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The Expressional Changes of Nicotinamide Adenine Dinucleotide Phosphate-Diaphorase and Neuronal Nitric Oxide Synthase in the Rat Cerebral Cortex and Hypothalamus during Food Restriction

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ABSTRACT

In the present study, we investigated the changes in the nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) activity and neuronal nitric oxide synthase (nNOS) expression during food restriction in the rat cerebral cortex and hypothalamus. The rats were placed on a restricted feeding schedule consisting of half the *ad libitum* quantity for 1, 2, 4, 6 and 9 weeks, and a free feeding schedule for 1 week. The loss of body weight peaked at 1 week after food restriction and persisted during the entire 9-week period of food restriction. In the hypothalamus, the NADPH-d activity and nNOS immunoreactivity were found to be significantly higher at 1 week and gradually decrease thereafter. 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. Our study provides evidence that food restriction has a significant effect on the nitric oxide synthesizing system of the hypothalamus. This suggests a possibility for the relative functions of the nNOS-positive neurons after food restriction.

Key words: Neuronal nitric oxide synthase, NADPH-diaphorase, hypothalamus, cerebral cortex, food restriction

INTRODUCTION

The level of nutrition affects important brain functions (Gibson and Bloss, 1999) such as the regulation of hormonal and central neurotransmitter ac-

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tivities (Schwartz et al., 2000). 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 (Duffy et al., 1997; Bruce-Keller et al., 1999).

Nitric oxide (NO) acts as a neurotransmitter as well as a biological messenger molecule. The role played by NO as a neurotransmitter in modulating the food intake has been well documented in rats (O'Shea and Gundlach, 1996; Stricker-Krongrad et al., 1996; Kanda et al., 2002), mice (Morley and Flood, 1991; Calignano et al., 1993) as well as other mammals (Vozzo et al., 1999). For example, complete food deprivation for 1 or 2 d increases the brain NOS levels and depresses the brain serotonin levels in the hypothalamus of rats (Squadrito et al., 1994; O'Shea et al., 1996). In addition, complete food deprivation for 2 d increases the NADPH-d (Jang et al., 2002) levels in the hypothalamus of rats. There is also evidence showing that NO has an inhibitory effect on food intake possibly via hypothalamic mechanisms (Ueta et al., 1995; Schwartz et al., 2000). Recently, it was reported that the cerebral cortex is also involved in the adaptation to food restriction (Olivenza et al., 2000; Shi et al., 2002). Therefore, investigating the influence of food in the hypothalamic area as well as the cerebral cortex is of considerable interest.

Until now, most studies have concentrated on the hypothalamus and the appetite-regulating areas, and have generally been performed using physiological examinations. Furthermore, despite the increasing amount of information published on the changes in the NOS levels during food deprivation (Jang et al., 2002; Ueta et al., 1995), there is a paucity of research on the possible morphological changes in the hypothalamus and cerebral cortex of rats during food restriction. Therefore, our present study was designed to investigate the changes in the neuronal NOS and NADPH-d levels during moderate 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/dark cycle. Prior to commencing the food restriction experiment, the rats were fed a purified diet containing 48% carbohydrate and 32.5% fat *ad libitum* for 4 weeks and the daily voluntary intake of purified diet was recorded over this period in the 6-week-old rats. 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 d, 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. After that, the brains were postfixed with 4% paraformaldehyde for 24 h and then were stored with 30% sucrose in 0.1 M phosphate buffered saline. Forty micrometer thick frozen sections were then made in the coronal plane using a cryostat.

NADPH-d activity was detected on the tissue section using the method reported by Vincent and Kimura (Vincent et al., 1992). Free-floating sections were incubated at 37°C 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 nNOS 24 h in PBS (4°C) 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), diluted 1 : 200 for 90 min. This was followed by incubation with avidin-biotin-peroxidase complex (1 : 100 dilution, 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 steps, the sections were washed with PBS for 5 min three times.

The rat brain analyses were carried out using the atlas reported by Paxinos and Watson (Paxinos et al., 1997). The slides were quantified using a com-

puter-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.

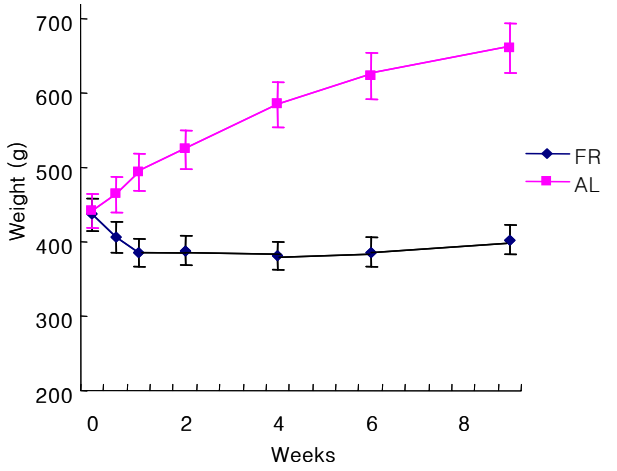

Fig. 1. Body weight changes in food restricted rats (FR) and *ad libitum*-fed rats (AL). The loss of the body weight reached the steady state level at 1 week after food restriction. The data represent as a mean±SEM (n=6).

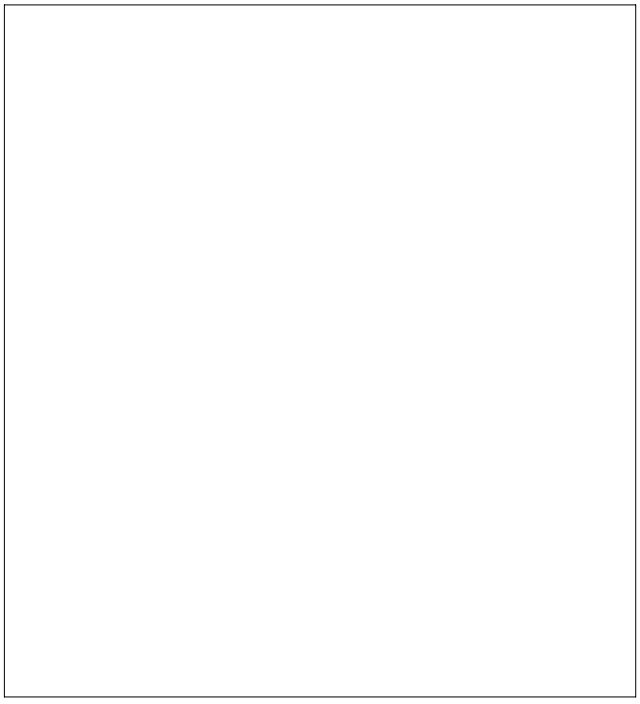

Fig. 2. Increase of NADPH-diaphorase activity and nNOS immunoreactivity in the paraventricular nucleus at 1 week after food restriction. NADPH-d activity in control (A), in 1 week of food restriction (B), and in 2 weeks of food restriction group (C). nNOS immunoreactivity in control (D), in 1 week of food restriction (E) and 2 weeks of food restriction group (F). Scale bar, 80 μm.

RESULTS

All the food-restricted groups showed a decrease in body weight. In the food-restricted group, a dramatic weight change showed in the first week group (12%). Afterwards, the change in weight was not apparent (Fig. 1).

NO synthase (NOS) is the key enzyme responsible for the generation of NO (Moncada et al., 1991; Bredt, 1999). NOS can utilize nicotinamide

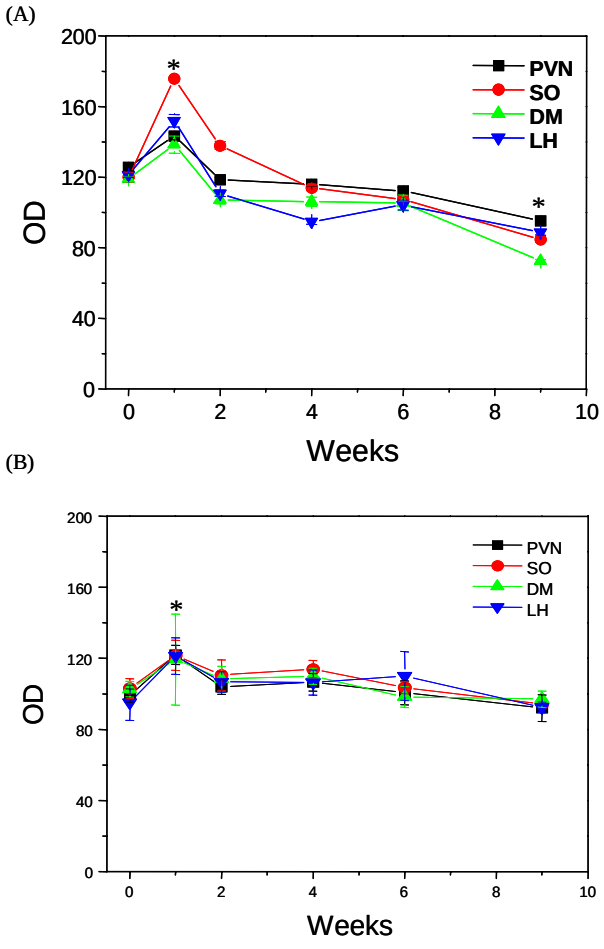

Fig. 3. The relative optical density of the NADPH-d-positive neurons (A) and nNOS-positive neurons (B) in the various regions of the hypothalamus; the paraventricular (PVN), the hypothalamic dorsomedia (DM), the supraoptic nuclei (SO), and the lateral hypothalamic area (LH). Each point represents as a mean±SEM. *$P<0.05$ compared with the control group.

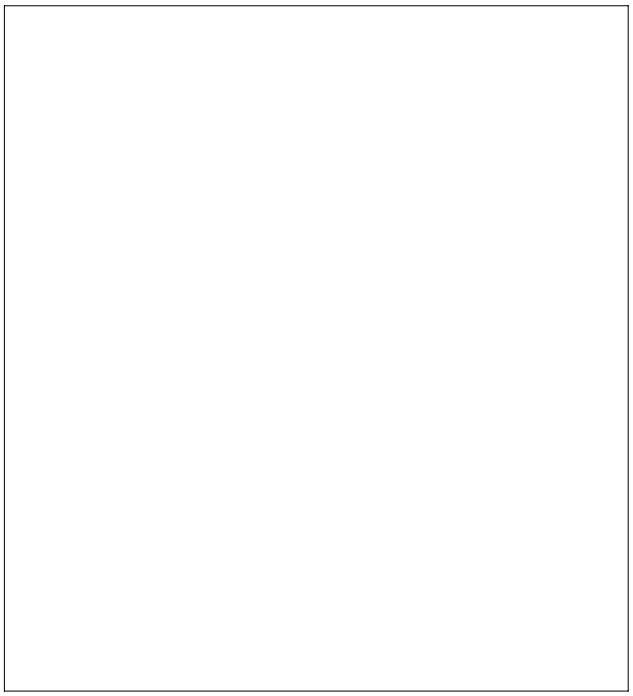

Fig. 4. No significant changes of NADPH-diaphorase activity and nNOS immunoreactivity in the primary motor cortex after food restriction. NADPH-d activity in control (A), in 1 week of food restriction (B) and 2 weeks of food restriction group (C). nNOS immunoreactivity in control (D), in 1 week of food restriction group (E) and 2 weeks of food restriction group (F). Scale bar, 80 μm.

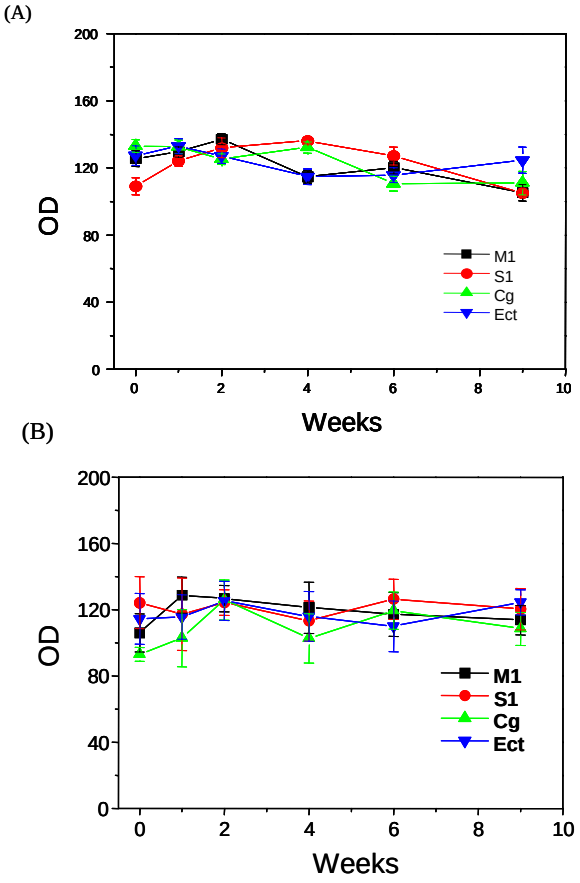

Fig. 5. The relative optical density of the NADPH-d- (A) and nNOS-positive neurons (B) in the various regions of cerebral cortex; the primary motor cortex (M1), the primary somatosensory cortex (S1), the cingulate cortex (CG) and the ectorhinal cortex (Ect). Each point represents as a mean ±SEM.

adenine dinucleotide phosphate-diaphorase (NADPH-d). For this reason, the NADPH-d reaction has been used to detect the changes in the NOS activity in the neurons and glial cells (Morris et al., 1997). NADPH-d- and nNOS-positive neurons were observed in the hypothalamus and cerebral cortex 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 at the 1 week-food restricted group than in the *ad libitum* fed control and 2 weeks group (Fig. 2). The optical density gradually decreased from 2-weeks to 9-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 the *ad libitum* fed control (Fig. 3)

However, in the cerebral cortex, there was no significant change in the optical densities of the NADPH-d- and nNOS-positive neurons at 1-week group (Fig. 4, 5).

DISCUSSION

The maximum loss of body weight was shown at 1 week after food restriction and this weight loss did not continue afterwards, which suggests that the major changes occurred at the earlier phase of the food restriction and a certain adaptation mechanism was maintained during the subsequent period of food restriction. The optical densities of the nNOS- and NADPH-d-positive neurons were significantly higher in the hypothalamus at 1week after food restriction. Interestingly, this change occurred concurrently with the maximum loss of body weight

recorded during the period of food restriction. Considering the suggested possibility that nitric oxide controls the appetite for food increase of optical densities of the nNOS- and NADPH-d-positive neurons might reflect a desire for food of food-restricted animals (Hui and Chan, 1995; Stricker-Krongrad et al., 1997).

In addition, the optical density of NOS-positive neurons in hypothalamus was significantly decreased afterwards. It has been reported that the inhibition of NOS reduces the food intake in rodents and chickens (Vozzo et al., 1999). It has also been established that the dietary-restricted rats adapted to chronic food restriction (Schwartz et al., 2000; Shi et al., 2002). Based on these and our present observations, it is plausible that the decrease of NOS activity underlies an adaptation mechanism to the chronic food restriction.

Although it is known that food restriction reduces brain damage and the behavioral outcomes (Bruce-Keller et al., 1999), we did not observe any significant change in the NOS and NADPH-d-positive neurons in the cerebral cortex despite their tendency to change. These findings imply that the hypothalamus is the main region affected by food restriction and that the hypothalamus as an appetite-regulating area plays a pivotal role in the short- and long-term regulatory loops controling the food intake. In conclusion, we suggest that food restriction can change the activity of NOS in the hypothalamus, which in turn plays an important role in regulating food intake.

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