

Immunohistochemical Changes in Orexigenic and Anorexigenic Neuropeptides in the Rat Hypothalamus after Capsaicin Administration

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(Received 11 May 2009/Accepted 15 June 2009)

ABSTRACT. Capsaicin has effects on the adiposity by increasing energy and lipid metabolism, and decreases appetite and fat intake. In the present study, we investigated changes in food intake and body weight after capsaicin treatment. We also observed changes in orexigenic and anorexigenic neuropeptides-agouti-related peptide (AgRP), α -melanocyte-stimulating hormone (α -MSH), adrenocorticotrophic hormone (ACTH) and orexin-immunoreactivities in the rat hypothalamus after capsaicin administration. Only one day after capsaicin treatment, the mean food intake was significantly decreased. There was no significant difference in the mean body weight between vehicle- and capsaicin-treated groups. In addition, after capsaicin treatment, numbers of AgRP- and orexin-immunoreactive (+) cells were significantly decreased in the arcuate nucleus (ARC) and lateral hypothalamic area, respectively. In contrast, the number of α -MSH⁺ and ACTH⁺ cells in the ARC of the capsaicin-treated rats was higher than in the vehicle-treated rats. These results indicate that capsaicin reduces food intake, not body weight, transiently, and decreases AgRP and orexin immunoreactivities, whereas it increases α -MSH and ACTH immunoreactivities in rat hypothalamic nuclei.

KEY WORDS: arcuate nucleus, capsaicin, lateral hypothalamic area, orexigenic and anorexigenic neuropeptides.

J. Vet. Med. Sci. 71(10): 1337–1342, 2009

Capsaicin is a major pungent ingredient present in a variety of capsicum fruits, such as red peppers [34]. Since its initial identification in 1919, numerous pharmacological effects of capsaicin have been investigated and reported [20, 34, 39]. It has been known that capsaicin reduces adiposity by increasing energy and lipid metabolism in rats and decreases appetite and fat intake in humans [15, 39]. It was recently reported that the capsaicin receptor, vanilloid receptor subtype 1 (VR1), was found throughout the central nervous system, including the hypothalamic nuclei [19, 33].

Many researchers have focused on specific neuropeptides, neurotransmitters, receptors and neuronal circuits that both neuroanatomically and neurophysiologically control food intake in the central nervous system [4, 31, 32]. The hypothalamus is thought to be a major center for controlling feeding behavior in the brain [10]. In many hypothalamic nuclei, the arcuate nucleus (ARC) and the lateral hypothalamic area (LH) are regarded as important centers for regulating food intake and energy homeostasis [4, 10]. In the hypothalamus, many neuropeptides, such as neuropeptide Y, orexin, agouti-related peptide and proopiomelanocortin, are known to participate in regulating food intake [10].

Agouti-related peptide (AgRP) is expressed only in the ARC of the hypothalamus and that AgRP-containing neurons project to various hypothalamic nuclei, such as the

paraventricular nucleus and LH [7]. AgRP is known to be a potent stimulant of food intake and to be involved in the maintenance of feeding behavior [37].

On the other hand, it has been reported that AgRP is an endogenous antagonist of melanocortin receptor (subtype 4) (MC4-R), which functions to control food intake and energy homeostasis in the hypothalamus [9, 12]. Proopiomelanocortin (POMC) is synthesized and expressed in the specific neurons of the ARC in the hypothalamus [8, 12, 23]. POMC is a precursor to some peptides such as adrenocorticotrophic hormone (ACTH) and α -melanocyte-stimulating hormone (α -MSH), and both the α -MSH and ACTH are known to play important roles in the regulation of food intake via the MC4-R [11, 18, 35, 36]. Orexin, a ligand of G-protein coupled receptor, is exclusively expressed in the LH and posterior hypothalamus, especially in the perifornical nucleus [6, 30]. Orexin neurons are known to have a reciprocal connection with various nuclei, including the ARC and to participate in the regulation of feeding behavior, sleep and neuroendocrine homeostasis [5].

Although there are many studies on pharmacological effects of capsaicin, few studies have been reported on its effects on the regulation of food intake and on immunohistochemical changes in orexigenic and/or anorexigenic neuropeptides in the rat hypothalamus. We, therefore, investigated changes in food intake and body weight and in AgRP, α -MSH, ACTH and orexin immunoreactivity in the rat hypothalamus after capsaicin administration.

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MATERIALS AND METHODS

Experimental animals: Forty male Sprague-Dawley rats, at 6 weeks of age, were obtained from Daehan Biolink (Eumsung, South Korea). The animals were housed in a conventional state under adequate temperature (23°C) and humidity (60%) control with a 12-hr light/12-hr dark cycle, and provided with free access to food and tap water. The procedures for handling animals and their care conformed to the guidelines that are in compliance with current international laws and policies (NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85-23, 1985, revised 1996). All of the experiments were conducted to minimize the number of animals used and the suffering caused by the procedures used in the present study.

Capsaicin treatment: Capsaicin (8-methyl-N-vanillyl-6-nonenamide, Sigma, St. Louis, U.S.A.) was given as a vehicle solution consisting of Tween 80 (polyoxyethylenesorbitan monooleate, Sigma), absolute ethanol and saline (10:10:80, v/v). Eye-wiping test was performed to examine the destruction of primary sensory afferents by the capsaicin pretreatment [14, 16, 28]. In brief, one drop of 1% NaOH solution was applied to both eyes by a Pasteur pipette, and the number of eye wipes during a 10-s period after application was recorded. To examine the effect of capsaicin on food intake and body weight, ten rats were injected intraperitoneally once with a 5 mg/kg dose of capsaicin, and the control animals were injected with vehicle. Baseline intake of pelleted chow and the initial body weight were established one day (day 0) before capsaicin or vehicle injection. Food intake and body weight were measured until 10 days following the injection.

Immunohistochemistry: To investigate the effect of capsaicin on immunohistochemistry for orexigenic and anorexigenic neuropeptides in the hypothalamus, ten rats were injected intraperitoneally once with a 5 mg/kg dose of capsaicin, and ten rats were injected with vehicle. All the animals were treated with a stereotaxic intracerebroventricular infusion of colchicine (50 µg/B.W. 100 g) over a period of 10 min under anesthesia with pentobarbital sodium at 6 hr after being injected with capsaicin or vehicle. Forty-eight hours after the colchicine treatment, the animals were anesthetized with pentobarbital sodium and perfused transcardially with 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in 0.1 M PBS (pH 7.4). The brain tissues were sectioned with a cryostat at 30 µm, and consecutive sections were collected in six-well plates containing PBS. The sections were sequentially treated with 0.3% hydrogen peroxide (H₂O₂) in PBS and 10% normal goat serum in 0.05 M PBS. They were next incubated with diluted rabbit anti-AgRP (1:500, Phoenix Pharmaceuticals, U.S.A.), rabbit anti-α-MSH (1:500, Phoenix Pharmaceuticals), rabbit anti-orexin (1:500, Phoenix Pharmaceuticals) or mouse anti-ACTH (1:200, Oncogene, U.S.A.) antiserum 48 hr at 4°C and subsequently exposed to biotinylated goat anti-rabbit or mouse IgG (diluted 1:200, Vector, Burlingame, CA) and streptavidin

peroxidase complex (diluted 1:200, Vector). Then, the sections were visualized by staining with 3,3'-diaminobenzidine (Sigma) in 0.1 M Tris-HCl buffer (pH 7.2) and mounted on gelatin-coated slides. A negative control test was carried out using pre-immune serum instead of primary antibody in order to establish the specificity of the immunostaining. The negative control resulted in the absence of immunoreactivity in all structures. The studied tissue sections were selected according to anatomical landmarks corresponding to Bregma -2.3 ~ -3.3 mm of rat brain atlas [25]. Twenty sections per animal were selected to quantitatively analyze AgRP, α-MSH, orexin and ACTH immunoreactivity. Digital images were captured with an Axiom1 light microscope (Carl Zeiss, Germany) equipped with a digital camera (Axiocam, Carl Zeiss, Germany) connected to a PC monitor. The number of AgRP, α-MSH, orexin and ACTH immunoreactive cells was counted as a mean number per section.

Statistical analysis: The data shown here represent the means ± SEM. Differences among the means were statistically analyzed by two-tailed Student *t*-test in order to elucidate the changes on immunoreactivities and differences on food intake and body weight between vehicle- and capsaicin-treated groups. The results of food intake and body weight were analyzed separately at each individual time point after injection. Statistical significance was considered at *P* < 0.05.

RESULTS

Changes on Food intake and body weight after capsaicin treatment: Before capsaicin treatment, the mean food intake was 27.2 ± 1.8 g and 28.1 ± 2.3 g in the vehicle- and capsaicin-treated group, respectively. One day after capsaicin treatment, the mean food intake was significantly decreased (Fig. 1A). However, there was no difference in food intake between two groups from 2 days after capsaicin treatment (Fig. 1A). In addition, there was no significant difference in the mean body weight between the vehicle- and capsaicin-treated groups (Fig. 1B).

AgRP, α-MSH and ACTH immunoreactivity in the ARC after capsaicin treatment: AgRP, α-MSH and ACTH immunoreactions were found in the ARC of the vehicle- and capsaicin-treated groups, and altered in the ARC after capsaicin treatment. The number of AgRP-immunoreactive (+) neurons in the ARC was significantly decreased in the capsaicin-treated group compared to that in the vehicle-treated group (Table 1, Fig. 2A and 2B). On the other hand, α-MSH⁺ and ACTH⁺ neurons were significantly increased in the capsaicin-treated group compared to those in the vehicle-treated group (Table 1, Fig. 2C-2F).

Orexin immunoreactivity in the LH after capsaicin treatment: Orexin immunoreaction was observed in the LH. In the vehicle-treated group, strong orexin immunoreactivity was observed in cell bodies (Fig. 3A). In the capsaicin-treated group, the number of orexin⁺ neurons in the LH was significantly decreased compared to that in the vehicle-

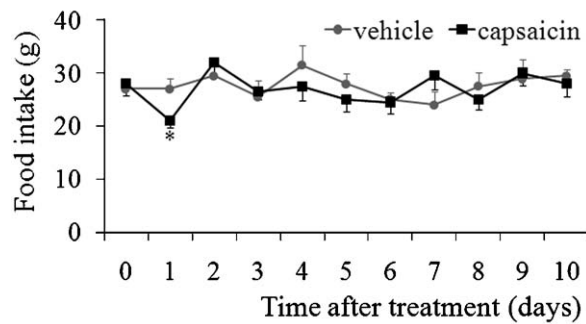
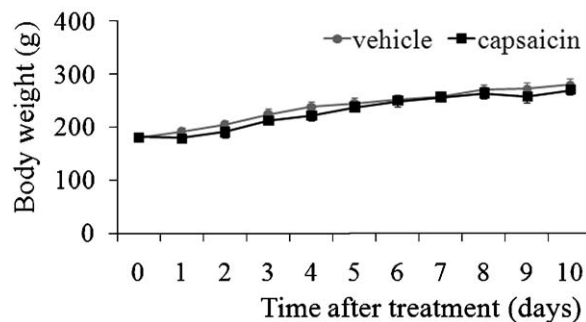
(A) Food intake**(B) Body weight**

Fig. 1. Food intake (A) and body weight (B) after vehicle and capsaicin treatment. Only 1 day after capsaicin treatment, the mean food intake is significantly decreased (A). However, from 2 days after capsaicin treatment, there is no difference in food intake between 2 groups (A). There is no significant difference in the mean body weight between vehicle- and capsaicin-treated groups (B).

treated group (Table 1, Fig. 3B).

DISCUSSION

In the present study, we examined changes in mean food intake and body weight after capsaicin treatment. We found that the mean food intake was significantly decreased at one day after capsaicin treatment. From 2 days after capsaicin treatment, there was no difference in food intake between two groups. Our present result was not inconsistent with other previous studies which capsaicin attenuated the suppression of food intake [16, 28]. On the other hand, it was also reported that capsaicin reduced mean body weight and mean food intake in high-fat diet model [24]. We presumed that this inconsistency was due to capsaicin dose. In this study, we used 5 mg/kg capsaicin, and observed that 5 mg/kg capsaicin did not destruct primary sensory afferents by eye-wiping test.

A previous study showed that the administration of capsaicin decreased the expression of neuropeptide Y (NPY) and increased the expression of cholecystokinin in the rat

Table 1. The number of AgRP, α -MSH, ACTH and orexin immunoreactive neurons in the arcuate nucleus and lateral hypothalamic area of vehicle- and capsaicin-treated rats

	Vehicle-treated rats	capsaicin-treated rats
AgRP	136.5 \pm 5.0	58.1 \pm 4.3*
α -MSH	38.3 \pm 2.5	69.4 \pm 3.4*
ACTH	33.9 \pm 2.3	62.5 \pm 3.0*
orexin	52.7 \pm 3.6	11.6 \pm 0.9*

The number of AgRP, α -MSH and ACTH immunoreactive neurons was counted in the arcuate nucleus of each section.

The number of orexin immunoreactive neurons was counted in the lateral hypothalamic area of each section.

Values indicate means \pm SEM.

* $P < 0.05$, significantly different between vehicle- and capsaicin-treated rats

hypothalamus [17]. They suggested that capsaicin may function to control food intake and obesity. In the present study, we examined immunoreactivities of AgRP, α -MSH, ACTH and orexin in the ARC and LH. The number of AgRP⁺ neurons was markedly decreased after capsaicin treatment. It was reported that the intracerebroventricular infusion of AgRP increased feeding behavior and body weight in rats [29, 37]. It was also reported that AgRP expression was increased in ob/ob mice and that the overexpression of AgRP in transgenic mice resulted in hyperphagia and obesity [23]. In addition, the basal levels of AgRP release and the expression of AgRP mRNA from the hypothalamus were elevated in fasted rats as compared to fed rats [21].

In this study, the number of both α -MSH⁺ and ACTH⁺ neurons in the ARC was significantly higher in the capsaicin-treated group than in the vehicle-treated group. It was reported that the intracerebroventricular administration of α -MSH inhibited food intake and increased energy expenditure in rodents [8, 22, 26, 35]. In addition, when ACTH was injected into the lateral ventricle or into the ventromedial hypothalamus, food intake was reduced in both fed and fasted rats [1, 36]. Its inhibitory effect on food intake was also confirmed in rabbits [2, 26].

We also observed change in orexin⁺ neurons in the LH after capsaicin treatment: The number of orexin⁺ neurons was markedly decreased after capsaicin treatment. It has been known that orexin mRNA levels are elevated in the LH of fasted rats and that the central administration of orexin stimulates food intake in a dose-dependent manner [13, 30, 31]. Therefore, orexin is thought to play a role in the hypothalamic regulation of feeding behavior [3, 6, 30, 31]. It was reported that orexin⁺ neurons in the rat and human LH received a dense innervation from NPY⁺, AgRP⁺ and α -MSH⁺ fibers [7]. Moreover, orexin⁺ neurons are known to project back to POMC⁺ and NPY⁺ neurons in the ARC neurons, where they act as an inhibitor of POMC⁺ neurons and as an activator of NPY⁺ neurons [27, 30]. It was also reported that α -MSH inhibited the stimulatory effect of AgRP [29]. These findings indicate that integrated and reciprocal connections of orexigenic and anorexigenic neu-

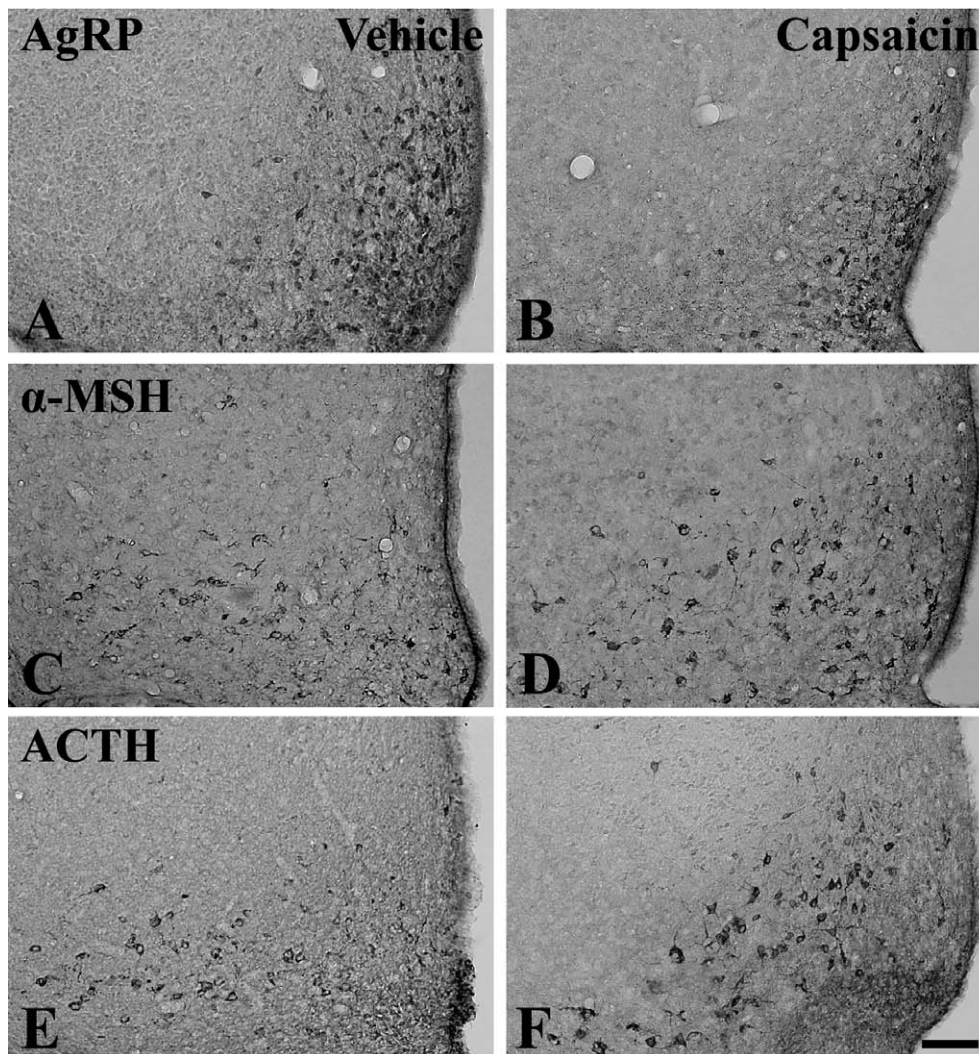


Fig. 2. Immunohistochemistry for AgRP (A, B), α -MSH (C, D) and ACTH (E, F) in the rat ARC. Left column (A, C, E): vehicle-treated group. Right column (B, D, F): capsaicin-treated group. AgRP⁺ neurons in the ARC are decreased in the capsaicin-treated group (B). α -MSH⁺ (D) and ACTH⁺ neurons (F) in the capsaicin-treated group are increased when compared to the vehicle-treated group. Bar=50 μ m.

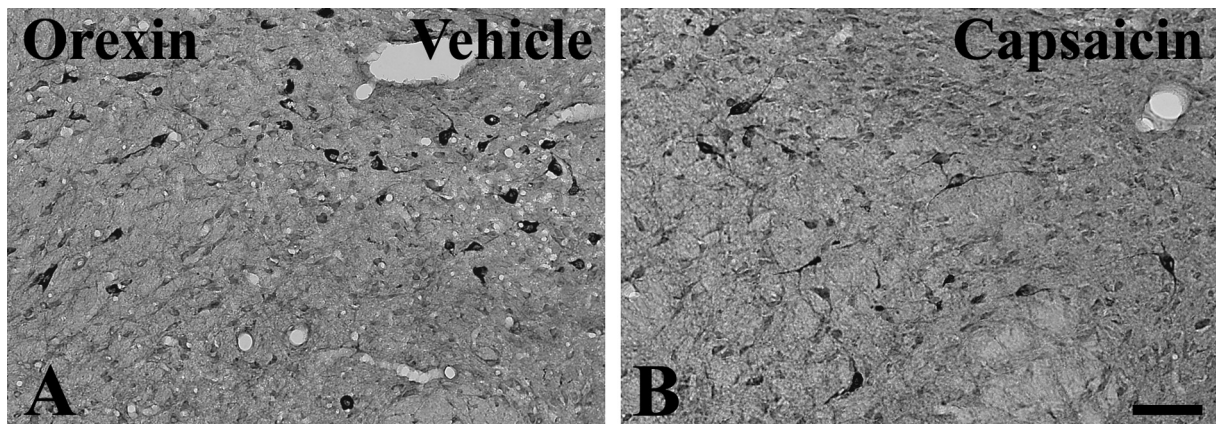


Fig. 3. Immunohistochemistry for orexin in the rat LH of vehicle- (A) and capsaicin-treated group (B). Orexin⁺ neurons are decreased after capsaicin treatment (B). Bar=50 μ m.

ropeptides play important roles in the regulation of food intake and energy homeostasis in the hypothalamus. The decrease of AgRP and orexin and the increase of α -MSH and ACTH in the rat hypothalamus after capsaicin treatment are thought to be related closely to these integrated and reciprocal connections [7, 27, 29, 30], and the effect of capsaicin may be mediated via VR1 in the hypothalamus [19]. Therefore, we presume that mean food intake was decreased at one day after capsaicin treatment in consequence. However, we cannot exclude the possibility that the decrease of mean food intake at one day was related to the capsaicin-induced pain or inflammation rather than the direct effect of capsaicin on the hypothalamus; because capsaicin is known as a major pungent ingredient [34] and capsaicin injection causes stimulation of chemosensitive pain receptors [38]. It is too difficult to explain the relationship between capsaicin administration and food intake exactly because there are few studies on it. Therefore, in further study, it will be necessary to elucidate whether capsaicin acts directly on the hypothalamus and food intake or not.

In conclusion, our present study indicates that mean food intake was significantly decreased at one day after 5 mg/kg capsaicin treatment. In addition, administration of 5 mg/kg capsaicin decreased AgRP⁺ and orexin⁺ neurons, whereas it increased α -MSH⁺ and ACTH⁺ neurons in the rat hypothalamus.

ACKNOWLEDGMENTS. The authors would like to thank Mr. Seung Uk Lee and Ms. Hyun Sook Kim for their technical help in this study. This work was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD) (KRF-2007-412-J00502).

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