Beneficial effects of lactoferrin on the blood glucose regulation in rats

（ラットの血糖調節機構に及ぼすラクトフェリンの有益な効果）

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Contents

General introduction

Several types of hypoglycemic medication are currently given to patients with type 2 diabetes mellitus. The applications presently available include insulin preparations and drugs for the improvement of insulin resistance, the promotion of insulin secretion and/or the suppression of postprandial hyperglycemia. Agents targeting incretin, a gastrointestinal hormone secreted by the stimulation of nutrients passing through the small intestinal lumen, have been a focus of attention in recent years based on the differences in their active mechanisms from those of other medications. While those methods established more precise treatment according to the causal factors, they have some disadvantages such as exorbitant costs, or adverse effects including gastrointestinal side effects and hypoglycemia (Nathan et al. 2009). It is due to these drawbacks that concomitant lifestyle interventions are needed, along with the development of other pharmaceutical cures.

Blood glucose level is come out as a consequence of the amount of glucose moved into blood stream and that disappeared from blood stream. Glucose absorption in small intestine, particularly in jejunum, depends on the activities of two types of glucose transporter; sodium-dependent glucose cotransporter 1 (SGLT1) and glucose transporter (GLUT) 2. Glucose transfer from lumen side to the epithelial cell is brought about by active transport through SGLT1, while that from the epithelial cell to hepatic portal vein is by passive

diffusion through GLUT2. GLUT2 is known not to require insulin stimulation for its function, and it is also reported that the passive diffusion through GLUT2 is a minor route in the process of small-intestinal glucose absorption (Krehbiel et al. 1996). These facts are thought to indicate that the amount of glucose transferred into blood stream in normal condition, is resulted from the efficiency of glucose transfer into the epithelial cells via SGLT1. Glucose in blood stream is cleared largely accompanied by the glucose uptake into skeletal muscle and adipose tissue, where insulin stimulates the translocation of GLUT4 from inside of the cell onto the plasma membrane. In case of oral glucose administration, it results in a larger volume of insulin secretion than intravenous glucose injection. It is predominantly thought to be due to the enhancement of postprandial insulin secretion by incretin. This response has been called the "*incretin effect"*, which is exerted via different pathway from that of basic insulin secretion. Previous study revealed that incretin effect accounts for 50-70% of the total insulin secretion (Baggio and Drucker 2007).

Maintenance of normoglycemia has a close relationship with an interaction between insulin and so-called "*stress-induced hormones"*; catecholamines (norepinephrine and epinephrine), glucagon and glucocorticoids. Secretions in those hormones are conventionally adjusted depending on the feeding condition of the individual, while excessive external stress results in hyperresponsiveness of the two main axes; the

sympathetic-adrenal medullary (SAM) system and the hypothalamic–pituitary–adrenocortical (HPA) axis, leading to an increase of those hormones' secretion. The epinephrine-induced increase in plasma glucose is thought to be brought about by enhanced output of hepatic glucose in the fed condition, and attenuated glucose clearance (Issekutz and Allen 1972; Deibert and DeFronzo 1980). It has been demonstrated that glucagon is involved in glucose intolerance by facilitating hepatic glycogenolysis in the fed condition and gluconeogenesis in the fasted condition (Gastaldelli et al. 2000). Glucocorticoids are also known to promote glucose intolerance, with its effect in opposition to insulin action (Lambillotte et al. 1997; Andrews and Walker 1999). The secretion of epinephrine is considered to be resulted from the heightened stimulation of SAM system, while that of glucocorticoids is from HPA axis. Transient hyperglycemia via stress is an outcome of those responses, becoming an aggravating or crucial risk factor for type 2 diabetes mellitus.

Lactoferrin (Lf) is a glycoprotein found in various mammalian body fluids, including blood, tears, saliva, and bile, and is especially abundant in milk (Masson et al. 1966; Baker 1994). The potential of Lf has been evidenced by its physiologically pleiotropic properties, as represented by biophylactic responses such as anti-inflammatory and anti-cancer effects (Tsuda et al. 2000; Ishikado et al. 2005). The fact that Lf receptors are expressed in many organs also supports that multifunctionality, and introduces the possibility of

as-yet-unknown influences (Suzuki et al. 2005). In recent years, interests have also been developed in the involvement of Lf in metabolic reactions. Lf supplementation improves lipid metabolism associated with reductions in the contents of hepatic cholesterol and triacylglycerol, accompanied by a suppressive effect on fat accumulation in the mesenterium and liver in mice (Takeuchi et al. 2004; Morishita et al. 2013).

Moreno-Navarrete et al. (2009) have shown the direct relevance of Lf for diabetes mellitus: namely, the Lf concentration in blood is positively correlated to insulin sensitivity, and negatively to blood glucose levels in humans with altered glucose tolerance. Furthermore, it has been demonstrated a direct interaction of Lf with the active site of dipeptidyl peptidase IV (DPP-IV), an endogenously existing enzyme known to involve in incretin degradation and activity, indicating a potential of Lf for incretin preservation (Aroor et al. 2013; Nongonierma and FitzGerald 2014). Recent studies have also shown a close relationship between Lf and stress; Lf exerts its anxiolytic and analgesic effects accompanied by an increase in nitric oxide production or activation of the μ-opioid system (Takeuchi et al. 2003; Kamemori et al. 2004; Tsuchiya et al. 2006), and immobilization-stress-induced modification of the immune response is normalized by Lf via its anti-inflammatory effect or cytokine regulatory action (Zimecki et al. 2005). These facts may suggest the advantage of Lf for the correction of glucose metabolism and diabetic care,

whereas it is still unknown whether Lf treatment can actually influence glucose tolerance in a normal or stressed individual.

Based on those backgrounds, the present study aims to investigate whether treatment with bovine Lf (bLf) induces any changes in blood glucose regulation in normal rats and those under stress condition. In non-stress condition, oral glucose tolerance test (OGTT) and intravenous glucose tolerance test (IVGTT) were performed, in order to examine the effects of Lf on comprehensive glucose kinetics, including glucose clearance and absorption. In addition, the glucose absorption in small intestine and the influence of Lf treatment on it were estimated by conducting an in vitro study using the everted sac method. In stress condition, OGTT combined with restraint stress (RS) was performed for the investigation whether the treatment of Lf involves in stress-induced hyperglycemia and parameters.

Chapter 1

Effects of lactoferrin on blood glucose regulation and glucose absorption in the small intestine in normal rats

Introduction

The extent of alteration in glucose tolerance has been compartmentalized by fasting blood glucose level. OGTT has been a simple and useful test, in which temporal change in blood glucose level is observed after a certain amount of oral glucose administration (Andrikopoulos et al. 2008; Bartoli et al. 2011). OGTT enables to evaluate comprehensive glucose tolerance and insulinotropic response, while this test itself does not clearly represent the situation in the glucose absorption in small intestine. In contrast, IVGTT has been helpful in assessing glucose clearance after exogenous glucose delivery to blood circulation. That is to say, IVGTT allows indirect determination of the glucose tolerance for a certain volume of glucose injected.

Responses to oral and intravenous glucose administration are different, and that is mainly caused by enhanced insulin secretion through incretin action (incretin effect). The effect is attributed to two types of incretin: glucose-dependent insulinotropic polypeptide (GIP) from K cells in the upper small intestine and glucagon-like peptide-1 (GLP-1) from L cells in the lower small intestine. Recent numerous studies have shown the strong relationship between incretin effect and glucose homeostasis; namely the promotion of insulin secretion or synthesis in pancreatic β cells, the increase of insulin sensitivity and the reduction of gastric emptying (Drucker 2006; Marathe et al. 2013).

This chapter deals with the examination whether treatment with bovine Lf (bLf) causes any changes in blood glucose regulation, including plasma glucose, insulin or incretin secretion. Two types of glucose tolerance test, oral glucose tolerance test (OGTT) and intravenous glucose tolerance test (IVGTT), were conducted to assess the influence of Lf on blood glucose and insulin kinetics in relation to incretin effects. In addition, the effect of Lf on the efficiency of glucose absorption in the small intestine was determined by performing an experiment with the everted sac method using a piece of the jejunum.

Materials and methods

Materials

BLf, and aprotinin from a bovine lung were purchased from Wako Chemical Co., Ltd. (Osaka, Japan). The purity of bLf was more than 95.0% with a 0.005-0.035% iron saturation level, as determined by HPLC analysis, and neither LPS contamination nor other poisonings were reported from the bLf. Isoflurane was purchased from Intervet Inc. (Tokyo, Japan). The titanium vascular access port (SWIRL-MIN), dedicated 25-gauge needle and 3-Fr polyurethane catheter were purchased from Primetech Co. (Tokyo, Japan). Urethane was purchased from Sigma-Aldrich (St.Louis, MO, USA). Sodium pentobarbital (Somnopentyl) was purchased from Kyoritsu Pharmaceutical Co., Ltd. (Tokyo, Japan). A 3-Fr polyvinyl chloride catheter (atom nutrition catheter) was purchased from Atom Medical Co. (Tokyo, Japan). A 4-Fr polyethylene tube (Hibiki) was purchased from Kunii Co. (Tokyo, Japan). A tube containing ethylenediaminetetraacetic acid (EDTA-2K) was purchased from FUJIFILM Medical Co., Ltd. (Tokyo, Japan).

Animals

All experimental protocols in this study were approved by the Animal Research Committee of Tottori University (approval numbers: 13-T-15 and 15-T-7). Male Wistar rats

were obtained at 5 and 7 weeks of age from the Institute for Animal Reproduction (Ibaraki, Japan). The former were used for the experiment of glucose absorption; the latter for the glucose tolerance tests. The rats were acclimatized to their surroundings for at least one week before the experiments. The animal room was controlled with a 12/12 hr light/dark cycle (lights on 7:00-19:00) at an ambient temperature of 24 ± 1 ℃. The rats were housed in plastic cages, with at most three rats per cage, and were allowed free access to water and standard chow (CE-2; Nihon Clea, Tokyo, Japan). The standard chow contains 24.9% protein, 4.6% fat, 4.5% fiber, 6.6% ash, 51.0% nitrogen free extract, and consists of 3.45 kcal/g. All experiments were conducted after overnight (16 hr) fasting.

Surgical operation for OGTT

Prior to OGTT, rats were fully anesthetized with 2–2.5% isoflurane gas, and then implanted with a vascular access port on their dorsal region, as previously described (Higuchi et al. 2013). Subsequently, a 3-Fr polyurethane catheter connected to the titanium vascular access port was inserted into the exposed right external jugular vein. These approaches enabled me to obtain large volume of blood with less hemolysis. The catheter was prefilled and kept locked with heparin-added physiological saline by flushing the solution twice a day. OGTT was performed after 3-days of recovery from the operation.

Surgical operation for IVGTT

A series of surgical procedures involving in IVGTT was followed by reference to the protocol, described by Frangioudakis et al. (2007). Rats were anesthetized with 1 g/kg urethane (s.c.) and 6.48 mg/rat sodium pentobarbital (s.c.). A 3-Fr polyvinyl chloride catheter, as a cannula for blood collection, was inserted into the exposed right external jugular vein. Until the end of IVGTT, the catheter was prefilled and kept locked with heparin-added physiological saline. The solution was flushed after blood collection in volumes equal to the collected blood. In addition, a 4-Fr polyethylene tube with 2.5-cm length was subsequently placed in the trachea to keep the airway smooth.

OGTT

The rats that had been operated upon were divided into four groups: group 1 received 10 m*l*/kg of saline orally with saline at a dose of 1 m*l*/kg intraperitoneally; group 2 received 10 m*l*/kg of saline orally with bLf at 100 mg/kg intraperitoneally; group 3 received 2 g/kg of glucose orally with saline at 1 m*l*/kg intraperitoneally; and group 4 received 2 g/kg of glucose orally with bLf at 100 mg/kg intraperitoneally. With oral saline administration, the effect of bLf on the endogenous glycemic change was assessed. The rats received the oral

administration at 30 min after the intraperitoneal injection of saline or bLf. Blood samples were collected before the oral administration (0), and at 15, 30, 60, 90, 120, and 180 min after the oral administration. The volume of blood collected at each time point was 0.1 ml in groups 1 and 2, while in groups 3 and 4, it was 0.5 ml up to the 60 min point; after that, it was 0.1 ml. Twenty microliters of each obtained blood sample was used to measure plasma glucose just after the blood collection. The remainders of the blood, collected at every time point in group 1 and 2, and that up to the 60 min point in group 3 and 4, were transferred into an ethylenediaminetetraacetic-acid (EDTA)-containing tube (FUJIFILM Medical Co., Ltd., Tokyo, Japan) with aprotinin (final concentration, 500 KIU/mL, Wako Chemical Co., Ltd., Osaka, Japan), and centrifuged (5,600 × g, 5 min, 4 °C). All samples were stored at -80 °C until measurement for levels of insulin in groups 1 and 2; insulin, GIP and GLP-1 in groups 3 and 4, respectively.

IVGTT

IVGTT was conducted to examine the direct influence of bLf on glucose clearance, discounting the factor of glucose absorption from the small intestine. Under continuous anesthesia of urethane and pentobarbital, the operated rats were intraperitoneally injected with saline (1 ml/kg) or bLf (100 mg/kg). 30 min after the injection, they were given an

intravenous administration of glucose (1 g/kg) into the caudal vein. Blood samples were collected before the intravenous administration of glucose (0) and again at 5, 10, 20, 40, 60, 120 min after the intravenous administration. The volume of blood collected at each time point was 0.2 ml. The obtained blood samples were processed experimentally using the same protocol as for OGTT section, and were used for the measurement of plasma glucose and insulin.

Estimation of the glucose absorption across the everted sac of the small intestine

The composition of Krebs-Henseleit buffer used in this experiment was as follows: 119 mM NaCl, 21 mM $NaHCO_3$, 0.6 mM KH_2PO_4, 2.4 mM K_2HPO_4, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 6.0 mM L-glutamine and 15 mM D-glucose, as previously described with some modifications (Ishikawa et al. 2007; Han et al. 2013). Rats were sacrificed under sodium pentobarbital anesthesia (100 mg/kg, i.p.) at 1 hr after intraperitoneal injection of saline (1 ml/kg, control) or bLf (100 mg/kg). An everted sac was made using the piece of jejunum and filled with 0.25 ml of Krebs buffer (Wilson and Wiseman 1954). The sac was incubated for 1 hr at 37 °C in a 50-ml flask containing 30 ml of Krebs buffer. Sample solutions were collected from outside (mucosal side) and inside (serosal side) the sac, and glucose concentrations were analyzed before (M_b, S_b) and after (M_a, S_a) the incubation, respectively.

In addition, the sac weight was measured before (W_b) and after (W_a) collection of the serosal solution. The value of W_b-W_a (mg) was treated as the volume of the serosal solution after the incubation (μl). These parameters were used to calculate the total amount of glucose absorbed into the sac (*TA*) according to the following equation:

$$TA \text{ (mg/g intestinal tissue)} = [S_a \text{ (mg/dL)} \cdot \{W_b\text{-}W_a \text{ (μl)}\} \cdot 10^{-5} - M_b \text{ (mg/dL)} \cdot 0.25 \text{ (ml)} \cdot 10^{-2}] / W_a \text{ (mg)} \cdot 10^3$$

Analysis of biochemical parameters

Plasma glucose was measured by a portable electrode-type blood glucose meter (ANTSENSE III; Horiba, Ltd., Kyoto, Japan). Plasma insulin was determined by the enzyme immunoassay method using an ELISA kit (AKRIN-010T; Shibayagi Co., Ltd., Gunma, Japan). The total GIP level in plasma was measured using an ELISA kit (EZRMGIP-55K; Millipore Corp., Billerica, MA, USA), while total GLP-1 was measured with an EIA kit (YK160; Yanaihara Institute Inc., Shizuoka, Japan). The glucose concentration in the glucose absorption experiment was estimated using an enzymatic assay kit (Glucose CII-test WAKO; Wako Pure Chemicals, Osaka, Japan).

Statistical analysis

Data are represented as means ± SEM. Differences between the glucose concentrations of the mucosal and serosal sides of the everted sac, and those between the amounts of absorbed glucose into the sac in the bLf-treated group and the control group were statistically investigated using two-way ANOVA, followed by Bonferroni's post hoc test. In other experiments, the differences between the bLf-treated group and the control group were examined by Welch's *t*-test (GraphPad Prism version 6.0 for Windows; GraphPad Software Inc., La Jolla, CA, USA), and the score variation within a group was compared by a Univariate approach with a division model (JMP; SAS Institute Inc., Cary, NC, USA). Statistical significance was accepted at a probability (P) < 0.05.

Results

Changes in plasma glucose, insulin, total GIP and GLP-1 in OGTT

As can be seen in Fig. 1A, plasma glucose varied in the range of about 25 mg/dL after the oral saline administration in both group. Within both groups, no significant changes were observed at each time point from basal level of plasma glucose. Although there were no significant differences in plasma glucose at each time point between the two groups, plasma glucose tended to be decreased 90 min after the oral saline administration. The Area Under the Curve (AUC) from 0 to 180 min also did not differ between the two groups (20,707 ± 1245 mg/dL × 3 hr in group 1 and 18,975 ± 1091 mg/dL × 3 hr in group 2).

Similarly to the changes in plasma glucose, plasma insulin did not significantly changed at each time point from basal level (Fig. 1B). There were also no significant differences in plasma insulin at each time point between the two groups, while group 2 showed a trend of higher levels of plasma insulin at and after 60 min from the oral saline administration, The Area Under the Curve (AUC) from 0 to 180 min also tended to be increased in the group 2 (47.2 ± 30.7 ng/mL × 3 hr in group 1 and 108.4 ± 41.6 ng/mL × 3 hr in group 2).

In OGTT, plasma glucose was lower at 0 min in group 4 ($P = 0.046$) (Fig. 1A). In both groups, plasma glucose immediately increased and reached a peak at 15 min after oral

glucose administration (bLf: $P < 0.05$, control: $P < 0.05$). In group 3, the increment of plasma glucose appeared to be sustained from the peak at 15 min to 60 min. In group 4, meanwhile, a tendency of mild decline in plasma glucose was observed in the same time period, and a significant reduction in plasma glucose was found at 180 min ($P < 0.05$). The AUC for plasma glucose tended to be lower in group 4 than in group 3 (29,082 ± 969 mg/dL × 3 hr in group 3 and 27,543 ± 564 mg/dL × 3 hr in group 4, respectively).

Plasma insulin was also immediately increased and reached a peak at 15 min after oral glucose administration in both groups ($P < 0.05$, resprectively, Fig. 1B). In group 3, plasma insulin gradually decreased after the peak to 120 min, and then plateaued until 180 min. In group 4, plasma insulin tended to remain elevated even after the peak, up to 90 min, and then showed plateauing values. At 30 min, the increasing width of insulin from 0 min was significantly larger in group 4 than in group 3 (group 3: 0.80 ± 0.072 ng/mL, group 4: 1.44 ± 0.108 ng/mL, $P < 0.05$). The AUC for plasma insulin also tended to increase in group 4, compared with that in group 3 (195.3 ± 25.1 ng/mL × 3 hr in group 3 and 212.4 ± 28.9 ng/mL × 3 hr in group 4, respectively).

Total GIP in plasma increased within 15 min after oral glucose administration in both groups ($P < 0.05$, respectively, Fig. 2A). The high concentration was sustained until 60 min in group 3, whereas the level in group 4 showed a tendency of gradual decrease and was

lower at 60 min ($P < 0.05$). The AUC for total GIP also tended to decrease in group 4, compared with that in group 3 (7,527 ± 512 pg/mL × 1 hr in group 3 and 6,312 ± 757 pg/mL × 1 hr in group 4, respectively).

In group 3, total GLP-1 in plasma tended to show a gradual increase, and was higher at 30 min compared with that from 0 min in OGTT ($P < 0.05$, Fig. 2B). On the other hand, the level in group 4 showed an immediate increase at 15 min ($P < 0.05$). Nevertheless, the AUC for total GLP-1 was almost same between the two groups (329.9 ± 11.5 ng/mL × 1 hr in group 3 and 331.3 ± 25.1 ng/mL × 1 hr in group 4, respectively).

Changes in plasma glucose and insulin in IVGTT

In IVGTT, plasma glucose reached a peak just after the injection of glucose ($P < 0.05$, respectively), rapidly decreased after the peak, and then continued to be flat after 40 min in both groups (Fig. 3A). At each time point, no significant difference was observed in plasma glucose between the two groups. The AUCs for plasma glucose in the bLf and the control groups were nearly the same (29,805 ± 2360 mg/dL × 2 hr in the control group and 29,077 ± 2149 mg/dL × 2 hr in the bLf group, respectively).

Plasma insulin showed a similar transition to that of plasma glucose ($P < 0.05$, respectively, Fig. 3B). Although there was no significant difference in plasma insulin at any

time point between the two groups, the bLf-treated group yielded a sustained increase in plasma insulin, which differed significantly from that at 0 min ($P < 0.05$). The AUC for plasma insulin did not also differ between the two groups (252 ± 17.8 ng/mL × 2 hr in the control group and 296 ± 26.6 ng/mL × 2 hr in the bLf group, respectively).

Alteration in the glucose absorption in the everted sac of jejunum

Fig. 4A shows the alteration in the glucose concentration inside and outside the everted sac of jejunum piece through incubation. Two-way ANOVA revealed significant effects of incubation ($F_{1, 19} = 126.7$, $P < 0.01$) and bLf treatment ($F_{1, 19} = 8.333$, $P = 0.020$) on the glucose concentration in the mucosal side of the sac: the incubation for 1hr and the bLf treatment resulted in significantly less glucose concentration on the mucosal side of the sac compared to saline, respectively. Significant effects were also noted in the serosal side (incubation: $F_{1, 19} = 34.90$, $P < 0.01$, bLf treatment: $F_{1, 19} = 5.852$, $P = 0.042$): the incubation for 1hr and the bLf treatment resulted in significantly more glucose concentration on the serosal side of the sac compared to saline, respectively. An interaction between incubation and bLf treatment was observed in the serosal side ($F_{1, 19} = 6.791$, $P = 0.031$), but not in the mucosal side ($F_{1, 19} = 5.039$, $P = 0.055$): the bLf treatment significantly enhanced an increase of glucose concentration in the serosal side of the sac, which was resulted from the

incubation for 1hr, but not in that in the mucosal side of the sac.

Significant effects on the calculated amount of glucose absorbed into the serosal side were found for incubation ($F_{1, 19}$ = 72.51, P < 0.01) and bLf treatment ($F_{1, 19}$ = 6.037, P = 0.040): the incubation for 1hr and the bLf treatment resulted in significantly more glucose amount in the sac compared to saline, respectively (Fig. 4B). An interaction between incubation and bLf treatment was seen ($F_{1, 19}$ = 9.186, P = 0.016): the bLf treatment significantly enhanced an increase of glucose amount in the sac, which was resulted from the incubation for 1hr. In the bLf group, the amount of glucose absorption after incubation was 1.7-fold that in the control group (6.1 ± 0.89 mg/dL in the control group and 10.6 ± 1.39 mg/dL in the bLf group).

Discussion

In this study, OGTT measured the effect of Lf on the movement of glucose from the intestine into the blood, combined with the movement of glucose out of the blood, including incretin effect. On the other hand, IVGTT measured the effect of Lf on the movement of glucose out of the blood itself. The obtained results revealed the potential of Lf for blood glucose regulation as follows: Lf can decrease blood glucose in relation to the enhancement of insulin secretion by the incretin effect, despite the fact that Lf promotes glucose absorption in the small intestine. To my knowledge, this is the first study to investigate the effects of direct application of Lf on the whole glucose metabolism. The present findings may open up new possibilities for the treatment of glucose intolerance.

Moreno-Navarrete et al. (2009) have suggested that the lower circulating Lf concentration in the humans with impaired glucose tolerance is relevant to the decreased number of neutrophils (the origin of endogenous Lf secretion). They also indicated the cause may be associated with the degranulation of neutrophils by hyperglycemia (Stegenga et al. 2008). The results in IVGTT demonstrated that the Lf treatment retained slightly higher plasma insulin levels in case of intravenous glucose administration. Though the possibility of Lf as the stimulant for insulin secretion could not be denied completely based on this finding, Lf was shown to result in no significant change in plasma glucose itself,

indicating that Lf yielded negligible effects on glucose disposition in blood circulation in IVGTT. These facts suggest that there is much truth in the interpretation that Lf does not facilitate normal pancreatic function, and the change in blood glucose itself does not activate the effects of Lf.

The experiment on oral saline administration was conducted to investigate the direct effect of Lf on endogenous blood glucose. Although it resulted in no significant effects of Lf on endogenous blood glucose changes and insulin secretion, the trend of lower plasma glucose was observed in later stage after the oral saline administration in the bLf-treated group. This change may provide the possibility that Lf contributes to the factors progressively required on fasted condition such as glucose uptake into muscle or adipose tissue and subsequent energy production. However, a tendency of increased insulin secretion was also observed temporally consistent with the changes in plasma glucose. As far as I know, there has been no information about direct involvement of Lf in endogenous insulin secretion. This regard is considered to be open to further discussion.

Based on this suggestion, Lf tended to decrease plasma glucose persistently after oral glucose administration, and significantly reduced the value closer to the basal level. These results are consistent with the trend for higher transition and the significantly larger increasing width of insulin at 30 min in the Lf-treated group in OGTT. The enhancement of

the total GLP-1 level in plasma at 15 min in Lf-treated group corresponded to the above results. Basic insulin secretion, triggered by glucose transport into pancreatic β cells, is routed through Ca^{2+} influx by the closure of the K_{ATP} channel. On the other hand, the K_{ATP}-independent pathway of insulin secretion is closely related to the increased cAMP concentration in β cells, mediated by adenylate cyclase (Yajima et al. 1999). The potentiation of insulin secretion by the incretin effect proceeds through this pathway. Accordingly, it seems reasonable to conclude that the rapidly augmented GLP-1 secretion contributed to the simultaneous enhancement of insulin secretion by Lf. Until recently, the question of whether Lf is tied with incretin secretion seemed to be unresolved. However, in the past several years, evidence has accumulated that whey containing Lf shows DPP-IV inhibitory action and exerts acute effects on incretin effect and glucose metabolism (Graf et al. 2011; Nongonierma and FitzGerald 2013). These findings do not negate the possibility that Lf plays a part in the preservation or direct acceleration of the incretin effect. In other words, Lf may be a useful candidate both to stimulate incretin secretion and to inhibit its degradation.

Attention must be directed toward the finding that basal plasma glucose was increased in the control group, compared with the Lf-treated group in OGTT. Superficially regarded, it can be inferred that Lf ameliorated mild stress arising from the surgical operation or the

manipulation during OGTT. In fact, Lf has anxiolytic and analgesic effects accompanied by an increase in nitric oxide production or activation of the μ-opioid system (Takeuchi et al. 2003; Kanemori et al. 2004; Tsuchiya et al. 2006). These effects suggest that Lf can give rise to a reduction in stress hormone secretion, brought about by interrupting the signaling pathway of those hormones past the cerebral cortex. On the other hand, the result in OGTT showed a trend toward lower basal level of plasma insulin in Lf-treated group, seemingly conflict with higher plasma glucose at the same time point. This contradiction may be related to the previously reported fact that acute physical stress leads to hyperglycemia without affecting insulin secretion (Romeo et al. 2007). Although it is possible that the physical stress, accompanied by the surgical operation, might not be fully passed off after 3days of recovery, that impact could be considered to be continued acutly or subacutely. In this regard, it raises the possibility that there was no close interaction between the basal levels of plasma glucose and insulin.

Lf did not change the total GIP in plasma just after the oral glucose administration, but did significantly lower that at 60 min. Meanwhile, plasma insulin tended to be higher over the same period. These results appear to contradict the previously assumed association between both incretins and insulin secretion. According to previous research using K cell signaling-modified mice, the GIP response to orally administered glucose was depressed,

while the glucose tolerance was not affected (Pedersen et al. 2013). This suggests that GIP makes only a slight contribution to glucose regulation, not only in individuals with impaired glucose tolerance, but also in normal individuals. The present finding that the plasma glucose-lowering effect and GIP secretion-lowering effect coexisted supports the above suggestion, and further strongly suggests that the immediate increase in GLP-1 secretion induced by Lf had an influence on blood glucose and insulin.

From the results in Fig. 4A, it can be regarded that typical glucose transport through small-intestinal epithelial cells occurred in the glucose absorption experiment. In addition, Lf increased both the glucose concentration in the serosal side after incubation and the calculated amount of glucose absorption into the sac. It would be incorrect to conclude that Lf increased the expression of GLUT2 in small-intestinal epithelial cells, since Lf was intraperitoneally injected to rats only 1 hr before isolating the whole of the small intestine. However, the promotion of small-intestinal glucose absorption is not always associated with quantitative GLUT2 expression. One finding suggesting that Lf directly contributes to the enhancement of glucose transport into small-intestinal epithelial cells is that Lf alleviated the inhibition of glucose transport into absorptive enterocytes through SGLT1, by down-regulating the Ca^{2+} and cAMP signaling pathway (Talukder et al. 2014). Although the fact that glucose uptake into muscle and adipose tissue is through the enhancement of the

translocation of GLUT4 to the plasma membrane triggered by insulin stimulation (Czech and Corvera 1999; Bryant et al. 2002), previous research has suggested that whey protein hydrolysate increases the translocation of GLUT4 to the plasma membrane in muscle tissue independently of insulin (Morato et al. 2013). With the consideration that the function of GLUT2 in the small intestine does not require insulin action, these findings may suggest that Lf augments the frequency of GLUT2 translocation to the surface of the plasma membrane of the epithelial cell, promoting glucose transfer to blood circulation. A detailed analysis of this possibility will be needed in future works.

The glucose absorption experiment was performed using a part of jejunum, where L cells do not locate but K cells do. Given the total GIP-lowering effect of Lf, it seems quite possible that Lf caused the decrease in the amount of glucose within the jejunum space, leading to lower GIP secretory stimulation. It is probable that Lf does not simply influence the receptor responsiveness of both incretins, given the inverse results between GIP and GLP-1 in this study. Ikoma-Seki et al. (2015) recently proposed that Lf induces lipolysis associated with the activation of hormone-sensitive lipase and adenylate cyclase in adipose tissue. The former activation is caused by the promotion of the intracellular cAMP signaling pathway, the latter by the cAMP response element binding protein. As mentioned above, the enhancement of insulin secretion by the incretin effect also has a close relation to the cAMP

concentration, which varies by the activation of adenylate cyclase after incretin binds to its receptor. There remains the possibility that Lf has a similar effect on the signaling pathway in the process of the insulin secretory promotion by the incretin effect, and that the change of GIP level in the early stage of OGTT is virtually cancelled out by the insulin signaling activation and decreased GIP secretory stimulation.

In conclusion, it was found that Lf does contribute to the elevation of plasma insulin and the transient acceleration of GLP-1 secretion, possibly leading to the attenuation of hyperglycemia, even with the enhancement of glucose absorption in the small intestine.

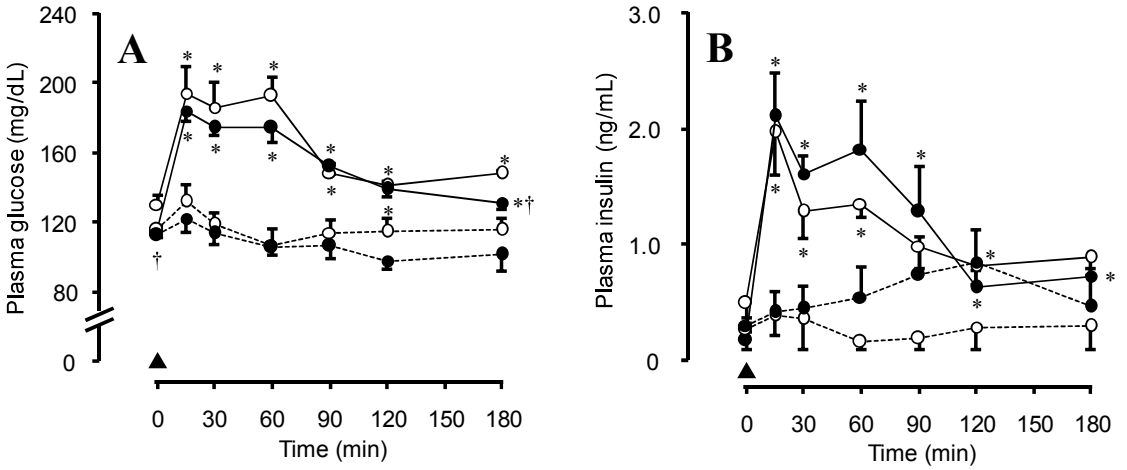

Fig. 1 Temporal change in plasma glucose and insulin following the oral administration of saline or glucose. The rats were intraperitoneally injected with saline (1 ml/kg) or bLf (100 mg/kg). 30 min after the injection, they were given an oral administration of saline (10 ml/kg) or glucose (2 g/kg). Each data point represents the mean ± SEM from 3 rats per group in case of oral saline administration, and 5-6 rats per group in case of oral glucose administration. (A) Changes in plasma glucose; (B) Changes in plasma insulin. Black triangle shows the time point of oral administration of saline or glucose. Dotted and Solid lines show the group of oral administration of saline and the group of oral administration of glucose, respectively. Open and filled circles show the group of intraperitoneal injection of saline and the group of intraperitoneal injection of bLf, respectively. *Significant difference from the value at 0 min in each group (based on a Univariate approach with a division model; $P < 0.05$). †Significant difference from the value at the same time point between the groups (unpaired Welch's *t*-test; $P < 0.05$).

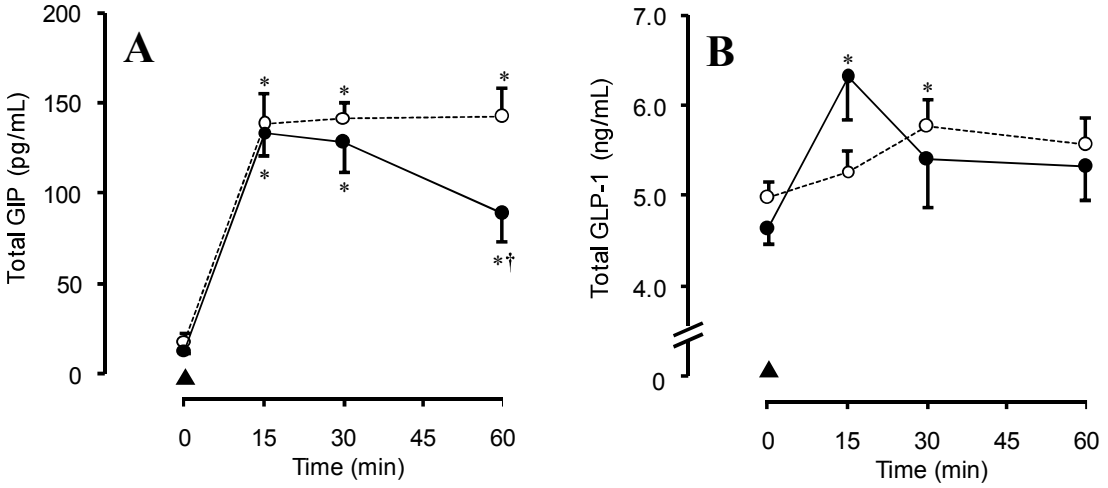

Fig. 2 Temporal change in total GIP and GLP-1 in plasma following the oral administration of glucose. The rats were intraperitoneally injected with saline (1 ml/kg) or bLf (100 mg/kg). 30 min after the injection, they were given an oral administration of glucose (2 g/kg). Each data point represents the mean ± SEM from 5 rats per group. (A) Changes in total GIP in plasma; (b) Changes in total GLP-1 in plasma. Black triangle shows the time point of oral administration of glucose (2 g/kg). Open and filled circles show the group of intraperitoneal injection of saline and the group of intraperitoneal injection of bLf, respectively. *Significant difference from the value at 0 min in each group (based on a Univariate approach with a division model; $P < 0.05$). †Significant difference from the value at the same time point between the groups (unpaired Welch's *t*-test; $P < 0.05$).

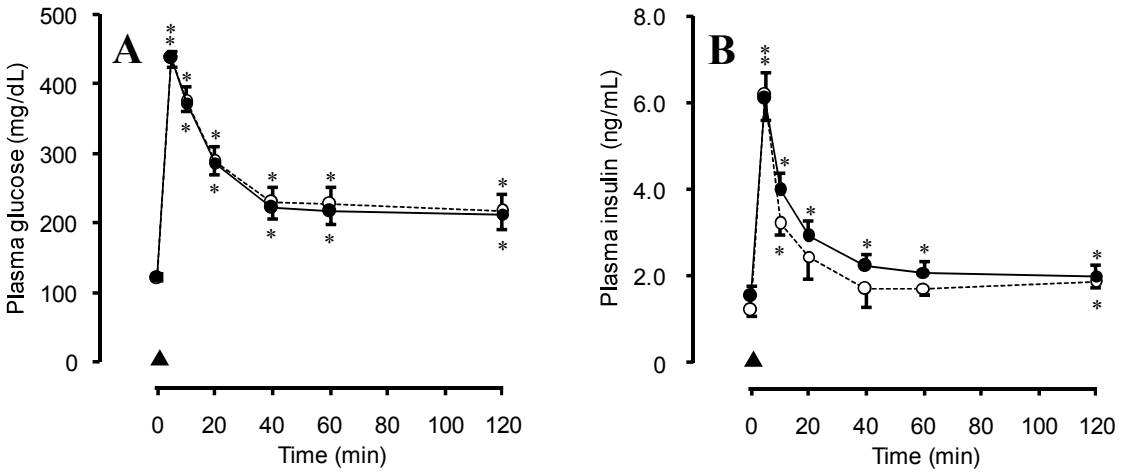

Fig. 3 Temporal change in plasma glucose and insulin following the intravenous injection of glucose. The rats were intraperitoneally injected with saline (1 ml/kg) or bLf (100 mg/kg). 30 min after the injection, they were given an intravenous administration of glucose (1 g/kg) into the caudal vein. Each data point represents the mean ± SEM from 5 rats per group. (A) Changes in plasma glucose; (B) Changes in plasma insulin. Black triangle shows the time point of intravenous administration of glucose (1 g/kg). Open and filled circles show the group of intraperitoneal injection of saline and the group of intraperitoneal injection of bLf, respectively. *Significant difference from the value at 0 min in each group (based on a Univariate approach with a division model; $P < 0.05$).

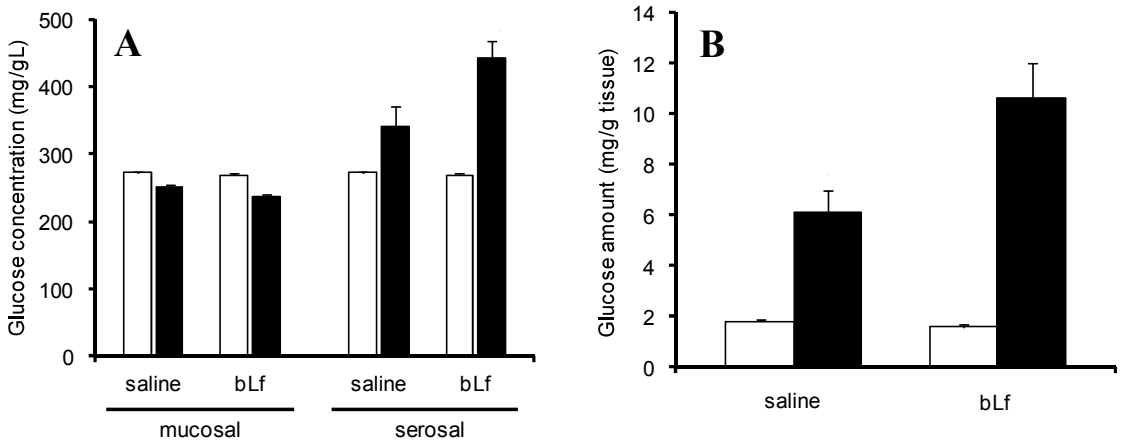

Fig. 4 Alteration in glucose absorption into an everted jejunum sac. The rats were intraperitoneally injected with saline (1 ml/kg) or bLf (100 mg/kg). 1 hr after the injection, they were sacrificed and the everted sacs were prepared. Each data point represents the mean ± SEM from 5 rats per group. (A) Glucose concentration outside (mucosal side) / inside (serosal side) the sac before and after the incubation. White and black squares show the glucose concentration before and after the incubation, respectively. (B) The amount of absorbed glucose into the sac per 1 g of jejunum tissue after the incubation. White and black squares show the amount of absorbed glucose before and after the incubation, respectively.

Chapter 2

Effect of lactoferrin on blood glucose regulation in rats under stress condition

Introduction

Normoglycemia is maintained by an interaction between insulin and catecholamines (norepinephrine and epinephrine), glucagon and glucocorticoids. The stress-induced hormones are known to increase endogenous level of blood glucose, accompanied by the enhancement of hepatic glucose output, glucose intolerance or insulin resistance, and with relation to the changes of glycogenolysis in fed condition and that of gluconeogenesis in fasted condition. Secretions in those hormones are conventionally adjusted depending on the feeding condition of the individual, while exposure to physical stressors also affects those secretions both acutely and chronically.

Restraint stress (RS) is a widely used method of the assessment of physical stress in rats. RS induces hyperglycemia accompanied by an increase in an adrenocorticotropic hormone, such as corticosterone (Giralt and Armario 1989; Tabata et al. 1998). Previous studies have shown that plasma glucose is not affected by the infusion of corticoid itself or by a subacutely repeated load of RS (24 hr) to rats, while acute RS (1 hr) induces hyperglycemia with increased plasma corticosterone (Torres et al. 2001). Therefore, it is believed that RS can be useful in evaluating the relationship between glucose homeostasis and the acute disorder in corticosterone-related pillar, namely the HPA axis. On the other hand, glucose endogenously synthesized by gluconeogenesis is known to account for >80 %

of the total glucose, even after 4 hr fasting in rodents, and strengthens the causal relationship between decreased levels in plasma glucose and corticosterone (Burgess et al. 2005; Kowalski and Bruce 2014).

Based on the finding in Chapter 1 that Lf facilitates potential glucose regulation accompanied by the enhancement of the incretin effect, the present study aims to examine whether treatment with Lf induces any changes in glucose regulation in rats under RS. In this chapter, OGTT was performed to assess the influence of Lf on the blood glucose and insulin kinetics in the RS load. Stress-induced hormones, such as plasma corticosterone, epinephrine and glucagon, were also measured to estimate the impact of RS on those parameters.

Materials and methods

Materials

BLf, and aprotinin from a bovine lung were purchased from Wako Chemical Co., Ltd.. Isoflurane was purchased from Intervet Inc.. The titanium vascular access port (SWIRL-MIN), dedicated 25-gauge needle and 3-Fr polyurethane catheter were purchased from Primetech Co.. Urethane was purchased from Sigma-Aldrich. A tube containing EDTA-2K was purchased from FUJIFILM Medical Co., Ltd.. A dedicated wire restraint cage (KN-468) was purchased from Natsume Seisakusho Co., Ltd. (Tokyo, Japan).

Animals

All experimental protocols in this study were approved by the Animal Research Committee of Tottori University (approval numbers: 16-T-6). Male Wistar rats were obtained at 7 weeks of age from the Institute for Animal Reproduction. The rats were acclimatized to their surroundings for at least one week before the experiments. The animal room was controlled with a 12/12 hr light/dark cycle (lights on 7:00-19:00) at an ambient temperature of 24 ± 1 °C. The rats were housed in plastic cages, with at most three rats per cage, and were allowed free access to water and standard chow (CE-2). All experiments were conducted after overnight (16 hr) fasting.

Surgical operation for OGTT

Prior to OGTT, rats were fully anesthetized with 2–2.5% isoflurane gas, and then implanted with a vascular access port on their dorsal region, as previously described (Higuchi et al. 2013). Subsequently, a 3-Fr polyurethane catheter connected to the titanium vascular access port was inserted into the exposed right external jugular vein. The catheter was prefilled and kept locked with heparin-added physiological saline by flushing the solution twice a day. OGTT was performed after 3-days of recovery from the operation.

OGTT

The rats that had been operated upon were divided into four groups: group 1 received 10 m*l*/kg of saline orally with saline at a dose of 1 m*l*/kg intraperitoneally; group 2 received 10 m*l*/kg of saline orally with bLf at 100 mg/kg intraperitoneally; group 3 received 2 g/kg of glucose orally with saline at 1 m*l*/kg intraperitoneally; and group 4 received 2 g/kg of glucose orally with bLf at 100 mg/kg intraperitoneally. With oral saline administration, the effect of bLf on the endogenous blood glucose change in the stressed condition and the impact of the RS itself on glycemic change were assessed. The rats received the oral administration at 30 min after the intraperitoneal injection, and then RS was applied for 60

min. RS was applied to the rats by getting them into a dedicated wire restraint cage with reference to the method of Hirata *et al.* [10]. Blood samples were collected before the intraperitoneal injection (-30), before the oral administration (0), and at 30, 60, 90, 120, and 180 min after the oral saline administration. The volume of blood collected at each time point was 0.1 ml in groups 1 and 2, while in groups 3 and 4, it was 0.5 ml at 0, 30, and 60 min; and 20 μl at -30, 90, 120 and 180 min. Twenty microliters of each obtained blood sample was used to measure plasma glucose just after the blood collection. The remainder of the blood, collected at 0, 30, 60 min, was transferred into an EDTA-containing tube with aprotinin (final concentration, 500 KIU/mL), and centrifuged (5,600 × g, 5 min, 4 ℃). All samples were stored at -80 ℃ until measurement for levels of insulin and corticosterone in groups 1 and 2; insulin, corticosterone, epinephrine and glucagon in groups 3 and 4, respectively.

Analysis of biochemical parameters

Plasma glucose was measured by a portable electrode-type blood glucose meter (ANTSENSE III). Plasma insulin was determined by the enzyme immunoassay method using an ELISA kit (AKRIN-010T). The corticosterone level in plasma was measured using an ELISA kit (ENC-ERKR7004; Endocrine Technologies Inc., Newark, CA, USA), and

epinephrine was measured with an ELISA kit (BA E-5100; LDN, Nordhorn, Germany). The glucagon level in plasma was measured using a competitive EIA kit (MK157; Takara Bio Inc., Shiga, Japan).

Statistical analysis

Data are represented as means ± SEM. The differences between the bLf-treated group and the control group were examined using Welch's *t*-test (GraphPad Prism version 6.0 for Windows), and the score variation within a group was compared by a univariate approach with a division model (JMP). Statistical significance was accepted at a probability (P) < 0.05.

Results

Changes in plasma glucose and insulin following the oral administration of saline or glucose, and RS load

As shown in Fig. 5A, plasma glucose varied in the range of about 30 mg/dL in groups 1 and 2. Significant increases in plasma glucose were observed at 30, 60 and 90 min compared with the level at the time of oral administration (0 min) in group 1, while the level in group 2 tended to show a gradual increase in the same comparison. In both groups, plasma glucose was not significantly changed at 0 min from the level at the time of intraperitoneal administration (-30 min). The area under the curve (AUC) from 0 to 180 min was almost the same in groups 1 and 2 (22,341 ± 545 mg/dL × 3 hr in group 1 and 22,734 ± 1315 mg/dL × 3 hr in group 2).

As for plasma insulin, in both groups, no significant change from basal level was observed at each time point during RS period (Fig. 5B). There were also no significant differences in the levels between the two groups. The AUC from 0 to 60 min was nearly the same in the two groups (37.9 ± 9.6 ng/mL × 1 hr in group 1 and 33.4 ± 5.7 ng/mL × 1 hr in group 2).

In OGTT, plasma glucose entirely maintained a higher transition in group 3, compared with that in group 4 (Fig. 5A). The peak level of plasma glucose was observed immediately

after the oral glucose administration in group 3 (30 min), and the level was sustained for at least 30 min. Meanwhile, a gradual increase in plasma glucose to its peak at 60 min was seen in group 4. Group 4 also exhibited significant decreases in plasma glucose at 0, 30, and 180 min relative to each level in group 3. The AUC was also significantly lower in group 4 than in group 3 (33,660 ± 935 mg/dL × 3 hr in group 3 and 27,798 ± 423 mg/dL × 3 hr in group 4).

Groups 3 and 4 displayed a peak of plasma insulin at 30 min and significant increases at 30 and 60 min after the oral glucose administration (Fig. 5B). Meanwhile, there were no significant differences in the level of plasma insulin at each time point, and the AUCs for insulin were nearly the same in the two groups (101.9 ± 9.0 ng/mL × 1 hr in group 3 and 101.1 ± 11.9 ng/mL × 1 hr in group 4).

Changes in plasma corticosterone following the oral administration of saline or glucose, and RS load

Significant increases in plasma corticosterone were seen at 30 and 60 min after the RS load within group 1, while there were no changes in the level within group 2 (Fig. 6). In addition, group 2 showed a significant decrease in the level at 30 min, and tended to lower the levels at 0 and 60 min. The AUC was significantly lowered in group 2 (10,937 ± 977

ng/mL × 1 hr in group 1 and 7,758 ± 673 ng/mL × 1 hr in group 2).

In OGTT, the increment of plasma corticosterone was seen immediately after the RS load in group 3, and the level appeared to be sustained from the peak at 30 min to 60 min at least (Fig. 6). On the other hand, a mild increase in and the sustaining of the plasma corticosterone level were observed within group 4 after the RS load, and significant reductions in the level were found at 30 and 60 min. The AUC for plasma corticosterone was also significantly lower in group 4 than in group 3 (12,763 ± 755 ng/mL × 1 hr in group 3 and 9,259 ± 365 ng/mL × 1 hr in group 4).

Changes in plasma epinephrine and glucagon following the oral administration of glucose, and RS load

Epinephrine in plasma tended to increase within 30 min after the RS load in group 3, whereas the level in group 4 showed a mild increment at the same time point (Fig. 7A). However, no significant difference was found in the AUC for epinephrine between the two groups (40,711 ± 10,148 pg/mL × 1 hr in group 3 and 36,652 ± 3,142 pg/mL × 1 hr in group 4). The results in Fig. 7B showed that plasma glucagon remained at about the baseline level during the RS load within groups 3 and 4. The overall transition of the level was also very similar between the two groups, and there were no significant differences in the levels or the

AUCs for glucagon between the groups (5,418 ± 503 pg/mL × 1 hr in group 3 and 5,143 ± 624 pg/mL × 1 hr in group 4).

Discussion

In the present study, the RS load with oral saline administration resulted in the increment of plasma glucose within the group receiving an intraperitoneal saline injection. This result was in accord with previously reported studies, which showed the hyperglycemia induced by 30-60 min of acute RS (Torres et al. 2001; Romeo et al. 2007). Contrary to the increase in those levels found within the intraperitoneal-saline-injected group, no significant changes were observed in plasma glucose within the intraperitoneal-bLf-injected group. These differences were temporally consistent with the changes in plasma corticosterone, while plasma insulin was not affected by RS load. These finding may suggest that Lf has the potential to attenuate the hyperglycemic responses brought about by the increase of corticosterone secretion following the RS. It can also be concluded that the RS load acted as a sufficient stressor to elicit hyperglycemia even in OGTT.

In OGTT, an initial rise in plasma glucose was found at 30-60 min after the oral glucose administration. This phase was overlapped by the RS period, and plasma corticosterone was significantly elevated in response to RS. It has been reported that acute RS (30 min) causes hyperglycemia without affecting plasma insulin in rats (Romeo et al. 2007), indicating that the RS load adopted in the present study could also have no impact on

endogenous insulin secretion. The data obtained in OGTT revealed no changes in plasma glucagon following the RS load, while a slight increase in plasma epinephrine was found at 30 min after the RS load was started only in the case of intraperitoneal bLf injection. Although the latter result does not negate the possibility that bLf enhanced epinephrine secretion, the largely lowered corticosterone and plasma glucose levels during RS are assumed to indicate a lower contribution to hyperglycemia by epinephrine. However, these results appeared not to be consistent with the previous research, which showed that acute immobilization (60 min) causes hyperglycemia accompanied by increases in the secretion of all of the three stress-induced hormones in rats under the fed condition (Yamada et al. 1993). The apparent contradiction in the secretary status of stress-induced hormones is considered to be brought about by the difference in stress severity between RS and immobilization. In addition, these facts lead to the possibility that the increment in plasma glucose, which was observed in the early stage in OGTT, was mainly associated with the actions yielded by increased corticosterone.

The obtained results suggest that Lf suppresses the increment of plasma glucose that occurs as a result of the combination of oral glucose administration and acute RS load. Although the suppressive change in plasma glucose was consistent with the decrease in plasma corticosterone, Lf did not induce such changes in plasma epinephrine and glucagon

during RS. These findings indicate that Lf may be involved in the hypoglycemic responses that occur under stress conditions, which can be attributed to the attenuated activation of the HPA axis rather than to that of the sympathetic nervous system. Southorn et al. (1990) have shown that insulin resistance caused by increased corticosterone is largely related to decreased insulin sensitivity. This finding is considered to support the result that Lf showed a hypoglycemic effect without affecting insulin secretion, and may suggest that Lf affects the improvement of corticosterone-related insulin resistance.

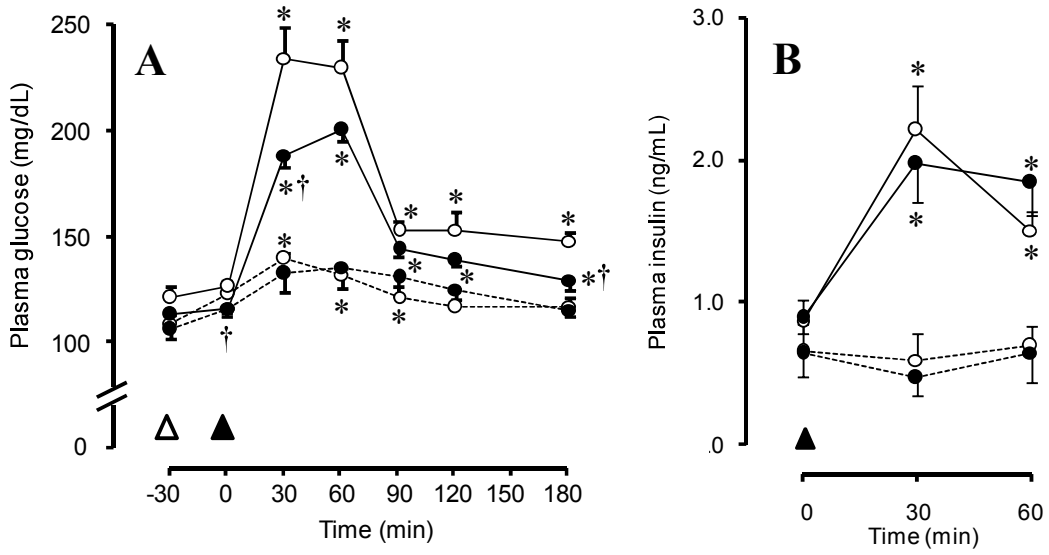

Fig. 5 Temporal change in plasma glucose and insulin following the oral administration of saline or glucose, and RS. The rats were intraperitoneally injected with saline (1 ml/kg) or bLf (100 mg/kg). 30 min after the injection, they were given an oral administration of saline (10 ml/kg) or glucose (2 g/kg), and then RS was applied for 60 min. Each data point represents the mean ± SEM from 5 rats per group. (A) Changes in plasma glucose; (B) Changes in plasma insulin. White triangle shows the time point of intraperitoneal injection of saline or bLf. Black triangle shows the time point of oral administration of saline or glucose. Dotted and Solid lines show the group of oral administration of saline and the group of oral administration of glucose, respectively. Open and filled circles show the group of intraperitoneal injection of saline and the group of intraperitoneal injection of bLf, respectively. *Significant difference from the value at 0 min in each group (based on a Univariate approach with a division model; $P < 0.05$). †Significant difference from the value at the same time point between the groups (unpaired Welch's *t*-test; $P < 0.05$).

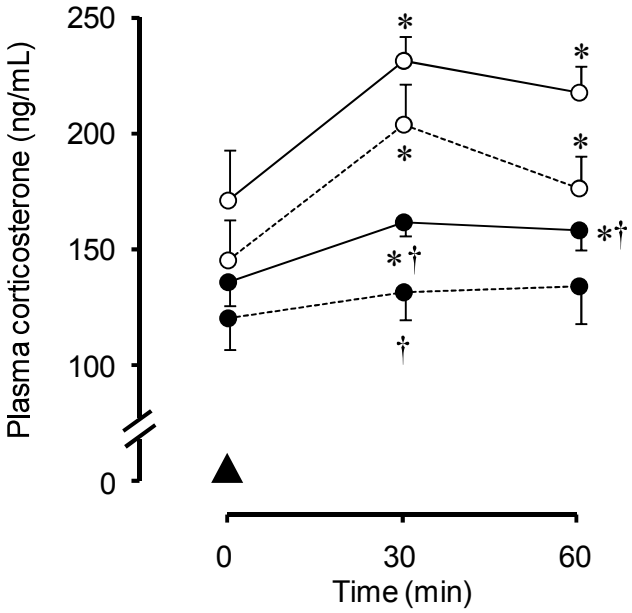

Fig. 6 Temporal change in plasma corticosterone following the oral administration of saline or glucose, and RS. The rats were intraperitoneally injected with saline (1 ml/kg) or bLf (100 mg/kg). 30 min after the injection, they were given an oral administration of saline (10 ml/kg) or glucose (2 g/kg), and then RS was applied for 60 min. Each data point represents the mean ± SEM from 5 rats per group. Black triangle shows the time point of oral administration of saline or glucose. Dotted and Solid lines show the group of oral administration of saline and the group of oral administration of glucose, respectively. Open and filled circles show the group of intraperitoneal injection of saline and the group of intraperitoneal injection of bLf, respectively. *Significant difference from the value at 0 min in each group (based on a Univariate approach with a division model; $P < 0.05$). †Significant difference from the value at the same time point between the groups (unpaired Welch's *t*-test; $P < 0.05$).

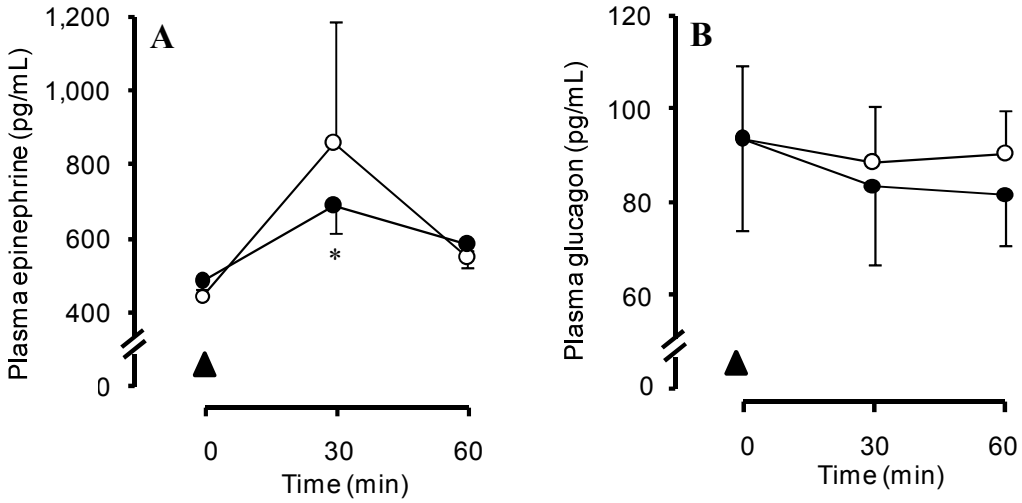

Fig. 7 Temporal change in plasma glucose, insulin and corticosterone following the oral administration of glucose. Each data point represents the mean ± SEM from 5 rats per group. The rats were intraperitoneally injected with saline (1 ml/kg) or bLf (100 mg/kg). 30 min after the injection, they received the oral administration of glucose (2 g/kg), and then RS was applied for 60 min. (A) Changes in plasma glucose; (B) Changes in plasma insulin; (C) Changes in plasma corticoste- rone. White triangle shows the time point of the intraperitoneal injection of saline or bLf. Black triangle shows the time point of the oral administration of glucose. Open and black-filled circles show the group of intraperitoneal injection of saline and the group of intraperitoneal injection of bLf, respectively. *Significant difference from the value at 0 min in each group (based on a univariate approach with a division model; $P < 0.05$).

General Discussion

LF is a glycoprotein found in various mammalian body fluids, and especially abundant in milk. Evidences of the physiological multifunctionality of Lf have been accumulated, as represented by biophylactic responses such as analgesia or anti-stress effect, and the recent interests have been focused on its involvement in metabolic reaction. However, the influence of Lf on blood glucose regulation still remains an open research problem. A major goal of this study has been to examine whether Lf affects glucose mechabolism in normal and stress condition. The effect of Lf on comprehensive glucose kinetics was investigated by performing OGTT and IVGTT, and that on small-intestinal glucose absorption was estimated by an in vitro study using the everted sac method. On the other hand, the involvement of Lf in the stress-induced hyperglycemia was assessed by conducting the OGTT, which is combined with RS.

I concluded that Lf acts as a temporal enhancer of GLP-1 secretion in L cell, accompanied by its exertion of the promotion of insulin secretion in normal condition. However, in the stress condition, the Lf treatment did never show any changes in insulin secretion in the RS period. Lf is known to promote the translocation of GLUT4 onto plasma membrane in muscle or adipose tissues independently on insulin action. From the

observation that the hypoglycemic action by Lf was thought not to be accompanied by the requirement for the enhancement of insulin secretion, the above action, which related to the promotion of glucose uptake into peripheral tissues, may resulted in the lower transition of plasma glucose in the stress condition. Furthermore, those results are considered to indicate that RS cancelled out the enhancement of GLP-1 secretion brought about by Lf. The central role of GLP-1 in stress condition has been focused on in previous studies, as yet known that both HPA axis and sympathetic nervous system are primed with the activation of central GLP-1 receptor (Holt and Trapp 2016). In addition, the other study has revealed the possibility of stress to enhance the physiological action of GLP-1; namely that GLP-1 neuron can be activated by a treatment with lithium chloride, lipopolysaccharide and cholecystokinin (Rinaman 1999). These findings are highly suggestive to explain that adaptive hypothalamic stress responses themselves results in hyperglycemic changes. On the other hand, restraint stress also affects the absorption of nutrients associated by changes in intestinal motility (Tsukada et al. 2002). In fact, glucocorticoids cause a decreased translocation of the intestinal GLUT2 and glucose absorption (Shepherd et al. 2004). As mentioned above, the secretion of incretin is followed by the nutrients stimulation on the specific cells, K cell and L cell, in the intestinal lumen. Based on the mechanism of GLP-1 secretion, one reason for no changes in insulin secretion in the stress condition can be lower

stimulation in L cell caused by the disorder of intestinal morphology, while the RS load might lead to the activation of GLP-1 receptor.

Conclusion

Lf has a potential to suppress hyperglycemia, accompanied by plasma insulin elevation via transiently accelerating GLP-1 secretion in normal condition, and that Lf even enhances glucose absorption from the small intestine. In stress condition, Lf suppresses the elevation of plasma glucose possibly accompanied by mediating HPA axis, not affecting SAM system or gluconeogenesis. These findings suggest that treatment by Lf might be useful as one method of decent glycemic control.

Summary

Firstly, the investigation whether lactoferrin (Lf) affects glycemic regulation of rats in normal condition was conducted. Focusing on the anti-stress effects of Lf, the influence of Lf on the glycemic changes, which was brought about by restraint stress (RS), was also evaluated. From carrying out the two experiments, I had the results as follows;

1. In normal condition, bovine Lf (bLf) pretreatment (100 mg/kg) had no significant effect on plasma glucose or insulin after the intravenous injection of glucose. On the other hand, in case of the oral glucose administration (oral glucose tolerance test, OGTT), the bLf treatment tended to show lower plasma glucose at and after the 15-min peak than the control group, and decreased at 180 min. In addition, the change in plasma insulin from 0 to 30 min was higher in the bLf group than in the control group.

2. In normal condition, total plasma glucose-dependent insulinotropic polypeptide (GIP) was lowered at 60 min by the bLf treatment, while an immediate increase in total plasma glucagon-like peptide-1 (GLP-1) was observed within the bLf group undergoing the OGTT.

3. The bLf pretreatment was associated with an increase in the amount of glucose

absorbed into the everted jejunum sac.

4. RS significantly raised plasma glucose, but the bLf pretreatment did not affect the level.

In the stress condition, plasma glucose showed an overall lower transition in the bLf group in OGTT, and the levels at 30 and 180 min or the area under the curve (AUC) were significantly decreased. Although the bLf treatment suppressed an increase in plasma corticosterone during RS, the levels of plasma insulin, epinephrine and glucagon were not changed by the bLf treatment.

Acknowledgement

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