Endocrine Journal 2000, **47** (3), 221–229

A Defect of Lipopolysaccharide-Induced Nitric Oxide Synthase Gene Expression in the Paraventricular Nucleus of Lewis Rats

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Abstract. The hypothalamo-pituitary-adrenal (HPA) axis activity of Lewis rats has been reported to be hyporesponsive to immune challenge, and nitric oxide (NO) has been demonstrated to be involved in the regulation of the HPA axis during the response to immune challenge. The present study investigates the effect of systemic injection of lipopolysaccharide (LPS) on NO production within the paraventricular nucleus (PVN) of immature female Fisher-344 (F344) and Lew/N rats (Lew/N), to clarify the pathophysiological role of NO in the dysregulation of the HPA axis in Lewis/N. Intraperitoneal injection of 25 mg/kg LPS significantly increased neuronal NO synthase (nNOS) mRNA expression in the PVN of F344 rats, but did not change nNOS mRNA levels in the PVN of Lew/N rats. CRF mRNA levels in the PVN significantly increased in response to LPS injection only in F344 rats. In contrast, inducible NOS (iNOS) mRNA increased similarly in both strains. These results demonstrated a defect of up-regulation of nNOS gene expression in the PVN of Lew/N rats following immune challenge. The defect appears to be associated with the dysfunction of the HPA axis in this strain. An increase in iNOS mRNA may partially restore NO production in the PVN.

Key words: Paraventricular nucleus, Lewis rat, Nitric oxide, NO synthase, Corticotrophin-releasing factor

(*Endocrine Journal* **47**: 221–229, 2000)

THE inflammatory-disease-susceptible Lewis (Lew/N) rat strain is prone to autoimmune diseases and its hypothalamo-pituitary-adrenal (HPA) axis is hyporesponsive [1–4]. Decreased biosynthesis of corticotropin-releasing factor (CRF), a main regulator of the HPA axis [5–7], has been regarded as one of the causes of the hyporesponsiveness of the HPA axis in Lew/N rat. CRF mRNA in the paraventricular nucleus (PVN) of Lew/N rats does not increase in response to streptococcal cell wall (SCW) injection as robustly as in immature female Fisher rats [2, 3].

Nitric oxide (NO) is a labile free gas that conveys biological information [8], and NO is now recognized as a potential neurotransmitter within the brain [9–12]. NO formation is under the control of the enzyme NO synthase (NOS), which occurs in several distinct isoforms [8]. The classic constitutive NOS (endothelial [eNOS] and neuronal NOS [nNOS]) pathways are localized in the brain [13]. The most prominent sites of localization of nNOS, apart from the cerebellum, are the hypothalamus, most notably the PVN [14, 15], suggesting a role for NO in biological function integrated within these areas. Systemic lipopolysaccharide (LPS) injection has been reported to increase nNOS gene expression in the PVN [16], suggesting that NO plays an important

Received: October 6, 1999
Accepted: March 3, 2000
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function in mediation of the HPA axis response to immune challenge.

Expression of iNOS has been demonstrated in the brain, besides the constitutive isoforms of nNOS. iNOS is preferentially expressed in macrophages, and up-regulated in response to a variety of inflammatory stimuli [17, 18]. However, Wong *et al.* demonstrated that profound induction of iNOS mRNA occurs in vascular glial and neuronal structures of the rat brain, including the PVN [19]. Recently, we reported that approximately half of the iNOS-producing PVN neurons of LPS-treated rats co-express CRF mRNA, based on the results of double in situ hybridization [20]. Thus, it is likely that NO derived from iNOS, as well as from nNOS, plays an important role in the neuroendocrine response to sepsis, especially in HPA axis activation.

Although Lew/N rats may provide a useful model for studying the relationship between the neuroendocrine and inflammatory response, NO production in the PVN following endotoxin administration has never been investigated. Therefore, the purpose of the present study was to investigate the activation of NO production in the PVN of the Lew/N rat in comparison to the F344 rat. Since most of the studies describing deficient hypothalamic CRF response to multiple inflammatory stimuli was performed in immature female rats, 5-week old female Lew/N and F344 rats were used in this study. We particularly focused on nNOS and iNOS gene expression in the PVN following intraperitoneal (ip) injection of LPS at a dose that induces clear manifestations of systemic inflammation.

Materials and Methods

Five-week-old female F344/N and Lew/N rats were used in the experiments. The animals were housed five per cage in a room with controlled temperature and a fixed lighting schedule (lights on from 8 : 00 am to 8 : 00 pm). Food and water were given *ad libitum*. Five rats were examined in each group.

Since we did not detect any significant increase in nNOS mRNA or iNOS mRNA levels in the PVN at 0.5, 2.5, or 5 mg/kg of LPS (L-2880, from E-coli, serotype 0111: B4, Sigma) in our previous study [20], we chose a dose of 25 mg/kg. After a one-week period of adaptation, the rats were intraperitoneally injected with 25 mg/Kg of LPS or sterilized normal saline and 5 hours later they were intraperitoneally injected with sodium pentobarbital (50 mg/kg) and perfused immediately after deep anesthesia (within 2 min) with ice-cold 4% paraformaldehyde in pH 9.5 0.1 M borate buffer [21]. This protocol was approved by the Animal Committee of Tokyo Women's Medical University. The excised brains were placed in 4°C fixative containing 20% sucrose for 2 days. Frozen sections (20 μm) were cut on a sliding microtome, and the sections were mounted onto silane-coated slides (Matsunami, Tokyo) and air-dried.

The hybridization protocol was basically the same as described previously [21]. Prior to hybridization, the sections were dried overnight under vacuum, digested with proteinase K (10 μg/ml, 37°C, 15–20 min), acetylated, and dehydrated. After vacuum-drying, 100 μl of the hybridization mixture (10^6 cpm/ml, with 10 mM dithiothreitol [DTT]) was spotted onto each slide, sealed under a coverslip, and incubated at 65°C overnight for the CRF and nNOS probes, and for 2 days for the iNOS probe. The coverslips were then removed, and the slides were rinsed in 4 × SSC (1 SSC; 15 mM trisodium citrate buffer, pH 7.0/0.15 M NaCl) at room temperature. Sections were digested with RNAase A (20 μg/ml, 37°C, 30 min) and washed in 0.1 × SSC for 30 min at 65°C. These sections were then exposed at 4°C to X-ray film for 5 to 21 days, dipped in NTB2 nuclear emulsion (1 : 1 with water, Kodak), exposed for 14 to 50 days, and developed. The slides were counterstained with thionin. An adjoining series of sections was stained with thionin to provide better cytoarchitectonic definition for analysis. All samples from a single experiment were assayed simultaneously.

The levels of CRF, nNOS mRNA, and iNOS mRNA in the PVN were semiquantitated by densitometric analysis of the autoradiograms produced on X-ray film by using an MCID image analysis system (Imaging Research, Inc., St. Catherines, Ontario, Canada) [22]. This system digitizes the continuous range of image gray shades into 256 discrete levels of gray, with the lower values being assigned to the darker groups. The levels obtained were converted to relative optical densities (ROD) by using the formula: $\mathrm{ROD} = \log_{10}(256/\mathrm{levels})$. The PVN was enclosed by a square (450 μm × 520 μm) as a fixed window that covered virtually the entire nucleus, and the ROD within the window was measured bilaterally.

The background was assessed by measuring the ROD within the window placed over another area of brain sections in which no RNA for CRF, nNOS, or iNOS was expressed. Data from the five rats in each group were analyzed. The sections analyzed were those that contained the dorsal aspect of the medial parvocellular subdivisions of the PVN, corresponding to plate 25 of the rat brain atlas of Paxinos and Watson [23].

The Pst I fragment of rat nNOS cDNA and a 217 bp fragment of the 5′-end of rat iNOS cDNA (a gift from Drs. S. Hirose and H. Hagiwara) were subcloned into p-Bluescript (Stratagene, La Jolla, CA, USA) and linearized with Bam HI and EcoRI, respectively. A pGEM-4 plasmid containing the rat CRF cDNA (1.2 kb, a gift from Dr. K. Mayo) was linearized with Hind III. Radioactive cRNA antisense copies were synthesized by incubation of 36 mM Tris, pH 7.5, 0.1 μg linearized plasmid in 6 mM $MgCl_2$, 2 mM spermidine, 8 mM DTT, 25 mM ATP/GTP/CTP, 5 mM unlabeled UTP, (α-^{35}S)UTP, 1 U RNAsin (Promega, Madison, WI, USA), and 10 U T7 polymerase for nNOS and iNOS probes, and SP-6 polymerase for CRF probe for 60 min at 37°C. A radiolabeled probe, with specific activity of 1.0×10^8 cpm/μg, was then purified on resin columns (Nensorb 20, NEN, Wilmington, DE, USA).

Results are presented as means ± SEM. Statistical significance of the data was initially established by two-way analysis of variance (ANOVA) (StatView 4.11). Strain and treatments were used as the main factors. When appropriate, post-hoc comparisons were carried out by using the Scheffe test. A level of $p < 0.05$ was regarded as statistically significant.

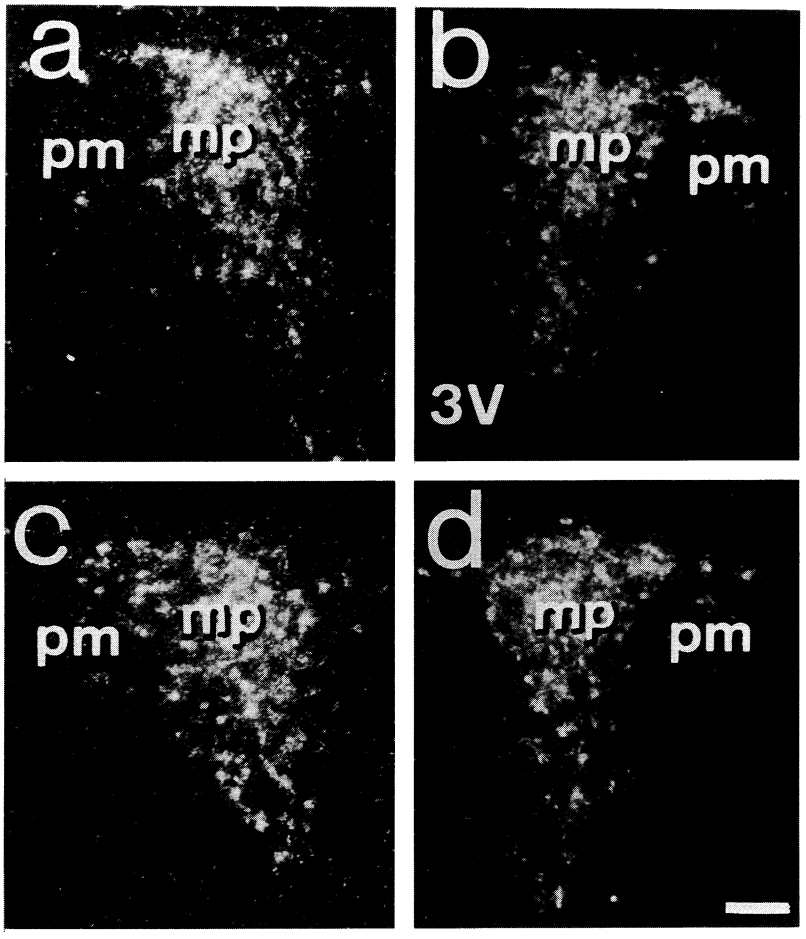

Fig. 1. Darkfield photomicrographs demonstrating the distribution of CRF mRNA expression in the PVN: (a) control F344 rat, (b) control Lew/N rat, (c) LPS-treated F344 rat, (d) LPS-treated Lew/N rat. mp: medial parvocellular PVN, pm: posterior magnocellular PVN, 3V: third ventricle. Bar = 100 μm

Results

CRF mRNA was mainly expressed in the parvocellular PVN in saline-injected rats (Fig. 1a, b). CRF mRNA levels of a F344 and Lew/N rats were almost similar in saline-injected group (Fig. 1a, b). CRF mRNA in the parvocellular PVN was increased following LPS injection in F344 rats (Fig. 1c). The CRF mRNA level appeared to be unchanged in Lew/N rats (Fig. 1d). nNOS mRNA was expressed mainly in the magnocellular division of the PVN, although a scattered signal was also detected in the parvocellular PVN (Fig. 2a, b). The nNOS mRNA level was not different between F344 (Fig. 2a) and Lew/N rats (Fig. 2b). The nNOS mRNA level in the PVN substantially increased in response to LPS injection in F344 rats (Fig. 2c), but only slight changes were observed in Lew/N rats (Fig. 2d). Hybridization for iNOS was indistinguishable from the surrounding areas within the PVN in the saline-injected control (Fig. 3a, b). iNOS mRNA was expressed in the PVN in the LPS-treated group of both F344 (Fig. 3c) and Lew/N rats (Fig. 3d). iNOS mRNA was clearly distributed in the parvocellular as well as the magnocellular division of PVN (Fig. 3c, d).

The results of the densitometric analysis are summarized in Fig. 4. The nNOS mRNA and CRF mRNA levels were normalized with respect to the saline-treated F344 rats. Since iNOS mRNA was undetectable in the control, the data are shown as ROD values. There was no difference in saline-induced CRF and nNOS mRNA levels in the PVN between F344 and Lew/N rats (Fig. 4b; ROD for CRF mRNA of F344: 0.423 ± 0.067, Lew/N: 0.388 ± 0.018, N.D.; ROD for nNOS mRNA of F344: 0.264 ± 0.035, Lew/N: 0.253 ± 0.008, N.D.). The CRF mRNA and nNOS mRNA levels in the PVN increased significantly in response to LPS injection in

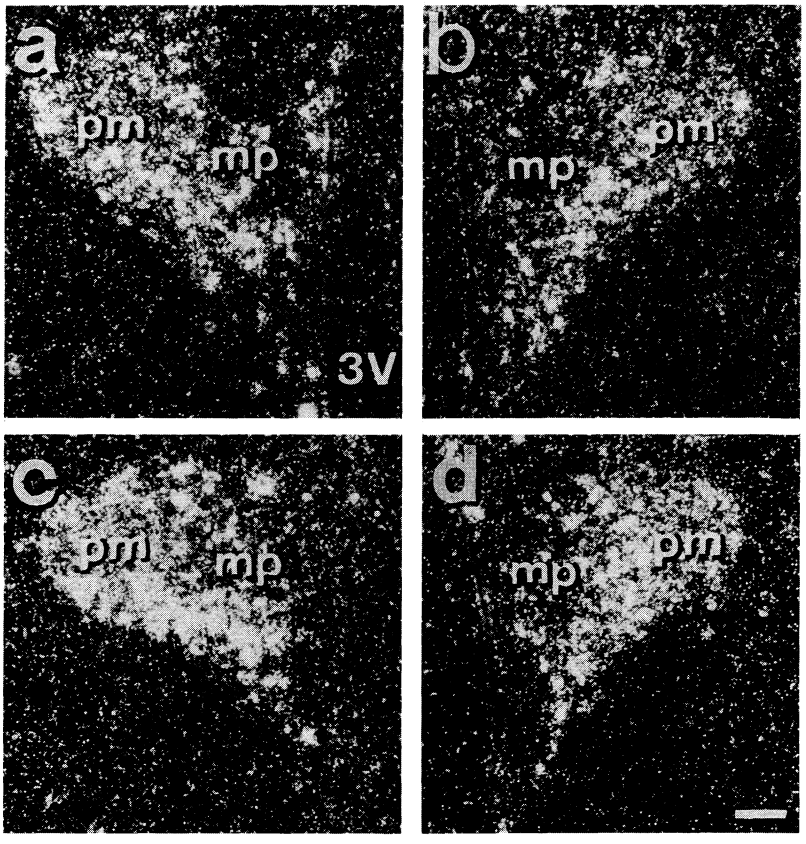

Fig. 2. Darkfield photomicrographs demonstrating the distribution of nNOS mRNA expression in the PVN: (a) control F344 rat, (b) control Lew/N rat, (c) LPS-treated F344 rat, (d) LPS-treated Lew/N rat. mp: medial parvocellular PVN, pm: posterior magnocellular PVN, 3V: third ventricle. Bar = 100 μm

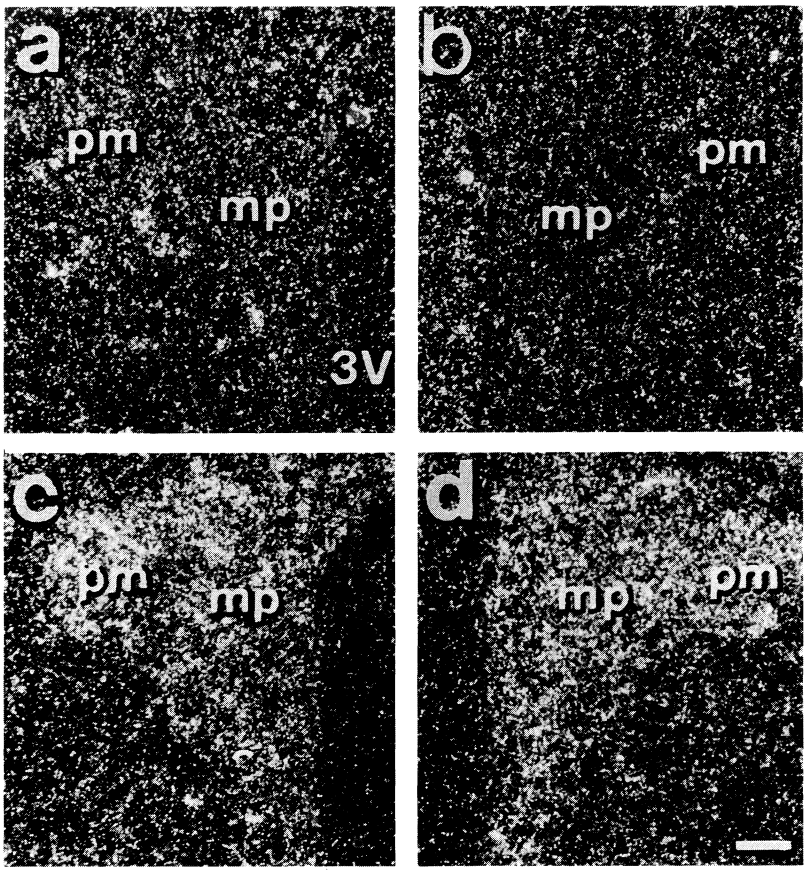

Fig. 3. Darkfield photomicrographs demonstrating the distribution of iNOS mRNA expression in the PVN: (a) control F344 rat, (b) control Lew/N rat, (c) LPS-treated F344 rat, (d) LPS-treated Lew/N rat. mp: medial parvocellular PVN, pm: posterior magnocellular PVN, 3V: third ventricle. Bar = 100 μm

the F344 rats, compared to the saline-treated control. In contrast, no significant changes in CRF mRNA or nNOS mRNA levels were observed in the Lew/N rats (Fig. 4a, b), and both CRF mRNA and nNOS mRNA levels in the PVN of the LPS-treated rats were significantly lower in the Lew/N rats than the F344 rats (Fig. 4a, b). There was a statistically significant correlation between CRF and nNOS mRNA levels in LPS-treated rats ($r=0.645$, $P=0.011$). The iNOS mRNA levels in the PVN were significantly increased in F344 as well as Lew/N rats (Fig. 4c). The iNOS mRNA levels in the PVN after LPS injection were almost the same in both strains (Fig. 4c).

In the supraoptic nucleus (SON), neither CRF nor iNOS mRNA was detected. There was no significant difference in nNOS mRNA in the SON among 4 groups of the rats (not shown).

Discussion

The present study demonstrated that both CRF and nNOS gene expressions in the PVN in response to LPS injection was significantly attenuated in Lew/N rats, as compared with F344 rats. In contrast, iNOS mRNA was similarly up-regulated in the PVN of both Lew/N and F344 rats. Thus, CRF gene expression induced by immune challenge in the PVN of Lew/N rats was decreased together with the reduction in nNOS gene expression.

The defect in up-regulation of CRF mRNA in the PVN induced by LPS injection is highly consistent with the findings in previous studies [2, 3]. Lew/N rats are reported to have a deficient hypothalamic CRF response to multiple inflammatory and non-inflammatory stimuli, whereas histocompatible F344 rats display a robust increase in hypothalamic CRF [1–4]. This study showed for the first time that a

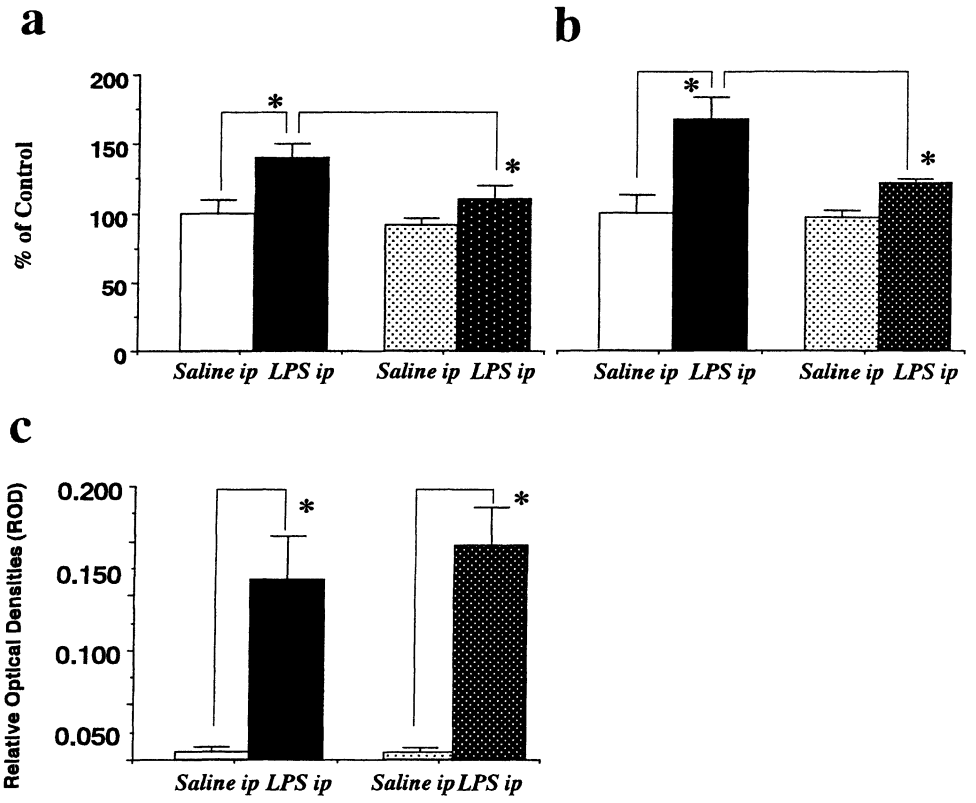

Fig. 4. Densitometric analysis showing CRF (a), nNOS (b), and iNOS mRNA (c) levels in the PVN of F344 rats (left columns) and Lew/N rats (right columns) in the saline-treated group and LPS-treated group, respectively. *$P < 0.05$ between the two groups.

defect of nNOS gene induction coincides with a reduction of up-regulation of the CRF gene in the PVN of Lew/N rats. This parallel regulation of CRF and NOS mRNAs has been previously described in response to different stimuli. Systemic injection of endotoxin reportedly increased the expressions of both the CRF and nNOS genes in the PVN [16]. Similarly, immobilization stress activates nNOS mRNA and protein in the PVN [24, 25]. Our finding of a defect of nNOS gene induction in Lew/N rats suggests another causative association between NO and CRF, and this could be regarded as one of the causes of the hyporesponsiveness of hypothalamic CRF following an immune challenge.

The results of in vitro experiments on the isolated hypothalamus indicate that LPS stimulates CRF release indirectly through various neuronal or humoral pathways [26, 27]. Among them, NO may be one of the factors involved in the effect of LPS on the hypothalamus, because NOS is localized in hypothalamic CRF neurons [28]. We have shown that both nNOS and iNOS are colocalized in CRF-producing neurons in the PVN following high-dose LPS injection by double in situ hybridization histochemistry [20]. The results show that iNOS was more robustly distributed and more frequently colocalized with CRF throughout the PVN after LPS injection. As a freely diffusible gas, NO may cause changes in a sphere approximately 100 μm in diameter, and thus NO produced within the parvocellular CRF-producing neurons may directly modulate the activity of these cells [29]. Activation of the PVN neurons may produce autofeedback inhibition or stimulation of its own activity as well as the surrounding neurons. The closer localization of iNOS to CRF neurons suggests the possibility that NO derived from iNOS may more readily reach CRF neurons and thus mediate HPA axis regulation directly, compared to that from nNOS.

Controversial findings, however, exist as to the direction of the changes in NO-induced CRF. Some groups have suggested a stimulatory influence of NO

on CRF release based on the results of in vitro studies [30, 31]. NO is also thought to exert a stimulatory effect on the activity of hypothalamic neurons, including CRF, in vivo [32]. In contrast, others have demonstrated an inhibitory influence of NO on CRF neurons in both in vitro [33, 34] and in vivo studies [35]. This discrepancy may be explained by the fact that NO can activate both guanylate cyclase and cyclo-oxygenase [36, 37]. These two enzymes have opposing effects, and thus the relation between their degree of activation would determine the net secretion of CRF. Alternatively, endogenous NO exerts a stimulatory influence on the activity of hypothalamic neurons, but an inhibitory influence at the level of neurosecretory nerve terminals [32]. There is another possibility, i.e., that NO may act both directly on CRF and indirectly through another intermediary having opposing effects [38]. Besides its role as a neuromodulator, NO in the PVN may modulate hypothalamic portal blood flow as a vasodilator molecule [39, 40].

nNOS activity is highly dependent on prevailing Ca concentrations [8]. Glutamate binding to N-methyl-D-aspartate (NMDA) receptors increases the level of intracellular Ca^{++}, which in turn activates nNOS through calmodulin for mediation of rapid events, such as neurotransmission [9]. iNOS, on the other hand, is relatively independent of the calcium milieu [8], and iNOS promoter elements confer LPS-inducibility [41]. Such a distinct manner of gene activation may contribute to the differential expression of the nNOS gene and iNOS gene in the PVN induced by immune challenge of Lew/N rats.

There are several possible pathways involved in the difference of response to LPS between Lew/N and F344 rats. First, production and release of inflammatory cytokines might be deficient in Lew/N rats. It is generally believed that peripheral injection of LPS stimulates macrophages to release interleukin (IL)-1 which in turn induces hypothalamic CRF release [42]. IL-2, which is also reported to activate NO synthase, leads to increased NO release that activates CRF release [43]. Second, neuronal pathway activated by LPS might be different among the two strains. Elmquist and Saper demonstrated, using immunohistochemistry for Fos coupled with retrograde transport technique, several cell groups activated by peripheral administration of LPS that directly project to the PVN [44]. These are the visceromotor cortex, median preoptic nucleus, ventromedial preoptic area, bed nucleus of the stria terminalis, parabrachial nucleus, ventrolateral medulla, and nucleus of solitary tract. Disturbance of neuronal pathway from any of these brain nuclei to PVN may be present in Lewis/N rats. Third, a defect of signaling through vagus nerve could explain the difference of responsiveness to intraperitoneal injection of LPS [45].

In summary, the present results point to possible involvement of nNOS in a defect of the HPA axis response in Lew/N rats. Reduction of the increase in nNOS gene expression in Lew/N rats may lead to a defect of up-regulation of the CRF gene induced by immune challenge in this species. Since iNOS induction was intact in the Lew/N rats, the NO derived from iNOS may represent a compensatory mechanism to maintain sufficient local release of NO in PVN neurons.

Acknowledgments

We are grateful to Dr. J. Imaki (Nippon Medical School) for helpful discussions, to Dr. S. Hirose and Dr. H. Hagiwara (Tokyo Institute of Technology) for the kind gift of rat nNOS and iNOS cDNA, Dr. K. Mayo (Northwestern Univ.) for the kind gift of rat CRF cDNA, and to Dr. S. Minami and Dr. H. Sugihara (Nippon Medical School) for the densitometric analysis.

References

1. Calogero AE, Sternberg EM, Bagdy G, Smith C, Bernardini R, Aksentijevich S, Wilder RL, Gold PW, Chrousos GP (1992) Neurotransmitter-induced hypothalamic-pituitary-adrenal responsiveness in inflammatory disease-susceptible Lewis rats: in vivo and in vitro studies suggesting a global defect in CRH secretion. *Neuroendocrinology* 55: 600–608.
2. Sternberg EM, Hill JM, Chrousos GP, Kamolaris T, Listwak SJ, Gold PW, Wilder RL (1989) Inflammatory mediator-induced hypothalamic-pituitary-adre-

nal axis activation is defective in streptococcal cell wall arthritis-susceptible Lewis rats. *Proc Natl Acad Sci USA* 86: 2374–2378.
3. Sternberg EM, Young WS III, Bernardini R, Calogero AE, Chrousos GP, Gold PW, Wilder RL (1989) A central nervous system defect in biosynthesis of corticotropin-releasing hormone is associated with susceptibility to streptococcal cell wall-induced arthritis in Lewis rats. *Proc Natl Acad Sci USA* 86: 4771–4775.
4. Sternberg EM, Glowa JR, Smith MA, Calogero AE, Listwak SJ, Aksentijevich S, Chrousos GP, Wilder RL, Gold PW (1992) Corticotropin releasing hormone related behavioral and neuroendocrine responses to stress in Lewis and Fisher rats. *Brain Res* 570: 54–60.
5. Rivier CL, Plotsky PM (1986) Mediation by corticotropin-releasing factor (CRF) of adenohypophysial hormone secretion. *Annu Rev Physiol* 48: 475–494.
6. Sawchenko PE, Swanson LW (1990) Organization of CRF immunoreactive cells and fibers in rat brain: immunohistochemical studies. In DeSouza EB, Nemeroff CB (eds) Corticotropin-releasing factor: Basic and Clinical Studies of a Neuropeptide, Boca Raton, CRC Uniscience, 29–51.
7. Vale W, Spiess J, Rivier C, Rivier J (1982) Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and β-endorphin. *Science* 213: 1394–1397.
8. Nathan C, Xie QW (1994) Nitric oxide synthase: roles, tools, and controls (review). *Cell* 78: 915–918.
9. Costa A, Poma A, Navarra P, Forsling ML, Grossman A (1996) Gaseous transmitters as new agents in neuroendocrine regulation. *J Endocrinol* 149: 199–207.
10. Garthwaite J (1991) Glutamate, nitric oxide and cell-cell signaling in the nervous system. *Trends Neurosci* 14: 60–67.
11. Moncada S, Palmer RMJ, Higgs EA (1991) Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev* 43: 109–142.
12. Snyder SH, Bredt DS (1992) Biological roles of nitric oxide. *Sci Am* 266: 68–77.
13. Bredt DS, Glatt CE, Hwang PM (1991) Nitric oxide synthase protein and mRNA are discretely localized in neuronal populations of the mammalian CNS together with NADPH diaphorase. *Neuron* 7: 615–624.
14. Grossman A, Rossmanith WG, Kabigting EB, Cadd G, Clifton D, Steiner RA (1994) The distribution of hypothalamic nitric oxide synthase mRNA in relation to gonadotropin-releasing hormone neurons. *J Endocrinol* 40: R5–R8.
15. Vincent SR, Kimura H (1992) Histochemical mapping of nitric oxide synthase in the rat brain. *Neuroscience* 46: 599–603.
16. Lee S, Barbanel G, Rivier C (1995) Systemic endotoxin increases steady-state gene expression of hypothalamic nitric oxide synthase: comparison with corticotropin-releasing factor and vasopressin gene transcripts. *Brain Res* 705: 136–148.
17. Butterry LD, Evans TJ, Springall DR Carpenter A, Cohen J, Polaj JM (1994) Immunochemical localization of inducible nitric oxide synthase in endotoxin-treated rats. *Lab Invest* 71: 755–764.
18. Minc-Golomb D, Tsarfaty I, Schwartz JP (1994) Expression of inducible nitric oxide synthase by neurons following exposure to endotoxin and cytokine. *Br J Pharmacol* 112: 720–722.
19. Wong ML, Rettori V, al-Shekhlee A, Bongiorno PB, Canteros G, McCann SM, Gold PW, Licinio J (1996) Inducible nitric oxide synthase gene expression in the brain during systemic inflammation. *Nature Med* 2: 581–584.
20. Harada S, Imaki T, Chikada N, Naruse M, Demura H (1999) Distinct distribution and time-course changes in neuronal nitric oxide (NO) synthase (nNOS) and inducible NOS (iNOS) in the paraventricular nucleus (PVN) following lipopolysaccharide (LPS) injection. *Brain Res* 821: 322–332.
21. Imaki T, Wang X-Q, Shibasaki T, Yamada K, Harada S, Chikada N, Naruse M, Demura H (1995) Stress-induced activation of neuronal activity and corticotropin-releasing factor gene expression in the paraventricular nucleus is modulated by glucocorticoids in rats. *J Clin Invest* 96: 231–238.
22. Imaki T, Wang X-Q, Shibasaki T, Harada S, Chikada N, Takahashi C, Naruse M, Demura H (1995) Chlordiazepoxide attenuates stress-induced activation of neurons, corticotropin-releasing factor (CRF) gene transcription and CRF biosynthesis in the paraventricular nucleus (PVN). *Mol Brain Res* 32: 261–270.
23. Paxinos G, Watson C (1986) *The Rat Brain in Stereotaxic Coordinates*, San Diego, Academic Press.
24. Calza L, Giardino L, Ceccateli A (1993) NOS mRNA in the paraventricular nucleus of young and old rats after immobilization stress. *Neuroreport* 4: 627–630.
25. Kishimoto J, Tsuchiya T, Emson PC, Nakayama Y (1996) Immobilization-induced stress activates neuronal nitric oxide synthase (nNOS) mRNA and protein in hypothalamic-pituitary-adrenal axis in rats. *Brain Res* 720: 158–171.
26. Milton NGN, Self H, Hillhouse EW (1993) Effects of pyrogenic immunomodulators on the release of corticotropin-releasing factor-41 and prostaglandin E2 from the intact rat hypothalamus in vitro. *Br J Pharmacol* 109: 8893–8898.
27. Pozzoli G, Costa A, Grimaldi M, Schettini G, Preziosi P, Grossman A, Navarra P (1994)

Lipopolysaccharide modulation of eicosanoid and corticotropin-releasing hormone release from rat hypothalamic explants and astrocyte cultures in vitro: evidence for the involvement of prostaglandin E2 but not prostaglandin F2α and lack of effect of nerve growth factor. *J Endocrinol* 140: 103–109.

28. Torres G, Lee S, Rivier C (1993) Ontogeny of the rat nitric oxide synthase and colocalization with neuropeptides *Mol Cell Neurosci* 4: 155–163.
29. Wood J, Garthwaite J (1994) Models of the diffusional spread of nitric oxide: implications for neural nitric oxide signaling and its pharmacological properties. *Neuropharmacology* 33: 1235–1244.
30. Brunetti L, Preziosi P, Ragazzoni E, Vacca M (1993) Involvement of nitric oxide in basal and interleukin β-induced CRH and ACTH release in vitro. *Life Sci* 53: 219–222.
31. Sandi C, Guaza C (1995) Evidence for a role of nitric oxide in the corticotropin-releasing factor release induced by interleukin-1β. *Eur J Pharmacol* 274: 17–23.
32. Kim CK, Rivier C (1988) Influence of nitric oxide synthase inhibitors on the ACTH and cytokine responses to peripheral immune signals. *J Neuroimmunol* 10: 353–362.
33. Costa A, Trainer P, Besser M, Grossman A (1993) Nitric oxide modulates the release of corticotropin-releasing hormone from the rat hypothalamus in vitro. *Brain Res* 605: 187–192.
34. Kostoglou-Athanassiou I, Costa A, Navarra P, Nappi G, Forsling ML, Grossman AB (1988) Endotoxin stimulates an endogenous pathway regulating corticotropin-releasing hormone and vasopressin release involving the generation of nitric oxide and carbon monoxide. *J Neuroimmunol* 86: 104–109.
35. Rivier C (1994) Endogenous nitric oxide participates in the activation of the hypothalamic-pituitary adrenal axis by noxious stimuli. *Endocr J* 2: 363–373.
36. Grossman AB, Costa A, Forsling ML, Jacobs R, Kostoglou-Athanassiou I, Nappi G, Navarra P, Satta MA (1997) Gaseous neurotransmitters in the hypothalamus. *Horm Metab Res* 29: 477–482.
37. Salvenimi D, Misko TP, Masferre JL, Seibert K, Currie MG, Needleman P (1993) Nitric oxide activates cyclooxygenase enzymes. *Proc Natl Acad Sci USA* 90: 7240–7243.
38. Mancuso C, Tringali G, Grossman A, Preziosi P, Navarra P (1988) The generation of nitric oxide and carbon monoxide produces opposite effects on the release of immunoreactive interleukin-1β from the rat hypothalamus in vitro: evidence for the involvement of different signaling pathways. *Endocrinology* 139: 1031–1037.
39. Ceccateli S, Lundberg JM, Fahrenkrug J, Bredt DS, Snyder SH, Hokfelt T (1992) Evidence for involvement of nitric oxide in the regulation of hypothalamic portal blood flow. *Neurosci* 51: 769–772.
40. Horn T, Smith PM, Mclaughlin BE, Bauce L, Marks GS, Pittman QJ. Ferguson AV (1994) Nitric oxide actions in paraventricular nucleus: cardiovascular and neurochemical implications. *Am J Physiol* 266: R306–R313.
41. Spitsin SV, Koprowski K, Michaels FH (1996) Characterization and functional analysis of the human inducible nitric oxide synthase gene promoter. *Mol Med* 2: 226–235.
42. Tilders FJ, DeRijik RH, Van Dam A, Vincent VA, Shitanus K, Persoons JH (1994) Activation of the hypothalamus-pituitary-adrenal axis by bacterial endotoxin, routes and intermediate signals. *Psychoneuroendocrinology* 19: 209–232.
43. Karanth S, Lyson K, McCann SM (1993) Role of nitric oxide in interleukin-2-induced corticotropin-releasing factor release from incubated hypothalami. *Proc Natl Acad Sci USA* 90: 3383–3387.
44. Elmquist JK, Saper CB (1996) Activation of neurons projecting to the paraventricular hypothalamic nucleus by intravenous lipopolysaccharide. *J Comp Neurol* 374: 315–331.
45. Bluthe RM, Walter V, Parnet P, Laye S, Lestage J, Verrier D, Poole S, Stenning BE, Kelley KW, Dantzer R (1994) Lipoplysaccharide induces sickness behavior in rats by a vagal mediated mechanism. *C R Acad Sci* 317: 499–503