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FULL PAPER

Changes in blood glucose and insulin responses to intravenous glucose tolerance tests and blood biochemical values in adult female Japanese black bears (*Ursus thibetanus japonicus*)

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Abstract

The metabolic mechanisms to circannual changes in body mass of bears have yet to be elucidated. We hypothesized that the Japanese black bear (*Ursus thibetanus japonicus*) has a metabolic mechanism that efficiently converts carbohydrates into body fat by altering insulin sensitivity during the hyperphagic stage before hibernation. To test this hypothesis, we investigated the changes in blood biochemical values and glucose and insulin responses to intravenous glucose tolerance tests (IVGTT) during the active season (August, early and late November). Four, adult, female bears (5–17 years old) were anesthetized with 6 mg/kg TZ (tiletamine HCl and zolazepam HCl) in combination with 0.1 mg/kg acepromazine maleate. The bears were injected intravenously with glucose (0.5 g/kg of body mass), and blood samples were obtained before, at, and intermittently after glucose injection. The basal triglycerides concentration decreased significantly with increase in body mass from August to November. Basal levels of plasma glucose and serum insulin concentrations were not significantly different among groups. The results of IVGTT demonstrated the increased peripheral insulin sensitivity and glucose tolerance in early November. In contrast, peripheral insulin resistance was indicated by the exaggerated insulin response in late November. Our findings suggest that bears shift their glucose and lipid metabolism from the stage of normal activity to the hyperphagic stage in which they show lipogenic-predominant metabolism and accelerate glucose uptake by increasing the peripheral insulin sensitivity.

Key words: bear, body fat, insulin, intravenous glucose tolerance test, metabolism

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Introduction

The Japanese black bear (*Ursus thibetanus japonicus*), a subspecies of the Asiatic black bear, undergoes annual cycles in body mass, food consumption, and reproduction. The annual cycle of body mass is characterized by an increase, mainly in adipose tissue, due to hyperphagia[15], in autumn, and a decrease in winter due to reliance on fat stores during hibernation[16]. Pregnant bears give birth and nurse cubs during hibernation[9,26], and the reproductive rates and the cub growth and survival rates are influenced by a sow's nutritional condition, which is correlated with food availability during autumn[6,7,19]. Previous studies suggest that sufficient body fat accumulation prior to hibernation is the key factor deciding the success of reproduction in bears. Food resources for Japanese black bears before hibernation are mainly nuts and fruits, which contain large amounts of carbohydrates, and it is speculated that bears convert these carbohydrates into body fat. However, the metabolic mechanisms involved in the regulation of body mass or body fat have yet to be elucidated.

The distinct seasonal accumulation of body fat without adverse health effects in hibernators makes them ideal models for studying body mass regulation and obesity. Previous studies have suggested that body mass gain is not simply a consequence of increased food intake, and that various factors, such as metabolic enzymes, hormones, and metabolic rate, are related to the seasonal change of energy balance and fat metabolism[5]. Basal insulin concentration, for example, has been reported to correlate with body mass in fat-storing hibernators[2,18,22]. In marmots, glucose uptake in peripheral tissues was significantly higher in summer (the period of rapid mass gain) compared to spring, autumn, and winter, and both hyperinsulinemia and peripheral insulin resistance were observed in autumn when the body mass peaks and animals cease to feed[22]. These results suggest that glucose metabolism, controlled by insulin, changes in relation to the change in body mass (body fat) in hibernators.

In this study, we focused on the fat-storing mechanism in the active season, especially during the hyperphagic stage, and hypothesized that bears efficiently convert carbohydrates into body fat by altering insulin sensitivity during this period. In order to test this hypothesis, we selected the intravenous glucose tolerance test (IVGTT) as a method to evaluate insulin action on glucose metabolism. We investigated the changes in blood biochemical values, glucose and insulin in response to IVGTT during the active season.

Materials and Methods

Animals: Four adult, non-pregnant, female Japanese black bears, between 5 and 17 years old, kept in Ani Mataginosato Bear Park, Akita Prefecture, Japan (N40°, E140.4°) were used in this study. In the bear park, all bears were provided approximately 2 kg of dried corn per day with some fruits, vegetables, and commercial bear pellets as supplements, and water was provided *ad libitum* during the active season from late April to November. The bears hibernated in the indoor rooms without feeding (water was available) from December to the following mid-April. The study was conducted in August and November 2010. Based on the definition of physiological stages by Nelson *et al*.[16], we defined August as the stage of normal activity, and November as the hyperphagic stage. The experiments were performed 3 times in August, early November, and late November.

This study was conducted in accordance with the guidelines of the Animal Care and Use Committee of Hokkaido University.

Induction and maintenance of anesthesia: The experiment was always performed indoors during the morning, and bears were not fed after

16:00 of the previous day. For induction of anesthesia, all anesthetic agents were administered intramuscularly by blow darts (2.5 ml dart syringe with 1.2 × 38 mm needle). We prepared a mixture of tiletamine HCl and zolazepam HCl (TZ; Zoletil® 100, Virbac, Carrors, France) at 300 mg/ml using 5 ml diluent to reconstitute the powdered TZ. The combination of 6 mg/kg TZ and 0.1 mg/kg acepromazine maleate (A.C.P. 10 Injection 100 ml, Delvet Pty Ltd., Australia, 10 mg/ml) was used for the immobilization of bears in this study. The dose of TZ in combination with acepromazine was based on results of our previous experiments[12]. Based on estimated body mass, we first administered acepromazine maleate, and TZ was given 30 min after the acepromazine administration. When the effect of TZ was insufficient, additional doses of TZ, approximately 1/3 of the original dose, were given. After immobilization, the bears were weighed and handled. Catheters (SurFlash® I.V. Catheters, TERUMO Co., Tokyo, Japan) were inserted into both external jugular veins: one for the infusion of anesthetic and glucose injection, and the other for blood sampling. The bears were kept immobilized throughout the experiment by continuous intravenous infusion of TZ dissolved in 500 mL of acetated Ringer's solution (SOLACET F, TERUMO Co., Tokyo, Japan) at a dose range of 0.3–2.2 mg/kg/hr. In November, we kept the bears warm by using an electric compact heater during the experiment so that the bears' body temperatures would not drop to 33°C or lower.

Blood sampling and IVGTT: Basal blood samples were first collected from the catheter and placed into vacuum tubes 10 min prior to glucose injection. Glucose (0.5 g/kg of body mass) was injected over 1–3 min as a sterile 50% solution. Time zero (base time) was defined as the endpoint of the injection period. Blood samples of 5 ml each for glucose and insulin determination were obtained at 0, 5, 10, 20, 30, 40, 60, 90, 120, and 150 min after glucose injection, from the catheter and placed into vacuum tubes. Immediately after each blood sampling, approximately 1 ml of heparinized physiological saline (approximately 2 U/ml of heparin) was used to flush the catheter.

Vacuum tubes containing a mixture of sodium fluoride, heparin sodium and EDTA-2Na as anticoagulant were used for glucose determination. Tubes containing EDTA-2K were used for triglycerides (TG) and total cholesterol (TCHO) determination, and heparin sodium was used as the anticoagulant for alanine aminotransferase (ALT) and aspartate aminotransferase (AST) determination. Blood was also collected from the catheter, placed in vacuum tubes without anticoagulant and allowed to clot prior to centrifuging and harvesting of serum. All collected blood samples were chilled on ice until centrifugation, and centrifuged at 1880Xg for 10 min within 10 min for plasma or 30 min for serum after blood sampling. Separated serum and plasma were stored at −30°C until assay.

Blood biochemistry and data analyses: Basal levels of blood biochemical values were analyzed using pre-samples obtained 10 min prior to glucose injection. Plasma concentrations of glucose, TG, TCHO, ALT, and AST were measured using an automatic blood analyzer (DRI-CHEM 7000, FUJIFILM Medical Co., Ltd., Tokyo, Japan). Serum non-esterified fatty acids (NEFA) were assayed using a commercial kit (NEFA C test, Wako Pure Chemical Industries, Ltd., Osaka, Japan). Serum insulin concentration was measured by enzyme-linked immunosorbent assay (ELISA) developed for dog insulin measurement (Insulin ELISA Kit/Dog, Morinaga Institute of Biological Science, Inc., Yokohama, Japan). Purified dog insulin was used as a standard. The intra- and inter-assay coefficients of variation were 4.5% and 10.4%, respectively.

The half-life for glucose in serum ($t_{1/2}$) and the fractional clearance rate (K) were determined from the results of IVGTT. In order to calculate the $t_{1/2}$, linear regression analysis of

a semilogarithmic plot of glucose concentration versus time between 10 and 30 min after glucose injection was used. The K value was calculated from the following formula: $K = (0.693 / t_{1/2}) \times 100 = \%/min$[13]. Peak values of glucose and insulin concentrations were calculated by subtracting the basal value from the highest value. Areas under the glucose and insulin curves ($AUC_{glucose}$, $AUC_{insulin}$) were calculated as total incremental area above zero value with Prism statistical software (GraphPad Software, version 5, San Diego, CA, USA). The $AUC_{glucose}$ was determined from 0 to 150 min. To evaluate the details of insulin secretion, the $AUC_{insulin}$ was determined from 0 to 10 min, 10 to 90 min, and 0 to 150 min after glucose injection as first phase secretion, second phase secretion and total secretion, respectively.

Statistical analyses: Differences in body mass, blood variables (glucose, insulin, TG, TCHO, NEFA, ALT, and AST) and the AUC among three groups (August, early November, and late November) were evaluated by one-way analysis of variance (ANOVA) for repeated measures. When a significant difference was determined, Tukey's multiple comparison test was performed. All statistical analyses were performed using Prism statistical software. A P value < 0.05 was considered significant. All data are expressed as mean ± SEM.

Results

The mean body mass and blood biochemical values in each group are presented in Table 1. The mean body mass in early and late November was heavier than that in August, but no significant differences were observed among the three groups. In contrast, the basal levels of TG in early (2.4 ± 0.2 mmol/L) and late November (2.4 ± 0.3 mmol/L) were significantly lower than in August (3.3 ± 0.2 mmol/L: $P < 0.05$). Although there was no significant difference in NEFA concentration among groups, the value (0.17± 0.08 mmol/L) in early November tended to be lower than those in the other groups (August: 0.77 ± 0.22 mmol/L, late November: 0.91 ± 0.36 mmol/L). The basal levels of TCHO, AST, and ALT were not significantly different among groups.

The changes in plasma glucose and serum insulin concentrations before and after glucose injection are presented in Figs. 1 and 2, respectively. Basal and peak values of glucose and insulin concentrations, $t_{1/2}$, and K in each group are presented in Table 2. Basal levels of

Table 1. Changes in body mass, basal plasma concentrations of triglyceride (TG), total cholesterol (TCHO), non-esterified fatty acids (NEFA), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) in 4 adult female bears.

Variables (units)	Physiological stage		
	Normal activity	Hyperphagia	
	Aug	Nov-a	Nov-b
Body weight (kg)	69.8 ± 5.5	74.9 ± 7.6	75.8 ± 7.0
TG (mmol/L)	3.3 ± 0.2[a]	2.4 ± 0.2[b]	2.4 ± 0.3[b]
TCHO (mmol/L)	7.6 ± 0.2	7.0 ± 0.4	7.3 ± 0.3
NEFA (mmol/L)	0.77 ± 0.22	0.17 ± 0.08	0.91 ± 0.36
AST (U/L)	82.5 ± 8.1	92.8 ± 3.6	101.0 ± 7.9
ALT (U/L)	20.0 ± 1.1	19.0 ± 1.4	18.3 ± 1.8

[a,b]Letters indicate significant differences among groups ($P < 0.05$).
Nov-a: early November, Nov-b: late November.
All data are expressed as mean ± SEM.

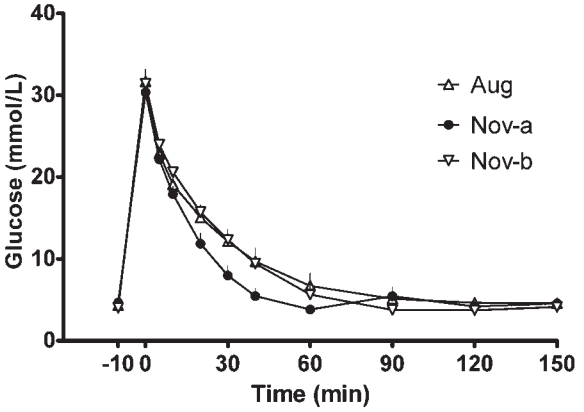

Fig. 1. Change in plasma glucose concentrations (mean ± SEM) in 4 adult female bears before and after intravenous administration of glucose. Basal blood samples were obtained 10 min prior to glucose injection. Glucose (0.5 g/kg of body mass) was injected at 0 min, and blood samples were obtained at 0, 5, 10, 20, 30, 40, 60, 90, 120, and 150 min after glucose injection. Nov-a: early November, Nov-b: late November.

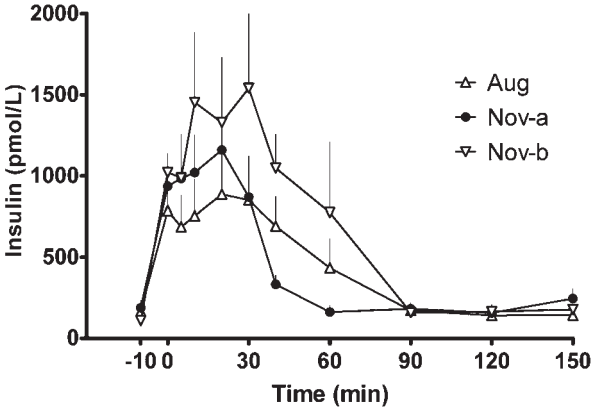

Fig. 2. Change in serum insulin concentrations (mean ± SEM) in 4 adult female bears before and after intravenous administration of glucose. Basal blood samples were obtained 10 min prior to glucose injection. Glucose (0.5 g/kg of body mass) was injected at 0 min, and blood samples were obtained at 0, 5, 10, 20, 30, 40, 60, 90, 120, and 150 min after glucose injection. Nov-a: early November, Nov-b: late November.

Table 2. Changes in basal (−10 min) and peak values of glucose and insulin concentrations, half-life for glucose disappearance from serum ($t_{1/2}$), and fractional clearance rate (K) in 4 adult female bears.

Variables (units)		Physiological stage		
		Normal activity	Hyperphagia	
		Aug	Nov-a	Nov-b
Glucose (mmol/L)	−10min	4.4 ± 0.1	4.7 ± 0.2	4.1 ± 0.1
	peak value	27.4 ± 1.6	25.7 ± 0.7	27.4 ± 0.8
$t_{1/2}$ (min)		31.8 ± 4.3[a]	17.3 ± 2.1[b]	28.5 ± 4.2[ab]
K (%/min)		2.4 ± 0.4[a]	4.2 ± 0.5[b]	2.6 ± 0.5[a]
Insulin (pmol/L)	−10min	167 ± 59	189 ± 46	109 ± 23
	peak value	871 ± 226[a]	1043 ± 252[ab]	1578 ± 446[b]

[a,b]Letters indicate significant differences among groups ($P < 0.05$).
Nov-a: early November, Nov-b: late November.
All data are expressed as mean ± SEM.

plasma glucose and serum insulin concentrations were not significantly different among groups. During IVGTT, plasma glucose concentrations peaked immediately after glucose injection (0 min) in all groups, then decreased gradually over time. There were no significant differences in the peak values of plasma glucose. Glucose disappearance rate was significantly different among groups. The $t_{1/2}$ in early November (17.3 ± 2.1 min) was significantly shorter than that in August (31.8 ± 4.3 min: $P < 0.05$), and the K in early November (4.2 ± 0.5%/min) was significantly higher than those in August (2.4 ± 0.4%/min) and late November (2.6 ± 0.5%/min: $P < 0.05$), although there was no significant difference in $AUC_{glucose}$ among groups. Serum insulin concentrations peaked within 30 min after glucose injection in all groups, and then decreased gradually over time. The peak value of serum insulin was tended to increase in

November and it was significantly higher in late November (1578 ± 446 pmol/L) compared to August (871 ± 226 pmol/L: $P < 0.05$). In early November, serum insulin concentration returned to basal level more rapidly than the other groups, and $AUC_{insulin}$ from 10 to 90 min in early November (37.2 ± 6.9 nmol/L/80 min) was significantly lower than that in late November (73.4 ± 18.3 nmol/L/80 min) ($P < 0.05$) (Fig. 3). The $AUC_{insulin}$ from 0 to 10 min and 0 to 150 min after glucose injection were not significantly different among groups.

Discussion

The blood biochemistry results in this study did not reveal any signs of disease, nor clinical abnormalities. Interestingly, plasma TG concentrations decreased significantly with increase in body mass. In general, blood TG is affected by the composition of nutrient intake[20,21]. However, in this study, all bears were provided roughly the same food in terms of both quantity and quality throughout the active season. Therefore, we speculated that the change in blood TG was due to endogenous metabolic changes. One possible explanation for the decrease in plasma TG is the reduction in TG synthesis in the liver. However, this explanation is unlikely for the following reason. Nakamura *et al*. observed a strong correlation between body mass and fat mass during hyperphagia in captive Japanese black bears[15]. Therefore, it is speculated that lipogenesis in the liver and adipose tissue are stimulated in bears during the hyperphagic stage. Another possible explanation for the decrease in plasma TG could be the activation of metabolic enzymes such as lipoprotein lipase (LPL) involved in lipid deposition. LPL is the rate limiting enzyme involved in the deposition of fatty acids into white adipose tissue from plasma TG contained within chylomicrons and very low-density lipoprotein particles[24]. Herminghuysen *et al*. reported that the adipose LPL activity increased in fall in American black bears (*Ursus americanus*)[11]. Similarly, yellow-bellied marmots (*Marmota flaviventris*), which are also fat-storing hibernators, showed the increase in activity[8] and relative mRNA concentrations[25] of LPL in adipose tissue during the mass gain phase (June-September). Therefore, it is possible that the decrease in plasma TG is due to the facilitation of TG removal from plasma by the activation of LPL. Since insulin acts as a major regulator of LPL and increases its activity and mRNA levels in adipose tissue[24], the relatively high basal insulin level in early November might have contributed to the decrease in plasma TG concentration. In addition, the relatively low value of serum NEFA concentration in early November might indicate a suppression of lipolysis in adipose tissue. These findings indicated that the metabolic state of bears in early November would shift toward lipogenesis. On the other hand, in late November, significantly low plasma TG was also observed, although the

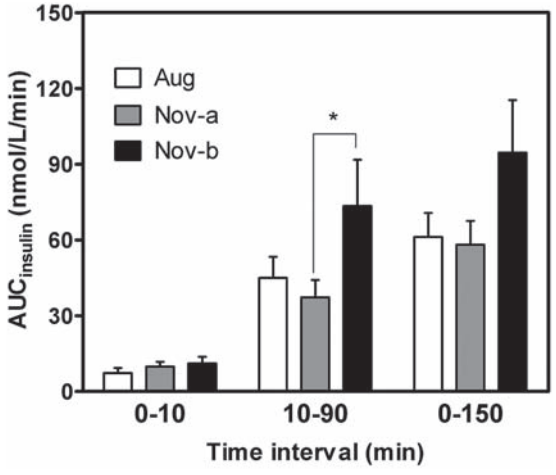

Fig. 3. Areas under the insulin curves ($AUC_{insulin}$) during selected time intervals after glucose injection in 4 adult female bears. Glucose (0.5 g/kg of body mass) was injected at 0 min. $AUC_{insulin}$ was calculated as total incremental insulin area above zero value, and determined from 0 to 10 min, 10 to 90 min, and 0 to 150 min after glucose injection as first phase secretion, second phase secretion and total secretion, respectively. Data are expressed as mean ± SEM. *Significant differences among groups ($P < 0.05$). Nov-a: early November, Nov-b: late November.

basal insulin value was relatively low compared with other groups. Given that the captive bears in Ani Mataginosato Bear Park gradually lose their appetite from mid-November and enter hibernation in December every year, it is conceivable that the metabolic state of bears changed already to the state of hibernation in late November. Therefore, the low plasma TG concentration in late November might arise from a reduction in TG synthesis in the liver.

When glucose concentration increases rapidly, as after a glucose bolus, insulin response shows a biphasic secretion pattern with a rapid increase to an initial peak, followed by an interpeak nadir, and a subsequent, slower, increasing phase[3]. The first phase of insulin secretion is important for glucose homeostasis and the loss of it is considered to be an important early marker for beta-cell dysfunction[10]. In healthy, lean humans and animals, the first phase of insulin secretion is observed during the first 5–10 min after bolus glucose injection[17,23] and serves to rapidly inhibit hepatic glucose production[4]. If the first phase of insulin secretion is impaired, the result is a higher blood glucose after glucose load, which will then elicit a greater second phase of insulin secretion[14]. In this study, the first phase of insulin secretion was not significantly different among groups, indicating that beta-cell glucose sensitivity was not impaired with increase in body mass. In addition, adequate inhibition of hepatic glucose production by insulin was indicated by the similar peak glucose values after glucose injection among groups. On the other hand, the second phase of insulin secretion was significantly different between early and late November. In early November, second phase of insulin secretion was significantly lower, and glucose disappearance was significantly faster than late November, as indicated by significantly low $AUC_{insulin}$ and high K in early November. In comparison with August, glucose disappearance in early November was significantly fast despite a lack of significant difference in second phase of insulin. These findings suggested that glucose tolerance in early November increased more than the other groups due to an increase in peripheral insulin sensitivity. In contrast, in late November, the glucose disappearance was comparable to August, although peak insulin concentration was significantly higher than August, suggesting a peripheral insulin resistance in late November. The results of IVGTTs revealed an alteration in glucose metabolism in bears between the stage of normal activity and the hyperphagic stage as well as between early and late November. Taken together with the results of blood biochemistry, we assume that early November may correspond to the late stage of hyperphagia and late November may correspond to the onset of hibernation. Consequently, we speculate that bears metabolically switch from hyperphagia to hibernation around mid-November.

In general, insulin sensitivity and insulin secretion are inversely related; insulin resistance is compensated by increased insulin secretion[1]. However, contrary to this theory, an increase in basal insulin concentration was not observed in late November, whereas the results of IVGTTs suggested a peripheral insulin resistance. A previous study in American black bears demonstrated that basal insulin values increased during the fall active phase and were returned to the normal range during early hibernation[18]. Because the main energy sources during hibernation are lipids, it is considered that glucose utilization in peripheral tissues decreases during this period and this change can cause a decrease in basal insulin secretion. Therefore, if bears had switched their metabolism toward hibernation in late November, the relatively low basal insulin value and peripheral insulin resistance are consistent with the adaptive changes for hibernation.

In conclusion, our findings suggest that bears shift their glucose and lipid metabolism from the stage of normal activity to the hyperphagic stage, in which they show lipogenic-predominant metabolism and accelerate glucose

uptake by increasing the peripheral insulin sensitivity. Additionally, our findings indicate that the bears at Ani Mataginosato may switch their metabolism from the hyperphagic stage to hibernation around mid-November. These results raise the possibility that bears may have a unique mechanism for fat accumulation during the hyperphagic stage. However, since there was no significant change in basal insulin levels in this study, the role of insulin in the regulation of body mass in bears is yet unclear. The principal limitation in this study was the large individual variability due to small sample size. Therefore, further study in larger numbers of bears is necessary to confirm our results and to elucidate the role of insulin in bears. In addition, we have not directly evaluated the peripheral insulin sensitivity. Thus, further study using a direct method, such as insulin injection experiment, is also needed to confirm the change in insulin sensitivity suggested by our results.

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