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Original Article

Obesity-related hypertension and enhanced plasma orexin-A level are attenuated by the consumption of thiamine water in diabetic rats under cerebral oxidative stress conditions

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ABSTRACT — Orexin-A has been suggested to control hypertension, feeding behavior, and obesity. We recently established that long-term consumption of thiamine water by obese diabetic rats leads to reduced obesity and metabolic disorders. In addition, we found that drinking thiamine water daily may modulate oxidative stress-related diseases, such as diabetes and its complications. In the present study, we focused on obesity-related hypertension and plasma orexin-A levels in Otsuka Long–Evans Tokushima Fatty (OLETF) rats under oxidative stress conditions and assessed their cerebral ADP-ribosylated protein expression after drinking thiamine water. The thiamine water-drinking group was administered 2 g thiamine/L in drinking water. Plasma orexin-A content was measured by ELISA testing. ADP-ribosylated protein expression was analyzed in the brain of OLETF rats using Western blotting. Primary experimental characteristics, body weight, and caudal blood pressure were similar among the groups. However, at 28 weeks of thiamine water-drinking, significant decreases in body weight and systolic blood pressure were observed in the diabetic-thiamine group compared to those in the diabetic-control group. Moreover, obese diabetic rats exhibited increased plasma orexin-A levels and poly-ADP-ribosylated protein levels in the brain. Notably, the enhanced plasma orexin-A level and cerebral oxidative stress conditions of the obese diabetic rats were attenuated by drinking thiamine water. The relationship between consumption of thiamine in drinking water and obesity-related hypertension and cerebral oxidative stress status via modulation of plasma orexin-A levels requires further investigation. It is noteworthy that the upregulation of orexin signaling may not only cause hypertension, but also maintain obesity in polyphagia-induced OLETF rats.

Key words: Thiamine, Plasma orexin-A level, Cerebral oxidative stress, Polyphagia-induced OLETF rat, Obesity, Hypertension

INTRODUCTION

Obesity is currently a worldwide pandemic (Ng *et al*., 2014; WHO, 2016, 2017; Afshin *et al*., 2017; Kohda, 2018). The rates of obesity and overweight are increasing annually. Obesity is poorly controlled and potentially hazardous to human health. Obesity is also a major modifiable risk factor for type 2 diabetes, which is also an epidemic (Tuomilehto *et al*., 2001; Knowler *et al*., 2002; Chiasson *et al*., 2002; Kawamori *et al*., 2009; DeFronzo *et al*., 2011; Muramoto *et al*., 2014). Further, obesity is a major contributor to the most predominant causes of premature death and disability, including cardiovascular disease (Hubert *et al*., 1983; Chei *et al*., 2008; Berlin and

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Colditz, 1990; Lu *et al*., 2014; Peirson *et al*., 2014), cerebral brain infraction (Rexrode *et al*., 1997; Bazzano *et al*., 2010; Bodenant *et al*., 2011), non-alcoholic fatty liver disease (Oza *et al*., 2009; DeLuis *et al*., 2010; Promrat *et al*., 2010; Lee *et al*., 2012), sleep apnea (Peppard *et al*., 2000, 2013; Jordan *et al*., 2014), chronic kidney disease (Foster *et al*., 2008; Thomas *et al*., 2011; Kainz *et al*., 2015), cancer (Renehan *et al*., 2008, 2010), and hypertension (Jamerson *et al*., 2008; Weber *et al*., 2013). Thus, obesity constitutes a global health crisis.

Blood pressure is mainly regulated by the sympathetic nervous system and various hypothalamic and brainstem neuronal groups (Guyenet, 2006; Zubcevic *et al*., 2011; Fisher and Paton, 2012). Orexin-A is a neuropeptide mainly produced by hypothalamic neurons (DeLecea *et al*., 1998; Sakurai *et al*., 1998). This peptide was initially thought to function in the central nervous system, such by regulating the sleep-wake cycle and feeding behavior (Sakurai *et al*., 1998, 2010; DeLecea, 2012). The orexin system is also involved in the regulation of sympathetic nerve activity and blood pressure (Matsumura *et al*., 2001; Shirasaka *et al*., 2002).

The status of B vitamin intake in a healthy population is generally satisfactory; however, some high-risk populations may have decreased intake or increased needs. Vitamin deficiencies are caused by a combination of inadequate ingestion, poor absorption, and increased excretion. Increased production of reactive oxygen species in the brain has been reported in individuals who have thiamine (vitamin B1) deficiency (Langlais *et al*., 1997). Patients with diabetes are reportedly thiamine-deficient, which is caused by the excretion of thiamine in the urine via osmotic diuresis; however, metabolic consumption of thiamine may be attributable to increased glucose metabolism in hyperglycemia (Yui *et al*., 1980; Seligmann *et al*., 1991).

We recently established that long-term consumption of thiamine water by obese diabetic rats leads to reduced obesity and metabolic disorders (Tanaka *et al.,* 2010; Kohda *et al.,* 2012, 2017). In addition, we found that drinking thiamine water daily may modulate oxidative stress-related diseases, such as diabetes and its complications (Kohda *et al.,* 2019). Orexin-A has been suggested to control hypertension, feeding behavior, and obesity (Sakurai *et al.,* 1998; Shirasaka *et al*., 1999; Zhou *et al*., 2015). In this study, we investigated the effects of the consumption of thiamine in drinking water on the ability of orexin to control blood pressure responses and on cerebral oxidative stress in a rat model of polyphagia-induced obesity.

MATERIALS AND METHODS

Chemicals

Thiamine hydrochloride was supplied by Kishida Chemical Co., Ltd. (Osaka, Japan). The Orexin-A ELISA kit was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). A glucose pilot meter and blood glucose test strips (Aventir Biotech, Carlsbad, CA, USA) were used for blood glucose testing. Anti-poly [adenosine diphosphate (ADP)-ribose] polymer antibody was purchased from Tulip Bio Labs (Lansdale, PA, USA). Horseradish peroxidase-conjugated anti-mouse IgG antibody was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Mammalian tissue lysis and extraction reagents and protease inhibitor cocktail were purchased from Sigma (St Louis, MO, USA). Blocking solution, signal enhancer solution, and enhanced chemiluminescence reagent were supplied by Nacalai Tesque (Kyoto, Japan). All other chemicals were of the highest purity available from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

Animals and experimental design

The animals were handled as per the institutional guidelines for animal research, and the experimental work was approved by the Experimental Animal Research Committee of Osaka University of Pharmaceutical Sciences. We chose Otsuka Long–Evans Tokushima Fatty (OLETF) rats that exhibit progressive obesity and metabolic disorders similar to that in human metabolic syndrome. Male OLETF rats (Japan SLC, Inc., Shizuoka, Japan) weighing 110–125 g and aged 5 weeks in the beginning, were used. Furthermore, Long–Evans Tokushima Otsuka (LETO) rats, non-obese/lean counterparts of OLETF rats, weighing 80–95 g and aged 5 weeks in the beginning, were also used for this study. The rats were housed in the animal facility in cages, received standard diet, and had access to water ad libitum; they were kept under temperature and humidity-controlled conditions with 12-hr/12-hr light/dark cycles. OLETF rats were randomly allocated to each the following drinking-water groups: drinking of thiamine water and drinking of tap water. The thiamine water-drinking group was administered 2 g thiamine/L in the drinking water. Individual daily water intake was recorded for OLETF and LETO rats. The body weights of the rats, as an assessment of obesity, were measured throughout the study period. Glucose levels, as an assessment of diabetes, were measured using blood collected from the tail vein of the rats. Glucose levels were determined using a blood glucose test strip and the random blood glucose level was measured in obese diabetic OLETF rats.

Blood pressure measurement

The non-invasive blood pressure parameter of systolic blood pressure was monitored using the tail-cuff method. The caudal blood pressure of obese and normal rats was measured with a tail-cuff blood pressure apparatus (BP-98A; Softron, Tokyo, Japan). Obese OLETF rats were supplemented with thiamine water and tap water from 5–38 weeks of age. In each drinking-water paradigm, at 5 and 33 weeks of age, blood pressure parameters were measured using the tail-cuff machine in the OLETF and LETO rats.

Preparation of protein extracts from rat brain

The obese and normal rats were sacrificed at 38 weeks of age. The rats were anesthetized with 50 mg/kg pentobarbital, and blood samples were collected from the ventral aorta. The whole brain tissue was collected immediately after the rats were exsanguinated. These tissues were homogenized at 4°C in tissue lysis and extraction reagent containing a protease inhibitor cocktail. The homogenates were centrifuged at 15,000 rpm for 15 min, and the supernatants were used for Western blot analysis to examine the poly ADP-ribosylated protein expression in the brain for oxidative stress assessment.

Western blot analyses

Protein samples were separated by 4–20% polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes. The membranes were blocked with blocking buffer for 1 hr at room temperature and incubated with the specific primary anti-poly (ADP-ribose) polymer antibody in signal enhancer solution overnight at 4°C. After washing the membranes three times with 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.1% Tween 20 to remove unbound antibodies, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody in signal enhancer solution for 1 hr at room temperature. Chemiluminescence for ADP-ribosylated protein expression was detected with the Ez-Capture MG machine (ATTO Corp., Tokyo, Japan) using enhanced chemiluminescence reagent.

Plasma orexin-A level measurement

Blood samples were collected from the ventral aorta into heparin tubes. The plasma was separated from whole blood by centrifugation in a refrigerated bench-top centrifuge (Kubota Corp., Tokyo, Japan), and plasma aliquots were stored at −80°C until analysis. Plasma orexin-A content was measured by enzyme-linked immunosorbent assay (ELISA) using orexin-A test kits. The kit does not cause cross-reaction with orexin-B. Plasma orexin-A assays were performed in duplicate. We also performed the spike and recovery test by ELISA using plasma samples from each drinking water group.

Statistical analyses

Data are expressed as the means ± S.E. Statistical analyses of the data from multiple groups were performed by analysis of variance followed by Tukey tests. All statistical analyses were performed using Pharmaco Basic software (Scientist Press Co., Ltd., Tokyo, Japan). A p-value < 0.05 was considered to indicate statistically significant results.

RESULTS

Effects of follow-up thiamine water-drinking on daily water intake and body weight gain in obese diabetic rats

At the start of water-drinking (thiamine or tap water) at 5 weeks of age, each obese diabetic rat had similar water intake and body weight level. The daily water intake and body weight of these rats was higher than that of the normal-control rats throughout the experimental period. However, substantial differences were observed in the daily water intake and body weight from the 28th week of drinking thiamine water until the end of 33 weeks of age in the study groups (Figs. 1 and 2). The body weights of the rats in the thiamine water, obese diabetic group were lower than those in the rats allocated to the tap water, obese diabetic group (Fig. 2).

Effects of drinking thiamine water daily on blood pressure parameters of the obese diabetic rats

Blood pressure parameters were recorded using the tail-cuff non-invasive method during the experimental period. The analysis of monitoring blood pressure parameter in obese diabetic rats showed an increase in the systolic blood pressure from 5 to 33 weeks of age (Fig. 3), suggesting the influence of increased body weight gain under obese condition (Fig. 2). The systolic blood pressure of obese diabetic rats in the tap water-drinking group at 33 weeks of age was higher than that in the non-obese diabetic rats. Notably, systolic blood pressure levels were significantly lower in obese diabetic rats that consumed thiamine water (Fig. 3).

Plasma orexin-A and random blood glucose levels in obese diabetic rats

We examined the levels of plasma orexin-A in diabetic rats that were overweight and had high blood pressure. The plasma orexin-A levels tended to be higher in

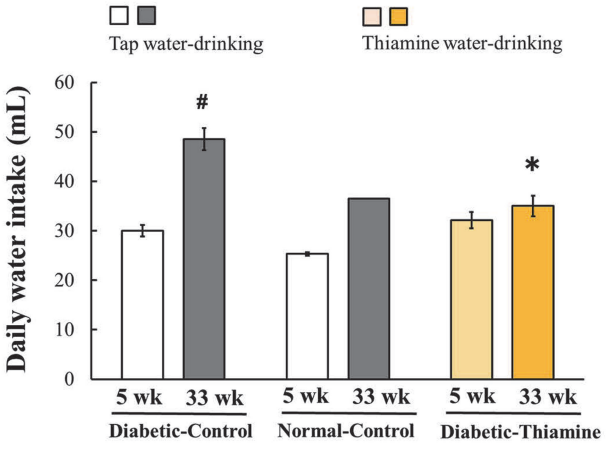

Fig. 1. Average daily water intake from 5 to 33 weeks of age by Otsuka Long–Evans Tokushima Fatty (OLETF) and Long–Evans Tokushima Otsuka (LETO) rats. Effect of drinking thiamine water on daily water intake at 5 and 33 weeks of age in OLETF rats with 2 g thiamine/L of drinking water. OLETF and LETO rats were randomly divided into the following groups: tap water-drinking OLETF group (diabetic-control, n = 6), thiamine water-drinking OLETF group (diabetic-thiamine, n = 6), and tap water-drinking LETO group (normal-control, n = 2/3). Significant differences were observed between the diabetic-control and diabetic-thiamine groups at 33 weeks of age. Significant differences were observed between the diabetic-control groups at 5 to 33 weeks of age. Each value represents the mean ± S.E. values. *p < 0.01 compared to the diabetic-control group at 33 weeks of age. #p < 0.01 compared to the diabetic-control group at 5 weeks of age.

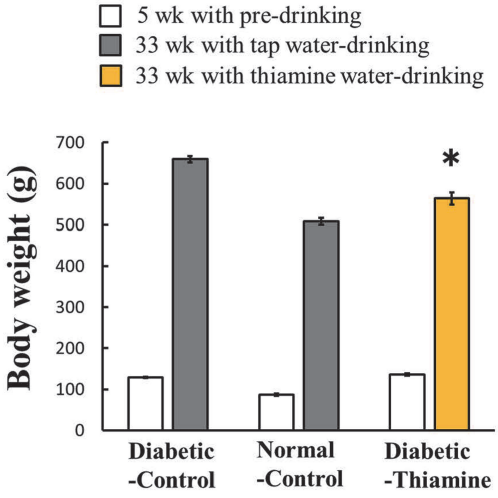

Fig. 2. Body weight changes from the pre-drinking stage (5 weeks of age) and at week 28 of thiamine supplementation (33 weeks of age) in Otsuka Long–Evans Tokushima Fatty (OLETF) and Long–Evans Tokushima Otsuka (LETO) rats. Effect of drinking thiamine water on body weight change from 5 to 33 weeks of age in OLETF rats with 2 g thiamine/L of drinking water. OLETF and LETO rats were randomly divided into the following groups: tap water-drinking OLETF group (diabetic-control, n = 6), thiamine water-drinking OLETF group (diabetic-thiamine, n = 6), and tap water-drinking LETO group (normal-control, n = 3). Significant differences were observed between the diabetic-control and diabetic-thiamine groups at 33 weeks of age. Each value represents the mean ± S.E. values. *p < 0.01 compared to the diabetic-control group at 33 weeks of age.

obese diabetic rats than in nonobese diabetic normal rats (Fig. 4A). Notably, in obese diabetic rats, the level of plasma orexin-A was significantly lower in the group that consumed thiamine versus that in the group consuming tap water (Fig. 4A).

The random blood glucose levels tended to be higher in obese diabetic rats than in nonobese diabetic normal rats (Fig. 4B). However, drinking of thiamine water had no effect on the random blood glucose levels of obese diabetic rats (Fig. 4B).

Cerebral poly ADP-ribosylated protein expression in obese diabetic rats

The expression ADP-ribosylated protein was analyzed in the brain of obese diabetic rats and normal rats by western blot analysis. A band positive for the anti-poly (ADP-ribose) polymer indicated the presence of ADP-ribosylated protein in the rat brain. Poly-ADP-ribosylated protein expression was higher in the tap water-drinking obese diabetic group than in the normal control group (Fig. 5). Cerebral poly-ADP-ribosylated protein expression was lower in the thiamine water-drinking group than in the tap water-drinking group among obese diabetic rats (Fig. 5).

DISCUSSION

We recently determined the beneficial effect of long-term consumption of thiamine water in obese diabetic rats (Kohda *et al.,* 2012, 2017; Tanaka *et al.,* 2010). Daily consumption of water containing thiamine may enable modulation of oxidative stress-related diseases, such as diabetes and its complications (Kohda *et al.,* 2019). In this study, after consuming thiamine in drinking water for 28 weeks, body weight gain and high blood pressure were significantly ameliorated in obese diabetic rats. Thus, reductions in body weight may improve the systolic blood pressure of obese diabetic rats.

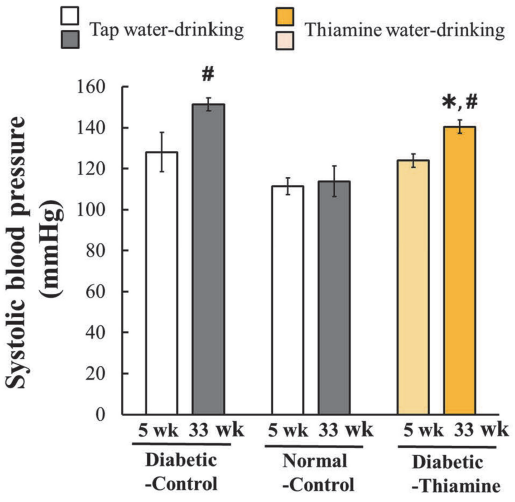

Fig. 3. Systolic blood pressure level from 5 to 33 weeks of age in Otsuka Long–Evans Tokushima Fatty (OLETF) and Long–Evans Tokushima Otsuka (LETO) rats. Effect of drinking thiamine water on systolic blood pressure at 5 and 33 weeks of age in OLETF rats with 2 g thiamine/L in the drinking water. OLETF and LETO rats were randomly divided into the following groups: tap water-drinking OLETF group (diabetic-control, n = 6), thiamine water-drinking OLETF group (diabetic-thiamine, n = 6), and tap water-drinking LETO group (normal-control, n = 2/3). Significant differences were observed between the diabetic-control and diabetic-thiamine groups at 33 weeks of age. Significant differences were observed between the diabetic-control groups at 5 to 33 weeks of age. Each value represents the mean ± S.E. values *p < 0.05 compared to the diabetic-control group at 33 weeks of age. #p < 0.05 compared to each diabetic group at 5 weeks of age.

Vitamins are natural constituents of food, and a well-balanced diet supplies all required vitamins. Most required vitamins cannot be produced in the body. Weight control and regular vitamin intake have health beneficial effects. Employing a combination of these strategies has benefits beyond blood pressure regulation. Vitamin supplementation has become popular among consumers for preventing or delaying illnesses (Lukaski, 2004; Ball, 2006). We recently found that despite the equivalent amount of food consumed, thiamine supplementation decreased the extent of body weight gain in obese diabetic rats (Kohda *et al.,* 2012; Tanaka *et al.,* 2010). This interesting finding could have great significance for reduction in obesity if thiamine administration shows a similar effect on human metabolism.

In this study, we examined the level of plasma orexin-A in diabetic rats that were overweight and had high blood pressure. Moreover, the cerebral oxidative stress status was assessed. In the target brain, under oxidative stress conditions, cerebral proteins are susceptible to obese diabetic reactive oxygen species-induced protein modifications, such as protein ADP-ribosylation. Obese diabetic rats exhibited increased plasma orexin-A levels and poly-ADP-ribosylated protein levels in the brain. The plasma orexin-A and cerebral oxidative stress levels in obese diabetic rats were decreased by drinking of thiamine-containing water. Our results suggest that thiamine-water drinking may exert a brain oxidative stress response by controlling the plasma orexin-A levels. Orexin was shown to elicit cardiovascular responses, hypertension, feeding behavior, and obesity (Sakurai *et al.,* 1998; Shirasaka *et al*., 1999; Zhou *et al*., 2015). Moreover,

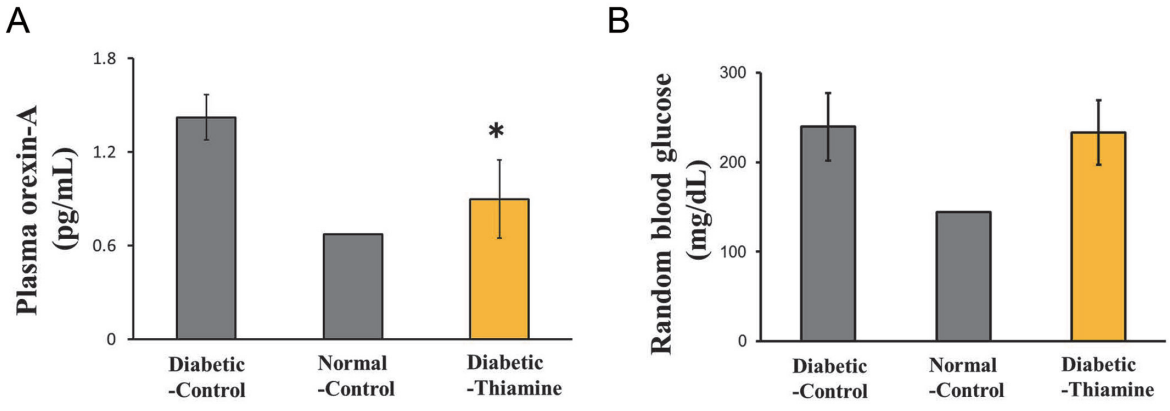

Fig. 4. Level of plasma orexin-A (A) and random blood glucose (B) in 38-week-old Otsuka Long–Evans Tokushima Fatty (OLETF) and Long–Evans Tokushima Otsuka (LETO) rats that consumed tap water or thiamine water with 2 g thiamine/L for 33 weeks. OLETF and LETO rats were randomly divided into the following groups: tap water-drinking OLETF group (diabetic-control, n = 6), thiamine water-drinking OLETF group (diabetic-thiamine, n = 6), and tap water-drinking LETO group (normal-control, n = 2). Each value represents the mean ± S.E. values. *p < 0.05 compared to the diabetic-control group.

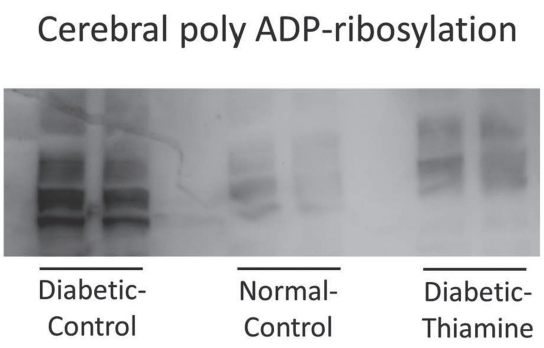

Fig. 5. Expression of ADP-ribosylated protein in the brain of 38-week-old Otsuka Long–Evans Tokushima Fatty (OLETF) and Long–Evans Tokushima Otsuka (LETO) rats that consumed tap water or thiamine water with 2 g thiamine/L for 33 weeks. OLETF and LETO rats were randomly divided into the following groups: tap water-drinking OLETF group (diabetic-control), thiamine water-drinking OLETF group (diabetic-thiamine), and tap water-drinking LETO group (normal-control). Brain samples were subjected to SDS-PAGE followed by western blot analysis with a specific antibody against the poly (ADP-ribose) polymer. Antibody-positive bands indicate the presence of poly-ADP-ribosylated protein in the brain.

orexin was reported to be involved in oxidative stress responses (Greene *et al*., 2016). The effects of thiamine consumption on obesity-related hypertension and cerebral oxidative stress via the modulation of plasma orexin-A levels require further investigation.

We found that an increased plasma orexin-A level was associated with high blood pressure in obese diabetic OLETF rats. We evaluated OLETF rats, which were developed by Kawano *et al*. (1991, 1992). Polyphagia-induced OLETF rats lack functional receptors for cholecystokinin-A, which is associated with satiety control mechanisms (Kawano *et al.,*1991,1992; Moran and Bi, 2006). Orexin-A was originally identified as a factor that enhanced feeding behavior (Sakurai *et al.,* 1998). We demonstrated that the plasma orexin-A level was significantly enhanced with increases in body weight gain and systolic blood pressure in obese OLETF rats. Our results suggest that upregulation of plasma orexin-A plays important roles in obesity-induced hypertension in obese OLETF rats.

In contrast, some studies showed that orexin-deficient animals are susceptible to obesity development (Hara *et al*., 2001, 2005). It has been reported that counterintuitive to the acute effect of orexin on promoting feeding, orexin deficiency causes obesity in animals, suggesting that it negatively regulates energy metabolism (Hara *et al*., 2001, 2005). In our study, thiamine supplementation decreased body weight despite the equivalent amount of food consumed by the OLETF rats (Kohda *et al.,* 2012; Tanaka *et al.,* 2010). In this study, thiamine consumption had no effect on the hyperglycemia of OLETF rats. Polyphagia-induced OLETF rats may not exhibit satiety, suggesting that cholecystokinin activation in the central satiety centers could not show satiety behavior regardless of the amount of food consumed. When orexin signaling is upregulated, OLETF rats might not exhibit the ability to self-regulate their food consumption. OLETF rats may have never stopped eating under lasting upregulation of orexin signaling. Thus, orexin-A may function in negative feedback under hyperglycemic conditions in normal rats but not in obese diabetic OLETF rats. It is noteworthy that the upregulation of orexin signaling may not only cause hypertension, but also maintain obesity in polyphagia-induced OLETF rats.

Conflict of interest---- The authors declare that there is no conflict of interest.

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