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Development of an organ bath technique for isolated rat pancreas preparations to assess the effect of 1,5-AG on insulin secretion

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Abstract: To investigate substances related to insulin secretion, we reported a convenient experimental method to reproduce insulin secretion from isolated rat pancreas preparations using an organ bath. While the method has experimental utility for investigating insulin secretion, optimization of the experimental design is still needed. The level of insulin outflow in the control decreased over time in our previous study. Decreasing serum 1,5-anhydroglucitol (1,5-AG) levels is also known to be shown in patients with worsening glycemic control. There is one *in vitro* report demonstrated that 1,5-AG induced insulin release. It appears that discussion needs to be deepened further on it. In this study, we investigated the effect of 1,5-AG on insulin secretion through to optimize the condition of endocrine function using the *ex vivo* organ bath technique. The level of insulin outflow in the control and 1,5-AG groups decreased over time in the organ bath experiment. To analyze the effect of trypsin on reduced insulin secretion, pancreas preparation was treated with soybean trypsin inhibitor (TI). Insulin outflow levels of the TI group were significantly higher than the control group. An enzyme indicator of tissue damage tended to be lower in the TI group. There was no significant enhancement of insulin secretion by 1,5-AG. The present study demonstrated the utility of TI application for the organ bath technique. This finding supported the development of an organ bath technique for the assessment of the effects of novel therapeutics on insulin secretion.
Key words: 1,5-anhydroglucitol, insulin, organ bath, trypsin, trypsin inhibitor

Introduction

Several types of antidiabetic drugs have been developed for use in patients with diabetes mellitus. However, definitive treatments for diabetes mellitus have not yet been established. Insulin is a peptide hormone secreted from β-cells in pancreatic islets that acts to lower blood glucose levels. Therefore, the continued search

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for substances related to insulin secretion for the advancement of pharmacotherapy in diabetes mellitus is being actively pursued. More recently, we reported an experimental method to reproduce insulin secretion from isolated rat pancreas preparations using an organ bath [19]. Our previous study showed that insulin secretion from rat pancreas tissue was stimulated by glucagon-like peptide-1 (GLP-1). Pancreatic organ bath preparations have been found to be sufficiently sensitive and reproducible to provide experimental utility as a method to assess the effects of novel therapeutics on insulin secretion.

Optimization of the experimental design for organ bath experiments using pancreas preparations is needed. The level of insulin outflow in the control decreased over time in our previous organ bath study [19]. Firstly, it is important to conduct the experiment under optimal conditions in order to mitigate the reduction in insulin secretion. Pancreatic juice contains digestive enzymes (i.e., amylase to digest starch, the proteolytic enzyme trypsin, lipase to break down triglycerides to fatty acids, etc.), which are produced and stored by exocrine acinar cells within the pancreas. In particular, trypsin release in the pancreas leads to autodigestive injury and pancreatitis [12]. When leakage of trypsin occurs in pancreatic tissues, inflammation-associated pancreatitis is induced clinically. As a result, pancreatitis leads to dysregulated glucose metabolism [4]. Thus, we predict that rat pancreas preparations are affected by pancreatic inflammation. Suppression of the early phase of acute pancreatitis is likely to be important for our experimental organ bath technique.

A previous study showed 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) in rats [30]. Therefore, our previous study was conducted mainly using the right lobe of the pancreas [19], despite reports that in rats the number of intralobular islets in the left lobe is more than in the right lobe [20]. We also investigated the insulin outflow between the right and left lobes of pancreas preparations in the presence of GLP-1 (unpublished). However, we have not confirmed if there are differences in pancreatic segments without stimulation. Hence, a comparison of pancreatic segments is required to verify this model of insulin outflow.

1,5-Anhydroglucitol (1,5-AG) is one of the major polyols in the human body. The concentration of 1,5-AG is maintained at approximately 20 μg/ml or greater in healthy humans [33]. It is also known that serum 1,5-AG levels have been shown to decrease in patients with diabetes mellitus [28], and 1,5-AG levels have been used as a glycemic marker. However, there are few reports on the physiological effects of 1,5-AG. A single *in vitro* report demonstrated that 1,5-AG induced insulin release in insulinoma cell lines [32]. On the other hand, previous studies, including ours, have demonstrated that serum 1,5-AG tends to decrease with age in rats [7] and humans [23]. More recently, research using high-resolution liquid chromatography-mass spectrometry demonstrated that 1,5-AG showed strikingly lower levels in healthy elderly subjects compared with healthy youths [2]. Older subjects have been found to have more disorderly insulin release, with decreased amplitude and mass of rapid insulin pulses compared to young subjects [17]. Therefore, we attempted to evaluate the effect of 1,5-AG on insulin secretion using an *ex vivo* organ bath technique as a pilot study.

This pilot study has two aims: to optimize the condition for quantitative analysis of insulin secretion in the organ bath experiment, and to investigate the effect of 1,5-AG on insulin secretion using the *ex vivo* organ bath technique. The outflow of insulin and amylase was measured as respective markers of endocrine and exocrine pancreas function.

Materials and Methods

Reagents and animals

1,5-AG and trypsin inhibitor from soybean (TI) were obtained from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Wistar-Imamichi rats (male, 21–31 weeks old) were purchased from the Institute for Animal Reproduction (Ibaraki, Japan). The rats were kept under a controlled temperature of 23 ± 3°C and a 12 h light/12 h dark cycle (lights on at 7:00). Laboratory diet (CE-2; Clea Japan, Inc., Tokyo, Japan) and water were provided *ad libitum*. The protocols were approved by the Institutional Animal Care and Use Committee, and the experiments in this study were conducted in accordance with the Guidelines for Animal Experimentation of Dokkyo Medical University. All efforts were made to minimize animal suffering and to reduce the number of animals used. Twenty rats were assigned to three age-matched groups: an untreated control group, which was used for comparing pancreatic regions (n=6; mean age, 174.2 ± 8.3 days), 1,5-AG treated group (n=8; mean age, 183.3 ± 6.9 days) and TI treated group (n=6; mean age, 183.3 ± 7.8 days). There was no significant difference in age among all groups (P=0.713).

Pancreas preparation

Rats were humanely euthanized with carbon dioxide and blood was taken from the caudal vein. Briefly, the

whole pancreas was removed from rats and 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, 3 mM EDTA, 11.9 mM $NaHCO_3$ and 5.56 mM glucose). The pancreas was divided into three parts, the right lobe located on the duodenal side, the left lobe near the hilum of the spleen and the residual pancreas. In anatomical descriptions of the human pancreas, the right lobe is considered the pancreatic head and the left lobe the pancreatic tail. Each lobe was wrapped in nylon mesh and suspended in an organ bath.

Organ bath technique

The organ bath technique was performed as described in a previous study [19]. The pancreas preparations were individually placed in a 5-ml organ bath with modified Tyrode's solution at 37°C under an atmosphere of 95% O_2-5% CO_2 and were equilibrated for 120 min with fresh replacement of the solution every 20 min. After equilibration, the incubation medium (2.3-ml) was sampled four times every 20 min for −20–60 min. To compare insulin outflow between the right and left lobes, each pancreas preparation was separated from an individual rat and the experiments were performed in tandem. To assess the effects of 1,5-AG on insulin and amylase outflow, the right lobe was stimulated with 1 mM 1,5-AG for 0–40 min. In a previous study, stimulation was conducted with the concentration range 0–0.61 mM of 1,5-AG in insulinoma cell lines [32]. A recent experiment *in vitro* was also incubated with 1,5-AG at concentrations of 0–0.975 mM [35]. Based on the above, we initially chose to examine the maximum effect of 1,5-AG at the concentration of 1 mM. To analyze the effect of trypsin on insulin secretion, the right lobe preparation was immersed in modified Tyrode's solution containing 0.1 mg/ml TI for the duration of the experiment. The same dose of TI was used in previous research with pancreatic acinar AR42J cells *in vitro* [14]. Based on the above, we initially chose to examine the effect of 0.1 mg/ml TI. The collected samples were transferred to Protein LoBind® tubes (Eppendorf, Hamburg, Germany) and stored at −80°C. After each sample collection, the pancreas preparation was weighed.

Quantitative determination of insulin and amylase outflow in organ bath samples

Insulin concentrations in the collected solutions were determined using an Ultra Sensitive Rat Insulin ELISA Kit (Morinaga Institute of Biological Science, Inc., Kanazawa, Japan) according to the manufacturer's instructions. A standard curve was generated by the ELISA assay and insulin concentrations in the samples were calculated using the standard curve.

Quantitative determination of amylase in the sampled medium was performed using a dry-chemistry analyzer (Fuji Dri-Chem 3500V and Fuji Dri-Chem Slide AMYL-PIII; Fujifilm Corp., Tokyo, Japan). The samples were diluted 10-fold with phosphate-buffered saline and warmed at 37°C. A 10-μl aliquot of solution was dropped on a slide. The value of insulin and amylase activity at each indicated time was presented relative to the weight of the unwrapped intact pancreas preparation.

Enzyme markers (glutamic oxaloacetic transaminase and lactate dehydrogenase) in organ bath samples

Activities of glutamic oxaloacetic transaminase (GOT) and lactate dehydrogenase (LDH) in the sampled incubation medium were detected using the dry-chemistry analyzer and Fuji Dri-Chem Slide (GOT/AST-P III and LDH-P III). The protocol was the same as for quantitative determination of amylase outflow. A 10-μl volume of the warmed stock solution without dilution was put on each slide. The value of GOT and LDH was presented relative to the weight of the unwrapped intact pancreas preparation.

Insulin secretion experiment using INS-1E cells

The rat insulinoma cell line INS-1E was grown in advanced RPMI-1640 medium (Thermo Fisher Scientific Inc., Waltham, MA, USA) containing 5% FBS, 0.5 mM monothioglycerol (FUJIFILM Wako Pure Chemical Corp.) and 2 mM L-alanyl-L-glutamine (FUJIFILM Wako Pure Chemical Corp.) [18]. Cells were seeded in 24-well plates (1×10^5 cells per well) and cultured for 2 days at 37°C in 5% CO_2. The medium was removed and INS-1E cells were preincubated for 1 h (5% CO_2, 37°C) in RPMI-1640 (glucose free; FUJIFILM Wako Pure Chemical Corp.) with 5% FBS and 100 mg/dl glucose. Next, the cells were incubated for 1 h in a fresh batch of the same composition medium with added 1,5-AG or TI. To determine the effects of 1,5-AG and TI on insulin secretion, INS-1E cells were incubated in RPMI-1640 containing various concentrations of 1,5-AG (0, 10, 100 and 1,000 μM) and TI (0, 1, 10 and 100 μg/ml). Samples were collected in Protein LoBind® tubes and insulin in the medium was determined with a Rat Insulin ELISA Kit (Morinaga Institute of Biological Science, Inc.) according to the manufacturer's protocol. To each well, 200-μl of M-PER™ Mammalian Protein Extraction Reagent (Thermo Fisher Scientific Inc.) was added to dissolve cells for the determination of cellular protein content with a Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific Inc.).

Statistical analysis

Statistical tests were carried out using IBM SPSS (version 25; International Business Machines Corp., Armonk, NY, USA). To assess changes over time in the organ bath study, a repeated-measures analysis of variance was performed. Significant differences in the experiment using INS-1E cells and experiments using organ baths were determined by one-way analysis of variance. Post hoc tests with Dunnett's test were used to detect significant differences between the control and treatment groups. Comparisons between the right and left lobes in organ bath experiments were performed using paired *t* tests. Comparisons between two groups in GOT and LDH determinations were performed with a Student's t-test. All values are expressed as means ± SE. *P*-values less than 0.05 were considered statistically significant.

Results

Insulin and amylase outflow in the organ bath experiment is shown in Fig. 1. Further, similar results are expressed as percentage of insulin and amylase outflow during 20–60 min incubation of samples in the individual experiments (Supplementary Table 1). In Table 1, the levels of insulin and amylase outflow from the right and left lobes from the untreated rat pancreas preparations are compared.

Insulin outflow in organ baths

There was a significant difference in insulin outflow values at 40 and 60 min among the 3 groups (control, 1,5-AG, and TI groups) (0 min: P=0.553, 20 min: P=0.159, 40 min: P=0.004, and 60 min: P=0.001, respectively) (Fig. 1A). Insulin outflow at 40 and 60 min in the TI group was significantly higher in the TI group than in the control group (40 min: P=0.005, and 60 min: P=0.001). There was no significant difference in insulin outflow between the control and 1,5-AG groups. The level of insulin outflow in the control and 1,5-AG groups decreased over time. In contrast, the insulin outflow of the TI group increased slightly from 20 min onward. However, there were no significant differences over time (control group: P=0.051, 1,5-AG group: P=0.069, and TI group: P=0.491, respectively). Furthermore, there were no significant differences in the values of insulin outflow between the right and left lobes (0 min: P=0.598, 20 min: P=0.848, 40 min: P=0.599, and 60 min: P=0.778, respectively) (Table 1). Similarly, there were no significant differences over time in the right (P=0.051) and left (P=0.166) lobes. Values of the right lobes were the same as control values presented herein.

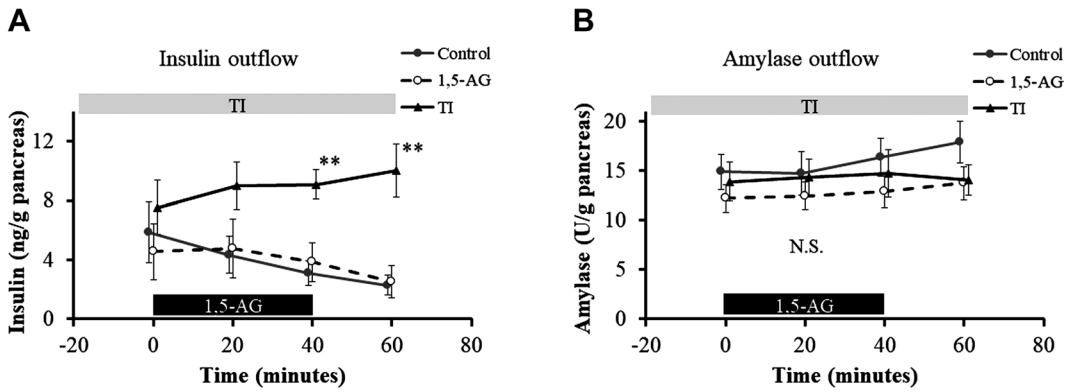

Fig. 1. Effects of TI and 1,5-AG on secretory responses from rat pancreas preparations. A shows insulin outflow. B shows amylase outflow. Each point is comprised of tissues from 6–8 rats. Values are expressed as mean ± SE. **<0.01; TI group vs. control group. N.S.: not significantly different. TI: soybean trypsin inhibitor (0.1 mg/ml). 1,5-AG: 1,5-anhydroglucitol (1 mM).

Table 1. Comparisons insulin and amylase outflow of right and left lobes from rat pancreas preparations.

		Time (min)			
		0	20	40	60
Insulin (ng/g pancreas)	Right lobes	5.85 ± 2.04	4.35 ± 1.26	3.08 ± 0.83	2.30 ± 0.70
	Left lobes	8.41 ± 4.55	4.75 ± 1.98	3.97 ± 1.73	2.68 ± 1.14
Amylase (U/g pancreas)	Right lobes	14.91 ± 1.79	14.74 ± 2.20	16.40 ± 1.93	17.93 ± 2.10
	Left lobes	5.64 ± 1.68**	5.35 ± 2.12**	5.96 ± 2.58**	5.89 ± 2.36**

Each value was obtained from 6 rats. Data are represented as means ± SE. ** P<0.01; right lobes vs. left lobes.

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Amylase outflow in organ baths

There were no significant differences in amylase outflow values among the three groups (control, 1,5-AG, and TI groups) (0 min: P=0.523, 20 min: P=0.604, 40 min: P=0.441, and 60 min: P=0.220, respectively) (Fig. 1B). There was a significant difference over time in the control group (P=0.004), whereas no significant difference was observed over time in the 1,5-AG (P=0.318) and TI (P=0.592) groups. Furthermore, there were significant differences in amylase outflow between the right and left lobes (0 min: P=0.003, 20 min: P=0.006, 40 min: P=0.003, and 60 min: P<0.001) (Table 1). A significant difference was observed over time in the right (P=0.004) but not in the left (P=0.705) lobe. Values of the right lobes were the same as the control values presented herein.

Enzyme marker of tissue damage (glutamic oxaloacetic transaminase and lactate dehydrogenase)

As shown in Fig. 2, there were no significant differences in GOT release between the control and TI groups at the three time points evaluated (−40 min: P=0405, 0 min: P=0.831, and 60 min: P=0.056). In contrast, at −40 min, LDH in the TI group was significantly lower than in the control group (−40 min: P=0.019, 0 min: P=0.094, and 60 min: P=0.177). There was no time-dependent alteration in GOT release in the control and TI groups (control group: P=0.350, and TI group: P=0.472). Similarly, there was no time-dependent alteration in LDH release in the control group (P=0.649). In contrast, there was a time-dependent alteration in LDH release in the TI group (P=0.038).

Insulin secretion from INS-1E cells

1,5-AG had no effect on insulin secretion in INS-1E cells (P=0.059) (Fig. 3A). TI had not effect on insulin secretion in INS-1E cells (P=0.365) (Fig. 3B).

Discussion

In this study, insulin outflow levels of the TI group

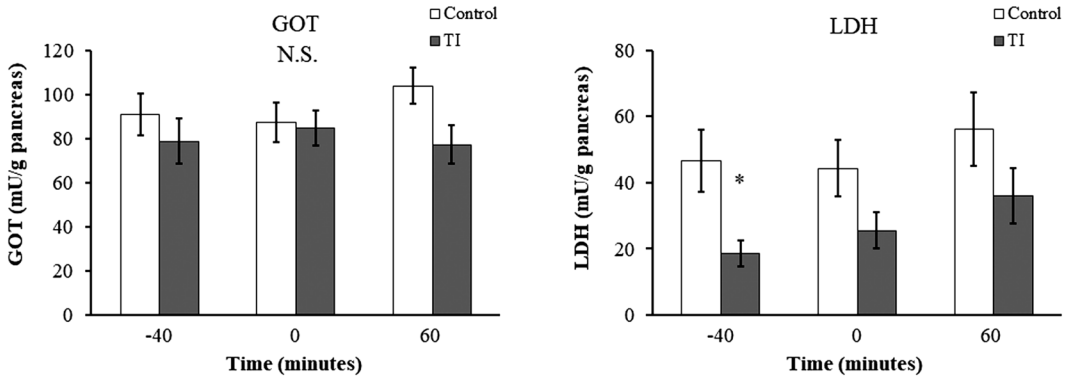

Fig. 2. Changes in tissue damage markers during organ bath incubation. The data was expressed as mean ± SE. from 5–6 rats. N.S.; not significantly different. *<0.05; TI group vs. control group. GOT: glutamic oxaloacetic transaminase. LDH: lactate dehydrogenase. White bar: control group. Grey bar: TI group. TI: soybean trypsin inhibitor.

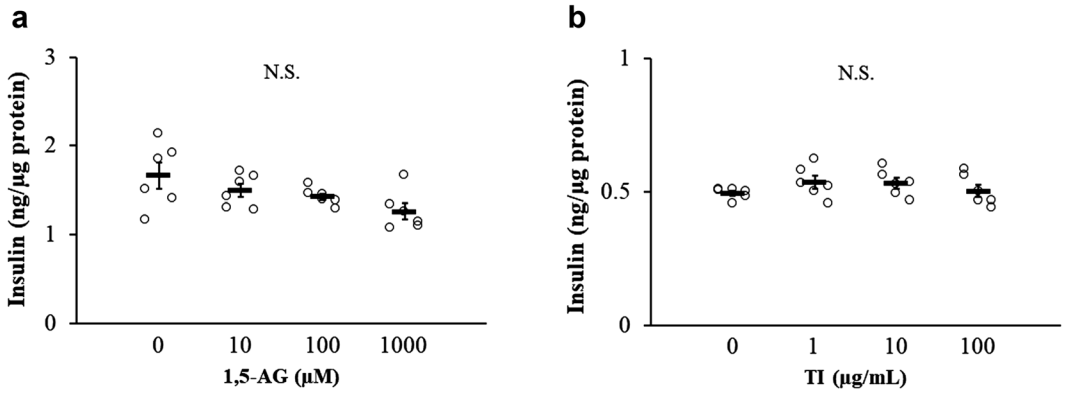

Fig. 3. Effects of 1,5-AG and TI on insulin release in INS-1E cells. A shows effect of 1,5-AG on insulin secretion. B shows effect of TI on insulin secretion. Each open circle represents an individual value. Each black bar represents the mean value ± SE. N.S.; not significantly different. 1,5-AG: 1,5-anhydroglucitol. TI: soybean trypsin inhibitor.

were significantly higher than the control group. Further, an enzyme indicator of tissue damage (LDH) tended to be lower in the TI group. On the other hand, there was no difference in insulin outflow between the control and 1,5-AG groups. 1,5-AG was not observed to induce insulin secretion in this study in either the *ex vivo* organ bath or *in vitro* INS-1E cell experiments.

In patients with acute pancreatitis, it is known that blood glucose levels and glucagon levels increase, and insulin levels decrease [4]. Kinami, *et al.* reported that insulin levels in homogenized pancreas tissue and glucose-stimulated insulin secretion from isolated islets decreased in rats with acute pancreatitis induced by bile duct ligation [11]. In the present study, insulin outflow from pancreas preparations in organ baths was improved by TI administration. Normally, trypsinogen is not converted to trypsin in pancreatic acinar cells. However, if trypsinogen is converted to trypsin in pancreas as a result of an accident (i.e., excessive pancreatic exocrine stimulation, disturbance of pancreatic duct flow, and the reflux of bile and intestinal fluid into the pancreatic duct), pancreatic secretory trypsin inhibitor (PSTI) is initiated to reduce trypsin activity [10, 24]. In situations where the production of trypsin exceeds the threshold of the inhibitory action of PSTI, autolysis of the pancreas occurs, which is thought to induce pancreatitis [9]. When preparing isolated pancreas samples, the pancreas is stimulated by cutting and separating, which may be responsible for the accidental conversion of trypsin. On the other hand, it is known that the protease kallikrein exists in the pancreas [6], which shows trypsin-like activity. Kallikrein is expressed in many tissues, including the human pancreas and salivary glands [3]. TI administration is an established method, and was reported to improve secretory function in exocrine gland secretion [31]. In fact, we added TI to the incubation medium to assess parotid exocrine function under a secretagogue-stimulated condition [27]. Liddle, *et al.* reported that rats fed TI exhibited increased amylase secretion resulting from elevated plasma cholecystokinin levels [13]. Similarly, it might be expected that amylase release is induced by cholecystokinin and activation of muscarinic receptors in our system.

The differences between *in vivo* and *ex vivo* systems of the pancreas on insulin secretion have been described in our previous report [19]. As with using TI, the previous *in vivo* study showed that oral administration of TI caused enlargement of the pancreas in rats [16, 34]. Also caused was a significantly greater amylase content of the pancreas [16]. By administering TI orally for 1 week, exocrine cells showed hypertrophy and hyperplasia [34]. Taken together, in the present study, the effectiveness time of TI might be short to affect for exocrine function. Moreover, because TI was administered orally for 1 week, islets of Langerhans were enlarged, and mitotic figures were found in β-cells [34]. Oral administration of TI is reported to significantly increase the insulin content of the pancreas [5]. However, to our knowledge, *in vivo* studies have not shown increased serum insulin levels after oral administration of TI.

It is known that GOT exists in liver, cardiac muscle, skeletal muscle, kidneys, brain, lungs, and pancreas [8, 25]. Moreover, GOT and LDH are essential indexes of Ranson's criteria for pancreatitis-associated mortality [1, 26]. A previous study has shown that high levels of GOT and LDH at the onset of disease may predict acute pancreatitis in elderly patients [15]. Therefore, to test whether TI treatment had protective effects on pancreas preparations, GOT and LDH release were determined. There was no significant difference in the values of GOT; however, a significant difference was observed in LDH values between the control and TI groups. A previous study of patients with acute pancreatitis showed no significant difference in GOT levels, whereas LDH levels were significantly higher in a severe state than in a mild state [1]. Essentially, Glasgow criteria (Blamey, *et al.*) [1] and Japanese severity score (JSS) for acute pancreatitis [36] do not have GOT as a variable. In addition, "LDH ≥ 2 times of upper limit of normal" was described as a prognostic factor in the JSS for acute pancreatitis [36]. In this system of suspended pancreas preparations, LDH is certainly a more sensitive predictor of pancreatic tissue damage than is GOT. A direct effect of TI administration was not observed with respect to insulin secretion *in vitro*. Toyota, *et al.* revealed that glucose-induced insulin secretion was significantly inhibited by trypsin administration in an isolated rat perfusion [29]. The present research suggested that insulin outflow was influenced and decreased by trypsin release, and that a trypsin inhibitor acted to reduce trypsin-induced inflammation.

A previous report employed 0.1 mg/ml TI to obtain more viable pancreatic exocrine cells from pancreas tissues [22]. TI was likely used to prevent activation of endogenous proteases. In experiments to assess the stimulation of insulin secretion, achievement of stable insulin outflow would be valuable. In the present study, the level of insulin outflow decreased over time in the control group, and increased over time in the TI group in the presence of 0.1 mg/ml soybean trypsin inhibitor. In future studies, we aim to optimize the conditions (i.e., horizontal transitions over time) for insulin secretion using the organ bath technique. Optimal conditions may not have been achieved in this study, which may account

for the discrepancy in TI concentration effects on insulin outflow.

A previous report showed that islets of Langerhans in the duodenal segment (right lobe) of the pancreas were more numerous than those the splenic segment (left lobe) in rats [30]. However, we confirmed that there was no significant difference in insulin levels between the right and left lobes in the *ex vivo* organ bath experiment. One possible explanation is that there was a uniform distribution of islets between each preparation in each rat. Another possibility involves the abundance of islets in these anatomical locations, which might be not detected in our organ bath technique. On the other hand, amylase outflow of the left lobe was significantly lower than that of the right lobe. The cut surface in pancreas preparations of the right lobe was greater than that of the left lobe. In addition, the shape of the left lobe of the pancreas is probably more diffuse than that of the right lobe. When the right lobe was prepared for organ bath experiments, a high uniformity in the pancreas preparations was observed (data not shown). Therefore, it may be advantageous to employ only one lobe of the pancreas preparations in the organ bath technique. Alternatively, the entire pancreas without separation could be utilized in a large organ bath.

Yamanouchi, *et al.* reported that 1,5-AG treatment induced insulin release in two insulin-secreting cell lines, MIN6 (mouse) and RINr (rat) [32]. However, 1,5-AG did not show effects on insulin secretion in the present study. This difference could be attributed to different responses of INS-1E cells compared to the cell types used in the previous report. It is possible that the cell line we used responds weakly to substrate-induced and glucose-induced insulin secretion compared to MIN6 or RINr cells. Therefore, there is a possibility that the inability of 1,5-AG to alterations insulin secretion has not been thoroughly investigated *in vitro*. Recently, Nakamura, *et al.* reported that serum insulin levels in healthy humans did not change after oral intake of 1,5-AG [21].

In conclusion, the present study demonstrated the utility of TI application for observing insulin secretion in rat pancreas preparations using an organ bath. We speculate that the optimal TI concentration is less than 0.1 mg/ml. This pilot study demonstrated that 1,5-AG had no effect on insulin secretion *ex vivo*. There was also no significant enhancement of insulin secretion by 1,5-AG *in vitro*. Further research is required to enhance knowledge of this topic and the physiological role of 1,5-AG.

Conflicts of Interest

None

Acknowledgments

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