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The Expressional Changes of Nitric Oxide Synthase in the Rat Brain Following Food Restriction

Kyounglan Kang[1,2], Youngbuhm Huh[3],Chan Park[3], Ryo Won Choue[1,2§]

[1]*Research Institute of Medical Nutrition, Kyunghee University, Seoul 130-701, Korea*
[2]*Department of Medical Nutrition, Graduate School of East-West Medical Science, Seoul 130-701, Korea*
[3]*Department of Anatomy, College of Medicine, Kyunghee University, Seoul 130-701, Korea*

This study investigated the changes in the neuronal nitric oxide synthase (nNOS) and nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) activities during food restriction in the rat brain such as cerebral cortex, cerebellum, caudate pautamen and hypothalamus. The rats were placed on a restricted feeding schedule consisting of half the *ad libitum* quantity for 3 days and 1, 2, 4, 6 and 9 weeks, and a free feeding schedule for 4 weeks. The loss of body weight peaked after 1 week of food restriction and persisted during the entire 9-week period of food restriction. The dramatic weight change in the first week (12%) and the reduction in weight changes thereafter suggest that major adaptation changes occur early and body maintenance occurs subsequently. In the hypothalamus, the optical densities of the NADPH-d and nNOS immunoreactivities were found to be significantly higher in the 1-week and lower in the 9-week food restricted group than in the *ad libitum* fed control rats. In contrast, in the cerebral cortex, the optical densities of the NADPH-d- and nNOS-positive neurons were not changed significantly during the period of food restriction. This study provides the morphological evidence showing that food restriction has a significant effect on the nitric oxide synthesizing system of the hypothalamus.

Key words: Nitric oxide synthase, Brain, Hypothalamus, Food restriction

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INTRODUCTION

The status of nutrition intake has important consequence for the brain function,[1)] the hormone regulation and the central neurotransmitter activity.[2)] There is increased understanding of the central pathways by which the known neurotransmitters affect food intake. However, the basic mechanism responsible for the adaptation to chronic food restriction is unclear, although many factors are believed to be involved.[3,4)]

Nitric oxide (NO) acts as a neurotransmitter as well as a biological messenger molecule (The activity of nitric oxide synthase is determined by NADPH-diapharase (NADPH-d)). The role played by NO as a neurotransmitter in modulating the food intake in rats,[5,6)] mice[7,8)] as well as other mammals[9)] has been well documented. For example, food deprivation for 1 and 2 days increases brain NOS levels and depresses brain serotonin levels in the hypothalamus of rats.[5,10)] In addition, food deprivation for 2 days increases the NADPH-d[11)] levels in the hypothalamus of rats. There is also evidence showing that NO has an inhibitory effect on food intake, possibly via hypothalamic mechanisms.[2,12)] Recently, it was reported that the cerebral cortex is also involved in adaptation to food restriction.[13,14)] For that reason, investigating the influence of food in the brain area is of considerable interest.

Most studies have concentrated on the brain and the regulating area of appetite and have generally been performed using not morphological but physiological examinations. Regardless of the increasing studies on the changes in NOS levels during food deprivation,[11,12)] there is a lack of research on the possible morphological changes in the brain of rats during food restriction. Hence, this study was designed to investigate changes in the body weight and NOS levels in the brain of SD rats following the period of food restriction.

MATERIALS AND METHODS

The experiments were performed using 10-week-old male Sprague-Dawley rats (housed two per cage in an environmentally conditioned animal facility with a 12 h light/

§ To whom correspondence should be addressed.
(E-mail : rwcho@khu.ac.kr)

dark cycle).[17] Prior to commencing the food restriction experiment, the rats were fed a purified diet containing 48% carbohydrate and 32.5% fat of total calories *ad libitum* for 4 weeks and the daily voluntary intake of purified diet was recorded over this period in the 6-week-old rats (Table 1). Thereafter, the food intake was reduced to half the voluntary intake (12 g instead of 24 g per rat per day, provided at 10:00 h during the period of food restriction). The control rats were sacrificed at the beginning of the experiment (n=6). The food-restricted rats were sacrificed at 3 days, and at 1, 2, 4, 6 and 9 weeks after the commencement of food restriction (n=6 per time point).

The animals were anesthetized with pentobarbital sodium (50 mg/kg, i.p.) and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS), pH 7.4 for 48 h. Forty micrometer thick frozen sections were then made in the coronal plane using a cryostat.

NO synthase (NOS) is the key enzyme responsible for the generation of NO.[15,16] NOS can utilize nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d).[15] For this reason, the NADPH-d reaction has been used to detect the changes in the NOS activity in the neurons and glial cells.[18] The sections were stained for histochemical NADPH-d detection using the method reported by Vincent and Kimura.[19] Free-floating sections were incubated at 37 ℃ for 60 min in 0.1 M PB, pH 7.4, containing 0.3% Triton X-100, 0.1 mg/mL nitroblue tetrazolium and 1.0 mg/mL β-NADPH. The sections were stained for the histochemical nNOS and incubated for 24 h in PBS (4 ℃) containing anti-nNOS antiserum (Transduction Laboratories, Lexington, USA; 1:1000 dilution), 0.3% Triton X-100, 0.5 mg/mL bovine serum albumin and 1.5% normal goat serum. They were then incubated with biotinylated secondary antibodies (Vector, Burlingame, USA), which were diluted 1:200, for 90 min. This was followed by dilution with avidin-biotin-peroxidase complex (1:100 dilutions, Vector) for 1 h at room temperature. Finally the sections were reacted with 0.02% 3,3'-diaminobenidine tetrahydrochloride and 0.01% H_2O_2 for approximately 3 min. After each of these incubation steps, the sections were washed with PBS for 5 min three times for a total of 15 min.

The rat brain analyses were carried out using the atlas reported by Paxinos and Watson.[19] The slides were quantified using a computer-assisted image analysis method (Multiscan, Fullerton, USA). A total of 16 sections from 8 different rats (two sections per animal) were used and a Student's *t*- test was used to evaluate the statistical significance of the differences between the means. A p value<0.05 was considered significant.

Table 1. Composition of experimental diets

Ingredients	(g/100g)
Casein	20
Corn oil	15
DL-Methionine	0.3
Sucrose	5.0
Cellulose	10.0
Corn Oil	15.0
Choline-C12	0.2
AIN-76 Mineral Mix	3.5
AIN-76 Vitamin Mix	1.0
% of Energy	17,365
Carbohydrate(%)[1]	48.0
Fat(%)[2]	32.5
Protein(%)[3]	19.5

1) Carbohydrate(%) as % of total calories
2) Fat(%) as % of total calories
3) Protein(%) as % of total calories

RESULTS AND DISCUSSION

At the beginning of experiment, the mean BW of the control group was 441.1±18.0 g while that of the food restricted group was 436.7±8.1 g. The mean BW of the 1-week control group was 492.9±10.1 g while that for the experimental group was 385.4±8.0 g. In the food-restricted group, a dramatic weight change was showed in the first week (12%). Afterwards, the change in weight was lower than that of the first week (Fig. 1). The dramatic weight change in the first week (12%) and the reduction in weight changes thereafter suggest that major adaptation changes occur early and body maintenance occurs subsequently. This remarkable change and the reduced degree of weight change afterwards suggest that

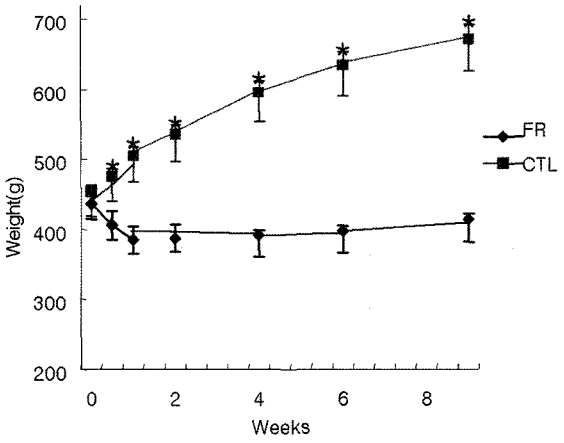

Fig. 1 The body weight change versus the food restriction period.
A dramatic weight change was showed in the 1-week of food-restricted group (12%). Afterwards, the change in weight was lower than the 1-week of group. The data is represented as a mean±SEM.
FR: Food restricted group, CTL: *ad libitum* control group (n=6).

the major changes occurred early and that the adaptation was maintained during the subsequent periods of food restriction.

NADPH-d- and nNOS-positive neurons were observed in the brain of all groups. The optical density of the NADPH-d- and nNOS-positive neurons in most hypothalamic regions such as the paraventricular, the hypothalamic dorsomedial, the supraoptic nuclei, and the lateral hypothalamic area, was significantly higher in the 1-week food restricted group than in the *ad libitum* fed control group (Fig. 2). The optical density gradually decreased between the second and ninth weeks of food restriction. In particular, after 9 weeks of food restriction, the optical density of the NADPH-d-positive neurons in the hypothalamus was significantly lower than that of the *ad libitum*-fed control group (Fig. 3). However, there was no significant change in the optical densities of the NADPH-d- and nNOS-positive neurons in the cerebral cortex such as motor cortex, somatosensory cortex, cingulated cortex, ectorinal cortex (Fig. 4, 5, 6).

Many studies have examined the effects of food restriction in the central nervous system using neurotransmitters and peptides.[21] Several reports have also detailed the relationship between food intake and the NOS levels in the brain.[6,22] For that reason, an investigation of the influence of food on the hypothalamic areas as well as on the other brain regions is warranted. Although the

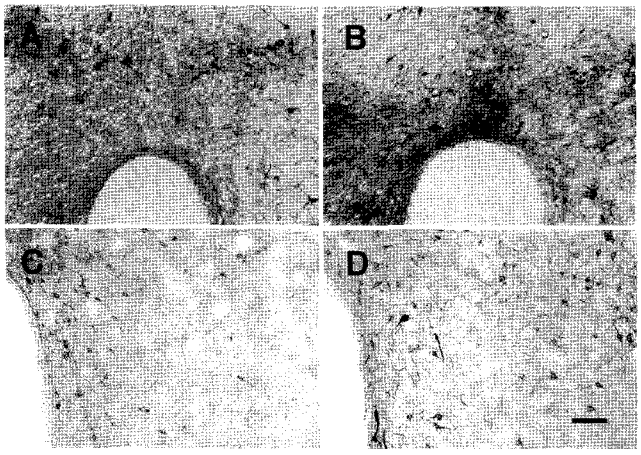

Fig. 2 At 1 week after food restriction, staining intensities of NADPH-d (B) and nNOS (D) positive neurons at the hypothalmus were markedly increased than control group (A, C).
Scale bar, 80 μm.

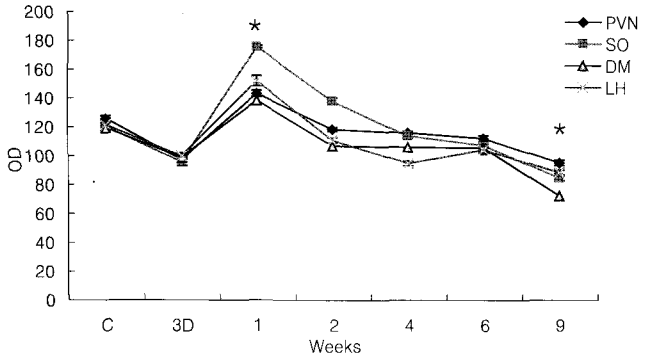

Fig. 3 The optical density of the NOS-positive neurons in the hypothalamus of rats was significantly changed at 1 and 9 weeks after food restriction.
The data is represented as a mean±SEM.
* $P<0.05$ comparedwith the control group.
The paraventricular (PVN), the hypothalamic dorsomedia (DM), the supraoptic nuclei (SO), and the lateral hypothalamic area (LH).
OD: optical density

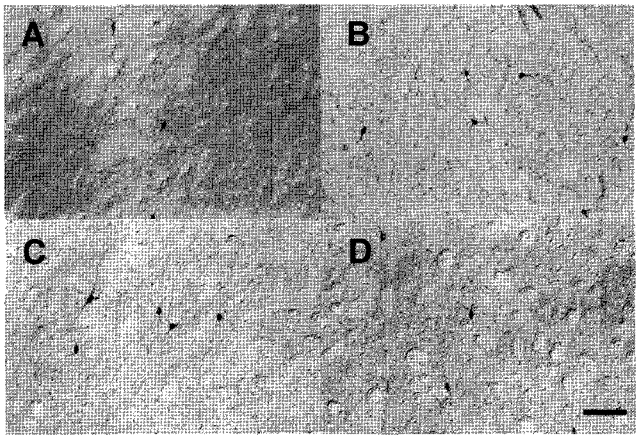

Fig. 4 The staining intensities of NADPH-d-positive neurons of the control group (A) were not significantly different from at 1 (B), 2 (C), 4 (D) weeks in the cerebral cortex during food restriction.
Scale bar, 80 μm.

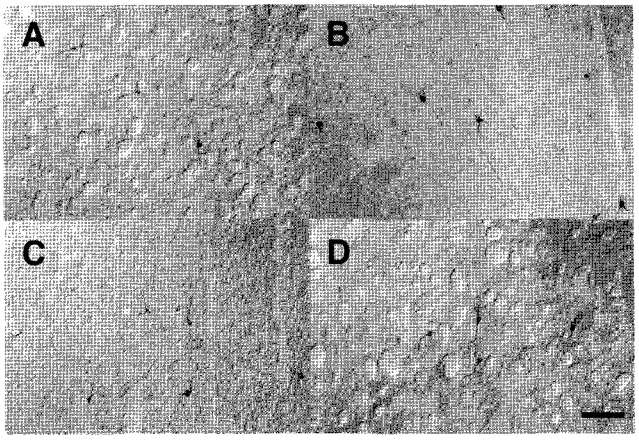

Fig. 5 The staining intensities of nNOS-positive neurons of the control group (A) were not significantly different from at 1 (B), 2 (C), 4 (D) weeks in the cerebral cortex following food restriction.
Scale bar, 80 μm.

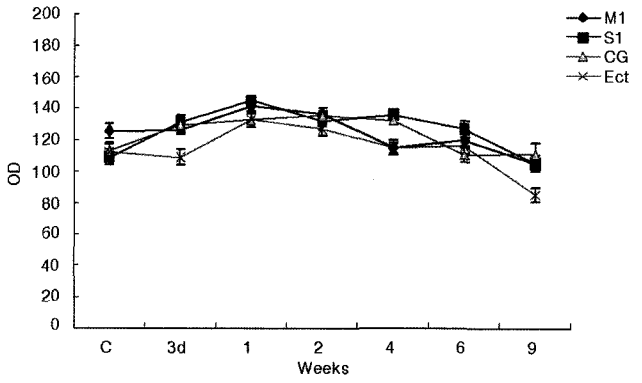

Fig. 6 The optical density of the NOS-positive neurons of the cerebral cortex was not significantly changed among the control and food restricted group.

The data is represented as a mean±SEM.
The primary motor cortex (M1), the primary somatosensory cortex (S1), the cingulate cortex (CG) and the ectorhinal cortex (Ect)
OD: optical density

underlying mechanisms are often discussed relative to food restriction, few reports have dealt with the morphological changes. Therefore, this study investigated changes in the neuronal NOS and NADPH-d during food restriction. The morphological data in this study showed that the change in the NOS activity were higher after 1 week of food restriction, suggesting that food restriction leads to alternations in NOS cells.

The body weight of SD rats during the period of food restriction was measured at various times. The loss of body weight peaked one week into the experiment. However, from that time until the end of the ninth week, there was a smaller decrease in body weight. The graph demonstrates that rats adapt to calorie restrictions (Fig. 1). Likewise, Widdowson *et al.* investigated the fact that dietary restricted rats adapted to a reduced caloric intake, which is consistent with the results of this report.[23] Short-term control is to ensure long-term regulation of the energy reserves, which is the principle determinant of body weight.[24] Takase *et al.* (2000) found that animals in a food restricted group showed a 16% reduction in body weight in the first week, followed by a continuous, slow rise in weight over subsequent days.[25] The result suggests that food restriction may be accompanied by the resistance of weight change aimed at the maintenance of energy homeostasis.

NO has been demonstrated to play an important role in the regulation of food intake and energy balance.[26,27] Moley (1999) reported that NOS may mediate the effects of NPY and leptin on food intake.[28] There has been accumulating evidence: Food deprivation increased NOS in the diencephalons. NO production is enhanced by a certain diet composition.[29] NOS activity was decreased in the hypothalamus and the fundus of Zucker rats.[30] These data suggest that NO might be a physiological mediator involved in the mechanisms that control feeding behavior. Also, NOS content changed significantly in the hypothalamus of food-restricted rats. NO has been determined to be a neurotransmitter which plays a role in modulating food intake.

The activity of nitric oxide synthase is determined by NADPH-diaphorase (NADPH-d). NADPH-d histochemistry, a fast and simple reaction often used by neuroscientists to visualize the expression of nNOS in the nervous system, offers a good alternative to the expensive and time-consuming immunohistochemistry.[31] Induced NADPH-d-positive neurons were not observed in the brains of the rats in the food-restricted group. Bicker reported that the formation of NO requires the presence of NADPH-d as a cofactor for the NOS enzyme.[32] Thus, a simple histochemical method for localizing NOS-containing cells is to stain fixed nervous tissue for NADPH-d. Although the intensity of NADPH-d stained cells was demonstrated among food-restricted dams, the pattern of NADPH-d staining across the brain was similar in all samples.

This study suggests that low levels of dietary calories may play a role in time and regional changes in NOS activity in the cerebral cortex of rats. Collectively, these data provide the first evidence that dietary restriction can alter synaptic homeostasis in a manner that enhances the ability of the synapses to withstand adversity. Taken together, the results of the present study suggest that food restriction-related changes in energy may influence the expression of NOS.

The optical density of nNOS- and NADPH-d-positive neurons was significantly higher in the hypothalamus after 1 week of food restriction. Interestingly, this change occurred concurrently with the maximum loss of body weight recorded during the period of food restriction. There is a report showing that NOS can be overproduced as a result of severe stress conditions[33] which is in agreement with our report.

This study showed significant changes in the optical density of NOS-positive neurons in the hypothalamus. This finding suggests that the hypothalamus is affected to a greater extent by food restriction than the cerebral cortex. The hypothalamus, which is an appetite-regulating area, plays a pivotal role in the short - and long - term regulatory loops that control food intake. Therefore, food restriction can change the activity of the NO system in the hypothalamus rather than in the cerebral cortex.

Our study provides morphological evidence showing

that food restriction has a significant effect on the nitric oxide synthesizing system of the hypothalamus. This shows that the growing resistance to weight change is aimed at maintaining energy homeostasis. NOS-positive neurons in the hypothalamus may also play an important role in the response to reduced caloric intake in the brain. This suggests some possibilities for the relative functions of nNOS-positive neurons after food restriction. In conclusion, NOS might play a role in regulating food intake in the hypothalamus of the brain.

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