—Original—

Systemic Endotoxin Induces Gene Expression of Inducible Nitric Oxide Synthase in Fetal Rat Brain

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Abstract

Background: Few studies have examined the response of the fetus under stress, such as with maternal infection. Recent work has indicated that nitric oxide (NO) modulates corticotropin-releasing hormone (CRH) secretion by the hypothalamus, but details of the action of NO on the fetus remain unclear. Therefore, we investigated the expression of inducible nitric oxide synthase (iNOS) mRNA and the response pattern following lipopolysaccharide (LPS) loading using a rat model of fetal infection.

Methods: Fetuses were delivered by cesarean section on day 20 of gestation and immediately placed in a chamber maintained at 37℃ and 100% relative humidity. The LPS group (n=12) was given 400 μg of LPS/100 g body weight, and the physiologic saline group (n=12) was given physiologic saline. Fetuses were then incubated for a further 3 hours. Fetuses were decapitated, the trunk blood was collected immediately after cesarean section or after 3 hours of incubation, and the fetal brains were fixed in formaldehyde and cryopreserved. Coronal cryosections of the brains were prepared, and a ^{35}S-uridine triphosphate-labeled antisense RNA probe for iNOS was then prepared. *In situ* hybridization was performed, and iNOS expression was evaluated semiquantitatively on the basis of optical density. In both groups, plasma corticosterone levels were determined with radioimmunoassay.

Results: Expression of iNOS mRNA was not noted in the physiologic saline group (3 hours postpartum). In the LPS group, iNOS mRNA expression was observed in the subfornical organ, but not in the paraventricular nucleus. Plasma corticosterone levels were significantly elevated in the LPS group.

Conclusions: In 20-day-old rat fetuses, the hypothalamic-pituitary-adrenal axis was already mobilized in response to LPS-induced stress. These results suggest that iNOS is not involved in the acute response of the hypothalamic-pituitary-adrenal axis to LPS challenge in 20-day-old rat fetuses.

(J Nippon Med Sch 2009; 76: 232–239)

Key words: fetus, inducible nitric oxide synthase, lipopolysaccharide, hypothalamic-pituitary-adrenal axis

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Introduction

When the body is subjected to psychologic or physical stress, the hypothalamus-pituitary-adrenal (HPA) system is activated, and glucocorticocoid secretion by the adrenal cortex is increased. The central role in the stress response is played by corticotropin-releasing hormone (CRH) in the paraventricular nucleus[1]. CRH is a peptide hormone consisting of 41 amino acids which is widely distributed in the nerves of the central nervous system; CRH local to the paraventricular nucleus stimulates ACTH secretion by the pituitary gland[2]. The fetus is thought to respond to a variety of stresses by activating its own HPA axis[3].

It was recently demonstrated that nitric oxide synthase (NOS) is involved in regulating the expression of CRH in the paraventricular nucleus during stress[4,5]. There are 2 types of NOS: constitutive NOS (cNOS), which is ordinarily present in cells and generates nitric oxide (NO) as needed, and inducible NOS (iNOS), which is not normally present but is produced as a result of gene activation by substances, such as endotoxins (elements that make up cell walls of *Escherichia coli*) and inflammatory cytokines (e.g., tumor necrosis factor γ and interleukin (IL) 1)[6,7].

iNOS mRNA is activated by a variety of stimuli, including the stress of infection, and is reported to be deeply involved in activation of the HPA axis[8,9]. In the fetus, however, the central nervous system is immature, and the regulation of the HPA axis function may differ from that after parturition. Indeed, Grino et al.[10] have reported that in rat fetuses, corticotropin-releasing factor (CRF) mRNA began to appear in the region of the paraventricular nucleus beginning on fetal day 17, increasing through day 20, and then decreasing on day 21, as birth approached. The levels of CRF mRNA became lowest levels on postpartum day 2 and then gradually increased. Although there have been reports on the effects of maternal CRF secreted in response to stress during gestation[11], to our knowledge there have been no reports on the role of NOS in the regulation of CRF secretion related to the stress response, and details of the role of NOS remain unclear.

It has recently been asserted that maternal infections, such as those resulting from premature rupture of membranes, increase the frequency of neurodevelopmental disorders, such as cerebral palsy[12]. Consequently, the fetal response to infectious stress is highly significant for perinatal management. We therefore investigated iNOS mRNA expression and the response of the HPA axis to lipopolysaccharide (LPS) administration using *in situ* hybridization in a rat model of fetal infection.

Materials and Methods

Animals and Tissues

All experiments were performed with the approval of the Nippon Medical School Animal Care Committee. Timed-pregnant Wister rats were purchased from Saitama Jikken Dobutsu Laboratory (Saitama, Japan). The morning that the vaginal plug was detected was defined as embryonic day 0 (E0). Pregnant Wistar rats were decapitated and the fetuses delivered by cesarean section on day 20 of gestation. An isothermal plate was maintained at 37℃, and a soft, dampened piece of paper was placed over it. The fetuses were immediately housed in glass containers at a humidity of 100%. The LPS group (n=12) was given LPS intraperitoneally (LPS from *E. coli* serotype 055: B5, 400 μg/100 g body weight). This dose was selected on the basis of the previous report that it elicits a robust activation of CRH, CRH receptors, and iNOS gene expression in the periventricular nucleus of adult rats[13,14]. The control group (n=12) was given physiologic saline intraperitoneally. Both groups were then incubated for 3 hours. Fetuses were decapitated immediately after cesarean section or after 3 hours of incubation, and were then exsanguinated from the head and trunk using a microcapillary tube (Terumo, Tokyo, Japan). Specimens were centrifuged at 4,000 rpm for 3 minutes, and plasma corticosterone concentrations were determined with radioimmunoassay. Fetal brains were then fixed in paraformaldehyde. One day before sectioning, embryos were immersed in Zamboni fixative containing 20% sucrose.

Sagittal or coronal serial sections (20 μm) were cut on a cryostat, thaw-mounted onto silane-coated slides, and stored at −70℃ until *in situ* hybridization histochemical studies were performed.

In situ Hybridization Histochemistry

In situ hybridization was performed as described previously[15,16]. Briefly, sections were dried for 0.5 to 1 hour, immersed in 1% Triton X-100 in 50 mM Tris/HCl buffer (pH 8.0) containing 25 mM EDTA for 30 minutes, and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine. Sections were dehydrated in an ascending ethanol series and air-dried. The probe (1×10^6 dpm/mL) was dissolved in buffer containing 50% formamide, 10% dextran, 1× Denhart's solution, 12 mM EDTA (pH 8.0), 10 mM Tris/HCl (pH 8.0), 30 mM NaCl, 0.5 mg/mL yeast tRNA, and 10 mM dithiothreitol; 100 μL of probe solution was applied to each slide. Slides were coverslipped and incubated at 60℃ overnight. Coverslips were then removed, and the slides were rinsed in 4x s— s— c— (SSC), digested with RNAase A (20 mg/mL) for 30 minutes at 37℃, and rinsed sequentially in 2 x SSC, 1 x SSC, and 0.5 x SSC, followed by 30 minutes of rinsing in 0.1 x SSC at 60℃, before being dehydrated again. Sections were used to expose X-ray film (Kodak X-omat, Eastman Kodak Co., Health Imaging Division, Rochester, NY, USA) for 7 days. The levels of iNOS mRNA were semiquantitatively determined with densitometric analysis of the autoradiogram produced on X-ray film using an MCID image analysis system (Imaging Research, St. Catherines, Ontario, Canada). The system digitizes a continuous range of image gray shades into 256 discrete levels of gray, in which the lower values are assigned to the darker groups. The levels obtained were converted to relative optical densities (ROD) using the following formula: $ROD=\log_{10}(256/levels)$.

iNOS Riboprobe

A 217-bp fragment of the 5'-end of rat iNOS cDNA was subcloned into pBlueScript vectors (Stratagene, La Jolla, CA, USA) and linearized with *Eco*RI. Radioactive cRNA antisense copies were synthesized by incubating 36 mM Tris (pH 7.5), 0.1 μg linearized plasmid in 6 mM $MgCl_2$, 2 mM spermidine, 8 mM dithiothreitol, 25 mM ATP/guanosine triphosphate/cytosine triphosphate, 5 mM unlabeled uridine triphosphate (UTP), (α-^{35}S) UTP, 1 U RNAsin (Promega, Madison, WI, USA), and 10 U T7 polymerase for 60 minutes at 37℃. Radiolabeled probe, with a specific activity of 1.0×10^8 c.p.m./μg, was then purified on resin columns (Nensorb 20, NEN, Wilmington, DE, USA).

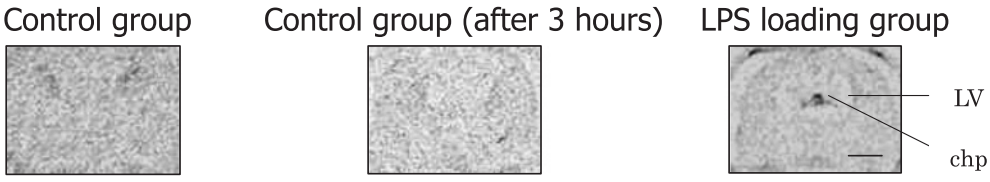
Statistical Analysis

Data are expressed as means ± SD. Significance was determined with a one-way analysis of variance with the Student's *t*-test to determine differences among groups. These tests were performed with SPSS 14.0J statistical software (SPSS, Inc., Chicago, IL, USA), and p values of less than 0.05 were considered to indicate significance.

Results

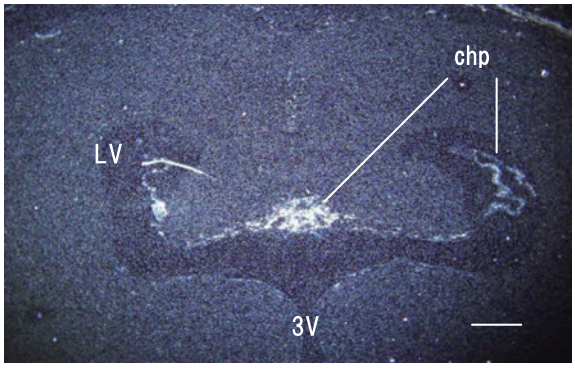
Autoradiography showed no iNOS mRNA expression in the physiologic saline group before physiologic saline administration or 3 hours after administration. In the LPS group, localized iNOS mRNA expression was noted in the subfornical organ, whereas no iNOS mRNA expression occurred in the paraventricular nucleus 3 hours after LPS administration (**Fig. 1**). Dark-field observation of tissue sections showed expression only in the choroidal plexus and subfornical organ (**Fig. 2**). On semiquantitative evaluation of iNOS mRNA expression on the basis of optical density, no expression was detected before or 3 hours after physiologic saline administration in the physiologic saline group, while an ROD value of 0.16 ± 0.03 was obtained in the LPS group; the difference between the groups was statistically significant (**Table 1**).

No significant increase in the plasma corticosterone levels occurred in the physiologic saline group with chamber incubation 3 hours after physiologic saline administration, but a significant increase was shown in the LPS group (357 ± 51.37 vs. 1,085 ± 283.92 ng/mL; **Table 2**).



bar 1mm
LV: light ventricle
chp: choroidal plexaus

Fig. 1 Comparison of iNOS mRNA expression in the fetal brain
Fetal brain sections were affixed to slides and observed with autoradiography. In the physiologic saline group, no iNOS mRNA expression was seen before physiologic saline administration or 3 hours after administration. In the LPS group, iNOS mRNA expression was noted in the subfornical organ.



bar 500 μm
LV: light ventricle
3V: 3rd ventricle
chp: choroidal plexaus

Fig. 2 iNOS mRNA expression observed with the dark-field method
Fetal brain sections were affixed to slides, a photographic emulsion was applied, and the specimens were developed by exposure to light in a dark box. In the LPS group, iNOS mRNA was expressed locally in the choroidal plexus and subfornical organ.

Discussion

CRH plays an important role as a coordinator of the HPA axis and is released from the paraventricular nucleus of the hypothalamus in response to stress[1].

The HPA axis is also thought to play an important role in the stress response in the fetus. *In vitro*, the fetal pituitary gland secretes ACTH in response to CRH, angiotensin, and vasopressin beginning on fetal day 17[17]. In addition, ACTH and corticosterone are present in the blood at measurable levels beginning on fetal day 16, and the concentrations of these substances increase on day 19. The ACTH content of the pituitary gland shows the same changes as do blood ACTH levels, and increases in blood ACTH and corticosterone levels are inhibited by administration of anti-CRF antibody to dams[3]. At the gene level, CRF mRNA appears in the small cells of the paraventricular nucleus beginning on fetal day 17 and remains at nearly the same level from fetal days 17 to 19. On day 21, however, CRF mRNA levels rapidly decrease and reach their lowest levels at parturition. By day 4 postpartum, CRF mRNA returns to the levels seen in mature rats[18]. In the fetus, the response to stress has been reported to include an increase in CRF mRNA in the paraventricular nucleus and an increase in blood ACTH in response to nonspecific stress[19]. In addition, increases in fetal blood ACTH and corticosterone levels occur in the fetus beginning on day 18 in response to stress placed on the dam[20,21]. These findings indicate that the physiologic role of CRH begins from fetal day 17[3]. On the basis of these reports, to investigate activation of the HPA axis and iNOS mRNA expression we used 20-day-old fetal rats, in which the HPA axis is already thought to respond to stress.

LPS is a structural component of the cell membrane of bacteria and is commonly used as a model of endotoxin infection. Peripheral administration of LPS stimulates production of tumor necrosis factor alpha, IL-6, and IL-1-beta, which are primary endogenous mediators[22]. It is

Table 1 Comparison of iNOS mRNA expression in the subfornical organ in the physiologic saline and LPS groups

	R.O.D (Mean ± SD)		
・Physiologic saline group (before saline administration)	0.01 ± 0.01	N.S.	＊
・Physiologic saline group (3 h after saline administration)	0.01 ± 0.01		
・LPS group (3 h after administration)	0.16 ± 0.03		

N.S. (not significant). ＊$P < 0.05$

Table 2 Comparison of blood corticosterone levels (ng/mL) in the physiologicsaline and LPS groups

	R.O.D (Mean ± SD)		
・Physiologic saline group (before saline administration)	357 ± 51.37	N.S.	＊
・Physiologic saline group (3 h after saline administration)	373 ± 54.54		
・LPS group (3 h after administration)	1,085 ± 283.92		

N.S. (not significant). ＊$P < 0.05$

thought that these cytokines cross the blood-brain barrier by means of the circumventricular organs, which lack the blood-brain barrier, or by active transport, and that they then stimulate the HPA axis. Moreover, it has been reported that cytokines are produced by neurons and glial cells in the brain and may activate the HPA axis[23]. The cytokines also stimulate production of secondary mediators in the brain, including NO, via cell-surface receptors on macrophages and vascular endothelial cells[4].

Although maternal LPS administration was considered as a model for examining the fetal response to maternal infections, the effects of the placental barrier could not be eliminated. Therefore, in the present study, we directly administered LPS to fetuses immediately after delivery to reproduce acute infection. iNOS mRNA expression was not seen in the paraventricular nucleus but was seen locally in the subfornical organ 3 hours after LPS adminstration. The subfornical organ is a circumventricular organ structure consisting of the organum vasculosum laminae terminalis, neurohypophysis, median eminence, area postrema, subcommissural organs, and pineal gland. The circumventricular organs are distributed along the cerebral ventricle of the central nervous system and detect osmotic pressure, salinity, endocrine hormones in the cerebrospinal fluid and blood constituents. The sites in the brain that are thought to participate in sensing salt concentrations are the organum vasculosum laminae terminalis and subfornical organ. These sites lack a blood-brain barrier. The subfornical organ, in particular, is directly affected by blood-borne stimulation and is, therefore, a suitable site for monitoring the concentrations of substances in the cerebrospinal fluid and blood[24].

The accumulated evidence strongly suggests that NO is involved in regulating the HPA axis by modifying CRH secretion from the neuroendocrine cells of the paraventricular nucleus[4,5,25]. However, the precise mechanism of action remains unclear. One possibility that has been suggested is that NO produced by iNOS activation after LPS administration stimulates CRH-producing neurons in the paraventricular nucleus[26]. In an anatomic investigation, Harada et al.[15] have found that intraperitoneal LPS administration induces expression of the iNOS gene in parvocellular neurosecretory cells, mainly in the paraventricular nucleus. Moreover, using double *in situ* hybridization, they found that 48.4% of the iNOS-positive cells were positive for CRF mRNA[15]. Pozzoli et al.[27] found in an *in vitro* investigation using a culture system for rat hypothalamic tissue that aminoguanidine, a selective inhibitor of iNOS, inhibits CRH secretion induced by the gp 120 coat protein of the immunodeficiency virus type I and the cumulative action of CRF mRNA but had no effect on selective inhibitors of nNOS and endothelial NOS[27]. In addition,

an investigation using iNOS knockout mice found that destruction of the iNOS gene induces the c-fos gene in the LPS-stimulated paraventricular nucleus, upregulates the CRH gene, and significantly decreases subsequent ACTH secretion[26].

On the other hand, induction of c-fos mRNA, used as a marker of neuroexcitation in the choroidal plexus and subfornical organ, and increased expression of IL-2 mRNA have been reported following LPS administration[28], and the subfornical organ is believed to play a role in activating the hypothalamic-pituitary system[29]. Consequently, a transmission pathway between the subfornical organ and paraventricular nucleus is thought to exist[30]. In monkeys, CRH fiber terminals are present in the subfornical organ, demonstrating a connection between the subfornical organ and CRH neurons in the paraventricular nucleus[31]. These findings indicate that cytokines released by LPS cross the blood-brain barrier by means of the circumventricular organs and active transport and subsequently activate iNOS. NO, which is produced in large quantities, stimulates the nerve activity of cells that contain CRH, facilitating the secretion of CRH and ACTH and upregulating the genetic expression of CRF mRNA in the paraventricular nucleus. This hypothesis is supported by the expression of iNOS mRNA in the subfornical organ and choroidal plexus seen after LPS loading in the present investigation.

In a study using mature male rats performed by Harada et al.[15], iNOS mRNA expression was demonstrated in the paraventricular nucleus 3 hours after LPS administration. In the present study, however, iNOS mRNA was not expressed in the paraventricular nucleus but was expressed locally in the subfornical organ 3 hours after LPS administration. In the fetus, CRH levels decrease after birth and do not increase even if the animal is subjected to stress, suggesting that this may be a so-called stress-nonresponsive period[10]. In the present study, however, fetal plasma corticosterone concentrations increased (**Table 2**), indicating that the HPA axis had already been activated by LPS at fetal day 20. Therefore, it is unlikely that NO alone is responsible for HPA axis activation by LPS in the fetus.

In addition to iNOS, prostaglandins and cytokines are thought to be involved in the HPA axis response induced by LPS[25,32]. Cyclooxygenase-2 and prostaglandin E2 have been reported to be induced in the choroidal plexus and subfornical organ after LPS administration[33,34], and LPS is believed to activate the CRH neurons of the paraventricular nucleus in the choroidal plexus and circumventricular organs. Moreover, marked increases in ACTH and corticosterone levels have been shown following LPS administration in CRH knockout mice, and LPS has been reported to act directly on the pituitary gland to stimulate ACTH secretion[35]. LPS may have also stimulated ACTH-corticosterone secretion without mediation by CRH in the present investigation. This finding suggests that iNOS induction in the paraventricular nucleus after LPS administration is strictly part of stress transmission and is not yet invoked through 3 hours after administration in the fetus, unlike in the adult animal. However, although the HPA axis can be stimulated for long periods by LPS administration, this study did not investigate beyond 3 hours after LPS administration. Consequently, NO may have had an effect beyond 3 hours after LPS administration, and the long-term effects of LPS in the fetus should be investigated further.

In the present investigation, the responsiveness of the HPA axis to LPS in the fetus differed from that reported for mature animals. The possibility that this difference was due to differences in experimental systems, as well as in the immaturity of the hypothalamus, cannot be ruled out. Although infection of the human fetus has been reported to cause severe damage to the central nervous system[12,36,37], this damage may be due to the immaturity of the defense mechanisms against infectious stress in the fetal brain. Further investigation of this issue is therefore desirable.

Acknowledgement: We thank Dr. Toshihiro Imaki for helpful discussions during the course of these studies and providing useful comments.

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(Received, December 17, 2008)
(Accepted, March 4, 2009)