Yonsei Medical Journal
Vol. 45, No. 1, pp. 123 - 128, 2004

Adrenalectomy Abolishes Fasting-induced Down-regulation of NADPH-diaphorase in the Rat Paraventricular Nucleus

Jeong Won Jahng[1], Jong-Ho Lee[2], Gun Tae Kim[1], Yun Mi Kim[1], Thomas A Houpt[3], and Dong Goo Kim[1]

[1]Department of Pharmacology and BK 21 Project for Medical Science, Yonsei University College of Medicine, Seoul, Korea;
[2]Department of Oral and Maxillofacial surgery, Seoul National University College of Dentistry, Seoul, Korea;
[3]Department of Biological Science, Florida State University, Tallahassee, FL, USA.

This study was conducted to define the molecular mechanism of fasting-induced down-regulation of neuronal nitric oxide synthase (nNOS) expression in the hypothalamic paraventricular nucleus (PVN). Rats were adrenalectomized (ADX), and then either underwent food deprivation or received varying doses of dexamethasone for 48 h. The brain tissues were processed for NADPH-diaphorase (NADPH-d) staining, a histochemical marker of nNOS enzyme activity. Both the ADX and the sham operated rats showed a significant weight loss after 48 h of food deprivation. Food deprivation decreased the number of NADPH-d containing cells in the PVN of sham rats, however, not in the ADX rats. Dexamethasone dose-dependently decreased NADPH-d cells in the PVN of ADX rats. The effect of ADX or dexamethasone was limited to the parvocellular subdivision of PVN. These results suggest that the adrenal glucocorticoids may down-regulate nNOS expression in the PVN during food deprivation.

Key Words: Hypothalamus, neuronal nitric oxide synthase, glucocorticoid

INTRODUCTION

Nitric oxide (NO) in the hypothalamic paraventricular nucleus (PVN) modulates a large number of neuronal, autonomic, and endocrine functions.[1,2] Many types of nutritional stresses, including food deprivation, regulate the expression as well as the enzymatic activity of neuronal nitric oxide synthase (nNOS) in the PVN. It has been reported that the intracellular intensity of nNOS mRNA, NADPH-diaphorase (NADPH-d) staining which considered as a marker for nNOS enzyme activity in the brain, and the apparent number of nNOS-immunopositive cells decrease in the PVN by food deprivation.[3,4] Food deprivation is accompanied by many autonomic and behavioral changes including compensatory hyperphagia,[5] decreased heart rate and blood pressure,[6] and decreased circulating levels of insulin[7] and leptin.[8] The hypothalamus, particularly the PVN, is potential site where metabolic and sensory signals may be integrated with neurochemical changes, such as decreased nNOS, to produce the integrated response to food deprivation.

Food deprivation has been reported to elevate the plasma glucocorticoid levels.[9-16] Glucocorticoid receptors are colocalized in NADPH-d stained cells in the hypothalamus,[17] and nNOS expression is down-regulated by glucocorticoids in the rat brain[18-20] as well as in the cell lines.[21]

Taken together, we hypothesized that the plasma glucocorticoids may down-regulate nNOS in the PVN during food deprivation. In this study, we firstly examined if adrenalectomy abolishes the fasting-induced down-regulation of nNOS, and then if the plasma glucocorticoids are sufficient to down-regulate nNOS in the PVN.

Received October 24, 2003
Accepted January 9, 2004

This research was supported by the Neurobiology Research Program from the Korea Ministry of Science and Technology (JWJ). Yun Mi Kim received scholarship support by BK21 Project for Medical Science.

Reprint address: requests to Dr. Dong Goo Kim, Department of Pharmacology, Yonsei University College of Medicine, 134 Shinchon-dong, Seodaemun-gu, Seoul 120-752, Korea. Tel: 82-2-361-5227, Fax: 82-2-313-1894, Email: dgkimpharm@yumc.yonsei.ac.kr

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 250-300g were purchased (Daehan Biolink Co., LTD., Chungbuk, Korea), and acclimated in a specific pathogen free (SPF) barrier area where the temperature (22 ± 1℃) and humidity (55%) were controlled constantly with a 12 h light-dark cycle (light between 07:00 and 19:00) in Yonsei university animal facility breeding colony. Rats were individually housed with ad *libitum* access to standard laboratory food (Purina Rodent Chow, Purina Co., Seoul, Korea) and tap water. Rats were cared according to the guide for animal experiments, 2000, edited by The Korean Academy of Medical Sciences, which is consistent with the NIH Guidelines for the Care and Use of Laboratory Animals 1996 revised. Animal experiments were approved by the Committee for the Care and Use of Laboratory Animals at Yonsei University.

Adrenalectomy and drug treatment

Bilateral adrenalectomy was performed with dorsal approach as previously described.[22] Sham adrenalectomy consisted of the same procedure without touching adrenal glands. After surgery, adrenalectomized rats received 0.9% saline instead of water to drink.

A week after surgery, 10 adrenalectomized and 9 sham-operated rats were food-deprived, but not fluid-deprived, beginning 1 h after lights-on, in order to determine if adrenalectomy abolishes fasting-induced down-regulation of nNOS in the PVN. Rats were transcardially perfused 48 h after the start of food deprivation, and the brains were processed for NADPH-d staining, which considered as a histologic marker for nNOS enzyme activity in the brain. As controls, the remaining 6 adrenalectomized and 5 sham-operated rats with free access to food were perfused in parallel with the deprived rats.

To determine if glucocorticoid is sufficient to down-regulate nNOS in the PVN, adrenalectomized rats were divided into 4 groups (n=4-6 rats/group) and received subcutaneous injection of dexamethasone (Chung-Wae Pharmaceuticals, Korea) at each different dose (0, 0.05, 0.5, or 5 mg/kg). Dexamethasone in aseptic physiologic saline was given 4 times over 48 h with 12 h intervals, beginning 1 h after lights-on. Rats were transcardially perfused 12 h after the last injection and the brains were processed for NADPH-d staining.

NADPH-diaphorase histochemistry

Transcardiac perfusion and NADPH-d histochemistry were performed as previously described.[23] For NADPH-d staining, alternate sections were collected through the rostral-caudal extent of the hypothalamic PVN (between bregma - 1.3 mm and - 2.1 mm) for NADPH-diaphorase histochmistry. The coordinates were based on Paxinos and Watson.[24]

Statistical analysis

The number of NADPH-d positive cells were blind-counted by hand after digitizing 720 × 540 micron images of two sections from the PVN (closest sections to bregma - 1.88 mm) from each brain using an Olympus BX-50 microscope (Olympus Co., Tokyo, Japan). Cell counts for the sections of each rat were averaged per section, and the individual mean counts averaged across rats within experimental groups. All data were analyzed by t-test or two way analysis of variance (ANOVA) and preplanned comparisons with the control were performed by *post-hoc* Fisher's PLSD.

RESULTS

Food deprivation after adrenalectomy

Both adrenalectomized (ADX) and sham-operated rats lost weight by 48 h of food deprivation (Fig. 1). A two-way ANOVA on changes in body weight revealed a main effect of deprivation ($p < 0.0001$), but no effect of surgery. Sham-operated rats, but not ADX rats, showed a decrease in the number of NADPH-d stained cells in the PVN after food deprivation, and the decrease was limited to the medial parvocellular (mP) subdivision (Fig. 2). A two-way ANOVA on the number of NADPH-d stained cells revealed a main effect of

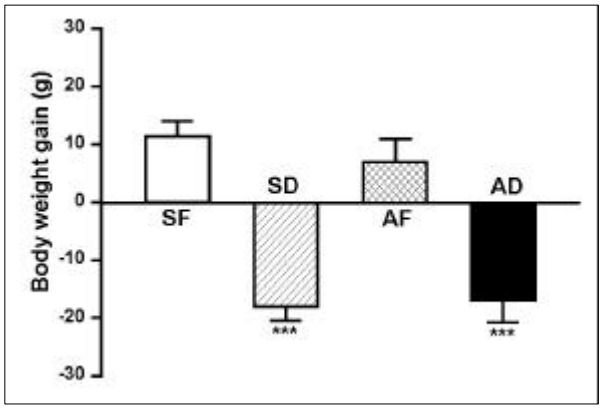

Fig. 1. Changes in body weight gain during food deprivation period. Both the adrenalectomized and the sham operated group showed a significant weight loss after 48 h food deprivation. Adrenalectomy did not affect the weight changes. SF, sham/fed; SD, sham/deprived; AF, adrenalectomy/fed; AD, adrenalectomy/deprived. ***$p<$ 0.0001 vs. sham/fed.

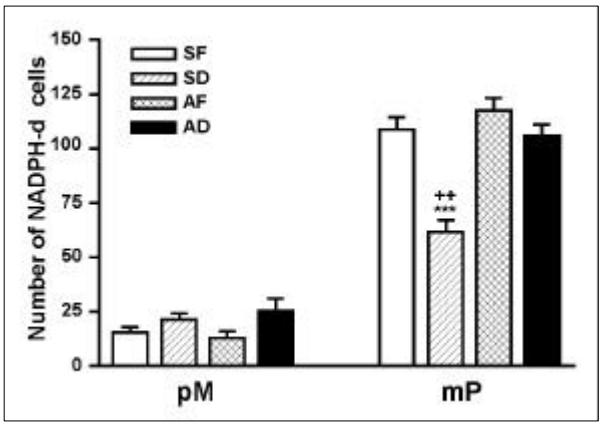

Fig. 2. Numbers of NADPH-d stained cells in the paraventricular nucleus (PVN) after 48 h of food deprivation. NADPH-d positive cells were decreased in the PVN of sham rats (SD), but not of the adrenalectomy rats (AD), by 48 h of food deprivation. Fasting-induced decrease in the number of NADPH-d cells was limited to the parvocellular subdivision (mP). Adrenalectomy showed no effect on NADPH-d staining in the non-deprived control group (SF vs. AF) either in the parvocellular (mP) or magnocellular (pM) subdivision of the PVN. SF, sham/fed; SD, sham/deprived; AF, adrenalectomy/fed; AD, adrenalectomy/deprived; pM, posterior magnocellular subdivision; mP, medial parvocellular subdivision. ***$p<0.0001$ vs. sham/fed, ++$p<0.01$ vs. ADX/deprived.

surgery ($p<0.01$) and a main effect of deprivation ($p<0.0001$). These results indicate that the adrenal glands are required for fasting-induced down-regulation of NADPH-d in the rat PVN.

Effect of dexamethasone on NADPH-d staining

To determine if nNOS down-regulation in the PVN is mediated by glucocorticoids, ADX rats received dexamethasone at varying doses (0, 0.05, 0.5 or 5 mg/kg, 4 times with a 12 h interval) with free access to rodent chow. Dexamethasone appeared to decrease NADPH-d staining in the PVN at all doses (Fig. 3A). NADPH-d positive cells in the PVN were reduced by dexamethasone in a dose dependent manner (Fig. 3B), and the reduction was limited to the medial parvocelluar (mP) subdivision (Fig. 3C). This result demonstrates that glucocorticoid is sufficient to down-regulate nNOS in the PVN, and supports the idea that the down-regulation may be mediated by glucocorticoid receptor.

DISCUSSION

It has been reported that food deprivation decreases the intracellular intensity of nNOS mRNA, NADPH-d staining which considered as a marker for nNOS enzyme activity in the brain, and the apparent number of nNOS-immunopositive or NADPH-d stained cells in the PVN.[3,4,25] In this study, adrenalectomy abolished fasting-induced decrease in the PVN-nNOS, i.e. numbers of NADPH-d stained neurons in the PVN *per se*, which reveals adrenal glands are required for the fasting-induced down-regulation of nNOS. We demonstrated that fasting-induced decreases in NADPH-d positive cells are limited to the parvocellular subdivision of the PVN. It has been reported that food deprivation elevates the plasma glucocorticoid levels,[9-16] glucocorticoid receptors are colocalized in NADPH-d stained cells of the hypothalamus,[17] and the immunoreactivities as well as mRNA expression of glucocorticoid receptor is predominantly detected in the parvocellular neurons, but not in the magnocellular, of the PVN.[26-28] We previously found that RU 486, glucocorticoids receptor antagonist, inhibits the fasting-induced down-regulation of nNOS immunoreactivity in the PVN, and the inhibition occurs only in the parvocelluar subdivision.[29] In the present study, dexamethasone, synthetic glucocorticoid, dose-dependently decreased the number of

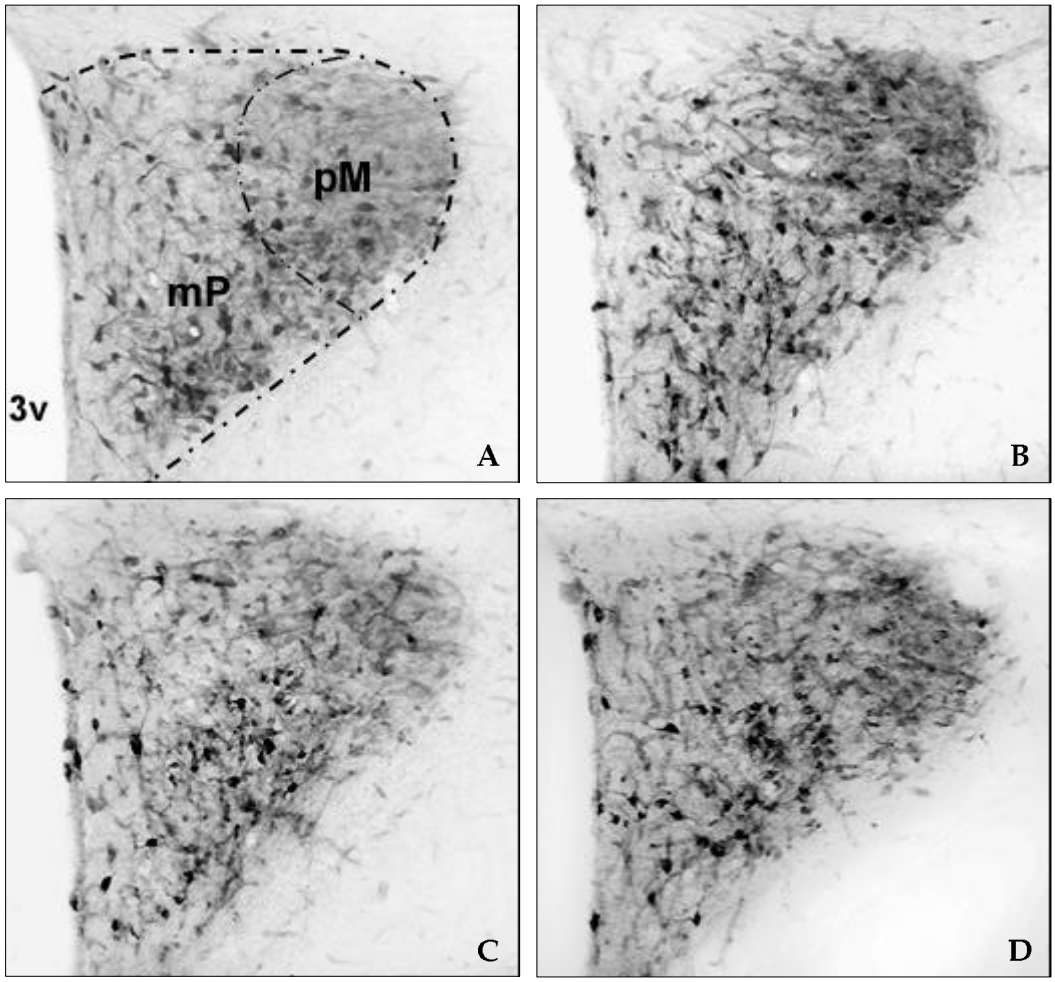

Fig. 3. Microphotographs of NADPH-d positive cells in the rat paraventricular nucleus. A week after adrenalectomy, rats received 4 injections of subcutaneous dexamethasone (A; 0, B; 0.05, C; 0.5, D; 5 mg/kg) with 12 h intervals. Twelve hours after the last injection, rats were sacrificed for NADPH-d histostaining. All doses of dexamethasone appeared to decrease NADPH-d attaining in the PVN. pM, posterior magnocellular subdivision; mP, medial parvocellular subdivision; 3V, third ventricle.

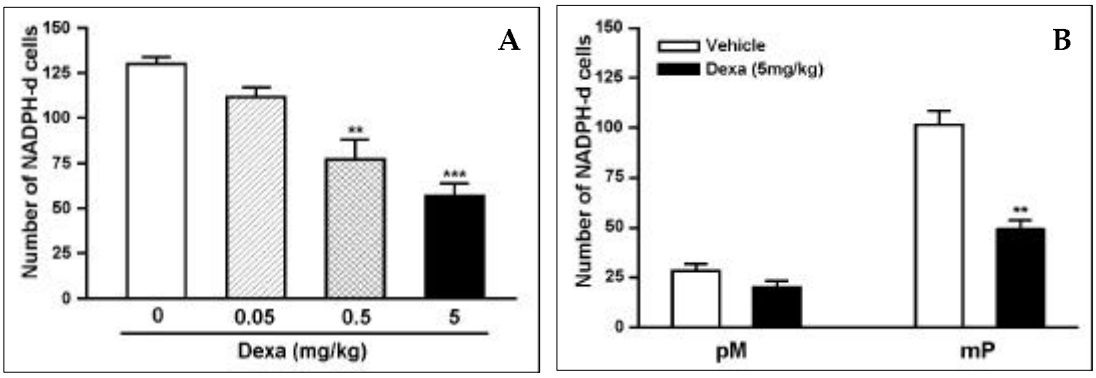

Fig. 4. Numbers of NADPH-d stained cells in the PVN of ADX rats after 48 h of dexamethasone administration. Dexamethasone decreased NADPH-d positive cells in the PVN in a dose-dependent manner (A). The inhibitory effect of dexamethasone on NADPH-d staining was limited to the parvocellular subdivision (B). pM, posterior magnocellular subdivision; mP, medial parvocellular subdivision. $^{**}p<0.01$, $^{***}p<0.0001$ vs. vehicle control.

NADPH-d stained cells in the PVN of freely fed rats with adrenalectomy. Furthermore, the decrease occurred only in the parvocellular subdivision where the glucocorticoid receptors are richly located,[26-28] but not in the magnocellular subdivision. These results indicate that the adrenal glucocorticoids are sufficient to down-regulate the PVN-nNOS. Taken all together, it is suggested that the plasma glucocorticoids, which increased during food deprivation, may suppress, perhaps, gene expression of nNOS in the parvocellular PVN, likely by a glucocorticoid receptor-mediated pathway.

Glucocorticoid receptor is known to be a transcription factor belonging to the superfamily of nuclear receptors, which modulate target gene transcription either after binding to DNA or by interfering with the activity of other transcription factors.[30] Thus one can expect that a negative glucocorticoid response element (GRE) may be implicated in the down-regulation of nNOS expression in the PVN, however, nNOS gene dose not contain a GRE in its upstream promoter region.[31] Instead, nNOS gene contains cAMP response element (CRE) in its upstream promoter[31-33] and nNOS expression was reported to be regulated by calcium influx through a CREB family transcription factor-dependent mechanism.[32] We previously reported that almost all the nNOS containing neurons in the PVN appears to have pCREB immunoreactivity as well, and furthermore, pCREB and NADPH-d staining shows a parallel change according to the feeding conditions, i.e. decreased by food deprivation and increased by refeeding.[34] Synthetic glucocorticoid suppresses both CREB phosphorylation[34] and nNOS expression[35] in the PVN, which induced by refeeding. Theses reports strongly support the idea that glucocorticoid-directed suppression of CREB phosphorylation may mediate the fasting-induced down-regulation of nNOS in the PVN. However, molecular mechanism by which glucocorticoids suppress CREB phosphorylation still needs to be explained.

In conclusion, adrenal glucocorticoids may mediate fasting-induced down-regulation of nNOS expression in the parvocellular subdivision of the rat hypothalamic paraventricular nucleus.

ACKNOWLEDGEMENTS

This research was supported by the Neurobiology Research Program from the Korea Ministry of Science and Technology (JWJ). Yun Mi Kim received scholarship support by BK21 Project for Medical Science.

REFERENCES

1. Krukoff TL. Central regulation of autonomic function: no brakes? Clin Exp Pharmacol Physiol 1998;25:474-8.
2. Lee S, Kim CK, Rivier C. Nitric oxide stimulates ACTH secretion and the transcription of the genes encoding for NGFI-B, corticotropin-releasing factor, corticotrophin-releasing factor receptor type 1, and vasopressin in the hypothalamus of the intact rat. J Neurosci 1999; 19:7640-7.
3. Ueta Y, Levy A, Chowdrey HS, Lightman SL. Inhibition of hypothalamic nitric oxide synthase gene expression in the rat paraventricular nucleus by food deprivation is independent of serotonin depletion. J Neuroendocrinol 1995;7:861-5.
4. O'Shea D, Gundlach AL. Food or water deprivation modulate nitric oxide synthase (NOS) activity and gene expression in rat hypothalamic neurons: correlation with neurosecretory activity? J Neuroendocrinol 1996;8: 417-25.
5. Schwartz MW, Dallman MF, Woods SC. Hypothalamic response to starvation: implications for the study of wasting disorders. Am J Physiol 1995;269:R949-57.
6. Casto RM, VanNess JM, Overton JM. Effects of central leptin administration on blood pressure in normotensive rats. Neurosci Lett 1998;246:29-32.
7. Schwartz MW, Marks J, Sipols AJ, Baskin DG, Woods SC, Kahn SE, et al. Central insuline administration reduces neuropeptide Y mRNA expression in the arcuate nucleus of food deprived lean (Fa/Fa) but not obese (fa/fa) Zucker rats. Endocrinology 1991;128:2645-7.
8. Maffei M, Halaas J, Ravussin E, Pratley RE, Lee GH, Zhang Y, et al. Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. Nat Med 1995;1:1155-61.
9. Pirke KM, Spyra B. Catecholamine turnover in the brain and the regulation of leuteinizing hormone and corticosterone in starved male rats. Acta Endocrinol 1982;100:168-76.
10. Garcia-Belenguer S, Oliver C, Mormede P. Facilitation and feedback in the hypothalamo-pituitary-adrenal axis during food restriction in rats. J Neuroendocrinol 1993;5:663-8.
11. Mitev Y, Almeida OF, Patchev V. Pituitary-adrenal function and hypothalamic beta-endorphin release *in vitro* following food deprivation. Brain Res Bull 1993; 30:7-10.

12. Kiss A, Jezova D, Aguilera G. Activity of the hypothalamic pituitary adrenal axis and sympathoadrenal system during food and water deprivation in the rat. Brain Res 1994;663:84-92.
13. van Haasteren GA, Linkels E, Klootwijk W, van Toor H, Rondeel JM, et al. Starvation-induced changes in the hypothalamic content of prothyrotrophin-releasing hormone (proTRH) mRNA and the hypothalamic release of proTRH-derived peptides: role of the adrenal gland. J Endocrinol 1995;145:143-53.
14. van Haasteren GA, Linkels E, van Toor H, Klootwijk W, Kaptein E, de Jong FH, et al. Effects of long-term food reduction on the hypothalamus-pituitary-thyroid axis in male and female rats. J Endocrinol 1996;150: 169-78.
15. Yoshihara T, Honma S, Katsuno Y, Honma K. Dissociation of paraventricular NPY release and plasma corticosterone levels in rats under food deprivation. Am J Physiol 1996;271:E239-45.
16. Hanson ES, Levin N, Dallman MF. Elevated corticosterone is not required for the rapid induction of neuropeptide Y gene expression by an overnight fast. Endocrinology 1997;138:1041-7.
17. Dufourny L, Skinner DC. Influence of estradiol on NADPH diaphorase/neuronal nitric oxide synthase activity and colocalization with progesterone or type II glucocorticoid receptors in ovine hypothalamus. Biol Reprod 2002;67:829-36.
18. Weber CM, Eke BC, Maines MD. Corticosterone regulates heme oxygenase-2 and NO synthase transcription and protein expression in rat brain. J Neurochem 1994;63:953-62.
19. Lopez-Figueroa MO, Itoi K, Watson SJ. Regulation of nitric oxide synthase messenger RNA expression in the rat hippocampus by glucocorticoids. Neurosciece 1998; 87:439-46.
20. Reagan LP, McKittrick CR, McEwen BS. Corticosterone and phenytoin reduce neuronal nitric oxide synthase messenger RNA expression in rat hippocampus. Neuroscience 1999;91:211-9.
21. Schwarz PM, Gierten B, Boissel JP, Foerstermann U. Expressional down-regulation of neuronal-type nitric oxide synthase I by glucocorticoids in N1E-115 neuroblastoma cells. Mol Pharmacol 1998;54:258-63.
22. Waynforth HB, Flecknell PA. Experimental and surgical technique in the rat. New York: Academic Press; 1992.
23. Jahng JW, Kim DG, Houpt TA. Subdiaphragmatic vagotomy induces NADPH diaphorase in the rat dorsal motor nucleus of the vagus. Yonsei Med J 2001;42: 215-9.
24. Paxions G, Watson C. The Rat Brain in Stereotaxic Coordinate. San Diego (CA):Academic Press; 1986.
25. Jahng JW, Kim DG, Houpt TA. NADPH diaphorase staining in the SON, PVN,and rostral NTS after food deprivation [abstract]. Soc Neurosci 1998;24:2129.
26. Liposits Z, Uht RM, Harrison RW, Gibbs FP, Paul WK, Bohn MC. Ultrastructural localization of glucocorticoid receptor (GR) in the hypothalamic paraventricular neurons synthesizing corticotrophin releasing factor (CRF). Histochemistry 1987;87:407-12.
27. Aronsson M, Fuxe K, Dong Y, Agnati LF, Okret S, Gustafsson JA. Localization of glucocorticoid receptor mRNA in the male rat brain by in situ hybridization. Proc Natl Acad Sci USA 1988;85:9331-5.
28. Uht RM, McKelvy JF, Harrison RW, Bohn MC. Demonstration of glucocorticoid receptor-like immunoreactivity in glucocorticoid-sensitive vasopressin and corticotrophin-releasing factor neurons in the hypothalamic paraventricular nucleus. J Neurosci Res 1988;19: 405-11.
29. Jahng JW, Kim YM, Park KI. Glucocorticoid receptor mediated down-regulation of nNOS in the rat PVN during food deprivation. Appetite 2002;39:82.
30. Beato M, Herrlich P, Schutz G. Steroid hormone receptors: many actors in search of a plot. Cell 1995;83: 851-7.
31. Jeong Y, Won J, Kim C, Yim J. 5'-Flanking sequence and promoter activity of the rabbit neuronal nitric oxide synthase (nNOS) gene. Mol Cells 2000;10:566-74.
32. Sasaki M, Gonzalez-Zulueta M, Huang H, Herring WJ, Ahn S, Ginty DD, et al. Dynamic regulation of neuronal NO synthase transcription by calcium influx through a CREB family transcription factor-dependent mechanism. Proc Natl Acad Sci USA 2000;97:8617-22.
33. Rife TK, Xie J, Redman C, Young AP. The 5'2 promoter of the neuronal nitric oxide synthase dual promoter complex mediates inducibility by nerve growth factor. Mol Brain Res 2000;75:225-36.
34. Lee JY, Lee JH, Kim DG, Jahng JW. Dexamethasone blocks the refeeding-induced phosphorylation of cAMP response element-binding protein in the rat hypothalamus. Neurosci Lett 2003;344:107-11.
35. Jahng JW, Houpt TA, Kim GT, Kim DG. Refeeding induced expression of neuronal nitric oxide synthase in the rat hypothalamic paraventricular nucleus. Appetite 2001;37:144.