preparations. Pancreas preparations for organ bath experiments do not require cannulation and are therefore less complex than isolated perfused pancreas preparations. However, previous research has generated almost no data on insulin secretion from pancreas preparations using organ bath preparations. The purpose of this study was to investigate the applicability of isolated rat pancreas preparations using the organ bath technique in the quantitative analysis of insulin secretion from β-cells. We found that insulin secretion significantly declined during incubation in the organ bath, whereas it was maintained in the presence of 1 μM GLP-1. Conversely, amylase secretion exhibited a modest increase during incubation and was not altered in the presence of GLP-1. These results demonstrate that the pancreatic organ bath preparation is a sensitive and reproducible method for the *ex vivo* assessment of the pharmacological properties of hypoglycemic agents.

Key words: amylase, glucagon-like peptide-1, insulin, organ bath, rat pancreas

Introduction

Diabetes mellitus is a lifestyle-related disease that is characterized by inappropriate or diminished insulin secretion. Recently, several hypoglycemic agents have been developed; however, a transformative therapy has not yet been established. Insulin is a peptide hormone that is secreted from pancreatic β-cells. In developing

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new pharmacotherapeutics for diabetes mellitus, it is important to explore candidate substrates that induce insulin secretion.

The pancreas is comprised of pancreatic exocrine tissue that produces digestive enzymes and endocrine tissue that secretes hormones to regulate glucose homeostasis. The endocrine tissue is comprised of cell aggregates known as the islets of Langerhans. The islets of Langerhans are composed of insulin-producing β-cells, α-cells that produce glucagon, δ-cells that produce somatostatin, and pancreatic polypeptide cells. Somatostatin inhibits insulin and glucagon secretion from the islets of Langerhans. The various cell types within the islets of Langerhans are intimately associated with each other and are thought to interact [12, 15]. On the one hand, glucagon-like peptide-1 (GLP-1) is one of the gastrointestinal hormones that increases insulin secretion and inhibits glucagon secretion in a glucose-dependent manner [5]. It was reported that plasma insulin levels rose significantly with GLP-1 infusion in human subjects [17]. In humans, GLP-1 analogues are used therapeutically to improve insulin secretion and treat type 2 diabetes mellitus.

To investigate the mechanisms of insulin secretagogues *ex vivo*, researchers have employed experimental methods utilizing insulinoma cell lines [2, 23] or isolated islets of Langerhans [18]. *In vivo*, extrapancreatic factors, such as neural factors in particular, cannot be excluded from the insulin secretion system. It is known that autonomic nerves are present in islets of Langerhans [8, 34], and have an effect on insulin secretion. Babkin and Starling described the exocrine function of the pancreas using perfused dog pancreas [3]. Furthermore, Long developed and demonstrated an experimental device for insulin measurement from isolated perfused pancreas [20]. To date, perfused rodent pancreas preparations have been widely used in the assessment of insulin secretion regulation [21, 33].

Experimental systems using an organ bath have been used to investigate the effects of hormones and drugs on organ responses [4, 29]. The organ bath technique is a traditional experimental system and has been frequently used for a number of organs, e.g., blood vessels [9, 24], and digestive tract [13, 16]. However, there are few previous studies on amylase secretion from pancreas preparations using organ baths [25, 31]. Pancreas preparations for organ bath experiments are less complex than isolated perfused pancreas preparations. Unlike the experimental use of isolated perfused pancreas preparations, pancreas preparations for organ bath experiments do not require cannulation. Drug effects on organ tissues can be observed directly in organ bath experiments. In light of these factors, we propose that this system would be more convenient and simpler, as well as sufficiently reproducible, for experiments on insulin secretion induced by substrates compared with isolated perfused pancreas.

However, previous research has generated almost no data on insulin secretion from pancreas preparations in organ bath experiments or on amylase secretion. The purpose of this study is to investigate the applicability of isolated rat pancreas preparations using the organ bath technique in the quantitative analysis of insulin secretion from β-cells, as well as amylase secretion.

Materials and Methods

Animals

Male Wistar-Imamichi rats (*Rattus norvegicus*; 13–32 weeks old) were obtained from the Institute for Animal Reproduction (Ibaraki, Japan). The rats were housed under controlled temperature and light conditions (lights on from 7:00 to 19:00) and supplied with laboratory food CE-2 (Clea Japan, Inc., Tokyo, Japan) and water *ad libitum* until just before the experiments. The experimental protocols were approved by the Animal Care and Use Committee, and the experiments were carried out according to the Guidelines for Animal Experimentation of Dokkyo Medical University. All efforts were made to minimize the number of animals used and their suffering. Twelve rats were randomly allocated to two groups: control group (n=6; mean age, 173.2 ± 20.8 days) and GLP-1 group (n=6; mean age, 167.5 ± 21.7 days).

Experimental protocol

The organ bath technique was performed according to a previous study [16]. Rats were humanely euthanized by carbon dioxide (CO_2). Whole blood was taken from the caudal vena cava to remove the blood from the pancreatic tissue. The pancreas was carefully separated from the other organs, i.e., the stomach, duodenum, and spleen (Figs. 1A1 and A2), and then immersed in modified Tyrode's solution (136.9 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1.05 mM $MgCl_2$, 0.42 mM NaH_2PO_4, 11.9 mM $NaHCO_3$, 5.56 mM glucose and 3 mM EDTA).

The fatty tissue surrounding the pancreas was almost

white in color and translucent, while the pancreas was light pink and opaque. The fatty tissue was removed and the collected pancreatic tissue was divided into two parts, the right lobe containing the body of the pancreas (approximately 3.0 cm), and the residual pancreas (Fig. 1A2). Next, a nylon mesh 5.0 cm^2 or greater was cut and used to wrap the right lobe with the body segment. The isolated pancreas preparation was tied using cotton thread on both sides of the nylon mesh, taking care not to catch the pancreatic tissue. The nylon mesh was cut 5.0 mm outside of both knots.

One end of the isolated pancreas preparation was anchored to a J-shaped acrylic stick using one side of the tied thread. Organ baths with 10 ml volumes were used. The preparation anchored to the acrylic stick was placed in the center opening of the organ bath, which was filled with modified Tyrode's solution at 37°C and aerated with 5% CO_2 and 95% O_2. The tissue was suspended between the hook using the other side of the tied thread, and then stretched to tension in a longitudinal direction (Fig. 1A3). The isolated pancreas preparations were incubated over a 120-min period.

To induce insulin outflow from the pancreas preparations, 1 μM GLP-1 (Human, 7–36 Amide, Peptide Institute, Inc., Osaka, Japan) was added to the organ bath medium during the 0–40-min period. Previous studies employed concentrations from 0.25 nM to 2.5 μM GLP-1 in isolated rat islets [26], and 25 nM GLP-1 in isolated perfused rat pancreas [21]. In addition, concentrations of 100 nM GLP-1 in a mouse insulinoma cell line and 1 μM GLP-1 in a rat insulinoma cell line [19, 27] have been used. Aliquots of the sampled solutions were transferred to Protein LoBind® tubes (Eppendorf, Hamburg, Germany) and frozen at −80°C until used in measurements. After each sample collection, the isolated pancreas preparation was placed on filter paper. At the end of the experiment, the pancreas was removed from the organ bath, dried on filter paper, and subsequently weighed.

Quantitative determination of insulin and amylase

Quantitative determinations of insulin in the collected organ bath samples were made with an Ultra Sensitive Rat Insulin ELISA Kit (Morinaga Institute of Biological Science, Inc., Kanazawa, Japan) according to the manufacturer's instructions. The collected samples were prepared using the dilution of the ELISA kit. Aliquots (100 μl) of standards and diluted samples were added to antibody-coated wells and incubated for 120 min at 4°C. Next, the wells were rinsed five times with wash buffer. A 100-μl aliquot of anti-insulin enzyme conjugate solution was added to each well and reacted for 30 min at room temperature. The wells were then washed seven times with wash buffer, 100 μl of substrate was added, and the enzyme reaction was allowed to proceed for 40 min under darkness. The reaction was stopped by adding sulfuric acid. The plate was immediately read at 450 nm as the measurement wavelength and 610 nm as the reference wavelength.

Dry chemistry analyzers (Fuji Dri-Chem 3500V and Fuji Dri-Chem Slide AMYL-PIII; Fujifilm Corp., Tokyo, Japan) were used to measure amylase in the collected samples. The collected samples were diluted with phosphate-buffered saline as necessary, and 10-μl aliquots were deposited on a Fuji Dri-Chem Slide. Levels of insulin and amylase are presented relative to the pancreas preparation weight.

The sampling design is shown in Fig. 1B. The pancreas preparations were equilibrated within the first 60 min of the study, with 5 ml fresh replacement of Tyrode's solution every 20 min (equilibration 1). After equilibration 1 and solution discharge, 2.3 ml of fresh solution was introduced into the empty organ bath with the pancreas preparation and incubated for 60 min, with fresh replacement of the solution every 20 min (equilibration 2). The whole pancreas preparation was immersed in 2.3 ml buffer in the organ bath. Samples were collected from the 2.3 ml of Tyrode's solution six times every 20 min (−20–100 min) after equilibration 1 and 2. Insulin levels of the collected samples during −20–60 min were measured at four sampling time points in each group. Amylase levels of the collected sample during −20–100 min were measured at six sampling time points in each group.

Insulin area under the curve (AUC) was determined from baseline to 60 min in each group. Similarly, amylase AUCs were determined from baseline to 60 min, and from baseline to 100 min in each group. Insulin AUC (%) and amylase AUC (%) are shown using calculated values from baseline as 100%.

Statistical analysis

Statistical tests were performed using IBM SPSS (version 12; IBM Corp., Armonk, NY). The Student's *t*-test was used for comparisons between two groups. A repeated-measures one-way ANOVA was performed to assess changes over time. *P*-values less than 0.05 were consid-

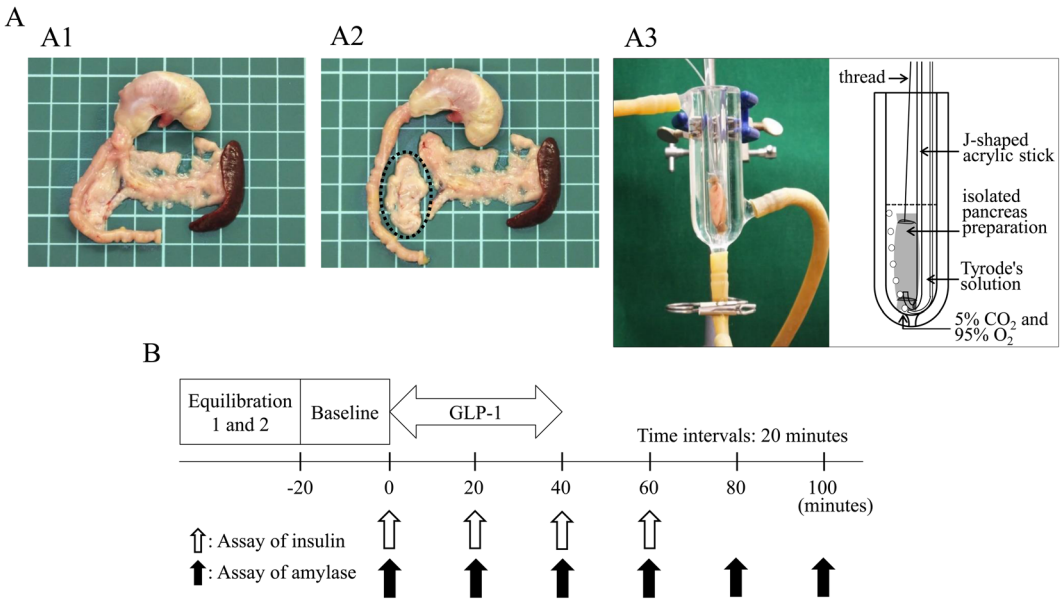

Fig. 1. Experimental system. (A) The experimental organ bath setup. A1: Stomach, duodenum, spleen, and pancreas were excised from the rat. A background grid with 1.0 cm^2 squares is shown. A2: The stomach and duodenum were separated and the pancreas was excised. The right lobe containing the pancreatic body (surrounded by a dotted line) was used. A3: Pancreas preparations in the organ bath. (B) Sampling design and GLP-1 drug treatment regimen. The pancreas preparation was suspended in an organ bath filled with Tyrode's solution for 60 min (20 min × 3 times) during equilibration 1. Next, the pancreas preparation was incubated with 2.3 ml of fresh Tyrode's solution for 60 min (20 min × 3 times) during equilibration 2. After equilibration, the organ bath buffer was replaced with 2.3 ml of fresh Tyrode's solution and incubated further. Samples of the incubation buffer were collected six times every 20 min in both groups. GLP-1 (1 μM) was prepared just before administration in 2.3 ml fresh Tyrode's solution to the organ bath. GLP-1 was administered for 40 min (20 min × 2 times). GLP-1: glucagon-like peptide-1.

ered to indicate statistical significance. All data are presented as means ± SE.

Results

There was no significant difference in age between the control and GLP-1 groups (P=0.854). Figures 2 and 3 show insulin secretion and amylase secretion, respectively. Values in the figures at each time point are presented as percentages, with the baseline value as 100%.

Insulin outflow

In the control group, the level of insulin outflow gradually decreased, and the insulin level at 60 min was less than 50% of that at baseline. The level of insulin outflow decreased significantly over time (P<0.001). On the other hand, the insulin levels at 20 and 40 min in the GLP-1 group were higher than baseline. However, there was no significant difference over time (P=0.457) in the GLP-1 group. Thereafter, the average level of insulin outflow at 60 min was the same as that of baseline in the GLP-1 group. The insulin outflow level at 40 min in the GLP-1 group was significantly higher than that of the control group (P=0.008). Table 1A shows that the insulin AUC (%) of the GLP-1 group was significantly greater than the control group.

Amylase outflow

There was no significant difference in the level of amylase outflow between the control and GLP-1 groups. Compared to the level of amylase outflow at baseline of both the control and GLP-1 groups, the average level of amylase increased at 60, 80, and 100 min in both groups, and also at 40 min in the control group. There were significant differences over time in both groups (P=0.030, respectively). Table 1B shows that the amylase AUC (%) of the control and GLP-1 groups did not significantly differ.

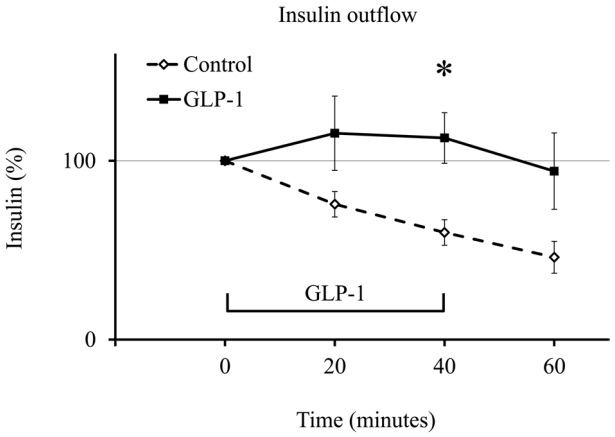

Fig. 2. Insulin outflow of rat pancreas preparations. Each group was comprised of tissues from 6 rats (◇, control group; ■, GLP-1 group). All data are expressed as percentages, and were normalized to the level of insulin at baseline in each group (100%). **P*<0.05; GLP-1 group vs. control group. GLP-1: glucagon-like peptide-1.

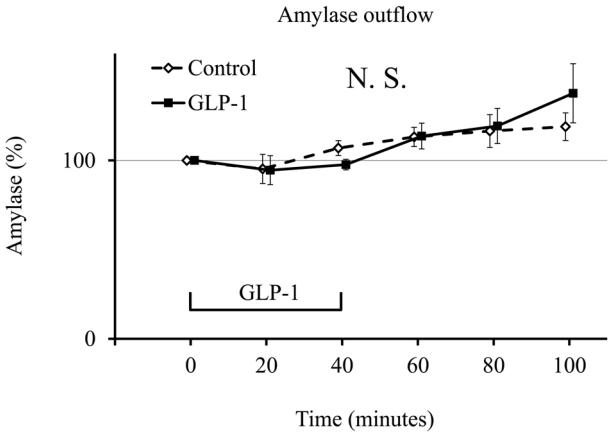

Fig. 3. Amylase outflow of rat pancreas preparations. Each group was comprised of tissues from 6 rats (◇, control group; ■, GLP-1 group). All data are expressed as percentages, and were normalized to the level of amylase at baseline in each group (100%). N.S.: not significantly different; GLP-1 group vs. control group. GLP-1: glucagon-like peptide-1.

Table 1. Insulin and amylase area under the curves

		Control group	GLP-1 group	*P* value
1A	Insulin AUC (%) [% • min]	41.72 ± 3.54	65.06 ± 8.91	0.035*
	Insulin AUC [ng • min/g pancreas]	91.10 ± 18.57	139.39 ± 36.45	0.265
1B	Amylase AUC (%) 0–60 minutes [% • min]	61.75 ± 2.53	59.81 ± 2.32	0.585
	Amylase AUC 0–60 minutes [U • min/g pancreas]	865.04 ± 193.79	494.80 ± 48.18	0.116
	Amylase AUC (%) 0–100 minutes [% • min]	108.26 ± 5.59	108.81 ± 5.90	0.947
	Amylase AUC 0–100 minutes [U • min/g pancreas]	1526.48 ± 350.98	901.71 ± 97.08	0.139

Data are expressed as mean ± SE. Analyses are performed by Student's *t*-test. 1A: Insulin AUC; insulin area under the curve, 1B: Amylase AUC; amylase area under the curve. GLP-1; Glucagon-like Peptide-1. **P*<0.05.

Discussion

In the present study, insulin levels of rat pancreas preparations of the control group gradually decreased in a time-dependent manner, while those of the GLP-1 group at 20 and 40 min increased compared to baseline. The results showed that there was a statistically significant difference between the control and GLP-1 groups for the 40 min insulin value and insulin AUC (%). The results of the organ bath experiment showed that insulin secretion from rat pancreas tissue was stimulated and induced by GLP-1. This is in agreement with previous studies using perfused rat pancreas preparations in which GLP-1 was shown to stimulate insulin secretion [21, 33]. While isolated perfused rat pancreas preparations have high experimental utility, they require additional techniques.

In general, insulin secretion is elicited with rising glucose concentrations; however, insulin secretion is not elicited at glucose levels at or below 6 mM [11]. When GLP-1 is administered to humans, insulin secretion is increased even under fasting blood glucose conditions, where blood glucose levels of approximately 5.1 mM are observed [32]. Based on the above, we examined the effect of 1 μM GLP-1 on insulin secretion under conditions of 5.56 mM (100 mg/dl) glucose. GLP-1 is known to increase insulin secretion in a glucose-dependent manner [5]. However, the results of the present study showed that insulin secretion is sensitive to GLP-1 in severe conditions. In some experiments, the level of insulin outflow in guinea pig pancreas preparations was uniformly low (data not shown). Isolated guinea pig pancreas preparations did not allow determination of insulin secretion, possibly because the stereostructure of insulin differs from that of rat (70% amino acid homology between guinea pig and rat).

Currently, the experiment of perfused pancreas preparations is said to be sensitive and confidence. However, the present technique has potential as a simple and convenient *ex vivo* system to examine the induction of insulin secretion in rats. In the present technique, operator proficiency is unnecessary. The setup time of the experiments using an organ bath is probably shorter than that of perfused pancreas preparations. In the experiments using an organ bath, it is easy to change solutions quickly. Therefore, it is possible to make experimental protocols under random conditions. In addition, the measurements of both insulin as endocrine and amylase as exocrine in the pancreas were assessable by using the samples from the solution in the organ bath. Unlike isolated perfused pancreas preparations, the present technique has the advantage of being able to ignore perfusion pressure which is regulated by pump-perfusion system.

We investigated amylase secretion from pancreas tissue in conjunction with insulin secretion because we wanted to know whether the quantitative measurement of amylase secretion was possible in rat preparations using an organ bath, and we wanted to evaluate pancreatic inflammation. While amylase is a digestive enzyme, serum amylase is used to help diagnose and monitor acute pancreatitis in humans. Some studies have reported that the administration of GLP-1 receptor agonists is related to the incidence of pancreatitis [7, 28], while others have noted conflicting results [6, 10]. In the present study, there was no significant difference between the GLP-1 and control groups at any time point. These results suggest that pancreatic tissue is not adversely affected by GLP-1 using the present technique. In the present study, the collected pancreatic tissue was divided and cut. The amylase level gradually increased in both groups, and the amylase level was likely to be affected by the duration *ex vivo*. It is possible that changes in the average level of insulin might be affected by pancreatic inflammation resulting from excision of the pancreas. The experimental design has a number of shortcomings, i.e., timing of sampling, non-optimized equilibration time, and the lack of trypsin inhibitor use. In the control group, the amylase level did not decrease in a time-dependent manner, regardless of the observed decrease in insulin levels. One possible reason for this is that reduced insulin secretion is associated with the effects of somatostatin. It is known that somatostatin inhibits insulin secretion [1, 14]. We aim to investigate the levels of somatostatin using this model in future studies.

The identification and precise excision of pancreatic segments is highly challenging. In the rat, it appears that unified terms for individual regions of the pancreas have not yet been established. In a previous report, Miyaki *et al.* described that the rat pancreas consisted of the body as well as the right and left lobes [22]. The present study was conducted mainly using the right lobe of the pancreas. This was done for two reasons. First, the left lobe of the pancreas is probably more diffuse than the right lobe. Second, Tsuchitani *et al.* described the rat pancreas as being comprised of a duodenal segment, a parabiliary segment, a gastric segment, and a splenic segment. They also demonstrated that the proportion of β-cell area to total pancreatic tissue in the duodenal segment (right lobe) was higher than that of the splenic segment (left lobe) [30]. This suggests that the entire pancreas can be examined without separation using a large organ bath.

In conclusion, the present study demonstrated that insulin secretion in rat preparations using an organ bath is reproducible and sensitive to GLP-1. Thus, the advantage of this bioassay of rat pancreas is that it facilitates the quantitative pharmacological analysis of insulin secretion from β-cells.

Conflict of Interest

None.

Acknowledgments

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