

Effect of Capsaicin on Cholecystokinin and Neuropeptide Y Expressions in the Brain of High-fat Diet Fed Rats

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ABSTRACT. Capsaicin, one of the pungent principles of hot pepper, has been reported to cause a cessation of increases in body weight and fat gain induced by high-fat feeding. Especially, in body weight and feeding control, cholecystokinin (CCK) has been well known as a satiety signal and neuropeptide Y (NPY) has been described as one of the most potent orexigenic signals. This study was carried out to investigate the effect of capsaicin on CCK- and NPY- immunoreactivities (IR) in the brain of high-fat fed rats. The animals were divided into normal-fat diet (NF), high-fat diet (HF) and high-fat diet containing capsaicin (HF-CAP) groups. Mean body weight gain (MBWG) of HF group was higher than that of NF group. However, in HF-CAP group, MBWG was lower than that of HF group. CCK-IR in suprachiasmatic nucleus (SCN), paraventricular nucleus (PVN), median eminence (ME), arcuate nucleus (ARC) and amygdala was not prominent in all the groups. In cerebral cortex, CCK-IR was more reduced in HF-CAP group than in the other groups. In the HF-CAP group, NPY-IR in the hypothalamic nuclei, amygdala and cerebral cortex was more poorly found than in the NF and HF groups. It is concluded that (1) NPY-IR may react more sensitively on capsaicin than CCK-IR, (2) no rapid increase of body weight in capsaicin treated rats may result from the diminished food intake through the low expression of NPY in hypothalamus in HF-CAP group.

KEY WORDS: capsaicin, cholecystokinin, high-fat diet, immunohistochemistry, neuropeptide Y.

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Capsaicin is a pungent principle of hot red pepper that has been studied because of its importance in spices, food additives and drugs, which was recently reviewed by Suzuki and Iwai [40]. The structure of capsaicin has been established as N-(4-hydroxy-3-methoxy-benzyl)-8-methylnon-trans-6-enamide [24]. Recently, the anti-obesity effect of capsaicin has received much attention. For example, Kawada *et al.* [20] suggested that capsaicin stimulate lipid mobilization from adipose tissue and lowers the perirenal adipose tissue weight and serum triglyceride concentration in lard-fed rats. Also, the supplementation of capsaicin was known to cause a cessation of increases in body weight and fat gain induced by high-fat dietary [6]. Among the various neuropeptides and hormones related in the control of feeding and body weight, particularly, cholecystokinin (CCK), an anorectic factor, and neuropeptide Y (NPY), an orexigenic factor are of interest.

CCK was first found by Ivy and Oldberg [17] in the pig small intestine, in which it is released by instillation of fat and activates gallbladder contraction. CCK is found in brain and various gastrointestinal segments and most abundant in the duodenum and jejunum [23]. The administration of CCK into the central nervous system has been reported to reduce food intake in several species [8, 31, 32, 43]. The

intraperitoneal injection of CCK also decreased food intake in rats [13]. It was reported that the alteration of feeding status might change not only brain CCK levels, but also CCK receptor concentrations in the specific regions of the mouse brain [29].

NPY, a peptide consisting of 36 amino acid residues, was first isolated from extracts of porcine brain [42], and is one of the most abundant peptides in the mammalian brain [1, 2, 7, 25]. In the rat brain, NPY-IR is appeared in most areas except the cerebellum. Especially, NPY is most numerous in perikarya of the cerebral cortex and in the nucleus of the hypothalamus, particularly the arcuate nucleus [1, 7]. John *et al.* [18] suggested that NPY has an important physiological role in the stimulation of feeding in the rat. In the hypothalamus, NPY is synthesized primarily by neurons in the ventromedial part of the arcuate nucleus, the cells of which are activated in response to negative energy balance such as caloric restriction or starvation [4, 11, 34]. NPY induces lipogenic enzymes in liver and white adipose tissue [5, 33, 38].

A few studies for the effects of the nutrient-related stimulation on the CCK and NPY have been carried out in the CNS of the rats and mice. Della-Fera *et al.* reported that intestinal infusion of a liquid diet altered CCK and NPY concentration in specific brain areas of rat [10]. Ziotopoulou *et al.* [44] showed that differential expression of hypothalamic neuropeptides in the early phase of diet-induced obesity in mice. Nevertheless the mechanism of cessation

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of increases in body weight in high-fat diet fed rats induced by capsaicin has not been fully understood. However, it has been postulated that the alteration of neuropeptides related to feeding mechanism may affect this cessation effect of body fat gain by capsaicin.

This study was carried out to investigate the effect of capsaicin on the expressions of CCK and NPY in the cerebral cortex and the hypothalamic nuclei of the high-fat diet fed and high-fat diet containing capsaicin fed rats by using immunohistochemistry.

MATERIALS AND METHODS

Experimental designs and animals: Male Sprague-Dawley rats (190–210 g body weight; 6–7 weeks; Samtako Co., Korea) were individually housed and maintained on a 12 hr light-dark cycle at $22 \pm 2^\circ\text{C}$ under 40–50% relative humidity. Feed and tap water were provided *ad libitum*. Animals were fed a normal diet for 3 days, prior to experiments, to allow them to adjust to the new environment and purified diet. All experimental procedures were carried out in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals. Animals were divided into three groups: the normal-fat diet (NF), the high-fat diet (HF) and the high-fat diet containing capsaicin (HF-CAP) groups ($n=5$ in each group). Animals in NF group were fed normal-fat diet as a control. Animals in HF group were fed high-fat diet containing 30% lard instead of some of maize starch of normal-fat diet. Animals in HF-CAP group were fed high-fat diet containing 30% lard plus 0.02% capsaicin (Sigma, U.S.A.). The dose of capsaicin used in this study was determined by the report of Choo and Shin and Kawada *et al.* [6, 20]. The compositions of the experimental diets were like Table 1 [6]. Animals of all experimental groups were fed for 14 days. The body weights and the amount of food intake were measured everyday at the same time during experimental period. For measuring the amount of food intake, animals were provided with a fixed quantity of diet (40 g/day/rat). And then each amount of the remaining feed was measured everyday. So, food intake was measured from the amount of the remaining feed and provided fixed quantity feed.

Tissue preparation: Animals were deeply anesthetized with a mixture of xylazine hydrochloride (Rompun[®], Bayer, Korea) and ketamin hydrochloride (Ketamin[®], Yuhan corporation, Korea), and then perfused intracardially with 0.9% saline and followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) at a rate of 70 ml/min with a perfusion pump (Masterflex, U.S.A.). After perfusion, the brains were dissected out and post-fixed overnight in the same fixative at 4°C . The brains were cryoprotected by transferring to 30% sucrose in 0.1 M PB and frozen in OCT embedding medium (Tissue-Tek, Sakura, U.S.A.). Coronal sections of 30 μm thickness were made with a freezing microtome (Leica, CM1850, Germany).

Immunohistochemistry: For immunostaining, free-floating tissue sections were rinsed with 0.01 M phosphate-buff-

Table 1. Composition of the experimental diets

Constituent	Normal-fat diet (g/kg)	High-fat diet (g/kg)
Casein	200	200
Corn starch	521	321
Sucrose	100	100
Corn oil	100	100
Lard	–	200
Cellulose	30	30
DL-methionine	2	2
Mineral mix ^{a)}	35	35
Vitamin mix ^{b)}	10	10
Choline bitartrate	2	2
Gross energy content (kcal/g)	4.25	5.2

a) American institute of nutrition (AIN) mineral mix containing (g/kg): calcium phosphate diabolic 500, sodium chloride 74, potassium citrate 220, potassium sulfate 52, magnesium oxide 24, mangnous carbonate 3.5, ferric citrate 6, zinc carbonate 1.6, cupric carbonate 0.3, potassium iodate 0.01, sodium selenite 0.01, chromium potassium sulfate 0.55.

b) AIN vitamin mix containing (g/kg): thiamin HCl 0.6, riboflavin 0.6, pyridoxine HCl 0.7, niacin 3, calcium pantothenate 1.6, folic acid 0.2, biotin 0.02, vitamin B₁₂ (0.1% trituration in mannitol) 1, dry vitamin A palmitate (500,000 U/g) 0.8, dry vitamin E acetate (500 U/g) 10, vitamin D₃ trituration (4,000,000 U/g), 0.25, manadione sodium bisulfite complex 0.15.

ered saline (PBS, pH 7.4), and then treated with 0.5% hydrogen peroxide in 0.01 M PBS for 15 min. The sections were washed with 0.01 M PBS 5 times for 7 min each, and then nonspecific binding sites were blocked by incubation in 10% normal goat serum in 0.1 M PBS for 20 min at room temperature. The sections were incubated overnight with rabbit anti-CCK antibody (Serotec Com., U.S.A.) at a dilution of 1:2,000 for visualization of CCK distribution or with rabbit anti-NPY antibody (Chemicon International, Inc., U.S.A.) at a dilution of 1:3,000 for visualization of NPY distribution. After incubation with primary antibodies, the sections were rinsed in 0.01 M PBS 5 times for 7 min each and then incubated for 2 hr at room temperature with biotinylated goat anti-rabbit secondary antibody (DAKO, Denmark). The sections were subsequently incubated with a streptavidin-HRP (DAKO, Denmark) for 1 hr at room temperature. Immunoreactivity was visualized by incubating the sections in 0.01 M PBS containing 0.05% 3'-diaminobenzidine tetrachloride (DAB) (Sigma, U.S.A.) and 0.003% hydrogen peroxide. The sections were washed with 0.01 M PBS for 35 min with 5 changes. Finally, the immunostained sections were mounted on gelatin-coated glass slides. Each specific areas of the brain were identified using brain maps by Swanson [41] and cresyl violet staining.

Statistical analysis: Statistical analyses of the data were performed using the StatView 4.5 (Abacus Concepts Inc., U.S.A.) program. The statistical significance of differences was assessed by one-way ANOVA followed by Bonferroni/Dunnett's test. Results are represented as mean \pm S.E.M. Differences were considered significant for $P < 0.05$.

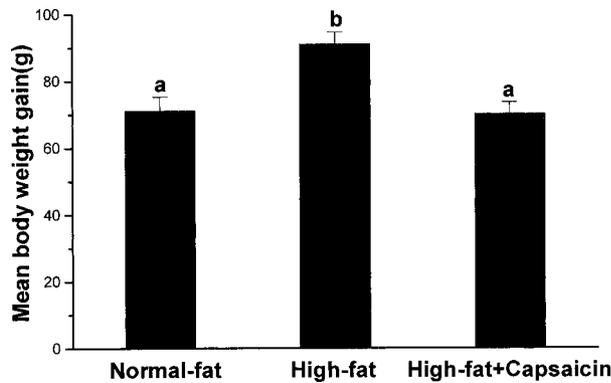


Fig. 1. The mean body weight gain was 71.2 ± 4.2 g in normal-fat, 91.0 ± 3.5 g in high-fat, and 70.1 ± 3.5 g in high-fat containing capsaicin diet fed groups, respectively. Bars not sharing a common letter were significantly different, $P < 0.05$.

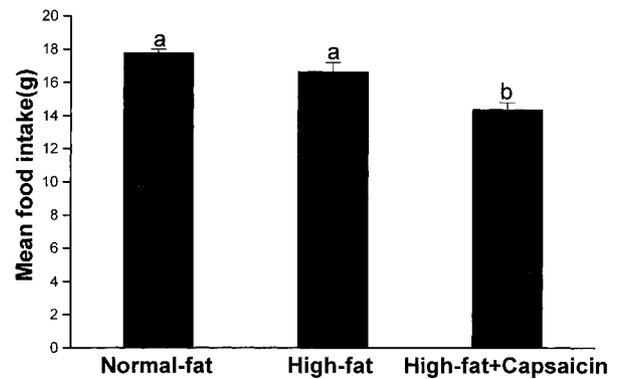


Fig. 2. The mean food intake weight was 17.8 ± 0.2 g in the normal-fat, 16.6 ± 0.6 g in the high-fat, and 14.4 ± 0.4 g in high-fat containing capsaicin diet fed groups, respectively. Bars not sharing a common letter were significantly different, $P < 0.05$.

RESULTS

Changes of body weight gain and food intake amount: In HF group, the mean body weight (MBW) increased from 211.4 ± 4.0 g to 302.4 ± 3.6 g, for a net gain of 43.0%. In HF-CAP group, MBW increased from 221.7 ± 0.4 g to 291.8 ± 3.6 g, for a net gain of 31.6%. In NF group, MBW increased from 225.2 ± 2.8 g to 296.4 ± 6.2 g, for a net gain of 31.6%.

The mean body weight gain (MBWG) of HF group was higher than that of NF group, however, MBWG of HF-CAP group was not different from that of in NF group. MBWG

of HF-CAP group was lower than that of HF group ($P < 0.05$; Fig. 1).

The mean food intake amounts of NF, HF and HF-CAP groups were 17.8 ± 0.2 g, 16.6 ± 0.6 g and 14.4 ± 0.4 g, respectively. The mean food intake amounts in both HF and NF groups were not different. However, the mean food intake amount of HF-CAP group was significantly less than those of NF and HF groups ($P < 0.05$; Fig. 2).

Expressions of CCK and NPY: In the suprachiasmatic nucleus (SCN) and paraventricular nucleus (PVN), CCK-immunoreactivities (IRs) were not prominent in all groups (data not shown). CCK-immunoreactive cell bodies were

Table 2. CCK and NPY immunoreactivities in the specific areas of the each group

		CCK						
		SCN	SON	PVN	ME	ARC	Amygdala	Cerebral cortex
NF	Cell body	-	++	-	-	+	-	+
	Fiber	-	-	-	++	-	-	-
HF	Cell body	-	-	-	-	-	-	+
	Fiber	-	-	-	++	-	-	-
HF-CAP	Cell body	-	-	-	-	-	-	±
	Fiber	-	-	-	+	-	-	-
		NPY						
		SCN	SON	PVN	ME	ARC	Amygdala	Cerebral cortex
NF	Cell body	-	++	-	-	+	+++	++
	Fiber	+++	-	++	++	-	-	±
HF	Cell body	-	+++	-	-	++	++	+
	Fiber	+++	-	+	+++	-	±	-
HF-CAP	Cell body	-	-	-	-	-	±	±
	Fiber	+	-	-	+	-	-	-

- ; No immunoreactive nerve cell bodies or fibers

± ; rare

+ ; few

++ ; moderate

+++ ; intensive

SCN (Suprachiasmatic nuclei); SON (Supraoptic nuclei); PVN (Paraventricular nuclei); ME (Median eminence); ARC (Arcuate nucleus).

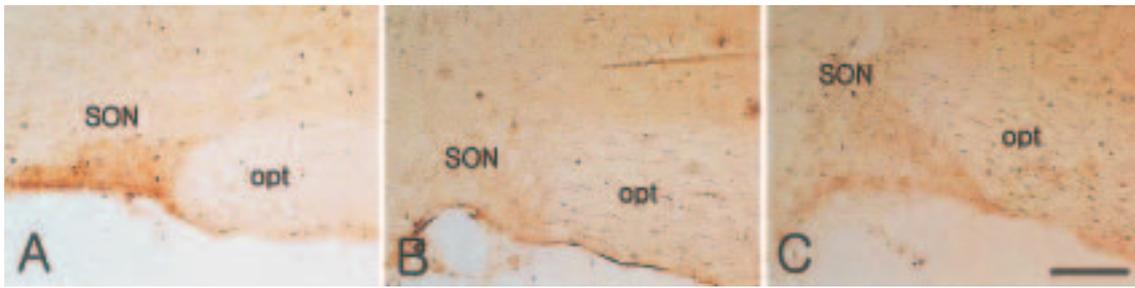


Fig. 3. Differences of CCK-immunoreactivity (IR) in the supraoptic nucleus. CCK-immunoreactive cell bodies were moderately found in normal-fat diet (NF) fed group, but not in the other groups. (A) NF; (B) High-fat; (C) High-fat diet containing capsaicin fed groups. SON (supraoptic nucleus), opt (optic tract). Scale bar = 200 μm .

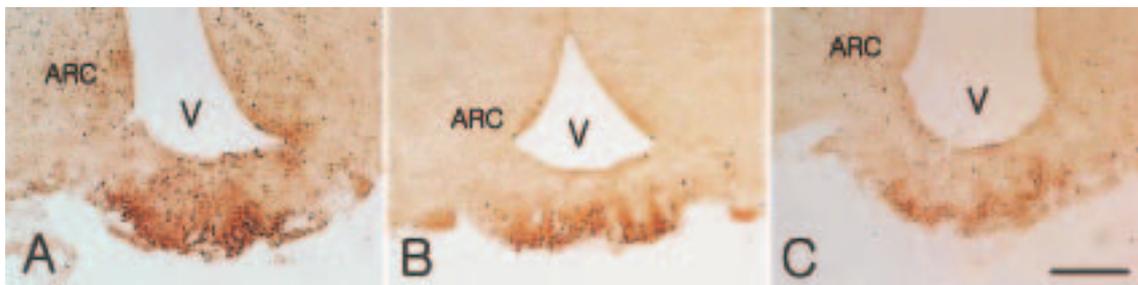


Fig. 4. Differences of CCK-IR in the arcuate nucleus (ARC) and median eminence (ME). CCK-immunoreactive nerve fibers were moderately found in the external lamina of the ME in normal-fat (NF) and high-fat diet (HF) fed groups, however, few in high-fat diet containing capsaicin (HF-CAP) group. CCK-IR cell bodies were observed only a few in the ARC of NF group only. (A) Normal-fat; (B) High-fat; (C); High-fat diet containing capsaicin fed groups. V (3rd ventricle). Scale bar = 200 μm .

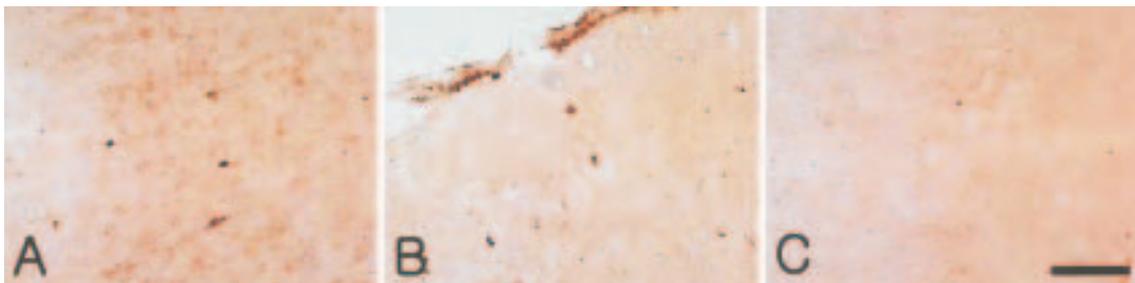


Fig. 5. Differences of CCK-IR in cerebral cortex (laminae V-VI). CCK-immunoreactive cell bodies were found in normal-fat (NF) and high-fat diet (HF) fed groups, but not in high-fat diet containing capsaicin (HF-CAP) group. (A) NF; (B) HF; (C) HF-CAP fed groups. Scale bar = 100 μm .

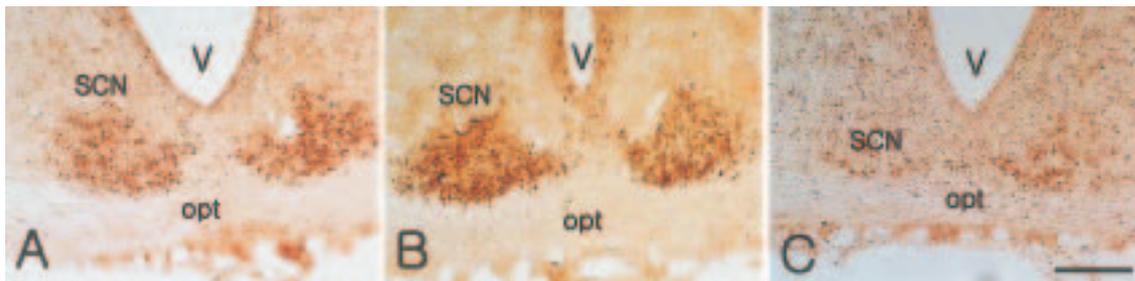


Fig. 6. Differences of NPY-IR in the suprachiasmatic nuclei (SCN). NPY-immunoreactive nerve fibers were intensively found in normal-fat diet (NF) and high-fat diet (HF) fed groups, but few found in high-fat diet containing capsaicin (HF-CAP) fed group. (A) NF; (B) HF; (C) HF-CAP fed groups. SCN (suprachiasmatic nucleus), opt (optic tract), V (3rd ventricle). Scale bar = 200 μm .

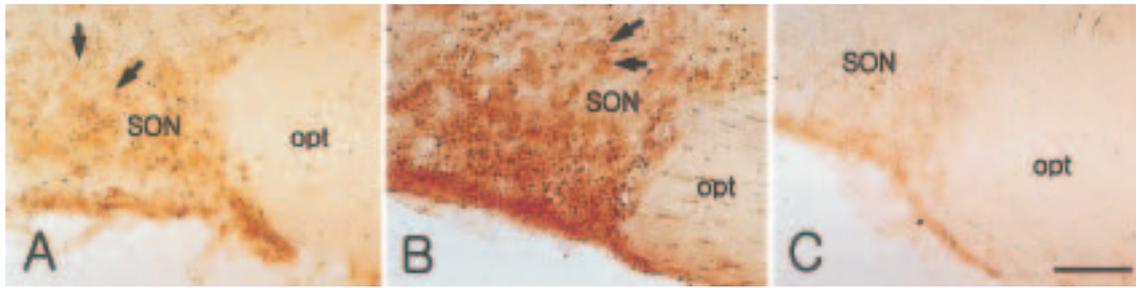


Fig. 7. Differences of NPY-IR in the supraoptic nucleus. NPY-immunoreactive cell bodies are observed in normal-fat diet (NF) and high-fat diet (HF) fed groups, but not observed in high-fat diet containing capsaicin (HF-CAP) fed group. Arrows indicate NPY-positive cells. (A) NF; (B) HF; (C) HF-CAP fed groups. SON (supraoptic nucleus), opt (optic nucleus). Scale bar = 200 μm .

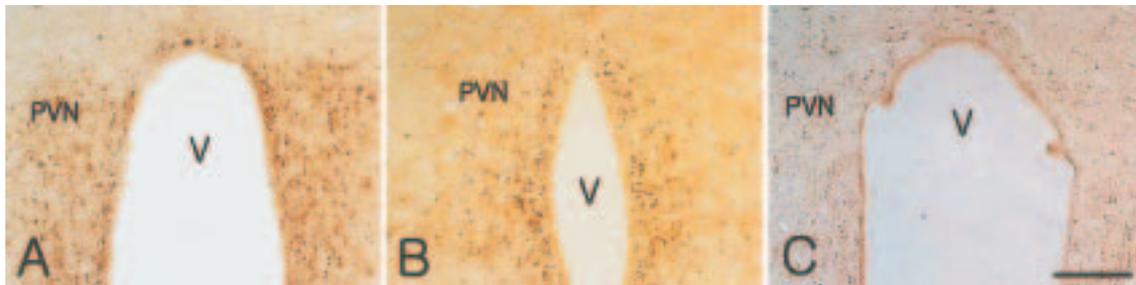


Fig. 8. Differences of NPY-IR in the paraventricular nucleus (PVN). NPY-IR was significantly found in high-fat diet (HF) fed and normal-fat diet (NF) fed groups, not in high-fat diet containing capsaicin (HF-CAP) fed group. (A) NF; (B) HF; (C) HF-CAP fed groups. V (3rd ventricle). Scale bar = 200 μm .

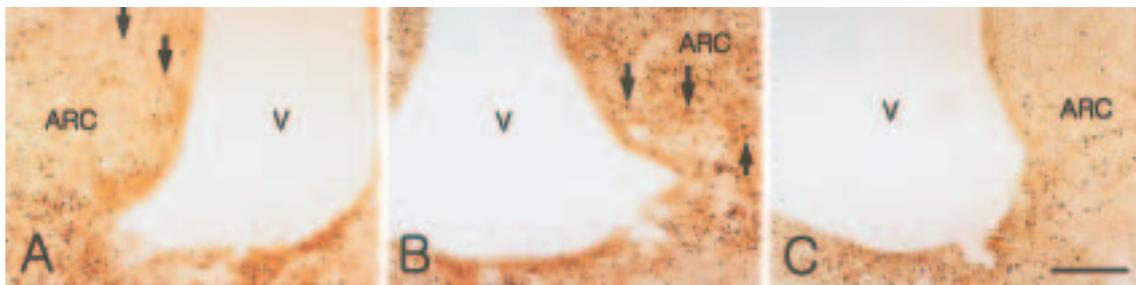


Fig. 9. Differences of NPY-IR in the arcuate nucleus (ARC) and median eminence (ME). NPY-immunoreactive cells were few and moderately found in the ARC of normal-fat diet (NF) fed and high-fat diet (HF) fed groups, respectively, however, not found in high-fat diet containing capsaicin (HF-CAP) fed group. NPY-IR nerve fibers were moderately and intensively found in the ME of NF and HF groups, however, few found in the ME of HF-CAP group. Arrows are NPY-positive cells. (A) NF; (B) HF; (C) HF-CAP fed groups. V (3rd ventricle). Scale bar = 200 μm .

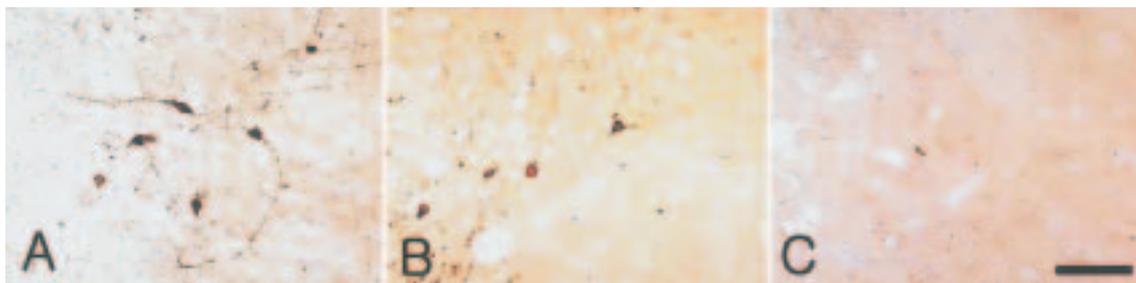


Fig. 10. Differences of NPY-IR in the amygdala (basomedial nucleus). NPY-immunoreactive cells were intensively found normal-fat diet (NF) fed group and moderately appeared in high-fat diet (HF) fed group, however, rare in high-fat diet containing capsaicin (HF-CAP) fed group. (A) NF; (B) HF; (C) HF-CAP groups. Scale bar = 100 μm .

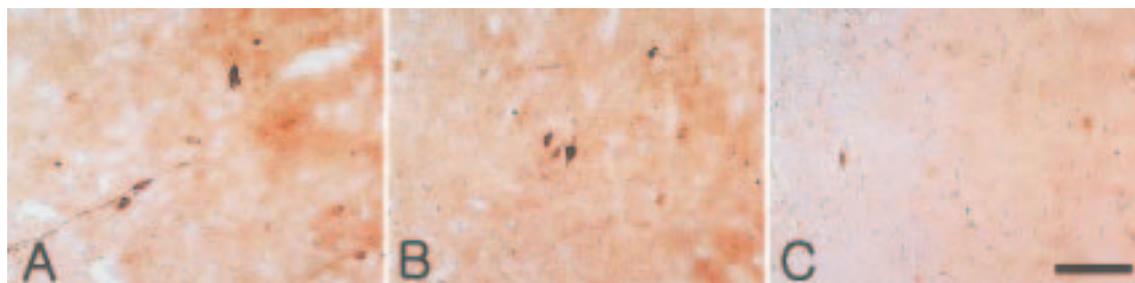


Fig. 11. Differences of NPY-IR in the cerebral cortex (laminae V-VI). NPY-immunoreactive cells were moderately found normal-fat diet (NF) fed group and more few appeared in high-fat (HF) diet fed group, however, rare in high-fat diet containing capsaicin (HF-CAP) fed group. (A) NF; (B) HF; (C) HF-CAP groups. Scale bar = 100 μm .

moderately found in the supraoptic nucleus (SON) of the NF group, but not in other two groups (Fig. 3). CCK-immunoreactive nerve fibers in the external lamina in the median eminence (ME) of HF-CAP group were more poorly found than in the same areas of the other groups. However, CCK-immunoreactive cell bodies were observed only a few in the arcuate nucleus (ARC) of NF group only (Fig. 4). CCK-immunoreactive cell bodies were found in the cerebral cortex in NF and HF groups, but not in HF-CAP group; the numbers of CCK-immunoreactive cell bodies per unit area (71.8 mm^2) were 5.7 ± 0.56 , 5.0 ± 0.58 , and 2.0 ± 0.37 , respectively ($P < 0.05$; Fig. 5). CCK-immunoreactive cell bodies and fibers in the hypothalamic areas of all groups were not prominent (Table 2).

NPY-immunoreactive nerve fibers were more significantly densely localized in the SCN of NF and HF groups than in the same area of HF-CAP group; the densities of NPY-immunoreactive nerve fibers per unit area ($99.2 \mu\text{m}^2$) were 173.3 ± 2.2 , 166.0 ± 3.7 , and 203.7 ± 0.7 , respectively ($P < 0.05$; Fig. 6). In the SON, NPY-immunoreactive cell bodies were observed in NF and HF groups, but not in HF-CAP group (Fig. 7). NPY-IR of NF and HF groups was significantly found in the PVN, but not in the same area of HF-CAP group; the densities of NPY-immunoreactive nerve fibers were 186.4 ± 1.0 , 194.1 ± 0.8 , and 217.4 ± 0.9 , respectively ($P < 0.05$; Fig. 8). NPY-immunoreactive nerve fibers were more significantly moderately found in the internal lamina of the ME in NF and HF groups than in HF-CAP group; the densities of NPY-immunoreactive nerve fibers per unit area ($99.2 \mu\text{m}^2$) were 169.2 ± 0.8 , 159 ± 1.4 , and 185.6 ± 3.0 , respectively ($P < 0.05$; Fig. 9). However, NPY-immunoreactive cell bodies were found in the arcuate nucleus (ARC) of NF and HF groups only (Fig. 9). NPY-immunoreactive nerve cells were found in the amygdala (basomedial nucleus) and cerebral cortex (laminae V-VI) of NF and HF groups. NPY-immunoreactive nerve cells were more rare in HF-CAP group than in HF group; NPY-immunoreactive cells were 1.8 ± 0.31 and 7.2 ± 0.31 in the amygdala, 2.0 ± 0.37 and 7.0 ± 0.58 in the cerebral cortex, respectively ($P < 0.05$; Figs. 10 and 11). In HF-CAP group, NPY-IRs of the SCN, SON, PVN, ME, ARC, amygdala (basomedial nucleus) and cerebral cortex (laminae V-VI) of

the brain were weaker than in the other groups (Table 2).

DISCUSSION

The satiety signal induced by the introduction of food into the intestine can be mimicked by systemic injections of CCK and its analogues [13, 16, 35, 36]. CCK is effective in reducing the amount of food intake when it is injected into the hypothalamus and the cerebral ventricle [8, 39]. On the contrary, NPY, a potent orexigenic agent, has been known to stimulate feeding when it is administered to the brain [19, 21]. When NPY is administered chronically, it has been shown to induce weight gain in rats [39]. Also, Stanley *et al.* [37] reported that the injection of peptide YY and neuropeptide Y into the paraventricular nucleus elicited a strong and selective increase in carbohydrate consumption, with little or no effect on protein or fat consumption.

Otherwise, many studies have been reported that the food-intake conditions (feeding, fasting, etc.) may have the effect on the contents of CCK and NPY in the CNS. For example, concentrations of CCK and NPY in specific brain areas have been changed by feeding and fasting, respectively [9, 26, 30]. Ziotopoulou *et al.* [44] reported that the body weight and hypothalamic expression of neuropeptides began to change significantly by 2 weeks after feeding of high- and low-fat diet in C57BL/6J mice, with final weight being higher in the high-fat (44.9 %) group than in low-fat (10 %) group. In the present study, there was no difference in the food intake between HF (30% fat) and NF (10% fat) groups, however, the significant difference in the mean body weight gain was observed, with final mean body weight gain being higher in the HF group. Although the animal strain and fat-content of the HF group of the present study were different with those of Ziotopoulou *et al.* [44], the results of the body weight increase in the HF group were consistent with those of Ziotopoulou *et al.*

CCK has been well known as a satiety signal [14, 16, 23, 33–35, 43]. Accordingly, it was anticipated that CCK-IR would be more prominent in the HF group in the specific areas of the brain, especially in the hypothalamic nuclei of brain in the HF group than in the HF-CAP group. In the present study, CCK-IR was not observed in the SCN, PVN,

and amygdala of all groups. However, CCK-immunoreactive cell bodies were observed in the SON and ARC only of NF group. These results indicate that hypothalamic CCK may be reduced by high-fat.

Schick *et al.* [31] suggested that CCK may not only initiate satiety messages. There are two types of CCK-receptors in CNS. Most receptors have been known to be type B, and type A receptors are only distributed in the hypothalamus and in the nucleus tractus solitarii. Accordingly, these brain regions may be implicated in the control of feeding behavior and mediation of peripheral CCK-induced satiety [15]. In the present study, the reduction of CCK-immunoreactive cell bodies in the cerebral cortex of the HF-CAP group indicates that expression of cerebral cortex CCK may be inhibited by capsaicin. These results suppose that capsaicin may affect CCK receptor type-B more susceptibly and consequently, may have little effect on the CCK-regulation of food intake. Recently, Farook *et al.* [12] strongly suggested that CCK-B receptors mediate the freezing behavior and the differential expression of these receptors underlie the strain difference in such behavior.

Ziotopoulou *et al.* [44] reported that NPY mRNA expression of hypothalamus was decreased 2 days after high-fat diet fed and return to baseline after 1 week high-fat diet fed. In the present study, NPY-IR was found in the SCN, SON, PVN, ME, ARC, amygdala (basomedial nucleus), and cerebral cortex (laminae V-VI) of HF and NF groups. The hypothalamic NPY-IR of HF group was similar to that of NF group after 2 weeks high-fat diet fed. In this study, NPY-IR of the HF-CAP group was less densely in the PVN, ME, SCN, ARC, amygdala (basomedial nucleus) and cerebral cortex (laminae V-VI) than in the same areas of HF group. These results suggest that capsaicin may reduce NPY expression in the brain and leads to limit the food intake. When leptin was administered to animals, animals showed far more sensitive to the meal-suppressing action of meal-generated signals such as CCK [3, 22]. Sahu [27, 28] reported that leptin make food intake decrease by depression of the expression of NPY.

From the present results, capsaicin appears to result in decreases in NPY expressions than in CCK expressions to diminish food intake through the low expression of NPY in hypothalamus in HF-CAP group.

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