［原著］Cholecystokinin was involved in the development of leptin resistance in OLETF rats

Cholecystokinin was involved in the development of leptin resistance in OLETF rats

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ABSTRACT

To investigate whether a feeding action of ghrelin and an antiobese action of leptin are modified by cholecystokinin (CCK), body weight, food intake, and plasma concentrations of CCK, active ghrelin, desacyl ghrelin and leptin were measured in 7 and 38 weeks old of Otsuka Long-Evans Tokushima Fatty (OLETF) rats which lack CCK1 receptor. Long-Evans Tokushima Otsuka (LETO) rats were used as normal counterpart. The mean body weight, the amount of food intake, active ghrelin and leptin were significantly increased, whereas CCK was significantly decreased in OLETF rats as compared to that in LETO rats at 7 and 38 weeks old. Multiple linear regression analysis revealed that active ghrelin and leptin were significant determinants for prediction of food intake in OLETF rats and that leptin was a significant determinant for body weight in LETO rats. Leptin resistance index (LRI) was calculated by a following equation; LRI = body weight × plasma leptin. The index was negatively correlated with concentration of CCK. Thus, the decreased plasma CCK in OLETF rats may associate with leptin resistance and increase body weight. *Ryukyu Med. J., 27(3,4) 105~114, 2008*

Key words: cholecystokinin; ghrelin; leptin; OLETF rat; obesity; leptin resistance

INTRODUCTION

Obesity can be resulted from greater food intake than energy expenditure. Many causes of obesity include sedentary lifestyle and abnormal feeding behavior. Abnormal feeding behavior is caused by mental stress, damage or dysfunction of ventromedial nuclei of hypothalamus, and genetic factors such as mutations of melanocortin receptor 4, leptin/leptin receptor, and cholecystokinin (CCK) receptors[1-3].

CCK has been known as a signal for satiation and termination of eating. CCK reduced meal size and meal duration, which resulted in an earlier appearance of a behavioral sequence of satiety similar to that seen following ingestion of a normal size meal[4]. Exogenous peripheral administration of CCK resulted in dose-related suppression of short-term food intake in a variety of species[5]. CCK receptors are expressed in the nodose ganglion and transported to the terminal ends of subdiaphragmatic vagal branches by axonal transport[6,7].

Ghrelin is one of gastrointestinal hormones and transmits a hunger signal via the vagal afferent. Ghrelin increases secretion of growth hormone, food intake, and body weight when administered peripherally or centrally. Ghrelin activates neuropeptide Y (NPY)-producing neurons localized in the arcuate nucleus of the hypothalamus. Secretion of ghrelin is up-regulated under conditions of negative energy balance such as fasting, insulin-induced hypoglycemia and cachexia[8].

Leptin, an adipocyte-derived hormone, has been known to regulate food intake and neuroendocrine functions and stimulates sympathetic nerve activity[9-12] via specific receptors (Ob-R) that are highly expressed in the hypothalamus. Usually, leptin has inhibitory effects on food intake, which is involved in intermediate hypothalamic neuropeptides such as pro-opiomelanocortin (POMC), NPY and orexin[13,14]. Plasma leptin was significantly increased in obese

animals and humans, and leptin resistance, rather than its deficiency, is suggested as the characteristic feature of obesity[15-17].

Although it has been clarified that many peptide hormones are involved in food intake and energy expenditure, the interaction of CCK with ghrelin or leptin has not yet been well understood. We hypothesized that CCK modulates the effect of leptin on control of body weight. To test the hypothesis, we measured plasma concentration of CCK, desacyl ghrelin, active ghrelin, and leptin in Otsuka Long-Evans Tokushima Fatty (OLETF) rats. OLETF rats lack CCK1 receptor and spontaneously onset non-insulin-dependent diabetes mellitus and mild obesity by overeating behavior[1].

MATERIALS AND METHODS

I. Animals and animal care

All animals were cared for following the Guidelines for Animal Experiments in Research Institutes (Notice No. 71 of the Ministry of Education, Culture, Sports, Science and Technology 2006) and the Guidelines for Animal Experiments issued by the University of the Ryukyus. All animal studies were reviewed and approved by the Animal Care Committee at the University of the Ryukyus. OLETF rat is a type 2 DM model with mild obesity and Long-Evans Tokushima Otsuka (LETO) rat is a nondiabetic counter part of the OLETF rats. Seven weeks old of those male rats were kindly donated by the Otsuka Pharmaceutical Tokushima Research Institute (Tokushima, Japan).

II. Measurement of amount of food intake and body weight

The amount of food intake during experimental periods was measured every day, and daily mean food intake was calculated every week. In a similar way, body weight was measured every day.

III. Measurement of plasma concentrations of CCK, ghrelin and leptin

The blood samplings were performed at the ages of 7 and 38 weeks old. The blood was collected from rat tail vein in the presence of 500 KIU/ml of aprotinin and 1/10 volume of 1.25 mg/ml ethylenediaminetetraacetic acid Na_2. Plasma was obtained by centrifugation (2000 × g, 10 min, 4 ℃) immediately after blood collection. For the ELISA measurement of ghrelin, 1/10 volume of 1 N HCl was added to the plasma.

CCK concentration was measured by using competitive ELISA method kit (Peninsula Laboratories Inc., CA, USA). The CCK peptide included in the sample plasma was competed against standard biotinylated CCK, which was reacted with horseradish peroxidase-conjugated streptavidin.

Desacyl and active ghrelin concentrations were measured by using sandwich ELISA method kit (Mitsubishi Kagaku Iatron, Tokyo, Japan). In brief, standard rat ghrelin peptide or plasma sample was added to pre-coated plate with anti-rat ghrelin, followed by the reaction with horseradish peroxidase-conjugated secondary antibody. Absorbance was measured after addition of substrate, 3,3',5,5'-Tetramethylbenzidine.

Leptin concentration was measured by using sandwich ELISA method kit (LINCO Research, MO, USA). After biotinylated anti-mouse leptin was reacted, horseradish peroxidase-conjugated streptavidin was reacted. Absorbance was measured after addition of substrate, 3,3',5,5'-Tetramethylbenzidine.

IV. Calculation of leptin resistance index (LRI)

Changes in body weight depend on the regulation of food intake and energy expenditure by feeding related hormones. Leptin negatively control body weight by decreasing feeding and by increasing energy expenditure. Thus, body weight can be simply and roughly estimated by a following equation: body weight = $1 / (k_{leptin} \times$ plasma leptin), where k_{leptin} is a coefficient of the sensitivity of feeding control or efficacy of energy expenditure. However, the majority of obese individuals have elevated rather than depressed levels of leptin and exogenously administered leptin did not induce substantial weight loss. That is, leptin seems to be ineffective in preventing obesity. The conception of leptin resistance is the reduced sensitivity and efficacy of leptin. Thus, we calculated reciprocal of k_{leptin} as a leptin resistance index (LRI).

V. Data Analysis

All data represent means ± standard error (SE). Stat View version 5 (SAS Institute Inc., North Carolina, U.S.A.) and Origin version 6.1J (OriginLab Corporation., Northampton, USA) were used to test statistically significant difference between two means by Student's unpaired t-test. $P<0.05$ was regarded

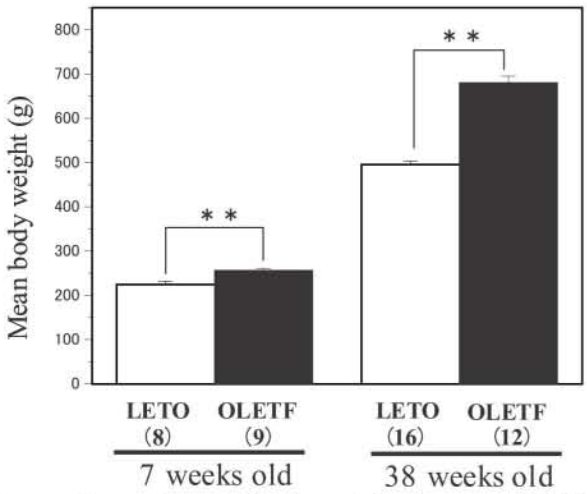

Fig. 1 Comparison of body weight between LETO and OLETF rats

Mean body weight of LETO (open bars) and OLETF (closed bars) rats were measured in 7 weeks old and 38 weeks old. Data represents mean ± SE. The numbers in parentheses represent the number of rats tested. **indicates statistically significant difference with $P<0.01$, as compared with age-matched LETO rats using Student's unpaired t-test.

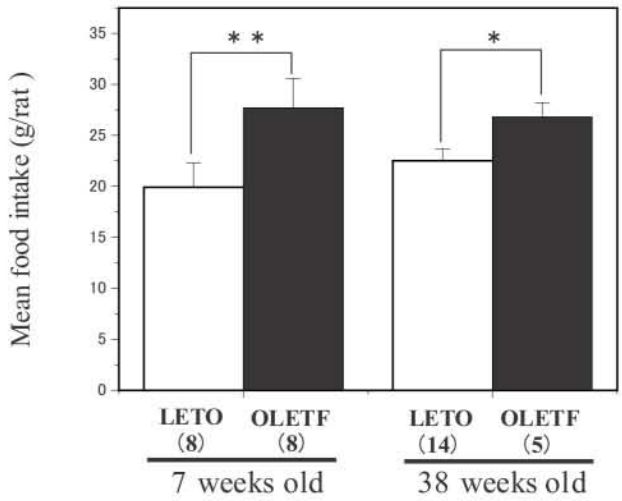

Fig. 2 Comparison of amount of food intake between LETO and OLETF rats

Mean food intake of LETO (open bars) and OLETF (closed bars) rats were measured in 7 weeks old and 38 weeks old. Data represents mean ± SE. The numbers in parentheses represent the number of rats tested. * and ** indicate statistically significant differences with $P<0.05$ and $P<0.01$, respectively, as compared with age-matched LETO rats using Student's unpaired t-test.

as a statistical significance. Multiple linear regression analysis was performed to identify a factor that contributes to the body weight and the amount of food intake. Simple regression analysis was used to find a correlation of LRI with body weight or plasma CCK. Furthermore, chi-square test for contingency tables and post-hoc cell contribution analysis was performed by StatView. To do this, each data of LRI, body weight and plasma concentrations of active ghrelin, leptin and CCK was categorized by comparing those mean values into the groups: *high* or *low*.

RESULTS

I. Body weight and food intake

The mean body weight of OLETF rats in 7 weeks old was 256.7 ± 9.2 g and it was significantly increased as compared with that of age-matched LETO rats (224.4 ± 5.4 g). The mean body weight of OLETF rats in 38 weeks old was 680.8 ± 14.5 g and it was significantly increased as compared with that of age-matched LETO rats (495.3 ± 7.7 g) (Fig. 1).

The mean daily food intake of OLETF rats in 7 weeks old was 27.7 ± 2.9 g and it was significantly increased as compared with that of age-matched LETO rats (19.9 ± 2.4 g). The mean daily food intake of OLETF rats in 38 weeks old was 26.8 ± 1.38 g and it was significantly increased as compared with

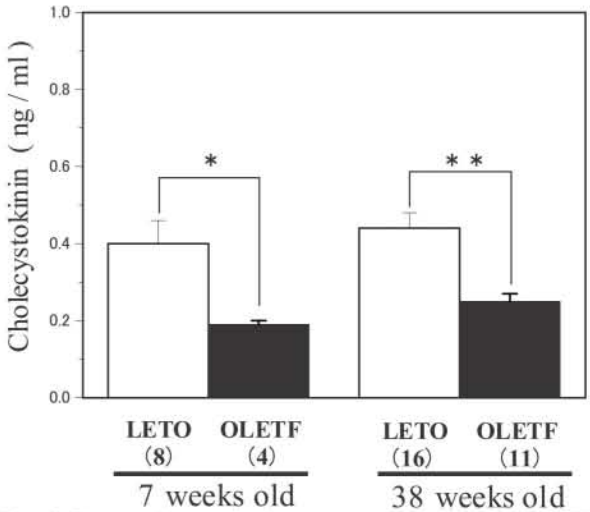

Fig. 3 Comparison of plasma concentration of CCK between LETO and OLETF rats

Mean plasma concentration of CCK of LETO (open bars) and OLETF (closed bars) rats in 7 weeks old and 38 weeks old. Data represents mean ± SE. The numbers in parentheses represent the number of rats tested. * and ** indicate statistically significant differences with $P<0.05$ and $P<0.01$, respectively, as compared with age-matched LETO rats using Student's unpaired t-test.

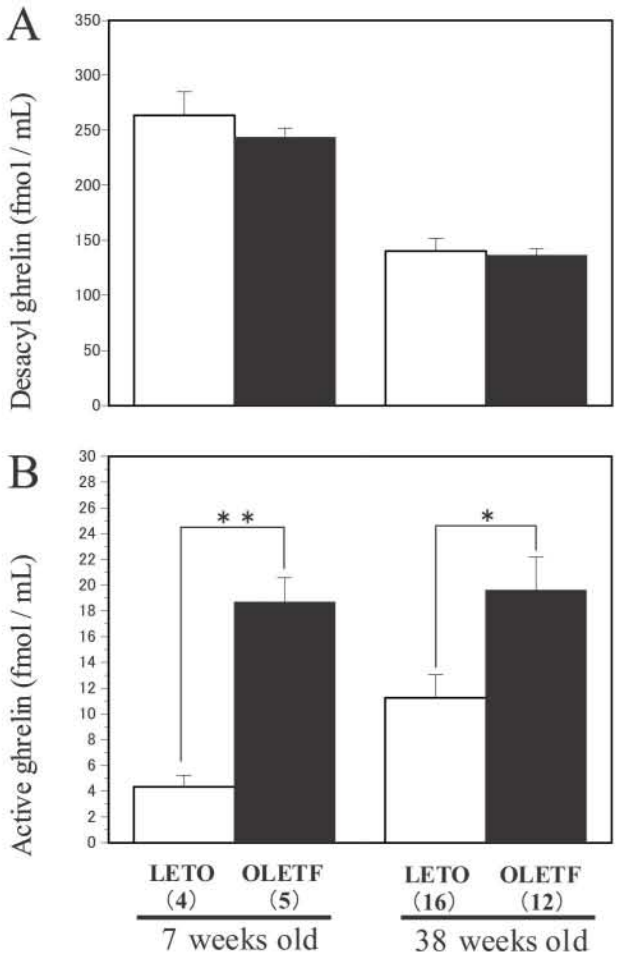

Fig. 4 Comparison of plasma concentration of desacyl and active ghrelin between LETO and OLETF rats
Mean plasma concentration of desacyl ghrelin (A) and active ghrelin (B) were measured in 7 weeks old and 38 weeks old in LETO (open bars) and OLETF (closed bars) rats. Data represents mean ± SE. The numbers in parentheses represent the number of rats tested. * and ** indicate statistically significant differences with $P<0.05$ and $P<0.01$, respectively, as compared with age-matched LETO rats using Student's unpaired t-test.

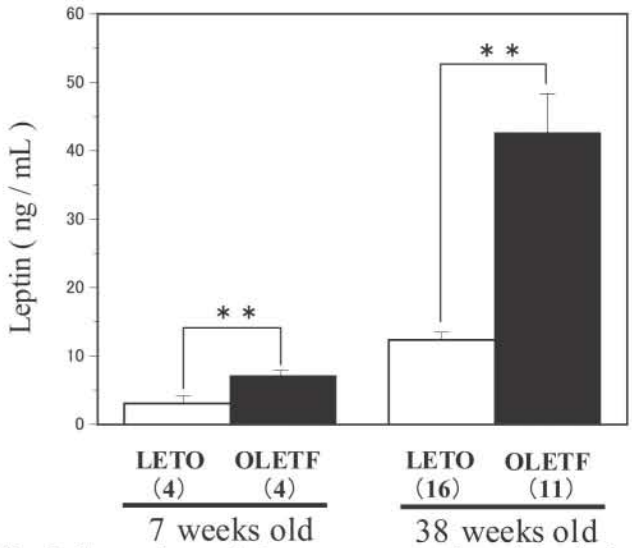

Fig. 5 Comparison of plasma concentration of leptin between LETO and OLETF rats
Mean plasma concentrations of leptin in LETO (open bars) and OLETF (closed bars) rats were measured in 7 weeks old and 38 weeks old. Data represents mean ± SE. The numbers in parentheses represent the number of rats tested. ** indicates a statistically significant difference with $P<0.01$, as compared with age-matched LETO rats using Student's unpaired t-test.

that of age-matched LETO rats (22.5 ± 1.18 g) (Fig. 2).

II. Plasma concentrations of CCK, ghrelin, and leptin

The plasma concentration of CCK in OLETF rats in 7 weeks old was 0.19 ± 0.01 ng/ml and it was significantly decreased as compared with that of LETO rats (0.40 ± 0.06 ng/ml). Similarly, the plasma CCK levels of OLETF rats in 38 weeks old was 0.25 ± 0.02 ng/ml and it was significantly decreased as compared with that of LETO rats (0.44 ± 0.04 ng/ml) (Fig. 3).

The plasma desacyl ghrelin level of OLETF rats in 7 weeks old was 243.6 ± 8.6 fmol/ml and there was no significant difference as compared with that of LETO rats (263.6 ± 21.6 fmol/ml). The plasma desacyl ghrelin level of OLETF rats in 38 weeks old was 136.4 ± 6.0 fmol/ml and there was no significant difference as compared with that of LETO rats (140.3 ± 11.8 fmol/ml) (Fig. 4A). However the plasma level of active ghrelin in OLETF rats at the age of 7 weeks old was 18.7 ± 1.9 fmol/ml and it was significantly increased as compared with that of LETO rats (4.3 ± 0.9 fmol/ml). The plasma level of active ghrelin in OLETF rats in 38 weeks old was 19.6 ± 2.6 fmol/ml and it was significantly increased as compared with that of LETO rats (11.2 ± 1.8 fmol/ml). In normal LETO rats, plasma active ghrelin in 38 weeks old was increased as compared with that of 7 weeks old, whereas there was no significant difference between these periods in OLETF rats (Fig. 4B).

The plasma concentration of leptin in OLETF rats at the age of 7 weeks old was 7.1 ± 0.2 ng/ml and it was significantly increased as compared with that of LETO rats (3.1 ± 0.2 ng/ml). The plasma concentration of leptin in OLETF rats at the age of 38 weeks old was 42.7 ± 5.7 ng/ml and it was

Table 1 Multiple linear regression analysis to predict food intake at the age of 38weeks old.

Variables	LETO (n = 14)		OLETF (n = 8)	
	Standardized regression coefficients	*P*-value	Standardized regression coefficients	*P*-value
CCK	0.315	0.458	- 0.269	0.194
Active ghrelin	0.368	0.370	0.662	0.018
Leptin	0.036	0.893	- 0.758	0.012

Table 2 Multiple linear regression analysis to predict body weight at the age of 38weeks old.

Variables	LETO (n = 20)		OLETF (n = 17)	
	Standardized regression coefficients	*P*-value	Standardized regression coefficients	*P*-value
CCK	0.176	0.306	0.221	0.398
Active ghrelin	0.033	0.840	- 0.117	0.652
Leptin	0.911	<0.001	0.355	0.184

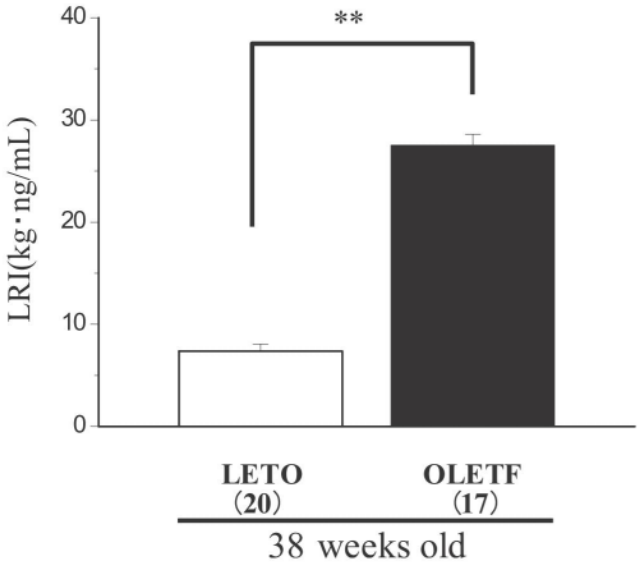

Fig. 6 Comparison of leptin resistance index between LETO and OLETF rats
Mean values of LRI in LETO (open bars) and OLETF (closed bars) rats were calculated from data in 38 weeks old. Data represents mean ± SE. The numbers in parentheses represent the number of rats tested.
** indicates a statistically significant difference with $P<0.01$, as compared with age-matched LETO rats using Student's unpaired *t*-test.

significantly increased as compared with that of LETO rats (12.4 ± 1.1 ng/ml) (Fig. 5).

III. Multiple regression analyses

To investigate whether the amount of food intake was regulated by the peptide hormones, multiple linear regression analysis was performed to find a contributing factor for food intake. The variables used were plasma concentrations of CCK, active ghrelin and leptin. As shown in Table 1, active ghrelin and leptin were significant determinants to predict the amount of food intake in OLETF rats. There were no such predictive factors for the amount of food intake in LETO rats.

In a similar way, multiple linear regression analysis for prediction of body weight was performed. The concentration of leptin was a significant predictor for body weight in LETO rats. However, there was no significant predictor in OLETF rats (Table 2).

IV. Comparison of LRI between LETO and OLETF rats

The LRI of OLETF rats in 38 weeks old was 27.5 ± 1.10 and it was significantly increased as compared with that of age-matched LETO rats (7.38

Table 3 Contingency table for chi-square test and post hoc cell contribution test for relation between LRI and variables

Variables		LRI	
		high	*low*
Body weight	*high*	17 (2.04)	0 (- 1.46)
	low	3 (- 2.87)	20 (1.13)
Active ghrelin	*high*	11 (0.24)	6 (0.17)
	low	6 (0.44)	16 (0.20)
Leptin	*high*	17 (2.04)	0 (3.02)
	low	0 (2.87)	23 (2.24)
CCK	*high*	2 (- 3.88)	15 (5.34)
	low	14 (4.35)	8 (- 3.19)

Each data was categorized into *high* or *low* group by comparing the mean value.
The numbers in parentheses represents the post hoc cell contribution test value.

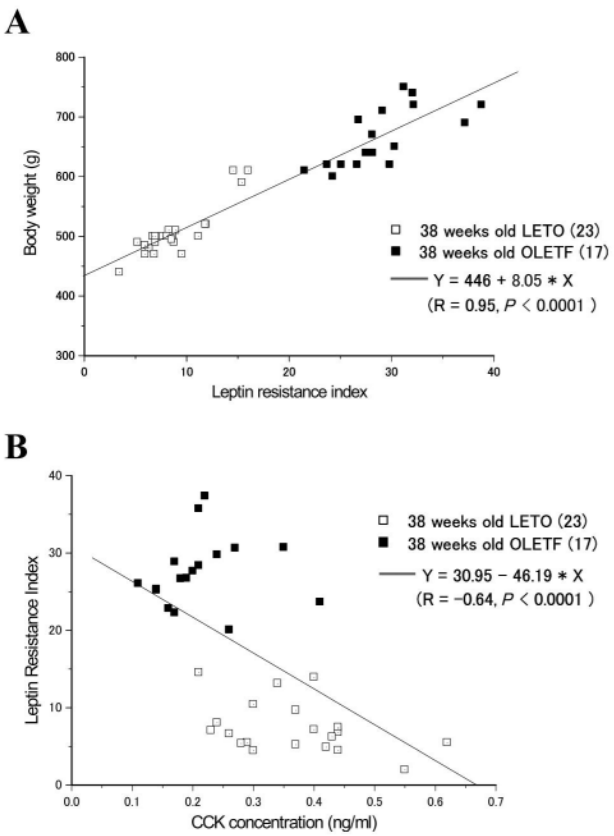

Fig. 7 Scatter diagrams of body weight versus LRI and LRI versus plasma CCK
Body weights (A) and plasma concentrations of CCK (B) were plotted with the function of LRI. Regression lines show a linear correlation between body weights and CCK concentrations and LRI. The equations of the regression lines, correlation coefficients (R) and its *P*-values are shown. Open squares: 38 weeks old LETO, closed squares: 38 weeks old OLETF.

± 0.68) ($P<0.01$, Fig. 6). Simple regression analysis revealed that LRI positively correlated with body weight of LETO and OLETF rats in 38 weeks old with a regression line of $Y = 446 + 8.05 \cdot X$ ($R = 0.95$, $P<0.0001$) (Fig. 7A). LRI negatively correlated with the plasma concentration of CCK in 38 weeks old LETO and OLETF rats with regression line of $Y = 30.95 - 46.19 * X$ ($R = -0.64$, $P<0.0001$) (Fig. 7B). Chi-square test for contingency table and post hoc cell contribution analysis were performed to assess the relationship between variables (including body weight, active ghrelin, leptin and CCK) and LRI. There was a significant relationship between LRI and those explanatory variables (chi-square value was 68.23, $P<0.01$). The best contributing variable for increasing LRI was the *low* group of CCK with a post hoc cell value of 4.35 (Table 3). Similarly, the best contributing variable for decreasing LRI was the *high* group of CCK with a post hoc cell value of 5.34 (Table 3).

DISCUSSION

Recent studies suggest that CCK, ghrelin and leptin have important roles in feeding related regulation; however, there are few studies on interrelationships among these hormones. In the present study, we investigated whether inhibitory effect of leptin on gaining body weight is modified by CCK by using OLETF rats. OLETF rats are known to lack CCK1 receptor[1,18,19].

Plasma concentration of CCK was not changed

in an age-dependent manner in both LETO and OLETF rats (Fig. 3). Therefore, homeostasis of plasma CCK seems to be independently regulated by development, feeding, and body weight. CCK is released from the I cells in the mucosa of the duodenum and upper jejunum mainly in response to fat entering the duodenum and CCK reduces feeding mainly by activation of melanocortin receptor in the hypothalamus[20-22]. Although it is not clear how the plasma concentration of CCK is significantly decreased in OLETF rats at the age of 7 and 38 weeks old (Fig. 3), deficiency of CCK1 receptor in OLETF rats might be involved. For example, if the secretion of CCK might be positively regulated by autocrine or paracrine via CCK1 receptor in the I cells or adjacent cells in duodenum, then lack of CCK1 would decrease the production of CCK in OLETF rats. Although there have been no reports on the co-localization of CCK and CCK1 receptor, CCK1 receptor was reported to exist in duodenum[23].

In OLETF rats, as compared with LETO rats, food intake and plasma concentration of active ghrelin were significantly increased in both 7 and 38 weeks old, whereas concentration of desacyl ghrelin was not changed (Figs. 2 and 4). In addition, plasma concentration of active ghrelin was increased in an age-dependent manner in LETO rats, whereas it was not changed during development in OLETF rats (Fig. 4). Taken together, although the production of total ghrelin was not enhanced, plasma concentration of active ghrelin was maximally elevated even at the age of 7 weeks old in OLETF rats. Interestingly, hyperphagia was demonstrated even at the age of 2 days in OLETF rats[24]. Therefore, abnormally elevated active ghrelin might be a main cause of hyperphagia in OLETF rats. Previous studies reported that plasma ghrelin levels were significantly decreased in human obese subjects and in obesity-model rats[25]. In contrast, plasma active ghrelin was significantly elevated from the age of 7 weeks old in OLETF rats. Active ghrelin is secreted from the X/A-like cells in stomach after being processed by n-octanoyl modification on Ser3[26]. Thus, in OLETF rats, the n-octanoyl modification might be significantly activated in the cells or desacylation of active ghrelin might be decreased in circulating blood.

Leptin regulates feeding and energy expenditures by acting at sites primarily within the central nervous system[27-29]. Production of leptin in adipocytes and plasma concentration of leptin are increased as the total amount of fat tissue in body is increased. Thus, the increase in plasma concentration of leptin in OLETF rats can be caused by the accumulation of fat tissue, which is evidenced by the fact that body weight was significantly increased from the age of 7 weeks (Fig. 1). However, obesity was not depressed even though plasma leptin was extraordinarily increased in OLETF rats (Fig. 5). The action of leptin is known to be suppressed by an unknown mechanism, phenomenon of which is called leptin resistance[30-32]. As shown in Fig. 6, LRI was significantly increased in OLETF rats, in which plasma concentration of CCK was decreased. In addition, LRI positively correlated with body weight of LETO and OLETF rats. Comparison of the four variants demonstrated that there were statistical differences in the numbers of *high* group and *low* group in LRI between the variables by chi-square test (Table. 3). CCK negatively contributes to increase LRI, whereas body weight and leptin positively do. For *high* group of LRI, CCK had the highest absolute value of post hoc cell contribution test. In addition, CCK had the highest absolute value for *low* group of LRI. Thus, LRI was varied with plasma concentration of CCK. This implies that CCK might be involved in the development of leptin resistance.

Several mechanisms for leptin resistance have been reported: Impaired leptin transport across the blood-brain barrier[33-38] and the presence of negative regulators of leptin signaling[39-42]. CCK1 receptor antagonist increased plasma leptin content by preventing plasma leptin from entering into the central nerves system via a specific leptin transport system located both in the blood brain barrier and in the choroid plexus[43-46]. Our data suggests that the decreased level of plasma CCK might be involved in the development of leptin resistance. The deficiency of CCK1 receptor in OLETF rats might cause leptin resistance by decreasing the production of CCK or by inhibiting its distribution to the brain.

In the present study, we demonstrated that CCK was significantly associated with the effect of leptin on body weight in OLETF rats. The decreased level of CCK in OLETF rats attenuated the inhibitory effect of leptin on gaining body weight by enhancing leptin resistance. Thus, the effect of ghrelin is more dominant than that of leptin, thereby manifesting obesity in OLETF rats. It is critically important to reveal the mechanism on the modulation of the effects of leptin by CCK in order

to establish the prevention and treatment of obesity.

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