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Increased secretion of insulin and proliferation of islet β-cells in rats with mesenteric lymph duct ligation

Ko Nagino [a,1], Junji Yokozawa [a,1], Yu Sasaki [a], Akiko Matsuda [a], Hiroaki Takeda [a], Sumio Kawata [a,b,*]

[a] Department of Gastroenterology, Faculty of Medicine, Yamagata University, Yamagata 990-9585, Japan
[b] Hyogo Prefectural Nishinomiya Hospital, 13-9 Rokutanji-cho, Nishinomiya 662-0918, Japan

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ABSTRACT

Background & aims: It has been suggested that intestinal lymph flow plays an important role in insulin secretion and glucose metabolism after meals. In this study, we investigated the influence of ligation of the mesenteric lymph duct on glucose metabolism and islet β-cells in rats.

Methods: Male Sprague–Dawley rats (10 weeks old) were divided into two groups: one underwent ligation of the mesenteric lymph duct above the cistern (ligation group), and the other underwent a sham operation (sham group). After 1 and 2 weeks, fasting plasma concentrations of glucose, insulin, triglyceride, glucose-dependent insulinotropic polypeptide (GIP), and the active form of glucagon-like peptide-1 (GLP-1) were measured. At 2 weeks after the operation, the oral glucose tolerance test (OGTT) and intravenous glucose tolerance test (IVGTT) were performed. After the rats had been sacrificed, the insulin content of the pancreas was measured and the proliferation of β-cells was assessed immunohistochemically using antibodies against insulin and Ki-67.

Results: During the OGTT, the ligation group showed a significant decrease in the plasma glucose concentration at 120 min ($p < 0.05$) and a significant increase in the plasma insulin concentration by more than 2-fold at 15 min ($p < 0.01$). On the other hand, the plasma GIP concentration was significantly decreased at 60 min ($p < 0.01$) in the ligated group, while the active form of GLP-1 showed a significantly higher level at 90 min (1.7-fold; $p < 0.05$) and 120 min (2.5-fold; $p < 0.01$). During the IVGTT, the plasma insulin concentration in the ligation group was significantly higher at 2 min (more than 1.4-fold; $p < 0.05$). Immunohistochemistry showed that the ratios of β-cell area/acinar cell area and β-cell area/islet area, and also β-cell proliferation, were significantly higher in the ligation group than in the sham group ($p < 0.05$, $p < 0.01$ and $p < 0.01$, respectively). The insulin content per unit wet weight of pancreas was also significantly increased in the ligation group ($p < 0.05$).

Conclusions: In rats with ligation of the mesenteric lymph duct, insulin secretion during the OGTT or IVGTT was higher, and the insulin content and β-cell proliferation in the pancreas were also increased. Our data show that mesenteric lymph duct flow has a role in glucose metabolism.

1. Introduction

Lymphatic vessels not only play a physiological role in maintenance of homeostasis and the immune response together with blood vessels, but also participate in the development of pathological states such as inflammation and cancer metastasis [1]. Mesenteric lymphatic vessels have a specific function, including absorption of long-chain fatty acids from the small intestine. Ligation of the mesenteric lymph duct is reported to prevent acute pulmonary dysfunction during ischemia/reperfusion and trauma/hemorrhage shock-induced cardiac contractile dysfunction [2,3]. Recently, it has been reported that impairment of lymphangiogenesis promoted by Prox1 haploinsufficiency leads to adult-onset obesity [4].

Incretins, glucose-dependent insulinotropic polypeptide (GIP), and glucagon-like peptide-1 (GLP-1) are produced in enteroendocrine K cells and L cells, respectively in the intestine in response to ingestion of carbohydrate and fat [5–7]. Both GIP and GLP-1 stimulate insulin secretion from pancreatic β-cells, accounting for ~50–70% of postprandial insulin secretion from the pancreas [8,9]. Recently, it has been shown that intestinal lymph is enriched with GIP [10] and GLP-1 [11], and studies using the lymph fistula rat model have demonstrated that the carbohydrate and lipid contents of food affect the secretion of GIP and GLP-1 into intestinal

Abbreviations: GIP, glucose-dependent insulinotropic polypeptide; GLP-1, glucagon-like peptide-1; OGTT, oral glucose tolerance test.

* Corresponding author. Address: Department of Gastroenterology, Faculty of Medicine, Yamagata University, 2-2-2 Iida-Nishi, Yamagata 990-9585, Japan. Fax: +81 23 628 5311.
E-mail address: Sumio_Kawata@pref.hyogo.lg.jp (S. Kawata).

[1] These authors contributed equally to this work.

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Table 1
Comparison of biochemical data between the lymph duct ligation and sham groups at 1 and 2 weeks after the procedure.

Characteristic	1 week after ligation		2 weeks after ligation	
	Ligation	Sham	Ligation	Sham
Body weight (g)	354 ± 19.4	370 ± 10.2	380 ± 25.3	394 ± 15.1
Epididymal fat/body weight (%)	ND	ND	1.3 ± 0.04	1.4 ± 0.08
Glucose (mg/dL)	112 ± 6.3	124 ± 4.5	115 ± 4.6	127 ± 4.8
Triglyceride (mg/dL)	66 ± 5.4	65 ± 5.1	53 ± 3.7	54 ± 4.0
Total cholesterol (mg/dL)	73 ± 3.6	76 ± 3.9	72 ± 3.3	75 ± 2.5
Insulin (ng/mL)	0.29 ± 0.03	0.42 ± 0.08	0.36 ± 0.06	0.45 ± 0.09
C-peptide (pg/mL)	534 ± 108	738 ± 270	388 ± 49.7	572 ± 113
GLP-1 (pmol/L)	5.4 ± 1.3	5.7 ± 2.8	4.3 ± 0.7	4.6 ± 1.8
GIP (pg/mL)	14.6 ± 2.8	14.8 ± 2.8	14.0 ± 2.2	15.0 ± 2.3
Adiponectin (μg/mL)	4.8 ± 0.3	4.8 ± 0.3	7.4 ± 0.4	6.4 ± 0.4

Data are presented as mean ± standard error.

lymph vessels [12,13]. The incretin concentrations in lymph are significantly higher than in portal or systemic blood [10,11]. These findings suggest that intestinal lymph flow plays an important role in insulin secretion and glucose metabolism after meals. However, the consequences of interference with intestinal lymph flow on glucose metabolism and insulin secretion have not been investigated.

In this study, we examined changes in glucose metabolism and insulin secretion resulting from blockage of mesenteric lymph flow by ligation of the thoracic duct in rats.

2. Materials and methods

2.1. Construction of an animal model

Seven-week-old male Sprague–Dawley rats were purchased from CLEA Japan, Inc. (Tokyo, Japan) and fed a normal diet after acclimation to the laboratory environment. At 10 weeks, the rats were subjected to lymph duct ligation above the cisterna chyli in the peritoneal cavity. To visualize lymph ducts, 5 ml/kg olive oil was orally administered to the rats 2 h prior to surgery [14]. Pentobarbital (40 mg/kg) was then injected into the peritoneal cavity to anesthetize the rats, and laparotomy was performed to expose the thoracic duct in the peritoneal cavity. The rats were divided into two groups: those subjected to lymph duct ligation (ligation group) and those subjected to a sham procedure, i.e. laparotomy only (sham group).

Preliminary experimental tests revealed that the tension of the cisterna chyli was lost by 3 weeks after surgery. Therefore, the entire study was conducted using rats no later than 2 weeks after surgery. This study was conducted after receiving approval from the Animal Center Ethical Committee of Yamagata University Medical School.

2.2. Methods for collection of blood samples and removal of pancreas tissue

A total of 16 rats, 8 from each of the ligation and sham groups, were used for the study. Blood samples were collected from the tail vein of rats before surgery (at 10 week of age) and at 1 week (11 weeks of age) and 2 weeks (12 weeks of age) after surgery. All samples were collected following an overnight (12 h) fast. Blood samples were placed into tubes containing ethylenediaminetetraacetic acid (EDTA), aprotinin, and dipeptidyl peptidase-IV (DPP-IV) inhibitor (Millipore, Billerica, USA) so that the final concentrations after addition of the sample became 1.25 mg/ml, 500 KIU/ml, and 50 μM, respectively. Plasma samples were stored at −80 °C.

At 2 weeks after ligation, a portion of tissue samples from the pancreatic body region was fixed in 4% paraformaldehyde for 24 h at room temperature and embedded in paraffin according to the conventional method. Unused pancreatic tissues were instantaneously frozen in liquid nitrogen and stored at −80 °C.

2.3. Blood biochemical tests

Plasma levels of glucose, triglyceride, total cholesterol, insulin, C-peptide, total GIP, activated GLP-1, and adiponectin were measured using glucose CII test Wako, triglyceride E-test Wako, Cholesterol E-test Wako (Wako, Osaka, Japan), Rebis Insulin-rat T, Rebis C-Peptide-rat U-type (Shibayagi, Shibukawa, Japan), and a Rat/Mouse GIP (Total) ELISA kit, and levels of activated GLP-1 using a GLP-1 (Active) ELISA kit (Millipore, Billerica, USA), and an Adiponectin (rat) ELISA kit (AdipoGen, Seoul, Korea), respectively.

2.4. Oral glucose tolerance test (OGTT)

OGTT was carried out on the ligation ($n = 8$) and sham ($n = 8$) groups with the animals under anesthesia. Blood samples were collected from the two groups via a tail vein at 2 weeks after surgery prior to glucose oral administration (0 min) and at 15, 30, 60, 90, and 120 min after administration of 2.0 g/kg glucose (gastric administration) using a gavage tube [15].

The insulinogenic index, an index of insulin secretion, was also calculated as described previously [16].

2.5. Intravenous glucose tolerance test (IVGTT)

IVGTT was carried out on each group with the animals ($n = 8$, respectively) under anesthesia. Blood samples were collected from the rats via a tail vein at 2 weeks after surgery prior to glucose administration (0 min) and at 2, 5, 7.5, 10, 15, 30, 45, 60, and 90 min after bolus injection of 1.0 g/kg glucose into the femoral vein.

2.6. Immunohistochemistry of pancreatic β-cells

Pancreatic tissue was collected 2 weeks after surgery and embedded in paraffin for immunohistochemical studies from a total of 16 rats: 8 from the ligation group and 8 from the sham group. The paraffin block containing pancreatic tissues was sliced into 3-μm-thick sections using a microtome (EMS-100L; ERMA Inc., Tokyo, Japan). Immunostaining of the thin-sectioned tissues was then performed as described previously [17]. For measurement of β-cell proliferation, double immunostaining for insulin and Ki-67 (a cell proliferation-associated nuclear antigen) was performed as described previously [18]. As the primary antibody, anti-rat Ki-67 mouse antibody (1:25, Dako, Glostrup, Denmark) was used. Nuclei were stained using hematoxylin.

AxioVision AutoMeasure (Carl Zeiss, Oberkochen, Germany) was used to analyze the images of the stained pancreatic tissue

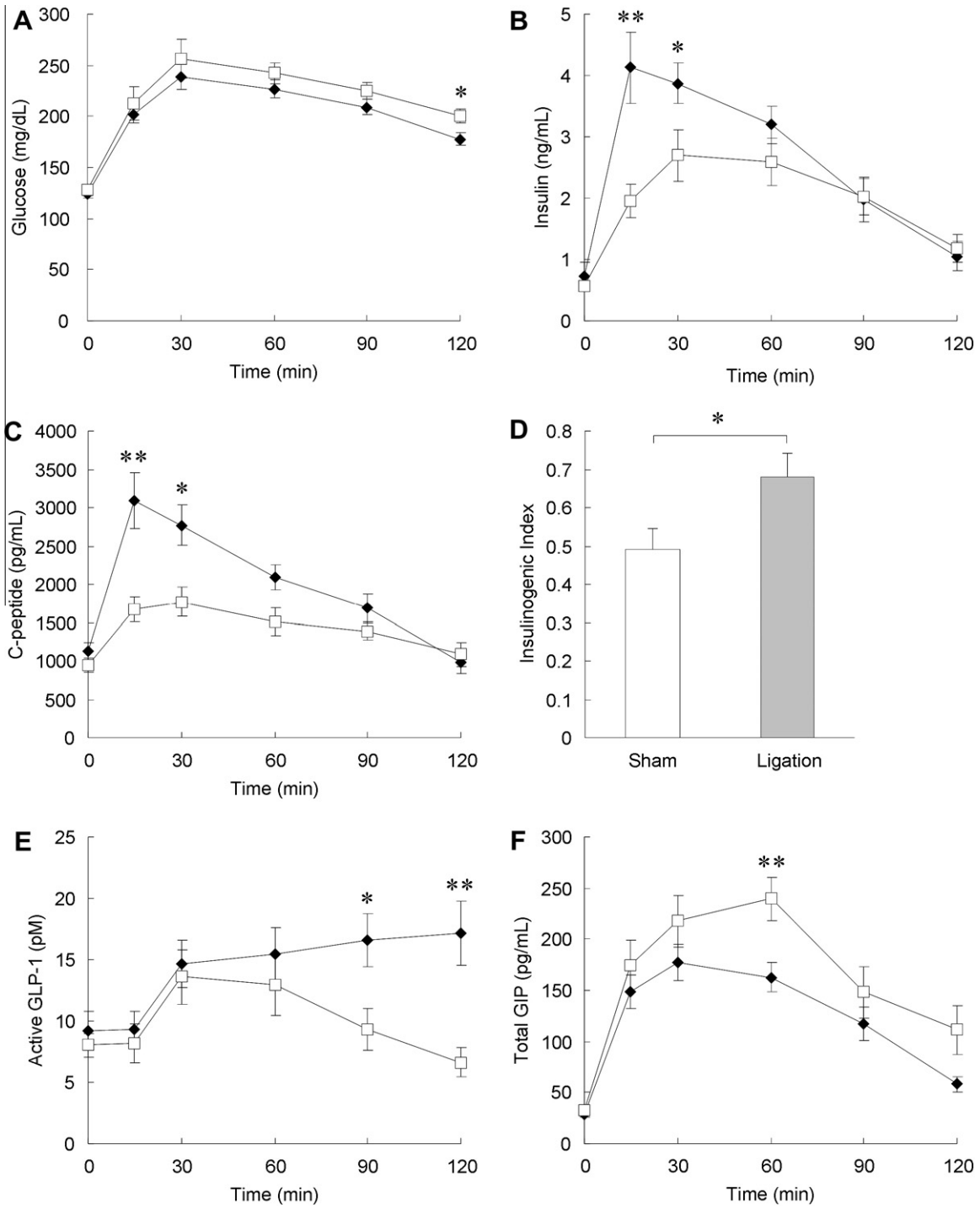

Fig. 1. Changes in plasma levels of glucose, insulin, C-peptide, activated GLP-1 and total GIP in rats with mesenteric lymph duct ligation during the oral glucose tolerance test (OGTT). (A) Plasma glucose levels, (B) plasma insulin levels, (C) plasma C-peptide levels in the ligation ($n = 8$, solid squares) and sham ($n = 8$, clear squares) groups. (D) Insulinogenic index in both groups ($n = 8$, respectively). (E) Activated GLP-1 and (F) total GIP in the ligation ($n = 8$, solid squares) and sham ($n = 8$, clear squares) groups. Data are presented as mean ± standard error ($^{*}p < 0.05$; $^{**}p < 0.01$).

samples. Cells stained with anti-insulin antibody were considered to be β-cells, and the total area of acinar cells and the area of islets and β-cells were measured in five microscopic fields per sample, for a total of 40 microscopic fields, at a magnification of ×100. The ratios of islets and β cells to the total area of acinar cells were measured for each sample [19]. The number of β-cells was calculated by counting the nuclei surrounded by cytoplasm positive for insulin staining. At least 1000 β-cells were counted per slide in each group ($n = 8$, respectively). The proliferation of β-cells per animal was expressed as a percentage according to the formula: 100× (number of Ki-67-positive β-cells/total number of β-cells).

2.7. Quantification of insulin in pancreatic tissues

A portion of frozen pancreatic tissue was weighed, then the tissue extract was prepared by acid–ethanol extraction method as described previously [20]. Insulin concentration per unit of pancreatic tissue (ng/mg wet weight of pancreas) was calculated

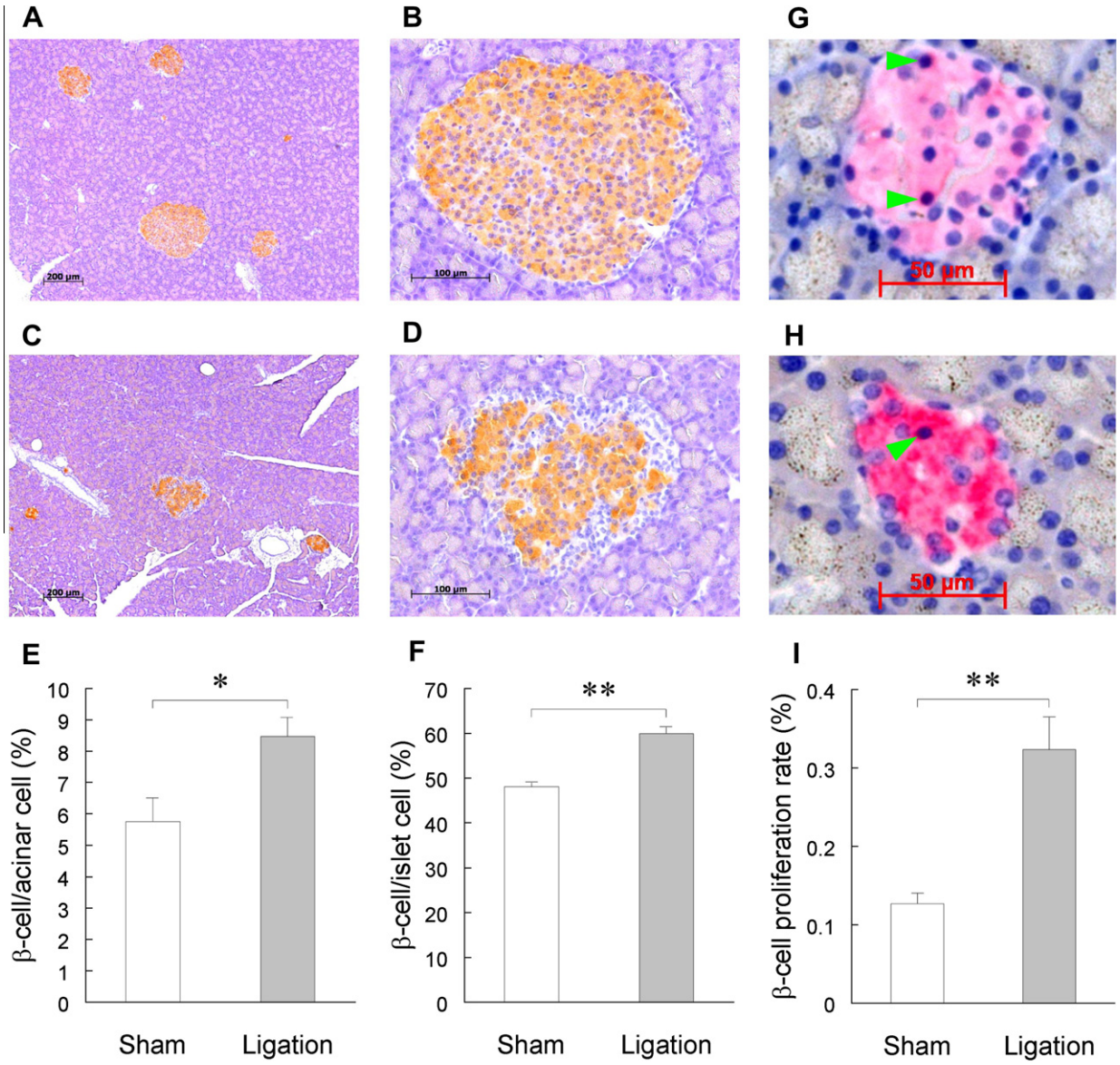

Fig. 2. Immunohistochemical study and morphometric analysis of islet β-cells of the pancreas in rats with mesenteric lymph duct ligation. Images of immunostained anti-insulin antibody-positive cells (β cells, brown) in the ligation (panel A at magnification of ×4; panel B, ×100) and the sham (panel C, ×4; panel D, ×100) groups. The ratio of the area occupied by β-cells to the total area of acinar cells (E) and to the islet (F) per microscopic field in both groups ($n = 8$, respectively). Pancreatic islets double-immunostained for insulin (red) and Ki-67 (brown/black) in the ligation (panel G at magnification of ×200) and sham (panel H, ×200) groups. Arrows denote Ki-67-positive nuclei. The proliferation of β-cells in both groups ($n = 8$, respectively) (I). Data shown in E, F and I are presented as mean ± standard error. ($^{*}p < 0.05$; $^{**}p < 0.01$).

by measuring the insulin level of the resulting supernatant by the ELISA method (Rebis Insulin-rat T; Shibayagi).

2.8. Statistical analysis

Measured values were presented as mean ± standard error (SE), and the SAS 9.1 software package (SAS Institute, Inc., Cary, USA) was used for statistical analysis. The two-sided *t* test was used to examine differences in average values between two independent samples at a significance level of $p < 0.05$.

3. Results

3.1. Baseline blood biochemistry data

Blood biochemistry data for the ligation group ($n = 8$) and the sham group ($n = 8$) at 1 and 2 weeks after surgery are shown in Table 1. Plasma levels of glucose, insulin, C-peptide, total GIP, GLP-1, adiponectin, triglyceride, and total cholesterol did not show any significant differences between the groups.

3.2. Changes in plasma insulin, GIP, and GLP-1 levels during OGTT

The plasma glucose level increased up to 30 min after glucose loading in both groups and then decreased, but remained higher than the initial level even at 120 min (Fig. 1A). Glucose levels in the ligation group tended to be lower than those in the sham group at any time point; a significantly lower glucose level was observed in the ligation group at 120 min (201 ± 6.9 mg/dL vs. 178 ± 6.0 mg/dL, $p < 0.05$).

The plasma insulin level was highest at 30 min in the sham group and at 15 min in the ligation group, and then continued to decrease up to 120 min, approximately reaching the pre-glucose load level (Fig. 1B). The insulin levels in the ligation group at 15

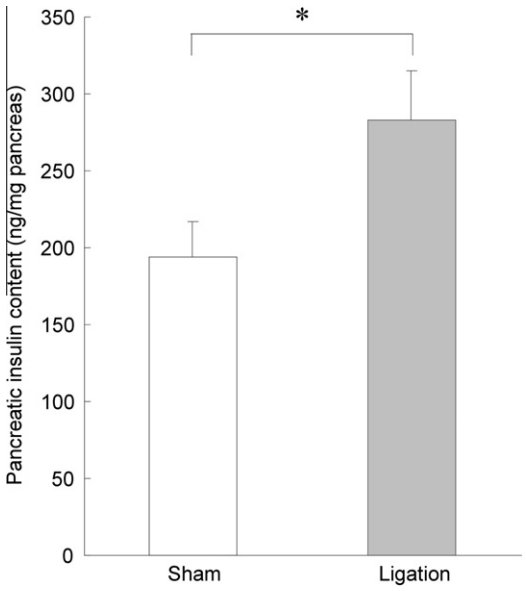

Fig. 3. Insulin contents of pancreatic tissues in rats with mesenteric lymph duct ligation. Insulin contents of pancreatic tissues in the ligation ($n = 8$) and sham ($n = 8$) groups ($^{*}p < 0.05$). Data are presented as mean ± standard error.

and 30 min were found to be 2 and 1.5 times higher than those in the sham group, respectively ($p < 0.01$ at 15 min; $p < 0.05$ at 30 min). The plasma C-peptide level was highest at 30 min in the sham group and at 15 min in the ligation group, and continued to decrease for up to 120 min to the level before glucose loading (Fig. 1C). C-peptide levels in the ligation group at 15 and 30 min were found to be 1.8 and 1.4 times higher than those in the sham group, respectively ($p < 0.01$ at 15 min; $p < 0.05$ at 30 min).

The insulinogenic index was 0.68 ± 0.06 in the ligation group and 0.49 ± 0.05 in the sham group, being significantly higher in the former ($p < 0.05$) (Fig. 1D).

The plasma level of activated GLP-1 in the ligation and sham groups quickly increased within 15–30 min after glucose loading, and then decreased for up to 120 min to reach the pre-test level in the sham group, although it continued to increase for up to 120 min in the ligation group (Fig. 1E). The level of activated GLP-1 in the ligation group tended to be higher than that in the sham group after 60 min, and was 1.7 and 2.5 times higher in the ligation group than in the sham group at 90 and 120 min, respectively ($p < 0.05$ at 90 min; $p < 0.01$ at 120 min).

The total GIP level in the plasma of the ligation and sham groups continued to increase, peaking at 60 and 30 min, respectively, and then decreased for up to 120 min, although the level at 120 min was still higher than the level prior to glucose loading

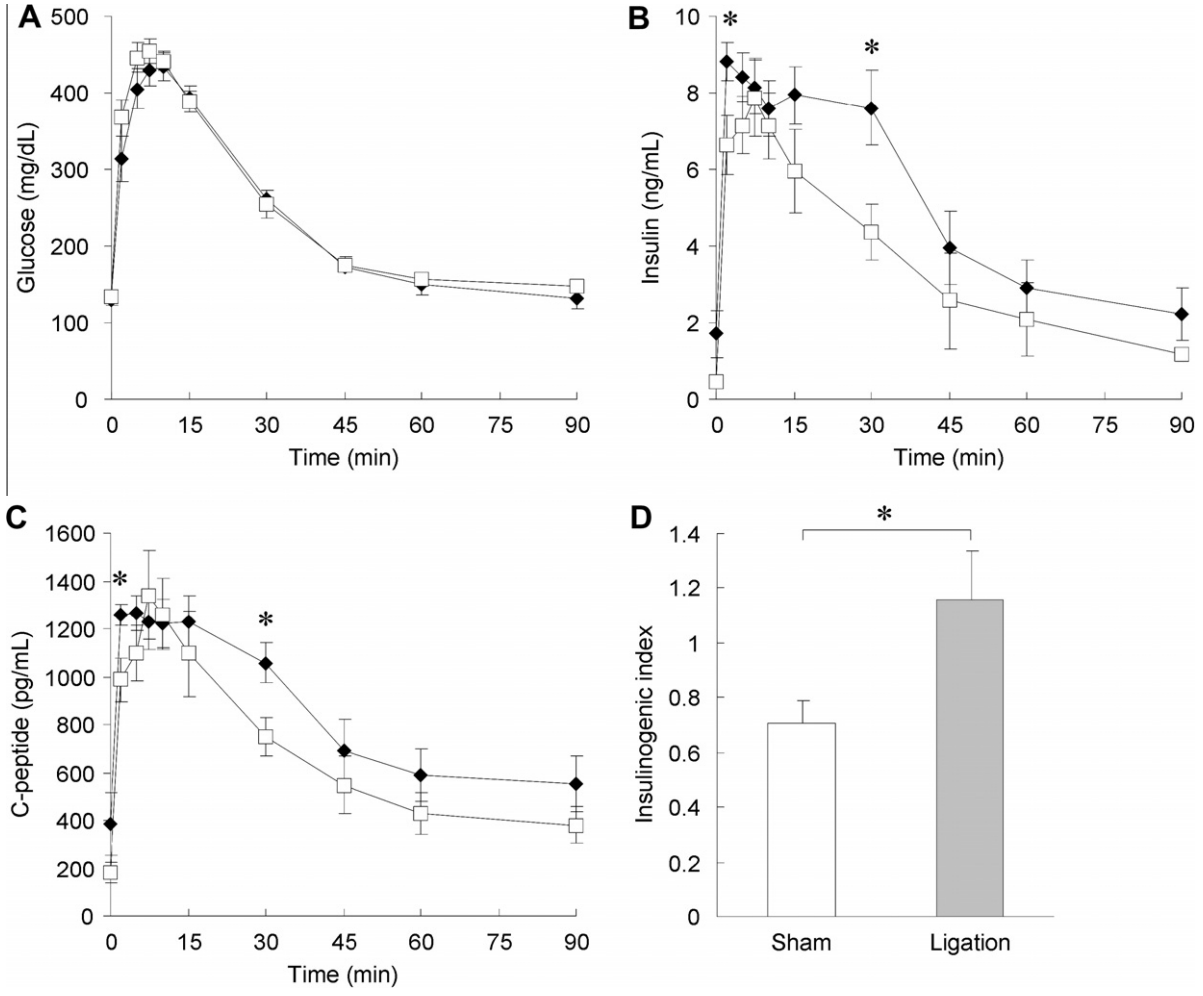

Fig. 4. Changes in plasma levels of glucose, insulin, and C-peptide in rats with mesenteric lymph duct ligation during the intravenous glucose tolerance test (IVGTT). (A) Plasma glucose levels, (B) plasma insulin levels and (C) plasma C-peptide levels in the ligation ($n = 8$, solid squares) and sham ($n = 8$, clear squares) groups. (D) Insulinogenic index at 2 min in both groups ($n = 8$, respectively). Data are presented as mean ± standard error ($^{*}p < 0.05$; $^{**}p < 0.01$).

(Fig. 1F). Overall, the level of total GIP in the ligation group was about 2/3 of that in the sham group ($p < 0.01$).

3.3. Immunohistochemistry of pancreatic β-cells

The area stained with anti-insulin antibody inside the islets was larger in the ligation group than in the sham group (Fig. 2A–D). The ratio of islets to the total area of acinar cells per microscopic field was 8.36 ± 0.75% in the ligation group and 5.75 ± 0.77% in the sham group, being significantly increased in the former ($p < 0.05$) (Fig. 2E). The ratio of the area occupied by β-cells to the area of islets was 59.8 ± 1.2% in the ligation group and 48.3 ± 1.1% in the sham group, the ratio being increased in the former ($p < 0.01$) (Fig. 2F).

3.4. Proliferation of β-cells

The percentage of Ki-67-positive β-cells was 0.32 ± 0.041% in the ligation group and 0.13 ± 0.013% in the sham group, indicating that the proliferation of β-cells had increased in the former ($p < 0.01$) (Fig. 2G–I).

3.5. Insulin levels in pancreatic tissues

The level of insulin in pancreatic tissue was 283.3 ± 31.8 ng/mg tissue in the ligation group and 193.8 ± 23.4 ng/mg tissue in the sham group, being significantly higher in the former ($p < 0.05$) (Fig. 3).

3.6. Changes in plasma insulin levels during IVGTT

Glucose levels in the ligation group tended to be lower than those in the sham group at 2 and 5 min, although not to a statistically significant degree (Fig. 4A).

The insulin levels in the ligation group at 2 and 30 min were 1.4 and 1.7 times higher than those in the sham group, respectively ($p < 0.05$ at each time point) (Fig. 4B). The plasma C-peptide level was highest at 2 and 5 min in the ligation group and at 7.5 min in the sham group, and then continued to decrease for up to 90 min to the level before glucose loading (Fig. 4C). C-peptide levels in the ligation group at 2 and 30 min were 1.3 and 1.4 times higher than those in the sham group, respectively ($p < 0.05$ at each time point).

Insulinogenic indices at 2 min were 1.16 ± 0.18 in the ligation group and 0.71 ± 0.08 in the sham group, being higher in the former ($p < 0.05$) (Fig. 4D).

4. Discussion

The present study showed that during the OGTT 2 weeks after ligation of the mesenteric lymph duct, plasma insulin levels were significantly higher at 15 and 30 min in the ligation group, and also that C-peptide levels were significantly higher at 15 and 30 min in the ligation group. These results indicated that insulin secretion increased in the early phase during the OGTT after ligation of the mesenteric lymph duct. This is the first report to indicate that postprandial insulin secretion is increased due to ligation of the mesenteric lymph duct.

The insulin content per unit wet weight of pancreas was significantly increased at 2 weeks after ligation of the mesenteric lymph duct, and the ratios of β-cell area/acinar cell area and β-cell area/islet area, as well as β-cell proliferation, were significantly higher in the ligation group than in the sham group. Moreover, we estimated the size of β-cells as described previously [21]. The mean size of β-cells did not differ significantly between the two groups (data not shown). These immunohistochemical and biochemical findings suggested that β-cells in the islets had proliferated, but were not hypertrophic, in the ligated rats.

To examine the mechanisms of insulin secretion in the early phase at 15 and 30 min during the OGTT, the ratios of β-cell area/acinar cell area and β-cell area/islet area, and also the proliferation of β-cells, which were increased in the ligation rat model, and the levels of incretins, GLP-1, and GIP were measured during the OGTT at 2 weeks after ligation. The level of the active form of GLP-1 did not differ between the two groups in the early phase, but was significantly higher at 90 min (1.7-fold) and 120 min (2.5-fold). The increased insulin secretion in the early phase could not be explained by GLP-1 secretion at this time. Instead, an increase of β-cells appeared to be responsible for the increased insulin secretion in the ligation group.

On the other hand, the plasma GIP concentration in the ligation group tended to be increased in the early phase at 15 and 30 min during the OGTT, although not to a significant degree, whereas it was significantly decreased at 60 min. There is a possibility that the slight increase of GIP stimulated insulin secretion in the early phase in the ligation group, in view of the enlarged islets and increased insulin content of the pancreas at 2 weeks after ligation.

To confirm the degree of insulin secretion resulting from the increase in β-cells, we carried out the IVGTT, which is not influenced by gut hormones such as incretin, in the ligation and sham groups (Fig. 2). The present study showed that during the IVGTT 2 weeks after ligation of the mesenteric lymph duct, the plasma levels of insulin and C-peptide were both significantly higher at 2 min. These results indicated that insulin secretion was elevated as a result of the increased number of β-cells without stimulation by incretins in the early phase of the IVGTT, suggesting that the increased secretion of insulin in the early phase of the OGTT in the ligation group was attributable mainly to these increased β-cells.

It is interesting that proliferation of β-cells in islets was observed in the rats with mesenteric lymph duct ligation. Incretins are reported to stimulate proliferation of β-cells by induction of cyclin D1 via activation of phosphatidylinositol 3-kinase and protein kinase A [22]. In this study the level of GLP-1 was significantly increased at 90 and 120 min during the OGTT in the ligation group. It is possible that the chronic postprandial elevation of the GLP-1 concentration stimulated proliferation of β-cells in the ligation group. On the other hand, GIP was significantly decreased in the later phase, and this could not have participated in the proliferation of β-cells in the ligation group.

Incretins, GIP and GLP-1, are secreted into the intestinal lymphatics and subsequently flow into the blood stream [10,11]. Incretins are known to regulate insulin activity via degradation with dipeptidyl peptidase-IV (DPP-IV). Recently, lymphatic flow has been shown to be an important system for delivery of incretins, which stimulate insulin secretion, since DPP-IV activity is much lower in lymph than in blood [10,11].

It still remains unexplained why rats subjected to lymph duct ligation showed an increase of GLP-1 in the later phase during the OGTT but a decrease of GIP. Secretion of GLP-1 and GIP may be influenced by retention of intestinal lymphatic fluid in the mesenteric lymph duct ligation model, for example through changes in their production by K and L cells of the intestine. It seems that GIP is secreted into lymphatic vessels preferentially over GLP-1 [10]. Recently, GLP-1 has been reported to reduce both intestinal lymph flow and triglyceride absorption [23]. To explain the difference between GIP and GLP-1 in their secretion responses to oral glucose intake in the lymph duct ligation model, further extensive investigation is needed.

In conclusion, this study has shown that mesenteric lymph flow plays a role in glucose metabolism. Ligation of the mesenteric lymph duct in rats did not influence fasting biochemical parameters, but increased β-cell proliferation, the ratios of β-cell area/aci-

nar cell area and β-cell area/islet area, and the insulin content in the pancreas. Insulin secretion was significantly increased during the OGTT and IVGTT. These observations suggest that ligation of the mesenteric lymph duct stimulates β-cell proliferation in the pancreas, possibly via increased postprandial secretion of GLP-1.

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Disclosures

None of the authors have any conflicts of interest (financial, professional, or personal) to disclose.

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