Yonsei Medical Journal
Vol. 45, No. 5, pp. 917 - 922, 2004

Fasting-induced Down-regulation of NADPH-diaphorase in the Magnocellular PVN of Rats

Joo Young Lee[1], Dong Won Kang[2], Dong Goo Kim[1], and Jeong Won Jahng[1]

[1]Department of Pharmacology, BK21 Project for Medical Science, Yonsei University College of Medicine, Seoul, Korea;
[2]Department of Pharmacology, Kwandong University College of Medicine, Gangwon-do, Korea.

In this study, we examined if glucocorticoids are required for the fasting-induced decrease of neuronal nitric oxide synthase (nNOS) in the magnocellular division of the paraventricular nucleus (PVN). Rats were adrenalectomized, subjected to 48 h of food deprivation with/without dexamethasone (5 mg/kg, 4 subcutaneous injections with 12 h intervals), and the brain slices were processed for NADPH-diaphorase (NADPH-d) staining, a histochemical marker for nNOS in neuronal cells. In food deprived adrenalectomized rats, but not in free fed intact rats, dexamethasone significantly decreased NADPH-d staining in the magnocellular PVN. We previously reported that food deprivation decreases nNOS in the magnocellular PVN of intact rats. Thus, the present results together with our previous report suggest that although glucocorticoids are required for fasting-induced nNOS down-regulation in the magnocellular PVN, glucocorticoids may not be directly involved and some other molecular signals produced by food deprivation may play a pivotal role over glucocorticoid in the regulatory pathway for nNOS expression in this brain region.

Key Words: Food deprivation, hypothalamus, neuronal nitric oxide synthase, glucocorticoid

INTRODUCTION

The nitric oxide (NO) pathway in the central nervous system has been reported to be involved in the control of feeding behavior and appetite.[1-3] Food deprivation suppresses gene expression of neuronal nitric oxide synthase (nNOS) in the hypothalamic paraventricular nucleus (PVN).[4,5] The hypothalamus, particularly the paraventricular nucleus (PVN), is a potential site where metabolic and sensory signals may be integrated with neurochemical changes to produce the integrated response to food deprivation. The PVN nitric oxide has been suggested to be involved in the regulation of autonomic functions.[6,7] Thus, nNOS down-regulation in the PVN may be a part of regulatory pathway for autonomic changes, such as decreased heart rate and blood pressure, which associated with food deprivation.[8] The mechanism by which food deprivation induces nNOS down-regulation in the PVN is largely unknown.

Food deprivation elevates plasma glucocorticoid levels,[9,10] and glucocorticoid receptors are colocalized in the hypothalamic neurons stained with NADPH-diaphorase (NADPH-d), a marker for nNOS enzyme activity in the brain.[11] Fasting-induced down-regulation of the PVN-nNOS is abolished either by adrenalectomy[12] or by treatment with glucocorticoid receptor antagonist RU486.[13] Synthetic glucocorticoid dexamethasone blocks refeeding-induced nNOS expression in the rat PVN.[14] We have also reported that dexamethasone decreases the number of NADPH-d stained cells in the parvocellular PVN of adrenalectomized rats.[12] Many reports have shown that glucocorticoid receptors are richly distributed in the hypothalamic nuclei, particularly the parvocellular subdivision of the PVN.[15-17] Taken all together, it is concluded that plasma glucocorticoids may suppress nNOS expression in the parvocellular PVN during food deprivation, via its re-

Received July 16, 2004
Accepted September 3, 2004

This work was supported by Yonsei University Research Fund of 2000, the Neurobiology Research Program from the Korea Ministry of Science and Technology (JWJ).

Reprint address: requests to Dr. Jeong Won Jahng, Department of Pharmacology, Yonsei University College of Medicine, Seoul 120-752, Korea. Tel: 82-2-361-5233, Fax: 82-2-313-1894, E-mail: jwjahng@yumc.yonsei.ac.kr

ceptor mediated pathway.

The existence of glucocorticoid receptors in the magnocellular subdivision of the PVN has been rarely reported. Indeed, exogenic glucocorticoids did not affect NADPH-d staining in the magnocellular PVN of adrenalectomized rats, contrary to a significant suppression in the parvocellular PVN.[12] Interestingly, we have found that food deprivation appears to rather increase NADPH-d staining in the magnocellular PVN of adrenalectomized rats,[12] but significantly decrease nNOS immunoreactivity in the magnocellular PVN of non-adrenalectomized intact rats.[13] These findings suggested a possible involvement of glucocorticoids in fasting-induced nNOS down-regulation in the magnocelluar PVN as well. In this study, we examined if food deprivation decreases NADPH-d staining in the magnocellular PVN of adrenalectomized rats when exogenic glucocorticoids are supplied.

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 temperature (22 ± 1℃) and humidity (55%) were controlled constantly with a 12 h light-dark cycle (light between 07:00 and 19:00) in the Yonsei University animal facility breeding colony. Rats were individually housed with free access to standard laboratory food (Purina Rodent Chow, Purina Co., Seoul, Korea) and tap water *ad libitum*. 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.

Drug treatment and adrenalectomy

To determine if glucocorticoid decreases NADPH-d staining in both the parvocellular and the magnocellular PVN in non-deprived intact rats, rats weighing 300 - 350g received 4 injections of subcutaneous dexamethasone (5 mg/kg, Chung-Wae Pharmaceuticals, Korea) or saline at 12 h intervals (n=5 in each group). All rats had free access to food and water during the injection period. The drug dose was chosen because we had previously found that NADPH-d staining in the parvocellular PVN, but not in the magnocellular PVN, is significantly suppressed by dexamethasone at this dose and injection schedule in free-fed adrenalectomized rats.[12]

To examine if food deprivation modulates the glucocorticoid effects on NADPH-d staining in each subdivision of the PVN, rats were food deprived along with dexamethasone treatment. Rats were adrenalectomized to exclude an influence from endogenic glucocorticoids. Bilateral adrenalectomy was performed with dorsal approach as previously described.[18] After surgery, rats received 0.9% saline instead of water to drink. After 1 week of post-operational recovery, adrenalectomized rats underwent 48 h of food deprivation, but not fluid deprivation, beginning 1 h after lights-on, and received 4 injections of subcutaneous dexamethasone (5 mg/kg) or saline over the deprivation period at 12 h intervals (n=7 in each group).

All rats were transcardially perfused 12 h after the last drug injection, which was 1 h after lights-on, and the brains processed for NADPH-d staining.

NADPH-diaphorase histochemistry

Transcardiac perfusion and NADPH-d histochemistry were performed as previously described.[19] 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). The coordinates were based on Paxinos and Watson.[20]

Statistical analysis

The number of NADPH-d positive cells was counted visually by an observation blind to the experimental procedure with 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 unpaired t-test using StatView software (Abacus, Berkeley, CA, USA).

RESULTS

Effect of dexamethasone on body weight gain and NADPH-d staining

Rats received 4 injections of dexamethasone (5 mg/kg) or saline over a 48 h period with free access to food and water. Dexamethasone induced a significant weight loss ($p < 0.0001$ vs. saline control) (Fig. 1A) and a marked decrease in the number of NADPH-d stained cells in the PVN (Fig. 1B and 1C). The effect of dexamethasone on NADPH-d staining in the PVN was limited to the parvocellular subdivision (mP) ($p < 0.01$ vs. saline control), where glucocorticoid receptors are predominantly located.[15-17] This result reveals that glucocorticoid is sufficient to decrease NADPH-d of the parvocellular PVN, and suggests that nNOS expression in the magnocellular PVN (pM) may not be influenced by glucocorticoids.

Effect of dexamethasone during food deprivation after adrenalectomy

We examined whether food deprivation modulates the effect of dexamethasone on the PVN-nNOS. Adrenalectomized rats underwent 48 h of food deprivation, and received 4 injections of dexamethasone (5 mg/kg) or saline at 12 h intervals over the deprivation period. A marked weight loss occurred by 48 h of food deprivation, and the fasting-induced weight loss was exacerbated by dexamethasone ($p < 0.05$ vs. saline control) (Fig. 2A). The number of NADPH-d stained cells was significantly decreased by dexamethasone treatment both in the parvocellular (mP) ($p < 0.0001$) and the magnocelluar PVN (pM) ($p < 0.05$), compared to each saline control (Fig. 2B and 2C). These results reveal that some other physiologic changes produced by food deprivation, besides elevated plasma glucocorticoids, may be involved in the down-regulation of nNOS expression in the magnocellular PVN, and suggest that the existence of glucocorticoids may still be

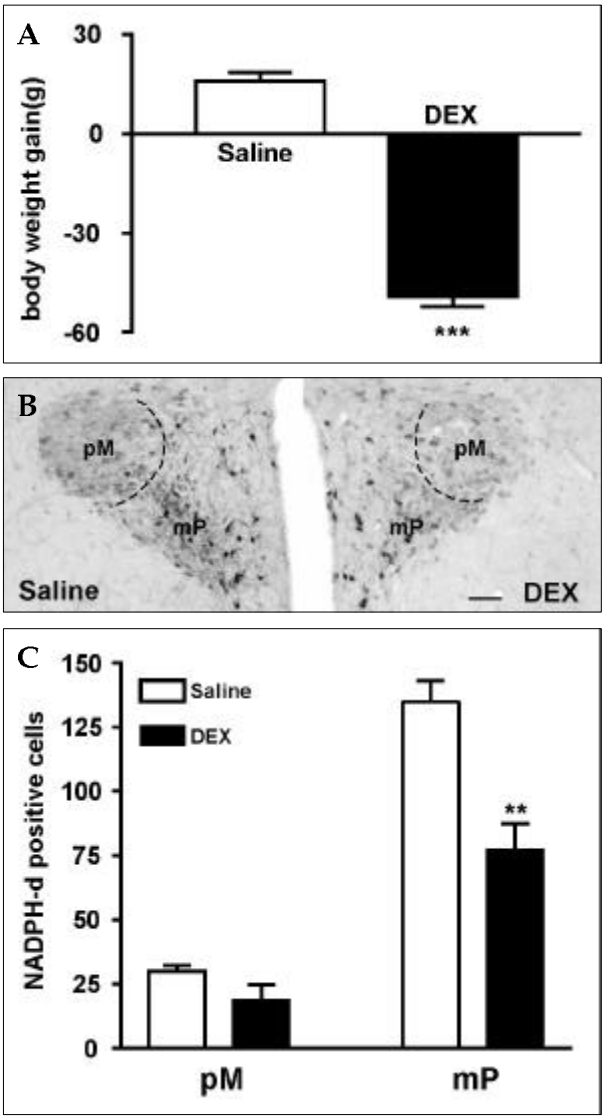

Fig. 1. Changes in body weight gain and NADPH-d stained cells in the paraventricular nucleus (PVN). Rats had free access to food and water, received 4 injections of dexamethasone (5 mg/kg) or saline at 12 h intervals. (A) Dexamethasone produced a significant weight loss over the 48 h treatment period. (B) NADPH-d staining in the PVN. (C) The number of NADPH-d stained cells significantly decreased in the mP, but not in the pM, of dexamethasone treated rats (DEX), compared to the saline controls. mP, medial parvocellular PVN; pM, posterior magnocellular PVN. $^{**}p < 0.01$, $^{***}p < 0.0001$ vs. each saline control, Scale bar; 100 μm.

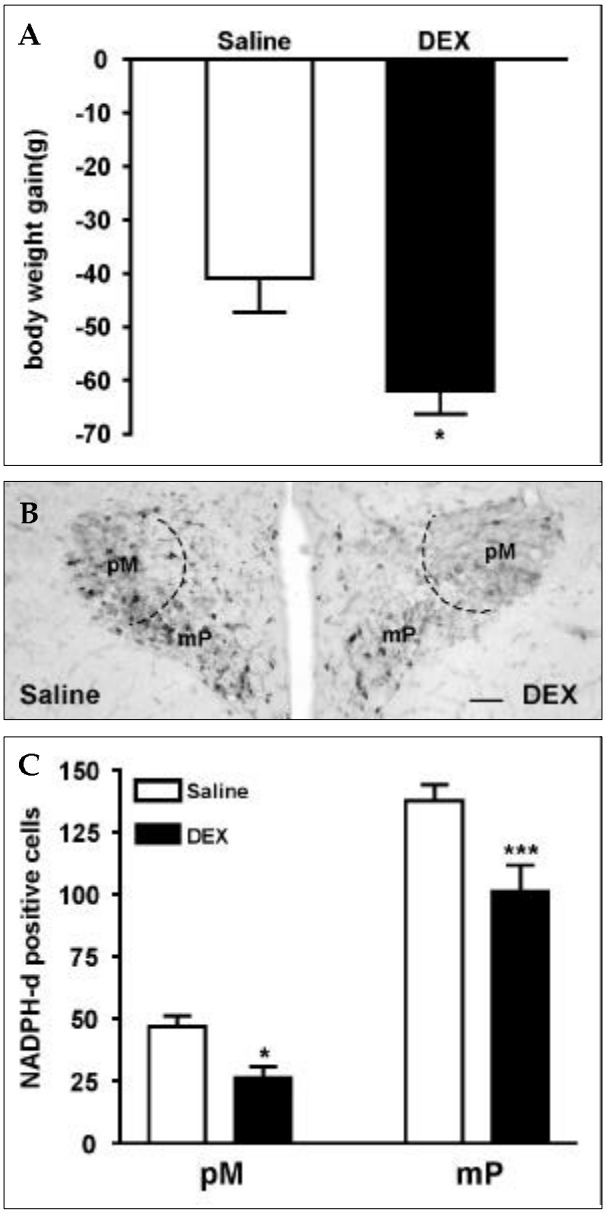

Fig. 2. Changes in body weight gain and NADPH-d stained cells in the PVN of adrenalectomized rats. One week after adrenalectomy, adrenalectomized rats underwent 48 h of food deprivation. Four injections of dexamethasone (5 mg/kg) or saline were given at 12 h intervals over the deprivation period. (A) Dexamethasone exacerbated fasting-induced weight loss. (B) NADPH-d staining in the PVN. (C) Dexamethasone significantly decreased the number of NADPH-d stained cells both in the parvo- (mP) and the magnocellular (pM) PVN, when it was given to the adrenalectomized rats during food deprivation. mP, medial parvocellular PVN; pM, posterior magnocellular PVN. *$p<0.05$, ***$p<0.0001$ vs. each saline control, Scale bar; 100 μm.

required for this regulatory pathway.

DISCUSSION

It has been reported that food deprivation down-regulates gene expression of neuronal nitric oxide synthase (nNOS) in the hypothalamic paraventricular nucleus (PVN),[4,5,21] and that plasma glucocorticoids, which are elevated during food deprivation, may play a pivotal role in the fasting-induced down-regulation of the PVN-nNOS, especially in the parvocellular subdivision.[12,13] In this study, we demonstrated that glucocorticoids are sufficient to suppress the PVN-nNOS in the parvocellular subdivision. That is, synthetic glucocorticoid dexamethasone significantly decreased the number of cells containing NADPH-d, a histochemical marker of nNOS, in the parvocellular PVN of free fed intact rats. This result concurs with our previous report that dexamethasone significantly reduces NADPH-d stained cells in the parvocellular PVN of adrenalectomized rats with free access to food and water,[12] and further supports the idea that plasma glucocorticoids may suppress nNOS expression in the parvocellular PVN during food deprivation.

We previously reported that food deprivation decreases nNOS immunoreactivity in the magnocellular PVN as well as in the parvocellular PVN.[13] The existence of glucocorticoid receptors in the magnocellular PVN has rarely been reported, while many reports have shown the abundant distribution of glucocorticoid receptors in the parvocellular subdivision of the PVN.[15-17] In this study, the number of NADPH-d containing neurons in the magnocellular PVN did not change by dexamethasone treatment in free fed rats. This result concurs with our previous report done in free fed adrenalectomized rats,[12] and suggests that glucocorticoid may not be an active inhibitory molecule in fasting-induced nNOS down-regulation in the magnocellular PVN. However, we have previously found that NADPH-d staining in the magnocellular PVN does not change by food deprivation in adrenalectomized rats, i.e. when endogenic glucocorticoids are depleted.[12] In this study, food deprivation significantly decreased NADPH-d cells in the magnocellular PVN in the adrenalectomized rats when glucocorticoids were supplied with dexamethasone treatment. Therefore, it is concluded that glucocorticoids are

required for fasting-induced nNOS down-regulation in the magnocellular PVN, however, glucocorticoids appear not to be the major inhibitory molecules in the regulation of nNOS expression in this brain region. In other words, some other physiologic changes produced by food deprivation, besides elevated plasma glucocorticoids, appear to play a major role in fasting-induced nNOS down-regulation in the magnocellular PVN.

Fasting-induced down-regulation of the magnocellular nNOS may be a part of the regulatory pathway for autonomic changes, such as decreased heart rate and blood pressure, which associated with food deprivation.[8] The PVN nitric oxide has been suggested to be involved in the regulation of autonomic functions,[6,7] and reported to modulate vasopressin release.[22-24] Vasopressin synthesized in the magnocellular neurons is known to be involved in the control of blood pressure and heart rate[25] as well as in the regulation of fluid and electrolyte balance.[26] It has been reported that mRNA expression of vasopressin in the magnocellular PVN is decreased by food deprivation, but not by glucocorticoid feedback suppression.[27] Taken together, it is postulated that the decrease in nNOS expression in the magnocellular PVN may take a role in the regulatory control for blood pressure and heart rate during food deprivation, perhaps via modulating the activity of vasopressin neurons.

It can be expected that decreased plasma levels of L-arginine, NO precursor, during food deprivation may contribute to the fasting-induced decrease of nNOS in the magnocellular PVN. However, it should be noted that the magnocellular nNOS is decreased by food deprivation only under the conditions of intact, sham adrenalectomy, or adrenalectomy with glucocorticoids supplement, but not in adrenalectomy with no glucocorticoids,[12] and that glucocorticoids only, without food deprivation, do not decrease the magnocellular nNOS. These results suggest that some adrenal components besides glucocorticoid, other than nutritional factors such as L-arginine, may take a role in the fasting-induced down-regulation of the magnocellular nNOS. Adrenomedullin, which was originally isolated from human pheochromocytoma, is abundantly found in the adrenal medulla, and exhibits potent vasodilating properties with a role in the control of fluid and electrolyte homeostasis.[28] Binding sites for adrenomedullin were found in the rat brain, including the hypothalamus,[29] and sympathetic outflow is stimulated by brain injection of adrenomedullin.[30] It has been reported that the nitric oxide-cGMP pathway is involved in the adrenomedullin-induced vasodilation,[31] and that intracerebroventricular adrenomedullin induces the activation of NOS containing neurons in the PVN, stimulates the hypothalamic production of nitric oxide.[30] Furthermore, it was suggested that a role of adrenomedullin in fluid homeostasis may overlay on the vasopressin-aquaporin system.[32] Taken all together, we suggest adrenomedullin as a tentative regulator molecule in the fasting-induced down-regulation of the magnocellular nNOS.

ACKNOWLEDGEMENT

We thank Mr. Gun Tae Kim for excellent technical assistances.

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