$Ins2^{Akita}$ mice exhibit hyperphagia and anxiety behavior via the melanocortin system

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Abstract. Elevated anxiety symptoms have been reported to be present in many patients with diabetes mellitus. The underlying mechanisms by which diabetes mellitus influences behavior remain to be determined. We assessed feeding and anxiety behaviors in spontaneously diabetic $Ins2^{Akita}$ mice. We measured blood glucose, body weight, and food and water intakes in C57BL/6 heterozygote $Ins2^{Akita}$ mice. The behavioral properties of $Ins2^{Akita}$ mice were assessed in an open-field test and an elevated plus-maze. The gene expression of hypothalamic neuropeptides was examined in non-food-deprived $Ins2^{Akita}$ mice. Body weights of the $Ins2^{Akita}$ mice were less than those of the age-matched C57BL/6 mice, as controls. Food and water intakes were increased in the $Ins2^{Akita}$ mice. In the open-field test, the $Ins2^{Akita}$ mice had decreased locomotor activity and increased immobilization time. The $Ins2^{Akita}$ mice exhibited anxiety behavior in the elevated plus-maze. RT-PCR analysis showed decreased proopiomelanocortin (POMC) mRNA expression and increased agouti-related protein (AGRP) mRNA expression in $Ins2^{Akita}$ mice. There were no significant differences in hypothalamic ghrelin mRNA expression. These observations indicate that $Ins2^{Akita}$ mice, which are characterized by hypoinsulinemia and hyperglycemia, exhibited hyperphagia and anxiety behavior; the mechanism of action involved the activation of hypothalamic AGRP and the inactivation of hypothalamic POMC. In addition, $Ins2^{Akita}$ mice are a useful model for understanding the mechanisms involved in the psychological complications of diabetes mellitus. Further, melanocortin systems may be therapeutic targets not only for diabetes but also for its associated complications.

Introduction

Diabetes mellitus currently affects more than 150 million adults worldwide (http://www.eatlas.idf.org). As a result of poor glycemic control, it can be a risk factor for various diseases, including myocardial infarction, stroke, Alzheimer's disease, bone fractures, and cancers of the colorectum, liver, pancreas, bladder and breast (1-5). It has been reported that elevated anxiety symptoms are present in 40% of patients with diabetes, with no conspicuous difference in prevalence between those with Types 1 and 2 (6). Anxiety is associated with poor glycemic control, but reversely its treatment improves glycemic control (7-9). A recent meta-analysis indicated that diabetes mellitus is a risk factor for depression (10). However, the underlying mechanisms through which diabetes mellitus influences psychological status remain to be determined.

$Ins2^{Akita}$ mice have an autosomal dominant mutation in the insulin 2 gene which results in hyperglycemia and hypoinsulinemia which are detectable after 4 weeks of age (11,12). The gradual accumulation of malfolded proinsulin-2 in the endoplasmic reticulum of pancreatic ß-cells causes the progressive loss of ß-cells due to proteotoxicity. Consequently, juvenile-onset hyperglycemia and hypoinsulinemia occur in the absence of obesity. Few studies have investigated the potential for the use of $Ins2^{Akita}$ mice as a model of diabetic complications. In the present study, we assessed feeding and anxiety behaviors in spontaneously diabetic $Ins2^{Akita}$ mice.

Materials and methods

Animals. The study protocol was approved by the Animal Research Ethics Committee of Kyoto University's Institutional Review Board. Animals were housed and handled according to the guidelines of the Animal Research Committee, Graduate School of Medicine, Kyoto University. We used male C57BL/6 heterozygote $Ins2^{Akita}$ mice and age-matched male C57BL/6 mice as controls (7-8 weeks of age; Japan SLC Inc., Shizuoka, Japan). The mice were housed individually in a regulated environment. A standard diet (F-2, 3.73 kcal/g; Funahashi Farm Corp., Chiba, Japan) was provided. Food and water were available *ad libitum* throughout this study. Mice were used for behavioral experiments after they were adapted to laboratory conditions for at least 7 days. Food and water intakes were measured for 24 h. Blood glucose was measured using the glucose oxidase method (Precision QID, MediSense Com., Tokyo, Japan).

Open-field test. Emotivity was tested using the standard open-field test (13,14). The apparatus consisted of a circular

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arena with diameters of 60 cm at the base and 80 cm at the top and a wall with a height of 50 cm. The apparatus was painted gray with black lines on the bottom, which divided the open-field into 25 parts of similar area by two concentric circles and a series of radii. The open-field test was conducted during the light phase of the light/dark cycle. A white noise provided a deep and uniform sonorous background. The open-field session involved placing a mouse in the center circle and monitoring its movement for 3 min. The following items were recorded: i) the time taken to leave the center circle; ii) the locomotor activity, in terms of the number of partitions crossed; iii) the time of immobilization; iv) the frequency of rearing; v) the frequency of grooming; and vi) the frequency of defecation.

Elevated plus-maze test. Anxiety was assessed in the standard elevated plus-maze 50 cm above the ground (15). The four arms were 27 cm long and 6 cm wide. Two opposing arms were enclosed by 15-cm-high walls (closed arms), while the other arms were devoid of walls (open arms). The elevated plus-maze test was conducted between 22:00 and 24:00. Each mouse was placed in the center of the maze facing one of the enclosed arms. The cumulative time spent in each arm and the number of entries into the open or closed arms were recorded during a 5-min test session. The time spent in the open arms was expressed as the percentage of total entry time (100 x open/open + closed) and the number of entries in the open arms was expressed as the percentage of the total number of entries (100 x open/total entries).

Real-time reverse transcriptase-polymerase chain reaction. Mice were sacrificed by cervical dislocation. The hypothalamic block was removed immediately, frozen on dry ice, and stored at -80˚C until preparation of real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Using the RNeasy mini kit (Qiagen Inc., Tokyo, Japan) RNA was isolated from the hypothalamic block. Quantification of mRNA levels was performed with SYBR-Green chemistry (Qiagen Inc.) using a one-step RT-PCR reaction on a sequence detection system (ABI PRISM 7700; Applied Biosystems Japan, Tokyo, Japan). The reaction was performed under the standard conditions recommended by the manufacturer. We used the mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as an internal control. All expression data were normalized to the GAPDH expression level from the same individual sample. The following primers were used for RT-PCR: GAPDH forward, ATGGTGAAGGTCGGTGTGAA and reverse, GAGTG GAGTCATACTGGAAC; neuropeptide Y (NPY) forward, TTTCCAAGTTTCCACCCTCATC and reverse, AGTGGT GGCATGCATTGGT; agouti-related protein (AGRP) forward, GAGTTCCCAGGTCTAAGTCTGAATG and reverse, ATCTAGCACCTCCGCCAAAG; melanin-concentrating hormone (MCH) forward, GGAAGATACTG CAGAAAGATCCG and reverse, ATGAAACCGCTCTC GTCGTT; ghrelin forward, AGCATGCTCTGGATGG ACATG and reverse, GCAGTTTAGCTGGTGGCTTCTT; cocaine- and amphetamine-regulated transcript (CART) forward, GCAGATCGAAGCGTTGCAA and reverse, TTG GCCGTACTTCTTCTCGTAGA; proopiomelanocortin (POMC) forward, GGCTTGCAAACTCGACCTCT and reverse, TGACCCATGACGTACTTCCG; corticotropin-releasing factor (CRF) forward, CGCAGCCCTTGAA TTTCTTG and reverse, TCTGTTGAGATTCCCCAGGC; urocortin 1 (UCN1) forward, ACTGTCCATCGACCTCAC CTTC and reverse, AAGGCTTTCGTGACCCCATA; urocortin 2 (UCN2) forward, CCTCAGAGAGCTCCTCA GGTACC and reverse, GGTAAGGGCTGGCTTTAGAGT TG; and urocortin 3 (UCN3) forward, CGCACCTCCAGA TCAAAAGAA and reverse, GGGTGCTCCCAGCTCCAT.

Table I. Comparison of body weight, blood glucose, food intake, and water intake in Ins2^{Akita} diabetic mice and age-matched C57BL/6 mice as controls.

	C57BL/6	C57BL/6 Ins2^{Akita}
Body weight (g)	26.0±0.66	24.0±0.51[a]
Blood glucose (mg/dl)	128±13.1	544±11.0[b]
Food intake (g/day)	4.48±0.59	8.16±0.61[b]
Water intake (ml/day)	4.68±0.26	33.28±3.36[b]

Results are expressed as mean ± SE (n=6). [a]P<0.05; [b]P<0.01 compared with age-matched C57BL/6 mice as controls by Scheffe's method.

Table II. Open-field behaviors of Ins2^{Akita} diabetic mice.

	C57BL/6	C57BL/6 Ins2^{Akita}
Time to move out (sec)	3.25±1.30	2.32±0.70
Locomotor activity	120.2±6.70	69.5±17.0[a]
Immobility time (sec)	2.61±0.77	17.18±5.86[a]
Rearing	18.33±4.26	6.83±3.18
Grooming	3.50±1.29	2.17±0.83
Defecation	1.17±0.48	1.83±0.48

Results are expressed as mean ± SE (n=6). [a]P<0.05 compared with age-matched C57BL/6 mice as controls by Scheffe's method.

Statistical analysis. Analysis of variance, followed by Scheffe's *post hoc* method, was used to assess differences among groups. Results are expressed as the mean value ± SE. P<0.05 was considered statistically significant.

Results

The heterozygote Ins2^{Akita} mice in the fed state had significantly elevated concentrations of blood glucose compared with the control mice (Table I). Their body weights were significantly less than those of the control animals. These data agree with results from previous reports. Food and water intakes were elevated by ~2- and 7-fold, respectively, compared with control mice.

In the open-field test, Ins2^{Akita} mice showed a significantly decreased locomotor activity and a significantly increased

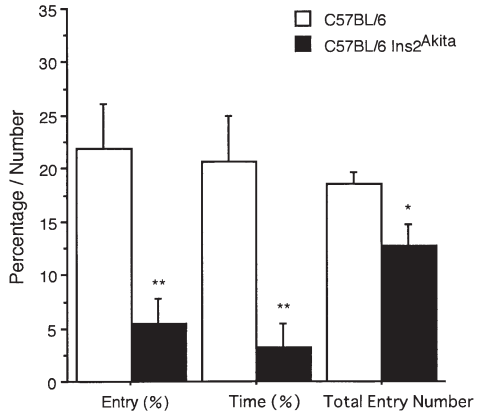

Figure 1. Anxiety behaviors in the elevated plus-maze. Results are expressed as mean ± SE (n=5). *P<0.05; **P<0.01 compared with age-matched C57BL/6 mice as controls by Scheffe's method.

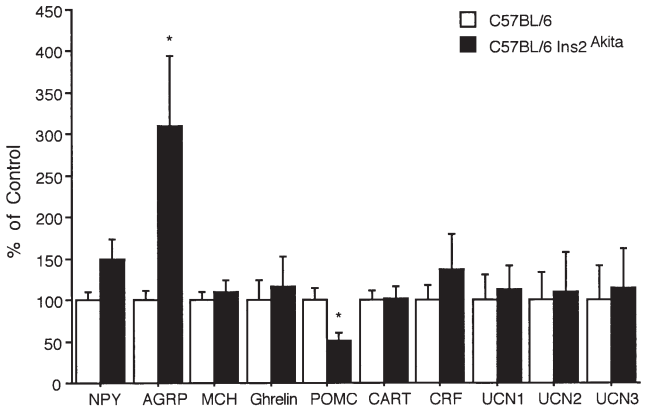

Figure 2. Hypothalamic peptide mRNA levels as assessed by RT-PCR in Ins2Akita diabetic mice, and expressed as the percentage of age-matched C57BL/6 mice as controls. Each bar represents the mean ± SE (n= 5-6). *P<0.05 compared with the C57BL/6 mice as age-matched controls by Scheffe's method.

time of immobilization compared with control mice (Table II). The frequency of rearing showed a tendency to decrease in Ins2Akita mice although this effect failed to reach statistical significance (P<0.056).

Ins2Akita mice exhibited greater anxiety behavior compared with control mice in the elevated plus-maze (Fig. 1). The percentage of the total number (% entry) and the total time (% time) of entries in the open arms were decreased compared with controls. The number of total entries, a crude measure of the overall locomotor activity, was also significantly decreased in Ins2Akita mice compared with controls.

The gene expression of hypothalamic neuropeptides was examined in Ins2Akita mice. RT-PCR analysis showed that the expression of AGRP in Ins2Akita mice was significantly increased by 210% compared with controls (Fig. 2). On the other hand, expression of POMC was significantly decreased by 49% compared with controls. There were no significant differences in hypothalamic ghrelin mRNA expression.

Discussion

This study clearly demonstrated that Ins2Akita mice exhibit hyperphagia and anxiety behavior. Cumulative evidence has demonstrated that abnormalities of eating behavior and mental disorders in patients with diabetes aggravate metabolic control and have a synergistic effect on mortality and the development of complications and disability (16,17). Treatment for eating disorders and depression can result in improved glycemic control as well as improved psychological well-being in patients with diabetes (18,19).

Streptozotocin (STZ)-induced diabetes mellitus is a highly reproducible rodent model of diabetes mellitus (20). Previous studies have reported that STZ-induced diabetic rats exhibited heightened anxiety in various experimental paradigms (21,22). Pretreatment with STZ increased anxiety in an elevated plus-maze test and reduced locomotor activity in an open-field test. Moreover, rat offspring of diabetic mothers showed anxiogenic activity in the elevated plus-maze test (23). We have assessed feeding and anxiety behaviors in a genetic model of spontaneous ß-cell loss in C57BL/6 mice, namely Ins2Akita diabetic mice. Although STZ causes robust hyperglycemia in rodents, pathological changes in organs except the pancreas are generally mild, resembling only the earliest pathological changes observed in humans (24,25). On the other hand, higher doses of STZ may impart greater toxicity in other organs. Moreover, STZ-treated animals tend to have lower blood pressure, which is contrary to characteristics observed in humans with diabetes mellitus (26). Previous reports have shown that there is a definite genetic susceptibility to the development of diabetes mellitus in humans (27,28). Although the metabolic characteristics of diabetic Ins2Akita mice have been characterized to some degree, little is known about the consequences of the mutation on their behavior and the underlying mechanisms thereof.

Since the discovery of leptin and ghrelin, much progress has been made in understanding the regulation of energy homeostasis (29-31). To date, hypothalamic neuropeptides, including NPY, AGRP, MCH, α-melanocyte stimulating hormone (α-MSH), CART, CRF, and urocortin, have been shown to play a crucial role in the regulation of food intake. α-MSH derived from the POMC precursor protein, and AGRP are synthesized in the arcuate nucleus of the hypothalamus and bind to specific melanocortin receptors (MCRs) (32,33). α-MSH suppresses feeding behavior and increases energy expenditure, while AGRP antagonizes the effects of α-MSH at MCR3 and MCR4 and causes hyperphagia (33,34). In our study, Ins2Akita mice exhibited increased AGRP mRNA expression and decreased POMC mRNA expression. Previous studies have reported that insulin activates POMC neurons, which produce α-MSH, thus reducing food intake (33,35). Therefore, hypoinsulinemia decreased the POMC mRNA level and increased the AGRP mRNA level in Ins2Akita mice, resulting in hyperphagic behavior.

It has been reported that inhibition of the melanocortin system may increase sensitivity to stress (36). A(y) mice, in which a spontaneous mutation results in ectopic agouti protein expression, lose more weight in response to stress. Transgenic mice overexpressing agouti protein show increased stress responsiveness, measured by an elevated plus-maze test (37).

These observations suggest that in Ins2^{Akita} mice, the activation of hypothalamic AGRP may play a key role in anxiety behavior. Alternatively, it has been shown that the deleterious mutation of POMC results in adrenal insufficiency (38). Likewise, acquired low POMC production may result in adrenal insufficiency, which has recently been shown to increase the risk of mood disorders (39).

In conclusion, Ins2^{Akita} mice are a useful model for understanding the mechanisms involved in the psychological complications of diabetes mellitus. Further, melanocortin systems may be therapeutic targets, not only for diabetes, but also for its associated complications.

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