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NOTE

Physiology

Lactoferrin ameliorates corticosterone-related acute stress and hyperglycemia in rats

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ABSTRACT. We aimed to assess the effects of lactoferrin (Lf) on glycemic regulatory responses under restraint stress (RS) in rats. Bovine Lf (bLf, 100 mg/kg) was intraperitoneally administered to rats before oral saline administration or oral glucose tolerance test (OGTT) following 60 min of RS load. In the case of oral saline administration, RS significantly raised plasma glucose, but bLf did not affect the level. Plasma glucose in OGTT showed an overall lower transition in the bLf group, and the levels at 30 and 180 min or the area under the curve (AUC) were significantly decreased. Although bLf suppressed an increase in plasma corticosterone during RS, the levels of plasma insulin, epinephrine and glucagon were not changed by the bLf treatment.

KEY WORDS: glycemic control, lactoferrin, restraint stress, stress-induced hormone

Normoglycemia is maintained by an interaction between insulin and catecholamines (norepinephrine and epinephrine), glucagon and glucocorticoids. 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 [6, 12]. 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 [7]. Glucocorticoids are also known to promote glucose intolerance, with its effect in opposition to insulin action [1, 15]. 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 [8, 20]. 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 [22]. Therefore, it is believed that acute RS can be useful in evaluating the relationship between glucose homeostasis and the hypothalamic-pituitary-adrenal axis (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 [4, 14].

Lactoferrin (Lf) is an iron-binding glycoprotein found in milk and body fluids in mammals; it is characterized by its multifunctionality, namely anti-bacterial, anti-inflammatory or anti-cancer effects [2, 11, 24]. Recent studies have shown a close relationship between Lf and stress as follows: Lf exerts its anxiolytic and analgesic effects accompanied by an increase in nitric oxide production or activation of the μ-opioid system, and immobilization-stress-induced modification of the immune response is normalized by Lf via its anti-inflammatory effect or cytokine regulatory action [13, 21, 23, 26]. Furthermore, the direct relevance of Lf for diabetes mellitus has been indicated in a previous study: namely, the Lf concentration in blood is positively correlated to insulin sensitivity and negatively to blood glucose levels in humans with altered glucose tolerance [17]. Although those findings are indirectly suggestive of the effects of Lf on physical-stress-induced disorder in homeostasis, possibly including glucose homeostasis, the details remain largely unknown.

The present study aims to examine whether treatment with bovine Lf (bLf) induces any changes in blood glucose regulation in rats under RS. An oral glucose tolerance test (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.

Male Wistar rats were obtained at 7 weeks of age from the Institute for Animal Reproduction (Kasumigaura, Japan). The rats were acclimatized to their surroundings for at least one week before the experiments. The animal room was controlled with a 12/12

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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; Nihon Clea, Tokyo, Japan). The standard chow contains 24.9% protein, 4.6% fat, 4.5% fiber, 6.6% ash and 51.0% nitrogen free extract, and consists of 3.45 kcal/g. All experiments were conducted after overnight (16 hr) fasting. The experimental protocols in this study were approved by the Animal Research Committee of Tottori University (approval numbers: 16-T-6).

Prior to each experiment, the rats were implanted with a vascular access port on their dorsal region under full anesthesia with 2–2.5% isoflurane gas (Intervet Inc., Tokyo, Japan), as previously described [9]. Subsequently, a 3-Fr polyurethane catheter, connected to the titanium vascular access port (Primetech Co., Nagoya, Japan), was inserted into the exposed right external jugular vein. These approaches enabled us to obtain large volume of blood with less hemolysis. After the operation, all rats were separately housed and were allowed to recover from the operation for three days. The catheter was prefilled and kept locked with heparin-added physiological saline by flushing the solution twice a day.

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 (Wako Chemical Co., Ltd.) 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 (KN-468, Natsume Seisakusho Co., Ltd., Tokyo, Japan) 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 administration. The volume of blood collected at each time point was 0.1 m*l* in groups 1 and 2, while in groups 3 and 4, it was 0.5 m*l* 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 and 60 min, was transferred into an ethylenediaminetetraacetic-acid (EDTA)-containing tube (FUJIFILM Medical Co., Ltd., Tokyo, Japan) with aprotinin (final concentration, 500 KIU/m*l*, Wako Chemical Co., Ltd.) and centrifuged (5,600 ×g, 5 min and 4°C). All plasma samples were stored at −80°C until measurement for levels of insulin and corticosterone in groups 1 and 2; insulin, corticosterone, epinephrine and glucagon in groups 3 and 4, respectively.

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., Shibukawa, Japan). The corticosterone level in plasma was measured using an ELISA kit (ENC-ERKR7004; Endocrine Technologies Inc., Newark, CA, U.S.A.), 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., Otsu, Japan).

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; GraphPad Software Inc., La Jolla, CA, U.S.A.), and the score variation within a group was compared by a univariate approach with a division model (JMP; SAS Institute Inc., Cary, NC, U.S.A.). Statistical significance was accepted at a probability (P)<0.05.

We first examined the effects of bLf administration and RS on endogenous change in blood glucose, insulin and corticosterone. As shown in Fig. 1A, plasma glucose varied in the range of about 30 mg/d*l* 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/d*l* ×3 hr in group 1 and 22,734 ± 1,315 mg/d*l* ×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.1B). 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*/m*l* ×1 hr in group 1 and 33.4 ± 5.7 *ng*/m*l* ×1 hr in group 2).

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.1C). 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*/m*l* ×1 hr in group 1 and 7,758 ± 673 *ng*/m*l* ×1 hr in group 2).

The results of the changes in plasma glucose, insulin and stress-related hormones in OGTT are shown in Fig. 2. Plasma glucose entirely maintained a higher transition in group 3, compared with that in group 4 in OGTT (Fig. 2A). 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/d*l* ×3 hr in group 3 and 27,798 ± 423 mg/d*l* ×3 hr in group 4).

As can be seen from Fig. 2B, 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. 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*/m*l* ×1 hr in group 3 and 101.1 ± 11.9 *ng*/m*l* ×1 hr in group 4).

The increment of plasma corticosterone was seen immediately after the RS load in group 3, and the level appeared to be

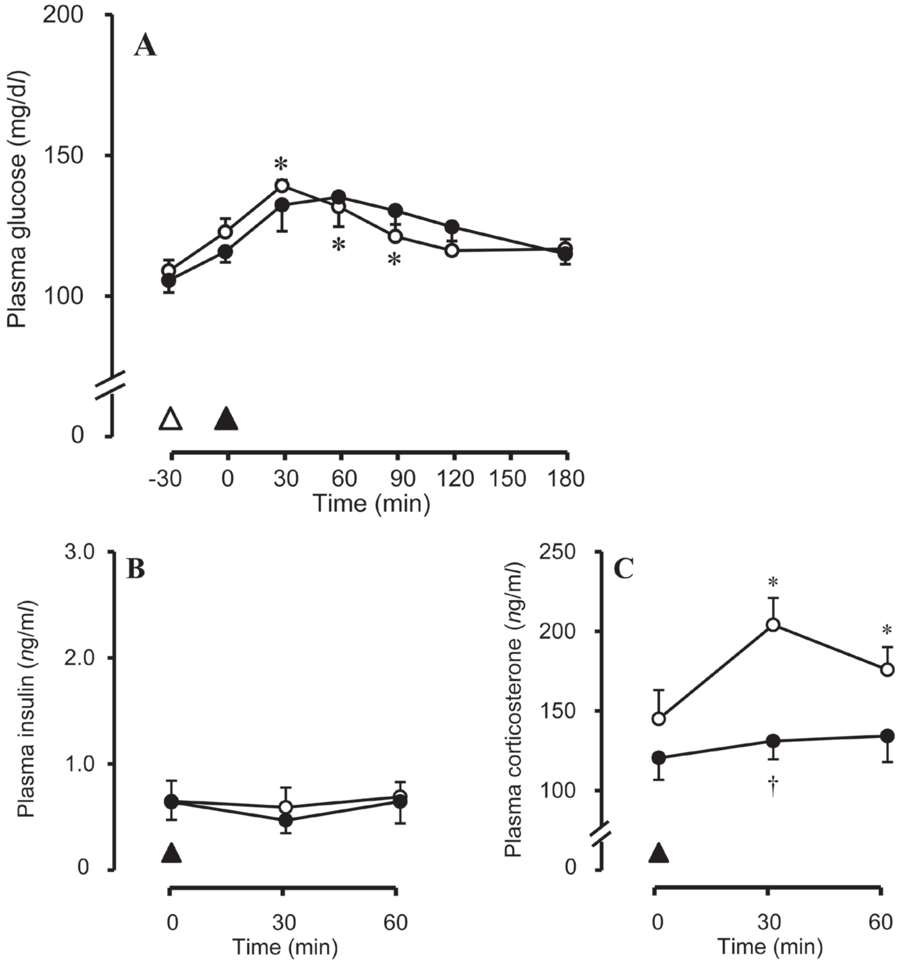

Fig. 1. Temporal change in plasma glucose, insulin and corticosterone following the oral administration of saline. Each data point represents the mean ± SEM from 5 rats per group. The rats were intraperitoneally injected with saline (1 m*l*/kg) or bLf (100 mg/kg). 30 min after the injection, they received the oral administration of saline (10 m*l*/kg), and then, RS was applied for 60 min. (A) Changes in plasma glucose; (B) Changes in plasma insulin; (C) Changes in plasma corticosterone. 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 saline. 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). †Significant difference from the value at the same time point between the groups (unpaired Welch's t-test; P<0.05).

sustained from the peak at 30 min to 60 min at least (Fig. 2C). 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 *n*g/m*l* ×1 hr in group 3 and 9,259 ± 365 *n*g/m*l* ×1 hr in group 4).

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. 2D). However, no significant difference was found in the AUC for epinephrine between the two groups (40,711 ± 10,148 *pg*/m*l* ×1 hr in group 3 and 36,652 ± 3,142 *pg*/m*l* ×1 hr in group 4). The results in Fig. 2E 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*/m*l* ×1 hr in group 3 and 5,143 ± 624 *pg*/m*l* ×1 hr in group 4).

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 [18, 22]. 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 findings 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

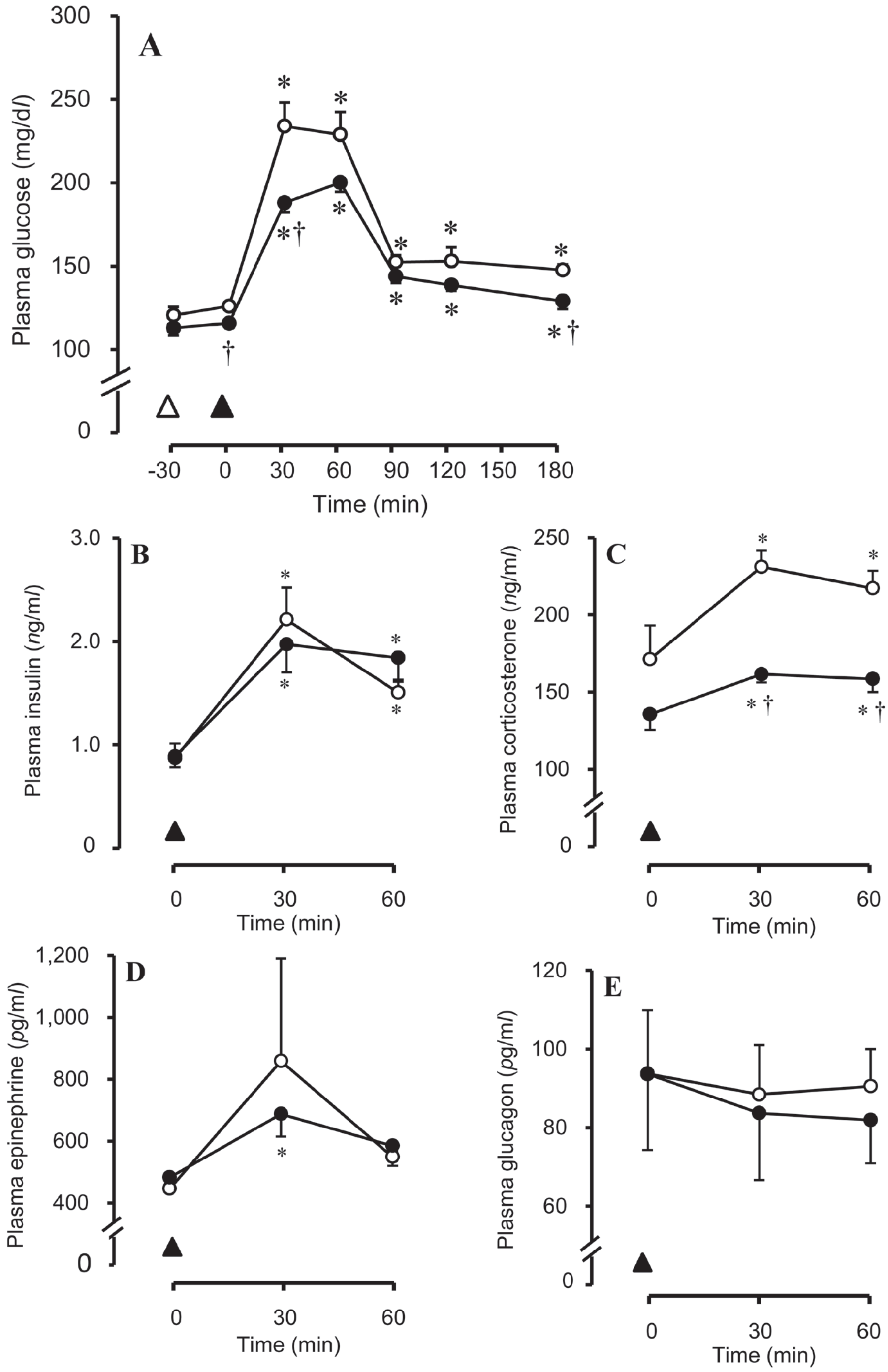

Fig. 2. Temporal changes in plasma glucose, insulin and stress-induced hormones following the oral administration of glucose. Each data point represents the mean ± SEM from 5–6 rats per group. The rats were intraperitoneally injected with saline (1 m*l*/kg) or bLf (100 mg/kg). 30 min after the injection, 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 corticosterone; (D) Changes in plasma epinephrine; (E) Changes in plasma glucagon. 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; $P<0.05$). †Significant difference from the value at the same time point between the groups (unpaired Welch's *t*-test; $P<0.05$).

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 [18], 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 [25]. 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.

Southorn *et al*. [19] 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 the Lf affects the improvement of insulin resistance. On the other hand, it is well-known that insulin promotes glucose uptake into muscle and fat tissue through the enhancement of the translocation of the insulin-responsive glucose transporter (GLUT4) to the plasma membrane [3, 5]. Although the possibility that Lf contributes to this mechanism could be raised, a recent study showed that whey protein-stimulated the translocation of GLUT4 to the plasma membrane in muscle tissue independently of insulin [16]. This suggests another possibility for the mode of hypoglycemic action by Lf. While Lf itself has a unique influence on the movement of GLUT4, the detailed mechanism remains to be determined in future studies.

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.

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