Corticotropin-releasing hormone (CRH) transgenic mice display hyperphagia with increased Agouti-related protein mRNA in the hypothalamic arcuate nucleus

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Abstract. Although glucocorticoid-induced hyperphagia is observed in the patients with glucocorticoid treatment or Cushing’s syndrome, its molecular mechanism is not clear. We thus explored the expression of neuropeptide mRNAs in the hypothalamus related to appetite regulation in CRH over-expressing transgenic mice (CRH-Tg), a model of Cushing’s syndrome. We measured food intake, body weight (including body fat weight) and plasma corticosterone levels in CRH-Tg and their wild-type littermates (WT) at 6 and 14 weeks old. We also examined neuropeptide Y (NPY), proopiomelanocortin (POMC) and Agouti-related protein (AgRP) mRNAs in the arcuate nucleus (ARC) using in situ hybridization. Circulating corticosterone levels in CRH-Tg were markedly elevated at both 6 and 14 weeks old. Body fat weight in CRH-Tg was significantly increased at 14 weeks old, which is considered as an effect of chronic glucocorticoid excess. At both 6 and 14 weeks old, CRH-Tg mice showed significant hyperphagia compared with WT (14w old: WT 3.9±0.1, CRH-Tg 5.1±0.7 g/day, p<0.05). Unexpectedly, NPY mRNA levels in CRH-Tg were significantly decreased at 14 weeks old (WT: 1571.5±111.2, CRH-Tg: 949.1±139.3 dpm/mg, p<0.05), and there were no differences in POMC mRNA levels between CRH-Tg and WT. On the other hand, AgRP mRNA levels in CRH-Tg were significantly increased compared with WT at both ages (14w old: WT 356.6±88.6, CRH-Tg 660.1±87.2 dpm/ mg, p<0.05). These results suggest that glucocorticoid-induced hyperphagia is associated with increased hypothalamic AgRP. Our results also indicate that hypothalamic NPY does not have an essential role in the increased food intake during glucocorticoid excess.

Key words: Cushing’s syndrome, Appetite, Obesity, Glucocorticoid, Agouti-related protein
similar to those of Cushing’s syndrome, it is proposed that glucocorticoid excess contributes to the pathogenesis of both states [10-12]. Obesity in Cushing’s syndrome is characterized by increased appetite [13] and body weight, especially in visceral-fat-dominant obesity with reduced lean mass.

There are several reports on the role of glucocorticoid-induced hyperphagia; central administration of glucocorticoid increases both food intake and hypothalamic neuropeptide Y (NPY) mRNA levels [14]. Furthermore, adrenalectomy reverses obese phenotype in ob/ob mice with a decrease in Agouti-related protein (AgRP) and an increase in proopiomelanocortin (POMC) mRNA levels in the hypothalamus [15]. These findings suggest that the central neuropeptides could be involved in the glucocorticoid-induced hyperphagia. Recently, Christ-Crain et al. has reported that systemic administration of glucocorticoid induced visceral obesity with increased hypothalamic adenosine monophosphate-dependent kinase (AMPK) activity [16]. However, the molecular mechanism of glucocorticoid-induced hyperphagia is not completely understood.

In this study, we tried to address this issue using the corticotropin-releasing hormone (CRH) transgenic overexpression mice (CRH-Tg), an animal model of Cushing’s syndrome with glucocorticoid excess [17]. We examined the mRNA expression levels of the appetite-related neuropeptides such as NPY, POMC and AgRP in the hypothalamic arcuate nucleus (ARC) both in CRH-Tg and wild-type littersmates. We found that CRH-Tg showed hyperphagia with an increase in hypothalamic AgRP mRNA, but unexpectedly, a decrease in NPY mRNA levels. The present results suggest that AgRP, but not NPY, could be responsible for the glucocorticoid-induced hyperphagia.

Materials and Methods

Animals

We used CRH-Tg, an animal model of Cushing’s syndrome, which was characterized previously [17], and also their wild-type littersmates (WT). All the experiments were carried out using male mice. All mice were maintained under controlled conditions of light (lights on, 0600-1800 h) at 24°C in a humidity-controlled room, and are allowed free access to standard chow and drinking water. All procedures were approved by the animal committee of Kochi Medical School.

Evaluation of the metabolic parameters in CRH-Tg mice

Body weights of CRH-Tg and WT littermates were measured once a week, until the end of the experiment. Their food weights were also measured, and mean daily food intake corrected by their body weight during the final week before decapitation were determined as an index of food consumption. For the determination of blood hormone levels, CRH-Tg mice and their WT littersmates (6 or 14 weeks old, n=6-8 in each group) were decapitated between 0900-1200 h. Trunk blood was collected for measuring plasma corticosterone and insulin levels using commercially available RIA/ELISA kits (corticosterone RIA kit, MP Biomedicals, OH, USA, and insulin ELISA kit, Morinaga Inc., Japan). Blood glucose levels were also determined by the enzymic method (FreeStyle: Nipro Inc., Japan).

In situ hybridization

After decapitation, the brain of each mouse was quickly removed and frozen by immersion in 2 methyl butane at –30 °C, and then stored at –80 °C until the tissue was sectioned on a cryostat. Frozen brain tissue was cut coronally in 20-µm thick sections for the in situ hybridization of the arcuate nucleus (ARC). The sections were taken from the following sites: ARC 1.40-1.90 mm posterior from the bregma. The sections were then thaw-mounted and air-dried on gelatin-coated slides, and were stored at –80 °C prior to the in situ hybridization.

For NPY, POMC and AgRP, a synthetic 48-base or 45-base oligodeoxynucleotide probe for each peptide mRNA was used (Table 1). The probes were labeled with [α-35S] dATP (>1000 Ci/mmol, Perkin Elmer Inc., Waltham, MA, USA) using terminal deoxynucleotidyl transferase (25 units/mL, Boehringer-Mannheim Biochemicals, Indianapolis, IN, USA) and tailing buf-

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Oligonucleotide sequence</th>
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<tbody>
<tr>
<td>NPY</td>
<td>5’-GTC CTC TCG TGG CGC GTC CTC GCC CGG ATT GTC CGG CTT GGA GGG GTA - 3’</td>
</tr>
<tr>
<td>POMC</td>
<td>5’-GCC CAC CGG CTT GCC CCA GCG GAA GTG CTC CAT GGA GTA GGA GGC CTT - 3’</td>
</tr>
<tr>
<td>AgRP</td>
<td>5’-TGC AGC AGA ACT TCT TCT GCT CGG TCT GCA GTT GTC TTC TTG AGG - 3’</td>
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Hyperphagia and AgRP increase in CRH-Tg

CRH-Tg shows hyperphagia with glucocorticoid excess

CRH-Tg showed marked hyperphagia compared with WT littermates at both 6 and 14 weeks old after correction by their body weight (Fig. 3). Absolute food consumption in CRH-Tg was significantly higher at 14 weeks old.

Hypothalamic mRNA levels of NPY, POMC and AgRP in CRH-Tg

NPY mRNA levels in the ARC were similar between CRH-Tg and WT at 6 weeks old, but, unexpectedly, those in CRH-Tg were significantly lower compared with those in WT littermates at 14 weeks old (Fig. 4). There were no differences in POMC mRNA levels in the ARC between WT and CRH-Tg at both ages (Fig. 5). In contrast, AgRP mRNA levels in the ARC in CRH-Tg were significantly higher compared with those in WT littermates at both 6 and 14 weeks (Fig. 6). NPY and AgRP mRNAs at 14 weeks old were tendency to decrease compared with those at 6 weeks old, which correspond to aging-related changes in hypothalamic neuropeptides [20, 21].

Discussion

In this study, we aimed to identify the hypothalamic neuropeptide(s), which is involved in the development of glucocorticoid-induced hyperphagia. The obtained data showed that CRH-Tg displayed hyperphagia with increased AgRP mRNA levels in the hypothalamic ARC. Obesity in the patients with glucocorticoid treatment or Cushing’s syndrome is characterized by increased energy intake [13]. Recent neuroendocrinological studies have clarified the involvement of a number of hypothalamic neuropeptides in appetite control, among which NPY, POMC-related peptide(s) and AgRP are assumed to play important roles [22, 23]. Indeed, in an animal model of Cushing’s syndrome, we observed significant increase in the expression of hypothalamic AgRP. These results support the idea that AgRP, an orexigenic neuropeptide, could be responsible for the increased appetite during glucocorticoid excess. Although CRH is known to be an anorexigenic neuropeptide [24, 25], CRH-Tg displayed hyperphagia and obesity, suggesting that glucocorticoid excess overcomes the effect of CRH in the
Fig. 1  Plasma concentrations of corticosterone (A) and insulin (B) in WT littermates (control; white bars) and CRH-Tg (black bars) at 6 and 14 weeks old (n=6-8, in each group). Values are means ± SEM. *p<0.05 vs. WT littermates.

Fig. 2  Body weight (A) and epididymal fat/body weight ratio (B) in WT littermates (control; white bars) and CRH-Tg (black bars) at 6 and 14 weeks old (n=6-8, in each group). Values are means ± SEM. *p<0.05 vs. WT littermates.

Fig. 3  Mean daily food intake (A) and mean daily food intake corrected by body weight (B) in WT littermates (control; white bars) and CRH-Tg (black bars) at 6 and 14 weeks old (n=6-8, in each group). Values are means ± SEM. *p<0.05 vs. WT littermates.
Fig. 4  NPY mRNA levels in the ARC (A) and film autoradiography of in situ hybridization for NPY mRNA in the ARC (B) in WT littermates (control; white bars) and CRH-Tg (black bars) at 6 and 14 weeks old (n=6-8, in each group). Values are means ± SEM. *p<0.05 vs. WT littermates.

Fig. 5  POMC mRNA levels in the ARC (A) and film autoradiography of in situ hybridization for POMC mRNA in the ARC (B) in WT littermates (control; white bars) and CRH-Tg (black bars) at 6 and 14 weeks old (n=6-8, in each group). Values are means ± SEM.

Fig. 6  AgRP mRNA levels in the ARC (A) and film autoradiography of in situ hybridization for AgRP mRNA in the ARC (B) in WT littermates (control; white bars) and CRH-Tg (black bars) at 6 and 14 weeks old (n=6-8, in each group). Values are means ± SEM. *p<0.05 vs. WT littermates.
animal model of Cushing’s syndrome. Further studies (e. g. effect of adrenalectomy) are necessary to distinguish glucocorticoid effect from central CRH over-expression in CRH-Tg.

Melanocortin 4 receptor (MC4R) plays a crucial role in the regulation of feeding behavior, as observed with both genetic and pharmacological technologies [26, 27]. MC4Rs on the hypothalamic neurons receive both agonistic and antagonistic signals. α-melanocyte-stimulating hormone (α-MSH) derived from the POMC gene stimulates the receptors and suppresses food intake with subsequent weight loss [28]. In contrast, AgRP, coexpressed in NPY neurons, is an endogenous antagonist of α-MSH and it promotes food intake and positive energy balance [29]. In the previous studies, adrenocortical insufficiency induced by either adrenalectomy or POMC-deficiency reduced the expression of hypothalamic AgRP mRNA, which were reversed by glucocorticoid replacement [15, 30, 31]. Our obtained data are in accordance with the reports, showing that the expression of hypothalamic AgRP mRNA was significantly higher in CRH-Tg with increased food intake. Since the expression levels of hypothalamic POMC were not changed, and those of hypothalamic NPY were rather decreased, we assumed that AgRP could be a key molecule in the glucocorticoid-induced hyperphagia and central obesity. Shimizu et al. have reported that glucocorticoid increased both AgRP gene expression and AMPK phosphorylation in hypothalamic culture, suggesting that glucocorticoid increased AgRP mRNA involving AMPK [32]. However, Claret et al. showed that AgRP mRNA levels in the ARC did not differ between mice lacking AMPKα2 in the AgRP neurons and their WT littermates [33]. Altogether, it is absolutely necessary to clarify the molecular mechanism(s) how glucocorticoid regulates AgRP gene expression in vivo.

It has been generally believed that NPY is one of the key molecule in glucocorticoid-induced hyperphagia [14, 32, 34], unexpectedly, however, we observed hypothalamic NPY mRNA expressions in CRH-Tg were rather decreased. This is of interest because NPY and AgRP are known to be co-expressed within the same neuronal cells of the ARC [35]. It is also recognized that AgRP is regulated more dynamically compared with NPY [15, 31], supporting the different regulation of NPY and AgRP gene within the same cell. Furthermore, it has been reported the possibility that CRH negatively regulates hypothalamic NPY expressions [36], which could explain the decrease of NPY mRNA in CRH-Tg. Intracerebroventricular injection of CRH will clarify direct effect of CRH on these hypothalamic neuropeptides. Our present results also indicate that NPY is not essential in the regulation of glucocorticoid-induced hyperphagia.

It is well known that glucocorticoid exerts negative effect on POMC mRNA in the anterior pituitary gland, but its effect on hypothalamic POMC expression is not completely understood. Indeed, the effects of adrenalectomy and/or glucocorticoid replacement on the expression of POMC mRNA in the ARC are contradictory [15, 30, 31]. In our current study, we could not find differences in hypothalamic POMC mRNA levels between CRH-Tg and WT littermates.

Considering the fact that glucocorticoid excess significantly contributes to the pathogenesis not only in Cushing’s syndrome but also in the metabolic syndrome [10-12], it is possible that hypothalamic neuropeptide(s) associated with glucocorticoid-induced hyperphagia play a role in the development of simple obesity. Accumulating evidences and our present study suggest that AgRP could be a key molecule in the glucocorticoid-induced hyperphagia and obesity. In addition, further studies will clarify the roles of other neuropeptides such as ghrelin, melanin-concentrating hormone, orexin, and other factor(s) in glucocorticoid-mediated appetite regulation.

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