Involvement of Neuropeptide Y in Hyperphagia in Human Growth Hormone Transgenic Rats

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ABSTRACT. We have previously produced human growth hormone (hGH) transgenic (TG) rats that show low circulating levels of both hGH and endogenous rat GH. Although body length of the TG rats is normal, they develop hyperphagia and severe obesity. The present study was undertaken to elucidate the causes of hyperphagia in the TG rats by focusing on temporal changes in plasma ghrelin levels and hypothalamic neuropeptide Y (NPY) contents. In both wild-type (WT) and TG rats, the highest value of plasma ghrelin levels was observed just before the dark phase, and thereafter plasma ghrelin levels were maintained higher in the TG than WT rats. Although NPY contents also showed the peak level just before the dark phase in both the arcuate (ARC) and paraventricular nuclei (PVN) of the hypothalamus, the values in the ARC, but not the PVN, of the TG rats was always lower than those of the WT rats, suggesting increased transport of NPY from the ARC to PVN in the TG rats. In addition, treatment with antagonists for Y1 and Y5 receptors for NPY reduced food intake much more effectively in the TG than WT rats. Intermittent treatment with recombinant hGH for a week significantly decreased food consumption, adipose tissue weight and plasma triglyceride concentrations in the TG rats. These results suggest that, in the TG rats, insufficiency in circulating GH stimulates the ghrelin-NPY system with a resultant increase in food intake.

KEY WORDS: ghrelin, growth hormone, hyperphagia, neuropeptide Y, transgenic rat.

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Growth hormone (GH) is secreted from the pituitary gland in a pulsatile manner and plays an important role in regulating metabolism as well as somatic growth [8, 10]. The actions of GH on metabolism involve both anabolism, such as protein synthesis in the muscle, and catabolism, such as lipolysis in the adipose tissue. Therefore, the role of GH is very important in terms of maintaining the balance of body composition. Indeed, GH-deficient (GHD) subjects become obesity with short stature and this obesity in GHD subjects is improved by GH-replacement therapy [9, 29]. Since the subjects of Prader-Willi syndrome (PWS), a type of GHD, typically represent hyperphagia [14], GH may also be involved in maintaining normal feeding.

We have previously generated transgenic (TG) rats expressing human GH (hGH) gene under the control of mouse whey acidic protein promoter [18]. Contrary to our expectation, serum hGH levels in the TG rats were relatively low and endogenous pulsatile secretion of rat GH was eliminated. While their body length was similar to that of the wild-type (WT) rats, the TG rats developed severe obesity [15] and hyperphagia [12], suggesting that they suffered from GHD. Additionally, the TG rats showed several symptoms that are linked to non-insulin dependent diabetes mellitus, e.g. hyperglycemia, hyperinsulinemia, and hypertriglyceridemia [16]. The TG rats, therefore, appear to be a good experimental model for the study of hyperphagia and resulting obesity and metabolic diseases under the condition of GHD.

Recently, ghrelin, a 28 amino acid peptide with n-

octanoylation in the 3rd serine residue, was isolated from stomach in humans and rats. Ghrelin was identified as a ligand of GH secretagogue receptor, and shown to stimulate the secretion of GH from the pituitary [24]. Its *n*-octanoylation in the 3rd serine residue was shown to be mandatory for the stimulatory effect on GH secretion. Furthermore, it became clear that ghrelin had a potent orexigenic action [27]. Ghrelin administered peripherally or centrally promoted not only GH secretion but also food consumption and body weight gain [24]. The orexigenic effect of ghrelin is thought to be mediated by hypothalamic peptides such as neuropeptide Y (NPY) and agouti-related protein (AgRP) [27]. NPY is known to be one of the most potent orexigenic factors [13]. NPY-producing neurons are located in the arcuate nucleus (ARC) of the hypothalamus and mainly project their fibers to the paraventricular nucleus (PVN) [2]. Although there are subtypes from Y1 to Y6 in NPY receptors, the Y1 and Y5 receptors are regarded to participate in the regulation of food intake [7, 19, 22, 23].

The present study was conducted to elucidate whether the ghrelin-NPY system is involved in hyperphagia in the TG rats with low circulating GH. To this end, diurnal changes in food intake, plasma ghrelin concentrations and NPY contents in the ARC and PVN were simultaneously examined. The effects of antagonists for Y1 and Y5 receptors, as well as recombinant hGH (rhGH), on food intake were also assessed.

MATERIALS AND METHODS

Animals: Generation of TG rats has been described previously [18]. Throughout the present study, the female TG

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rats (heterozygotes) and their WT littermates were used. They were housed in a room at controlled temperature of 23°C with a lighting schedule of 12 hr light/dark (light on at 0700 h). After being weaned from mothers at 3 weeks of age, they were individually housed with free access to laboratory chow and water. Body weight was recorded once a week from 4 to 14 weeks of age and food consumption was recorded from 5 to 14 weeks of age ($n=6$ for each group). At 12 weeks of age, all the WT and TG rats were ovariectomized under ether anesthesia. Two weeks after surgery, food consumptions over 24 hr were recorded every 2 hr ($n=6$ for each group), and the animals were further subjected to the experiments described below. All the experiments were conducted according to the Guideline for the Care and Use of Laboratory Animals, the University of Tokyo.

Determination of plasma ghrelin: For the measurements of plasma ghrelin, WT and TG rats ($n=5-7$ for each group) were decapitated at 1100, 1500, 1700, 1900 and 2300 hr. Blood samples were collected to the tubes containing EDTA·2Na (1.25 mg/ml) and aprotinin (Trasylol, Bayer AG, Munich, Germany, 500 U/ml blood), and centrifuged at $1,800 \times g$ for 20 min at 4°C. After centrifugation, plasma samples were added with 1N HCl (10% volume) immediately, and stored -80°C until used. Plasma ghrelin concentrations were measured using a commercial active ghrelin ELISA kit (Mitsubishi Kagaku Iatron, Inc., Tokyo, Japan), which did not recognize des-acyl ghrelin, following the manufacturer instructions.

Determination of NPY in the hypothalamus: WT and TG rats were decapitated at 1100, 1500, 1700, 1900 and 2300 hr ($n=4-6$, for each point), and the hypothalamic regions containing exclusively the ARC and the PVN were dissected out. The tissue samples containing the ARC and PVN were obtained using a brain matrix (RBM-4000c, Activational Systems, Inc., Forterra, MI). With the ventral surface of the brain facing up in the brain matrix that was prechilled on ice, a blade was inserted into a slot of the brain matrix at the place of 4.1 mm posterior from the bregma. The second blade was inserted into a slot 2 mm rostral from the first blade, and the third blade was inserted into a slot 1 mm rostral from the second blade. The tissue block containing the ARC was trimmed approximately 2 mm square at the ventral part of the third ventricle from the first slice, and the tissue block containing the PVN was trimmed approximately 1 mm square at the dorsal part of the third ventricle from the second slice. Each of the tissue samples were as homogenized with 0.1 N HCl and centrifuged at $18,000 \times g$ for 30 min at 4°C, and the supernatants were collected and stored -80°C until used. Amount of protein of the supernatants was measured by Bradford method (Protein Assay kit, Bio-Rad, Hercules, CA). The NPY assay was performed using commercial NPY RIA kit (Peninsula Laboratories, INC, San Carlos, CA) according to the assay protocol supplied by the manufacturer.

Administration of NPY Y1- and Y5-receptor antagonists: Antagonists for Y1-receptor [22] and Y5-receptor [23] were kindly supplied by Banyu Pharmaceutical Co., Ltd., Japan.

The WT and TG rats ($n=6$ for each group) were intraperitoneally (i.p.) administered with vehicle, 10 and 30 mg/kg body weight (BW) of antagonists (dissolved in ethanol/polyethylenglycol 600/saline, 10/25/65) at 1800 hr, and the food consumption of the whole dark phase (1900–0700 hr) was measured.

Administration of rhGH: The TG rats ($n=6$) were administered i.p. with rhGH (Novo Nordisk, Bagscaerd, Denmark) at a dose of 100 $\mu\text{g}/\text{rat}$ in 0.3 ml saline 4 times a day at 4 hr intervals between 0700 and 1900 hr for 7 days as described previously [15]. As a control, WT ($n=6$) and TG rats ($n=9$) were administered saline (0.3 ml) alone. During the experiment, food consumption was measured daily. Three hour after the last injection (2,200 hr on day 7), the animals were decapitated and their blood samples were collected. Body weight and the weight of corpus adiposum ovarii (CAO) were recorded, and plasma triglyceride concentrations were measured with commercial kit (Wako, Osaka, Japan).

Statistical analysis: All the data was analyzed by ANOVA followed by Tukey-Krameris test as a post-hoc test. Graphed data are expressed as mean $\pm$ SE. P values less than 0.05 were considered as statistically significant.

RESULTS

Food consumption in TG rats: In the TG rats, body weight (Fig. 1A) and food consumption (Fig. 1B) were significantly larger than those in the WT rats throughout the observation period between 4 and 14 weeks of age. These results were in consistent with our previous results in male rats [11, 15]. Food consumption of every 2 hr over 24 hr is shown in Fig. 2A. In both WT and TG rats, food consumption evidently increased at the beginning of the dark phase, which was the largest value throughout 24 hr, and there was not a significant difference in 2 hr food consumption between WT and TG rats. However, cumulative food consumption during the light phase, dark phase and whole day was significantly increased in the TG rats compared with the WT rats (Fig. 2B).

Plasma ghrelin concentrations: Changes in plasma ghrelin concentrations between 1100 and 2300 hr are shown in Fig. 3. At 1100 hr, plasma ghrelin concentration was significantly lower in TG than in WT rats. In both WT and TG rats, the highest value was observed just before the dark phase (1700 hr). Thereafter, plasma ghrelin concentrations were maintained higher in the TG than in WT, and the difference at 1900 hr was significant.

Hypothalamic NPY contents: Changes in NPY contents in brain tissues containing the ARC and PVN between 1100 and 2300 hr are shown in Fig. 4A and B, respectively. In the ARC of WT rats, NPY contents significantly increased at 1700 hr than before, and thereafter the contents declined. Similar changes were observed in NPY contents in the ARC of TG rats, but the values in TG were always lower than those in WT rats. In the PVN, the highest contents of NPY were also observed at 1700 hr in both WT and TG rats, the

Fig. 1. Changes in body weight (A) and food consumption (B) in the wild-type (WT) and transgenic (TG) rats with age. Each symbol and vertical bar represents mean $\pm$ SE (n=6). * $p < 0.05$ vs. WT.

Fig. 2. Food consumption every 2 hr for over 24 hr (A) and that during the light phase, dark phase and whole day (B) in wild-type (WT) and transgenic (TG) rats. Shaded area in (A) shows the dark phase. Each symbol, column and vertical bar represents mean $\pm$ SE (n=6). * $p < 0.05$ vs. WT.

values of which were significantly higher than those at 1100 and 1900 hr. There were no significant difference in NPY contents in the PVN between WT and TG rats except at 1700 hr, when the content was significantly higher in TG than in WT rats.

Effects of NPY antagonists on food consumption: The effects of Y1 and Y5 antagonists on food consumption of the dark phase are shown in Fig. 5A and B, respectively. In the WT rats, Y1 antagonist at a dose of 30 mg/BW significantly decreased food consumption, though that of 10 mg/BW did not affect food consumption. In the TG rats, Y1 antagonists at doses of 10 and 30 mg/BW significantly suppressed food consumption in a dose-dependent manner. On the other hand, Y5 antagonist at all the doses used did not affect food consumption in the WT rats. In contrast, Y5 antagonist at a dose of 30, but not 10 mg/BW significantly decreased food consumption.

Effects of rhGH administration on TG rats: Changes in

food consumption following rhGH or saline is shown in Fig. 6A. Food consumption in TG rats injected with saline was significantly larger than that in WT rats injected with saline throughout the experimental period. Treatment with rhGH significantly decreased food consumption of TG rats at days 5 and 6. There was no difference in food consumption between WT rats with saline treatment and TG rats with rhGH treatment at day 6. Body weight, adipose tissue (CAO) weight and plasma triglyceride levels were significantly greater in TG than WT rats (Figs. 6B, C and D). Although treatment with rhGH for 7 days did not affect the body weight of TG rats, it significantly decreased adipose tissue weight and plasma triglyceride levels.

DISCUSSION

In the present study, the increase in body weight and food consumption was confirmed in female TG rats, which is

Fig. 3. Changes in plasma ghrelin concentrations in the wild-type (WT) and transgenic (TG) rats between 1100 and 2300 hr. Shaded area represents the dark phase. Each symbol and vertical bar represents mean $\pm$ SE ($n=5-7$). * $p<0.05$ vs. WT. Values with different characters are significantly different ($p<0.05$).

consistent with our previous observations in male TG rats [12, 15]. The largest food consumption was observed right after the beginning of the dark phase (1900–2100 hr), which followed the peak levels of plasma ghrelin just before the dark phase (1700 hr), in both the WT and TG rats. The peak levels of plasma ghrelin before the dark phase was also reported by Murakami *et al.* [26], which may be related to the increase in food intake at the beginning of the dark phase. Although there was no difference in the peak values

of plasma ghrelin at 1700 hr between the WT and TG rats, the level at 1900 hr was significantly higher in the TG than WT rats. The level at 2300 hr was also higher in the TG than WT rats, though not significantly. This increase in plasma ghrelin levels in the TG rats may account for hyperphagia during the dark phase in the TG rats.

The contents of NPY in both the ARC and PVN were also highest at 1700 hr, which was consistent with a previous report by Jhanwar-Uniyal *et al.* [21] who showed that there was a peak in NPY contents in both the ARC and the parvocellular subdivision of the PVN just before the dark phase. These peaks of hypothalamic NPY levels were probably due to the increase in plasma ghrelin levels at this time, since it is known that ghrelin can increase mRNA expression and peptide production of NPY [30, 33]. Although the pattern of changes in NPY contents in the hypothalamic nuclei was similar between the WT and TG rats, the levels in the ARC were always higher in the WT than TG rats and those in the PVN at 1700 hr were higher in the TG than WT rats. The precise reasons for these differences in NPY contents in the hypothalamus currently remain unclear. However, since we have observed in males that NPY mRNA expression in the ARC of the TG is not different from that of the WT (our unpublished observation), we speculate that the differences in NPY contents are not attributable to the difference in NPY synthesis, but to the difference in the excitability of NPY neurons that may stimulate the transport of NPY from the ARC to PVN and its release from the PVN.

In support of above assumption, antagonists for both the Y1 and Y5 receptors for NPY were much more effective in TG than WT rats. This suggests that hyperphagia observed in the TG rats largely depends on the NPY system in the hypothalamus. It has been shown that NPY stimulates food intake by acting on the NPY receptors expressed in neurons in the PVN [4]. In addition, it has been also demonstrated that the orexigenic action of ghrelin disappears by adminis-

Fig. 4. Changes in NPY contents in the hypothalamic arcuate (ARC) (A) and paraventricular nuclei (PVN) (B) in the wild-type (WT) and transgenic (TG) rats between 1100 and 2300 hr. Shaded area represents the dark phase. Each symbol and vertical bar represents mean $\pm$ SE ($n=4-6$). * $p<0.05$ vs. WT. Values with different characters are significantly different ($p<0.05$).

Fig. 5. Effects of NPY Y1 (A) and Y5 (B) receptor antagonists on food consumption in the wild-type (WT) and transgenic (TG) rats. Animals were intraperitoneally administrated with vehicle, 10 and 30 mg/kg body weight of antagonists at 1800 hr, and the food consumption during the whole dark phase (1900–0700 hr) was measured. Each column and vertical bar represents mean $\pm$ SE (n=6). Values with different characters are significantly different ($p<0.05$).

Fig. 6. Changes in daily food consumption (A), and resultant body weight (B), corpus adiposum ovarii (CAO) weight (C), and plasma triglyceride concentrations (D) following rhGH treatment. The wild-type (WT) and transgenic (TG) rats were treated with saline or rhGH at a dose of 100 μ g/rat 4 times a day at 4 hr intervals between 0700 and 1900 hr for 7 days. Each symbol, column and vertical bar represents mean $\pm$ SE (n=6–9). * $p<0.05$ vs. WT/Saline. † $p<0.05$ vs. TG/Saline. Values with different characters are significantly different ($p<0.05$).

tration of Y1 or Y5 receptor antagonists [27]. Taken together, it is suggested that, in the TG rats, the excitability of NPY neurons in the ARC is facilitated during the dark phase at least partially by the increase in plasma ghrelin levels, and NPY released in the PVN in turn increases food intake through the Y1 and Y5 receptors.

In the present study, intermittent administration of rhGH decreased adipose tissue weight and plasma triglyceride levels in the TG rats, indicating that this treatment effectively caused lipolysis. This treatment also decreased food intake, suggesting that hyperphagia is caused at least partially by a decreased GH action in the TG rats. It has been shown that episodic administration of GH in rats decreased stomach ghrelin mRNA and plasma ghrelin levels [28], while hypophysectomy increased plasma ghrelin levels [31]. In human study, it is known that plasma ghrelin levels are high in PWS subjects who show GHD [14], whereas they are low in acromegalic subjects [5]. Therefore, although there are still some negative reports showing that GH treatment does not modify ghrelin secretion [20] and ghrelin levels are not influenced by GH levels in GHD subjects [25], endogenous GH appears to exert a negative feedback effect on stomach ghrelin production and secretion. If this is the case, intermittent treatment with rhGH may decrease plasma ghrelin levels, and thereby reduced food intake in the TG rats.

As discussed above, the present study suggests that the low circulating levels of GH are the primary cause for hyperphagia in the TG rats. At present, however, it is still difficult to simply correlate food intake with GH levels. For example, it has been reported that GH administration to intact rats increases food consumption [1], and that GH replacement increases NPY mRNA expression in the hypothalamus of hypophysectomized rats [6]. It may be noteworthy here to mention, however, that PWS subjects with GHD often develop eating disorders. Since there are several common features between PWS subjects and the TG rats used in the present study, including hyperphagia, obesity and gonadal dysfunction as well as GHD [3, 17, 32], dysfunction within GH-ghrelin-NPY system may also take part in eating disorders in PWS subjects. The TG rats could be used as good experimental model for the study of eating disorders and related metabolic diseases originating in inappropriate GH secretion like PWS.

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