Changes of hypothalamic $\alpha$-MSH and CART peptide expression in diet-induced obese rats

De-Run Tian$^{a,b}$, Xiao-Dong Li$^{a,b}$, Yu-Shun Shì$^a$, You Wan$^a$, Xiao-Min Wang$^a$, Jaw-Kang Chang$^c$, Jun Yang$^c$, Ji-Sheng Han$^{a,*}$

$^a$Neuroscience Research Institute, Peking University, 38 Xue-Yuan Road, Beijing 100083, PR China
$^b$Department of Anatomy, Tianjin Medical University, Tianjin 300070, PR China
$^c$Phoenix Pharmaceuticals Inc., Belmont, CA 94002, USA

Received 14 May 2004; received in revised form 10 August 2004; accepted 12 August 2004

Available online 5 October 2004

Abstract

Two hypothalamic peptides, cocaine and amphetamine-regulated transcript (CART) and $\alpha$-melanocyte-stimulating hormone (c-MSH), recognized as anorexigenic neuropeptides to suppress the feeding behavior, were monitored in rats fed with a high-fat (HIF) diet for 14 weeks. While half of the rats developed obesity (diet-induced obese, DIO), some did not (diet resistant, DR). Compared to the DR rats and the control rats (fed with standard chow), DIO rats were accompanied by a markedly higher energy intake and a decrease in the number of neurons carrying $\alpha$-MSH and CART peptide in the arcuate nucleus of the hypothalamus. Failure of hypothalamic anorexigenic peptides CART and c-MSH to increase their content in response to HIF diet may play a key role for overly high energy consumption, resulting in obesity.

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Keywords: Cocaine and amphetamine-regulated transcript peptide; $\alpha$-Melanocyte-stimulating hormone; Diet-induced obesity; High-fat diet; Hypothalamus

1. Introduction

Feeding behavior and body weight are controlled through complex interactions between the central nervous system and peripheral organs. Food intake, especially the meal size, is controlled by a series of short-term hormonal and neural signals which is derived from the gastrointestinal tract, such as cholecystokinin [3] and ghrelin [9,10,26]; while long-term energy stores are signaled by other hormones such as insulin and leptin [16]. These signals seem to modulate the expression of orexigenic and anorexigenic neuropeptides in the hypothalamus and other brain regions, culminating in changes in food intake and energy expenditure for the control of energy homeostasis [14,31]. In recent years, much evidence has accumulated on a number of hypothalamic neuropeptides, including neuropeptide Y (NPY), agouti-related protein, cocaine and amphetamine-regulated transcript (CART) peptide, and proopiomelanocortin (POMC)-derived peptides such as $\alpha$-melanocyte-stimulating hormone (c-MSH) [2]. Both CART peptide and c-MSH have been shown to inhibit feeding behavior when administered centrally. In addition, they increase sympathetic outflow and thermogenic activity resulting in an increased energy expenditure [7,13,25,28].

In animals and humans, a chronic high-fat (HIF) diet with- out a compensatory increase in energy expenditure leads to the progressive development of obesity [11,29]. The model of diet-induced obese (DIO) animal has been proven useful as a model for human obesity [18]. As in much of human obese cases, the animal model of DIO appears to follow a poly- genic mode in inheritance. Thus, the physiological changes observed in this animal model should provide a useful in- sight into the development of obesity in humans. Several previous experiments have dealt with diet-induced changes...
in hypothalamic gene expression of neuropeptides in rodents. Thus, CART mRNA is heavily expressed in the hypothalamus [6], which is reduced by fasting, and also by genetic leptin deficiency and leptin resistance [17,22]. Rodents prone to DIO are accompanied by an elevated level of NPY mRNA and decreased POMC mRNA in the arcuate nucleus (ARC). On the other hand, decreased NPY mRNA and increased POMC mRNA were found in diet resistant (DR) animals [4,19,21,23,35].

To gain further insight into the physiological response of the rat to an HIF diet, we investigated the food intake, energy intake, body weight and the expression of CART peptide and α-MSH in the hypothalamic arcuate nucleus (ARC) of the rats when they were chronically exposed to HIF diet. Emphasis was put on the possible difference in peptide expression between rats that did or did not develop obesity with the same HIF diet (DIO versus DR).

2. Research methods and procedures

2.1. Establishment of DIO model in rats

Three-week-old male (45–55 g) Sprague–Dawley (SD) rats were obtained from Vital Company, Beijing. Animals were housed in a facility with controlled temperature (22 ± 2 °C) and maintained in 12:12 h light-dark cycles (light on from 07:00 to 19:00 h). To acclimatize to the new environment, all rats were fed with standard laboratory chow and water available ad libitum during the first week of the experiment. All procedures were performed in accordance with institutional guidelines of the Animal Care Committee of the Peking University.

Animals were then randomly divided into two groups: (1) the control group (n = 20), fed with standard laboratory chow (Vital Company Beijing), consisting of 5% fat, 55% carbohydrates, 22% protein, 7% ash and 5% fiber (3.80 kcal/g); (2) the HIF diet group (n = 80), fed with HIF diet consisting of 50% standard laboratory chow, 15% lard, 10% sucrose, 5% powdered milk, 10% egg, 5% sesame oil and 5% peanut kernel. The HIF diet consisted of 30% fat, 40% carbohydrate, 15.5% protein, 4% ash, and 3% fiber, containing 4.76 kcal/g. Body weight was monitored every week.

After feeding for 14 weeks, rats in HIF diet-feeding group, showing higher body weights, were assigned to the DIO group (n = 40, 50%), their body weights surpassed the maximum body weights of rats in the control group. Rats of the HIF diet group with lower body weight were assigned to the DR group (n = 13, 16%), their body weights were less than the average of the control group. In the HIF diet group, the rats with body weight falling into the range between the average and the maximum body weights of the control group (n = 27, 34%) were discarded. Ten rats of DIO and DR group respectively, and 20 rats of control group were housed individually, and fed with the corresponding diet. Food intake and body weight were measured daily, for 4 weeks.

2.2. Immunohistochemical procedure

Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and were transcardially perfused with 150 ml of isotonic saline followed by 250 ml of 4% paraformaldehyde. After perfusion, the brains were removed from the skulls and placed in the same fixative overnight at 4 °C. After post-fixation, the brains were kept sequentially in 10, 20 and 30% sucrose solution respectively for 24 h each at 4 °C for cryoprotection, and were then frozen at −70 °C. Serial coronal sections of 40 μm thickness were prepared at ARC level with Cryostat (Leica, Nussloch, Germany). A consistent angle of cut was maintained by examining the shape of the third ventricle and anterior commissure. To ensure reliable comparisons among different groups and to maintain stringent tissue preparation and staining conditions, all the brains from different groups were processed at the same time.

Free-floating staining method was used. Tissue sections were pre-incubated in a solution containing 4% normal goat serum, 1% bovine serum albumin and 0.3% Triton X-100 at 37 °C for 30 min. After washing with PBS, sections were incubated with primary antibodies (rabbit-anti-rat antiserum against α-MSH or CART peptide at a dilution of 1:4000, Phoenix, CA) for 48 h at 4 °C. The sections were then incubated with anti-rabbit biotinylated IgG (1:200, Vector Labs, Burlingame, CA) for 4 h at room temperature. The sections were washed in PBS, and then incubated with the streptavidin–biotin–peroxidase complex (1:200, Vector Labs) for 2 h at room temperature. The sections were then washed in PBS, and then incubated with the streptavidin–biotin–peroxidase complex (1:200, Vector Labs) for 2 h at room temperature. The sections were then developed in 100 mmol/L acetate buffer containing 0.02% 3,3′-diaminobenzidine (DAB, Sigma), 4% nickel ammonium sulphate and 0.03% H2O2 for 10 min at room temperature. All of the incubations and washing steps were carried out on an orbital shaker. After the immunohistochemical procedure, the sections were mounted onto gelatin-coated slides, dehydrated through graded series ethanol solutions followed by xylene.

After coverslip, the sections were examined under an Olympus light microscope. All the α-MSH and CART peptide-immunoreactive neurons were counted under 10× magnification according to the atlas of the rat brain in stereotaxic coordinates by Paxinos and Watson. Counting was done in a blind manner as to the identity of the sample by a single observer. Only cell bodies that clearly exhibited cytoplasmic staining were scored as positive. Six sections were counted for α-MSH or CART peptide positive neurons per brain. Comparisons between groups were performed by analysis of five randomly assigned animals per group, and the cell count in each section was determined from both the left and right sides of the hypothalamic ARC.

2.3. Statistical analysis

Results were expressed as mean ± S.E.M. Food intake, body weight and the number of the immunoreactive neurons of the DIO, DR and control group were analyzed.
using one-way analysis of variance (ANOVA), followed by Newman–Keuls comparison test. Differences were considered statistically significant for $P < 0.05$.

3. Results

3.1. Effect of HIF diet on body weight and energy consumption in DIO and DR rats

Initial average body weight of the rats in the control group (52.2 ± 1.3 g) did not differ significantly from that in the HIF diet group (52.7 ± 0.6 g) ($P > 0.05$). Six weeks later, difference in body weight became apparent between the two groups ($P < 0.05$). At the fifteenth week, the average body weight of the control group was 551.7 ± 10.9 g, compared with 635.3 ± 11.6 g in HIF diet group, which was 14.6% greater than that of the control group ($P < 0.01$) (Fig. 1). In the HIF diet group, the average body weight of DIO rats was 698.9 ± 6.7 g ($n = 40$), as compared to 521.5 ± 6.6 g ($n = 13$) in DR rats ($P < 0.01$). Body weight of DIO rats was 34.0% greater than that of the DR rats ($P < 0.01$) (Fig. 2).

During the period of 16–19 week, a significantly higher energy intake per day was observed in DIO group (144.4 ± 2.9 kcal/day) as compared to the DR (119.7 ± 2.4 kcal/day) and the control group (108.0 ± 4.9 kcal/day) ($P < 0.001$) (Fig. 3A). The amount of food intake in the DIO group (29.1 ± 0.58 g/day) remained at the same level as the control group fed with the standard chow (29.0 ± 1.28 g/day), yet that of the DR group was significantly less than the DIO group (23.6 ± 0.49 g/day) (−17%, $P < 0.001$) (Fig. 3B).

3.2. CART peptide expression in ARC

Typical distributions of CART peptide-immunoreactive neurons in the hypothalamic ARC in the rat brain were shown in Fig. 4. A large number of CART peptide-immunoreactive neuronal perikarya and fibers were observed in the ARC. Compared to DR rats (105.7 ± 3.5 cell/section) and control rats (99.3 ± 4.4 cell/section), a significant reduction in the number of CART peptide neurons was observed in the DIO rats (89.3 ± 2.1 cell/section) ($P < 0.01$) (Fig. 5), showing a decrease of 15.5 and 10.1%, respectively.
3.3. α-MSH expression in ARC

In the ARC of hypothalamus, the number of α-MSH-immunoreactive neurons was less than that of CART-immunoreactive neurons (Fig. 4). Compared to DR rats (51.4 ± 1.0 cell/section) and control rats (57.6 ± 2.5 cell/section), a significant reduction in the number of α-MSH neurons was observed in the ARC of the DIO rats (37.6 ± 1.5 cell/section) (P < 0.001) (Fig. 6), showing a decrease of 26.8 and 34.79%, respectively.

4. Discussion

Obesity, one of the most prevalent diseases in the developed countries, is associated with the consumption of a...
The brain responds to altered energy homeostasis by adjusting feeding behavior and energy expenditure. The hypothalamus has long been recognized as a major site in the central nervous system where a spectrum of internal and external environmental information is integrated for energy homeostasis. Previous studies have examined the physiological regulation exerted by nutritional factors on the hypothalamic neuropeptides involved in energy homeostasis. Thus, acute food deprivation leads to down regulation of POMC mRNA in rodents [5]. Conversely, POMC mRNA expression is up-regulated in overfed rats [12]. Concerning the hypothalamic regulation of POMC mRNA expression in rodents feeding with an HIF diet, Ziotopoulou et al. found a marked increase in POMC mRNA levels, 2 weeks after feeding with an HIF diet in C57Bl/6J mice [39], which are among the more sensitive strains in response to dietary intervention [33,37]. However, Lin et al. demonstrated in the same strain of mice that after 19 weeks of HIF feeding, ARC POMC mRNA levels showed a 55% decrease [23], suggesting that the hypothalamic regulation bears a temporal course of response, i.e. an attempt of compensation in the early stage, and a failure of compensation in the late stage. In other words, when animals are fed with high fat diet chronically, a dysfunction of this compensatory mechanism occurs in some individuals, which leads to obesity.

In contrast to DIO mice, the DR mice showed an increased level of POMC mRNA after 14 weeks on an HIF diet [4]. Since POMC is the precursor of α-MSH, a potent inhibitor of food intake, the hypothalamic over-expression of POMC is a natural feedback mechanism in response to high fat diet, in order to maintain homeostasis of energy balance and body weight.

CART peptide is among the most recently discovered putative peptide neurotransmitter [8,32]. Robson found that the marked decrease in CART mRNA expression in the ARC in gold thioglucose-lesioned mice may contribute to the development of obesity [27]. Central injection of recombinant CART peptide is effective in inhibiting feeding behavior [17], favoring lipid oxidation and the reduction of fat storage both in normal and DIO rats [28]. CART-deficient mice were significantly heavier when fed an HIF fat diet than on a regular chow diet at the 14th week of the feeding studies [7]. Taken together, these data constitute strong evidence of a role for endogenous CART peptides in the regulation of feeding behavior and body weight. To our knowledge, a decrease of CART peptide positive neurons in ARC of DIO rats, shown in the present study, had not been reported before.

It should be noted that if the reduction of food intake of the DR rats is due to a compensatory mechanisms of over expression of CART and/or α-MSH peptide, then there should be an increase of CART/α-MSH positive neurons in hypothalamic ARC compared to normal rats. This was, however, not found in hypothalamic sections. An alternative possibility is that a seemingly unchanged tissue level of the neuropeptide is indeed a result of an accelerated synthesis and an exaggerated release. This uncertainty can only be resolved by the simultaneous assessment of localized peptide release and the level of gene expression.

In conclusion, the effect of long term HIF diet on the feeding behavior of rats seems to have significant individual variation. A small fraction of the rats remained in normal body weight by compensatory reduction of food intake to keep the energy consumption at a constant level. Most of the rats, however, showed an increase of body weight due to an increase of energy metabolism. This variation of response may be sensitive to diet-induced obesity, while others can maintain normal body weight on the same diet. The task of identifying causative or contributory factors is made difficult because of the multi-etiologic nature of obesity and the multiple metabolic perturbations that occur in obese individuals. The rat model of DIO has been useful in studying the role of the brain in regulating feeding behavior and energy homeostasis [15,24,30,38]. The metabolic profile of DIO rats bears resemblance to that of obese humans such as insulin resistance, dyslipidemia and hyperleptinemia [20,30,36].

The major purpose of the present study was to observe whether there is a significant difference in the expression of some anorexogenic neuropeptides (CART peptide and α-MSH) in ARC between DIO and DR rats. It was interesting to note that while there was a general trend of increase in energy intake and body weight in response to chronic HIF diet, SD rats fed with the same diet for 14 weeks diverged into two populations. About half of the rats developed DIO with body weight higher than the highest in the control group, and the rest are within the range of control group. About 16% of the rats were designated as diet resistant (DR) rats according to our stringent criteria (body weight less than the average of control group). It is evident from the dietary record that (a) the consumption of food and energy of the DIO rats was significantly higher than that of the DR rats; (b) the DIO rats took the same amount of food pellets despite the change in diet composition; (c) the DR rats consumed significantly less amount of HIF diet to keep the energy consumption equal to that of the control group. These results clearly show that the development of obesity is accounted for by both environmental factors (e.g., diet composition) and genetic factors (individual variation).

To explore the possible mechanisms, we tried to correlate the changes in the expression of hypothalamic neuropeptides. It was especially noticeable that DIO rats were accompanied by a marked down-regulation of α-MSH and CART peptide-positive neurons in ARC of hypothalamus, relative to the DR and control groups. Since α-MSH and CART peptide are known to have a potent effect of decreasing food intake and body weight [25,34], a reduction in the number of α-MSH and CART peptide-positive neurons in ARC of DIO rats may have contributed to the increase in food intake and body weight.

High-energy diet [30]. A subset of the population appears to be sensitive to diet-induced obesity, while others can maintain normal body weight on the same diet. The task of identifying causative or contributory factors is made difficult because of the multi-etiologic nature of obesity and the multiple metabolic perturbations that occur in obese individuals. The rat model of DIO has been useful in studying the role of the brain in regulating feeding behavior and energy homeostasis [15,24,30,38]. The metabolic profile of DIO rats bears resemblance to that of obese humans such as insulin resistance, dyslipidemia and hyperleptinemia [20,30,36].

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in energy intake. This is accompanied by a reduction in the number of neurons carrying the anorexigenic neuropeptides CART and MSH in the hypothalamus. It is speculated that a dysregulation of the compensatory mechanism of hypotalama-

mic anorexigenic neuropeptides may account for the obesity observed in rats, fed chronically with high fat diet.

**Acknowledgments**

This research was supported by the National Natural Science Foundation of China (30270024) to DRT, and a grant from the Ministry of Education (985 project) to JSH.

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