

## LETTER TO NEUROSCIENCE

# PERIPHERAL ADMINISTRATION OF LIPOPOLYSACCHARIDE ENHANCES THE EXPRESSION OF GUANOSINE TRIPHOSPHATE CYCLOHYDROLASE I mRNA IN MURINE LOCUS COERULEUS

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**Abstract**—GTP cyclohydrolase I is the first and rate-limiting enzyme for the *de novo* biosynthesis of tetrahydrobiopterin, which is the cofactor for tyrosine hydroxylase. Lipopolysaccharide can modulate tetrahydrobiopterin production by up-regulating GTP cyclohydrolase I protein expression in the locus coeruleus in the mouse brain. The increased supply of tetrahydrobiopterin in the locus coeruleus leads to increased tyrosine hydroxylase activity without affecting the level of tyrosine hydroxylase protein expression, resulting in an increase in norepinephrine turnover at the site. This study was performed to address whether the increase in GTP cyclohydrolase I protein is dependent on the *de novo* synthesis of GCH in the locus coeruleus. After *i.p.* administration of lipopolysaccharide, the mRNA expression of GTP cyclohydrolase I was examined. The expression level increased within 2 h, and reached to maximum level at 4 h after the lipopolysaccharide administration. However, the mRNA expression level of 6-pyruvoyl-tetrahydropterin synthase and sepiapterin reductase, both of which are involved successively after GTP cyclohydrolase I in tetrahydrobiopterin biosynthesis, were not affected by the lipopolysaccharide administration. These results suggest that GTP cyclohydrolase I upregulation alone is enough to modulate tetrahydrobiopterin production in the locus coeruleus. In addition, the mRNA level of tyrosine hydroxylase was also not affected by the lipopolysaccharide administration. Taken together, the data indicate that GTP cyclohydrolase I plays a crucial role in regulating norepinephrine biosynthesis by a pathway the activity of which is triggered by lipopolysaccharide *i.p.* administration. © 2003 IBRO. Published by Elsevier Science Ltd. All rights reserved.

**Key words:** (6*R*)-L-erythro-dihydroxypropyl-2-amino-4-hydroxy-5,6,7,8-tetrahydropteridine, GTP cyclohydrolase I, locus coeruleus, lipopolysaccharide, tyrosine hydroxylase.

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**Abbreviations:** ANOVA, analysis of variance; BH4, (6*R*)-L-erythro-dihydroxypropyl-2-amino-4-hydroxy-5,6,7,8-tetrahydropteridine; cDNA, complementary DNA; DIG, digoxigenin; GCH, GTP cyclohydrolase I; GTP, guanosine triphosphate; LC, locus coeruleus; LPS, lipopolysaccharide; NE, norepinephrine; PCR, polymerase chain reaction; PLST, protected least significance test; PTPS, 6-pyruvoyl-tetrahydropterin synthase; SR, sepiapterin reductase; TH, tyrosine hydroxylase; TPH, tryptophan hydroxylase.

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Tetrahydrobiopterin [(6*R*)-L-erythro-dihydroxypropyl-2-amino-4-hydroxy-5,6,7,8-tetrahydropteridine; BH4] is the essential cofactor for tyrosine hydroxylase (TH) (Nagatsu et al., 1964), tryptophan hydroxylase (TPH) (Lovenberg et al., 1967), and phenylalanine hydroxylase (Kaufman, 1959), all of which are enzymes that synthesize catecholamines (dopamine, norepinephrine [NE], and epinephrine), serotonin, and L-tyrosine, respectively. The pathway for the *de novo* biosynthesis of BH4 from guanosine triphosphate (GTP) involves GTP cyclohydrolase I (GCH), 6-pyruvoyl-tetrahydropterin synthase (PTPS), and sepiapterin reductase (SR) (Thöny et al., 2000). Among them, GCH is the first and rate-limiting enzyme for *de novo* biosynthesis of BH4 (Nagatsu et al., 1989).

It is well known that lipopolysaccharide (LPS), an endotoxin released from the outer membranes of gram-negative bacteria, induces the alterations in the biosynthesis of catecholamines at the specific nuclei in CNS (Lacosta et al., 1999, Lavicky and Dunn, 1995). We recently reported that the protein level of GCH in the locus coeruleus (LC) of the mouse brain increased in response to peripherally administered 5 µg LPS (Kaneko et al., 2001a). This increase led to the elevated content of BH4 and activated NE production rate in the LC. By contrast, TH protein expression was not affected by the LPS *i.p.* administration. Thus, we concluded that an increased supply of BH4 in the LC played a crucial role in activating the TH molecule, and that the activation resulted in increased NE production at the site.

This present study was aimed at substantiating the findings mentioned just above regarding the LC from the viewpoint of mRNA expression. The quantification of mRNA expression levels of GCH, PTPS, SR, and TH at the LC was carried out by using the quantitative real-time PCR method and *in situ* hybridization histochemistry techniques.

## EXPERIMENTAL PROCEDURES

### Chemical reagents

LPS from *Escherichia coli*, sero-type 026:B6, was purchased from Sigma Chemical Company (St. Louis, MO, USA). Reagents used in this study were of analytical grade and were purchased mainly from Sigma.

**Table 1.** Primers for real-time PCR

Gene name	Genbank accession number	Primer orientation	Nucleotide sequence (from 5' to 3')	Starting sequence position	Size of the PCR amplicon (bp)	References
Mouse GCH	L09737	Forward	GCAGCGAGGAGGAAAACCA	149	72	Nomura et al., 1993
		Reverse	CCAGCGAGAGCAGAATGGA	220		
Mouse TH	M69200	Forward	GGCTTCTCTGACCAGGCGTAT	580	68	Ichikawa et al., 1991
		Reverse	TGCTTGATTGGAAGGCAATCTC	647		
Mouse PTPS	NM_011220	Forward	TGGAGGAGGCCATCATGAA	236	87	Turri et al., 1998
		Reverse	TTTCTGTCGTGCTCACAGCAT	322		
Mouse SR	NM_011467	Forward	TCAACAACGCAGCCACTCTT	296	81	Ota et al., 1995
		Reverse	CCCAGTAGTTGTTACCTCAGCTA	376		

## Animals

Eight-week-old C3H/HeN male mice, which are LPS sensitive (Poltorak et al., 1998; Qureshi et al., 1999), were obtained from S.L.C. (Hamamatsu, Japan) and housed as previously reported (Kaneko et al., 2001a). The use of animals was kept to the minimum necessary to validate the data, and all animal protocols were carried out according to the National Institute of Health guidelines for the care and use of laboratory animals.

## Quantitative real-time PCR

Brain samples containing the LC (termed as "LC sample") were prepared at 2, 4 or 6 h after i.p. injection with 5  $\mu$ g or 500  $\mu$ g of LPS or saline as already reported (Kaneko et al., 2001b). Total RNA was extracted from the LC samples by using RNA isolation reagent (Isogen; Nippon Gene Co.; Tokyo, Japan). Complementary DNA (cDNA) was synthesized with 2  $\mu$ g of total RNA by reverse transcription reaction (Life Technologies; Tokyo, Japan). All the pairs of the primers used in quantitative real-time polymerase chain reaction (PCR) (Table 1) generated the single bands with the predicted size from cDNA on a conventional PCR (data not shown). cDNA prepared from the LC sample was also used to produce the standard samples.

Quantitative real-time PCR for the samples comparative to 100 ng cDNA was performed on an ABI 7700 PCR Instrument (Perkin Elmer Biosystems, USA) with SYBR Green (Perkin Elmer Biosystems) according to the manufacturer's instructions. Each sample was tested in triplicate on quantitative real-time PCR, and four mice for each group were used to calculate the means and S.D.

## In situ hybridization histochemistry

Mouse brain samples were prepared as previously described (Kaneko et al., 2001a) following i.p. administration with 5  $\mu$ g LPS or saline. Ten-micrometer sections were cut on a cryostat (Leica Jung CM1800; Finetec; Tokyo, Japan), thaw-mounted onto slides coated with 3-aminopropyltriethoxy silane (Matsunami Glass Ind.; Osaka, Japan).

A mouse GCH cDNA fragment was generated by PCR with a 5' primer (TCTAGACTTACCAAGGGATACCAGGA) and a 3' primer (CTCGAGCCTGGCAAGTTACTGAGACCA), using mouse GCH/pBS as a template. A mouse TH cDNA fragment was generated by PCR with a 5' primer (TCTAGAACCCTGGTCAACAAGTTTGACC) and a 3' primer (CTCGAGAGCATAGAGGCCCTTCAGCGT), using mouse TH/pBS as a template. Mouse PTPS and SR cDNA fragments were generated by PCR with 5' primers (TCTAGATGCAACAATCCGAATGGCCAC) and (TCTAGAAACGCAGCCACTCTTGGGGAT), and 3' primers (CTCGAGTTCTGTCGTGCTCACAGCATC) and (CTCGAGAGCCTTCCCGCACAGTACAGA), respectively, using mouse cDNA derived from mouse neuroblastoma cell line N1E-115 as a template. The

PCR products were subcloned into pGEM-T easy vector (Promega; Madison, WI, USA), respectively. Digoxigenin (DIG)-labeled RNA probes were synthesized by using a DIG RNA Labeling Kit (Roche; Mannheim, Germany).

*In situ* hybridization histochemistry was performed as described previously (Nomura et al., 1988; Tsukamoto et al., 1991). Briefly the sections were hybridized in freshly prepared hybridization buffer containing 500 pg/ $\mu$ l DIG-labeled probes after proteinase K treatment. After removal of excess unhybridized RNA by RNase A treatment, signals were detected by immunohistochemical staining with DIG Nucleic Acid Detection Kit (Roche). Photographs were taken with an Axio Vision system (Carl Zeiss; Tokyo, Japan). The semi-quantitative analyses of the microphotographs were carried out by using NIH Image Software. The specificity of the RNA signals was examined by the comparison between the brain sections hybridized with sense or antisense probes.

## Statistics

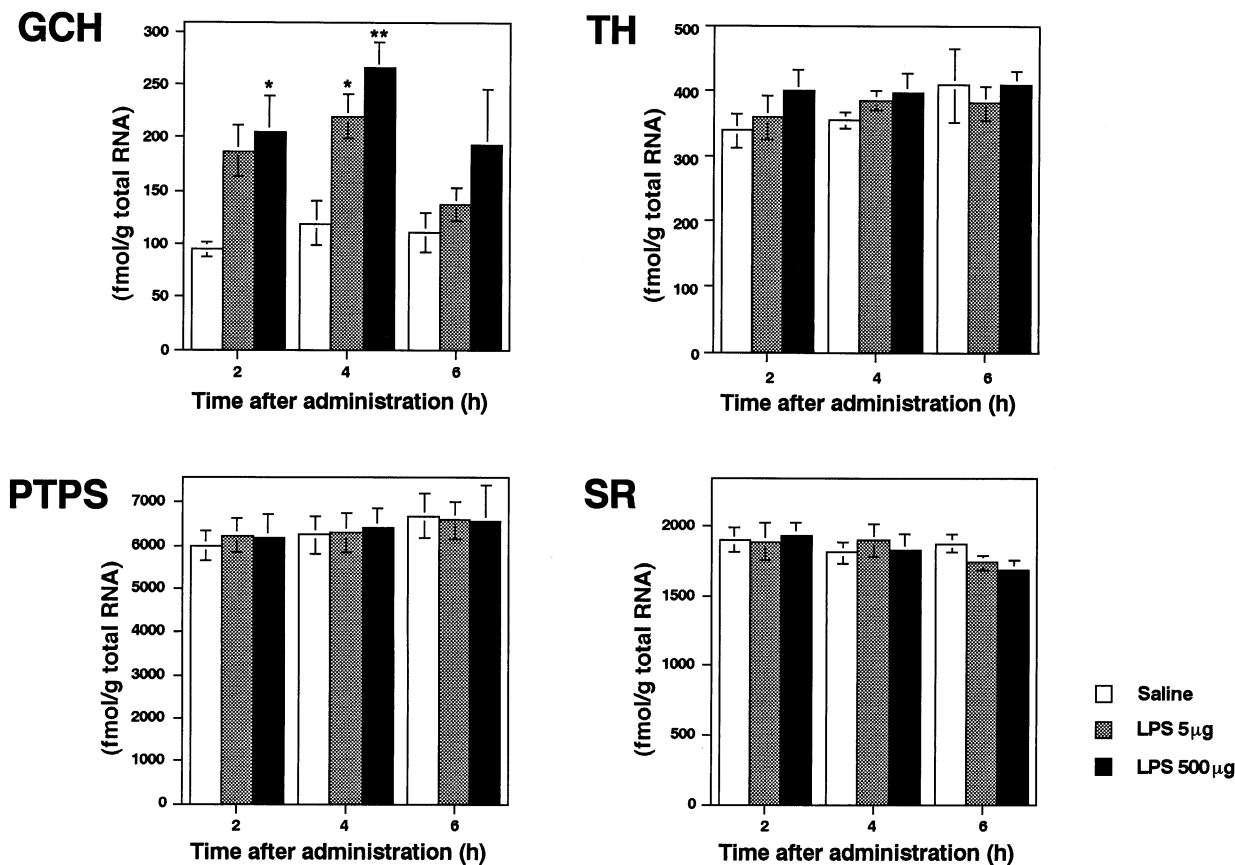
All numerical data were expressed as the mean  $\pm$  S.D. Analysis of variance (ANOVA) was used to analyze all data. If the ANOVA revealed a significant overall effect, the significance of the differences between results was determined by Fisher's protected least significance test as a post-hoc test. For all statistical analyses,  $P < 0.05$  was considered to be statistically significant.

## RESULTS AND DISCUSSION

We administered 5  $\mu$ g or 500  $\mu$ g of LPS to C3H/HeN male mice by i.p. injection and measured GCH, PTPS, SR and TH mRNA in the LC sample (see Experimental Procedures) by using the quantitative real-time PCR method (Fig. 1).

At 2 h after vehicle administration, the expression level of GCH mRNA in the LC sample was  $95 \pm 7$  femtomoles/g of total RNA. At 2 h after the administration, 5  $\mu$ g of LPS enhanced the expression levels of GCH mRNA in the LC sample to  $187 \pm 24$  femtomoles/g of total RNA; 500  $\mu$ g of LPS enhanced to  $206 \pm 33$  femtomoles/g of total RNA. Although the difference of GCH mRNA expression between vehicle-administered mice and 500  $\mu$ g of LPS-administered ones were statistically significant ( $P < 0.05$ ), the difference between vehicle-administered and 5  $\mu$ g of LPS-administered mice was just short of the statistical significance ( $P = 0.0506$ ).

At 4 h after the administration, the expression level of GCH mRNA in the LC sample obtained from LPS-administered mice reached its maximum value during 6-h-observation (vehicle-administered mice,  $119 \pm 21$  femtomoles/g



**Fig. 1.** Quantification of GCH mRNA, TH mRNA, PTPS mRNA, and SR mRNA level in the LC sample from LPS-administered C3H/HeN mice measured by using the SYBR Green real-time PCR method. Mice (four mice per each group) were i.p. administered 5 μg or 500 μg of LPS or saline, and decapitated at indicated time points. Immediately thereafter the brains were removed, total RNA was extracted from LC samples and reverse transcribed to cDNA. Quantitative real-time PCR was performed with the specific primers listed in Table 1 and cDNA as a template. Data are displayed as the mean (column) ± S.D. (bar) values. Each value marked with an asterisk was significantly greater than the corresponding value obtained from the saline-administered mice (control): \* $P < 0.05$ , \*\* $P < 0.01$ .

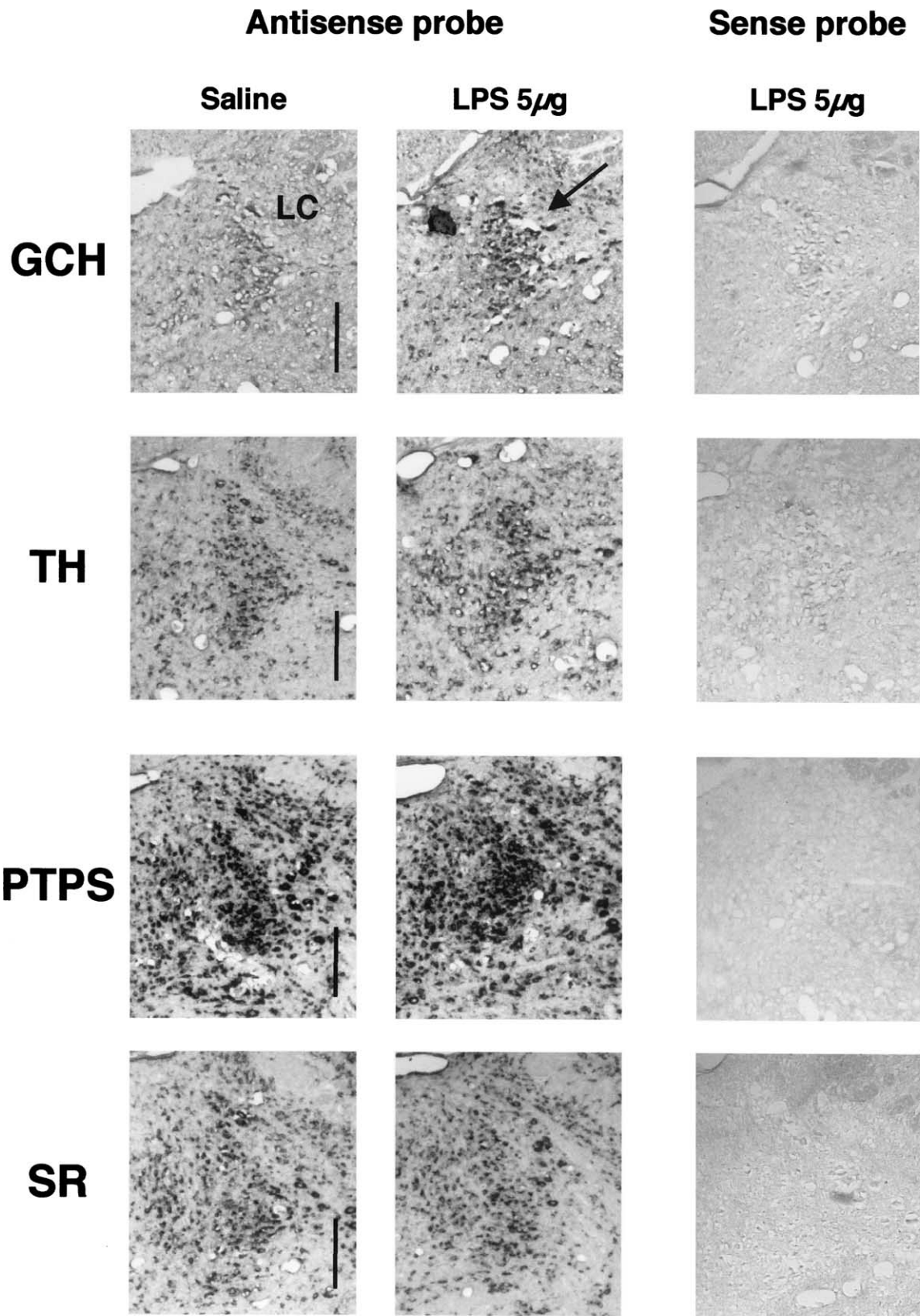
of total RNA; 5 μg of LPS-administered ones,  $220 \pm 21$  femtomoles/g of total RNA; 500 μg of LPS-administered ones,  $266 \pm 25$  femtomoles/g of total RNA). The differences between vehicle-administered and LPS-administered mice were statistically significant ( $P < 0.05$  for 5 μg of LPS,  $P < 0.01$  for 500 μg of LPS).

At 6 h after the administration, the expression level of GCH mRNA in the LC sample obtained from LPS-administered mice decreased close to that obtained from vehicle-administered ones (vehicle-administered ones,  $111 \pm 18$  femtomoles/g of total RNA; 5 μg of LPS-administered ones,  $137 \pm 15$  femtomoles/g of total RNA; 500 μg of LPS-administered ones,  $193 \pm 53$  femtomoles/g of total RNA). Although the values obtained from LPS-administered mice were higher than those obtained from vehicle-administered ones, the differences were not statistically significant.

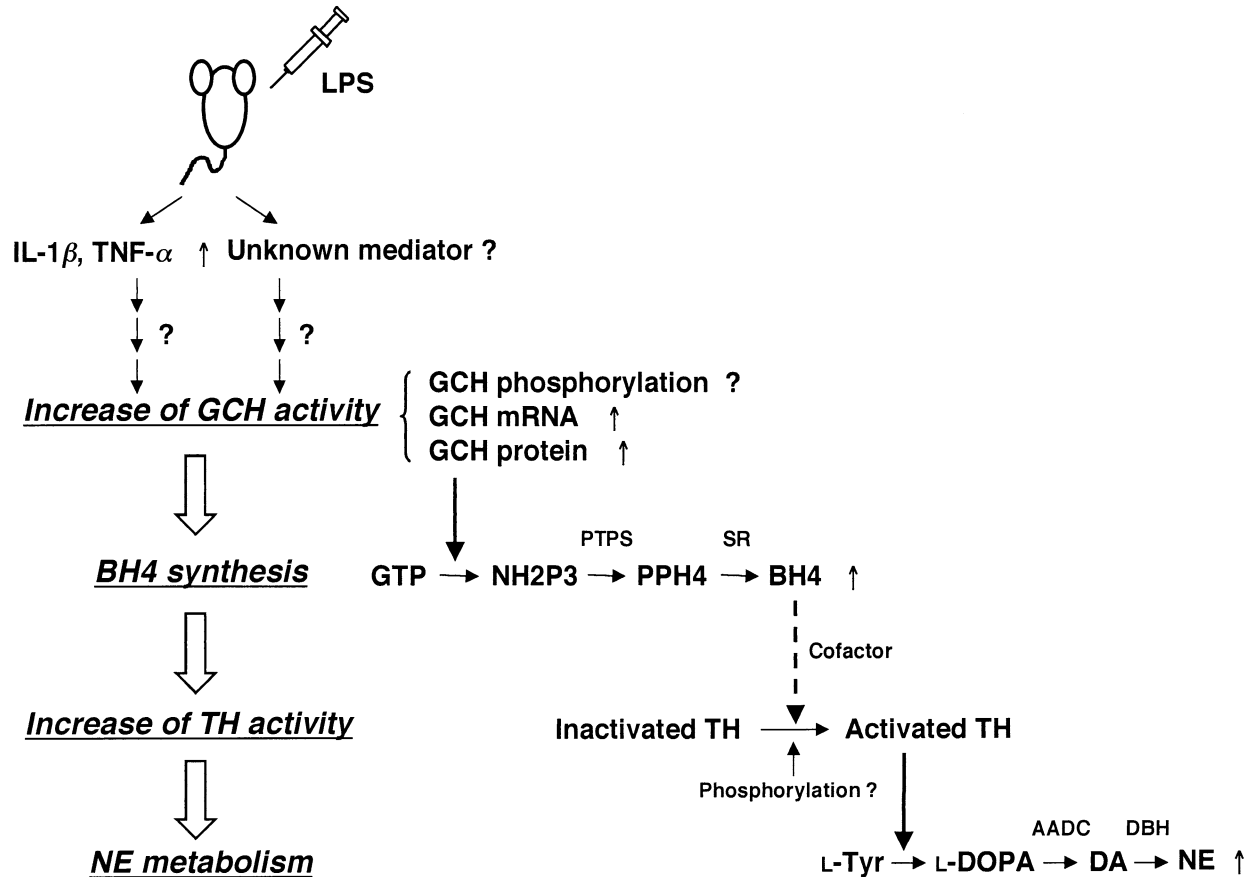
Interestingly, the results induced by 500 μg of LPS administration were only 1.1- to 1.2-fold higher than those induced by 5 μg of LPS. There was no statistical difference in the GCH mRNA expression between 5 μg of LPS-administered mice and 500 μg of LPS-administered ones. These results suggest that 500 μg of LPS was an excess dose, and that 5 μg of LPS was sufficient to induce sub-

maximal response of GCH mRNA expression in the LC sample. On the other hand, the mRNA expression levels of TH, PTPS and SR in the LC sample were not affected by 5 μg of LPS, nor were they altered by 500 μg of LPS (Fig. 1).

As a next step, the *in situ* hybridization histochemistry method was performed to evaluate the increase in GCH mRNA level in the LC sample after the LPS administration. The expression level of GCH mRNA in the LC was clearly enhanced at 4 h after LPS 5-μg administration (Fig. 2). The semi-quantitative analysis of microphotographs shown in Fig. 2 by using NIH Image software indicated that the density of GCH mRNA at the LC in LPS-treated mice was 2.0-fold higher than that at the LC in vehicle-treated ones (data not shown). In contrast, TH, PTPS and SR mRNA in the LC was not affected by LPS administration (Fig. 2) and the semi-quantitative analysis of their microphotographs by using NIH Image software indicated the density of mRNA in LPS-treated mice was as same as that in vehicle-treated ones (1.1-, 1.0-, and 1.1-fold, respectively; data not shown). These results correlated well with those obtained from quantitative real-time PCR method. Because no signals were detected when brain sections were hybridized



**Fig. 2.** High-magnification bright-field photomicrographs showing the labeling of GCH mRNA, TH mRNA, PTPS mRNA and SR mRNA in sections at LC. Brains were removed from C3H/HeN mice at 4 h after the i.p. administration of 5  $\mu$ g of LPS. The sections were hybridized with antisense or sense probe, respectively, and then immunostained with anti-DIG antibody. Scale bar=100  $\mu$ m. Four mice for each group were used to prepare brain samples.



**Fig. 3.** Schematic model of the activation of the NE biosynthetic pathway triggered by an i.p. administration of LPS. Inflammatory cytokines such as interleukin-1 $\beta$  (IL-1 $\beta$ ) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) or unknown mediator would be produced at first in the LPS-administered mice. The signal that reached to the CNS may increase the expression levels of GCH mRNA and protein in the LC. The increased and/or activated GCH would generate more BH4, which would activate TH and finally lead to the increase in NE turnover in the LC. AADC, aromatic L-amino acid; DA, dopamine; DBH, dopamine  $\beta$ -hydroxylase; L-Tyr, L-tyrosine; NH<sub>2</sub>P<sub>3</sub>, 7,8-dihydroneopterin triphosphate, PPH<sub>4</sub>; 6-pyruvoyltetrahydropterin.

with the sense probes (Fig. 2), signals obtained with the antisense probes were judged as specific.

In this study, we analyzed the alteration of mRNA levels of GCH and TH genes in the LC after an LPS administration to rationalize following two findings that had remained to be solved in our previous report; the one is of the LPS-induced increase in the GCH protein level in the LC and the other is of no effect of LPS on the TH protein level in the site. As shown in Figs. 1 and 2, the mRNA expression level of the GCH gene increased in the LC after the LPS administration. These observations suggest that the increase in GCH protein was due to increased de novo biosynthesis of GCH mRNA and/or due to increased stability of GCH mRNA. On the contrary, the mRNA expression level of TH gene in the LC was not affected by the LPS administration (Figs. 1, 2). Based on this observation, it is unlikely that the accelerated degradation of TH protein might be canceled by a concomitant enhancement of the rate of TH protein synthesis caused by increased mRNA expression level of TH gene, which might have kept the TH protein level constant.

Although it was reported that the catalytic activities of TH and TPH in rat frontal cortex and midbrain increased

after 2 h by peripheral administration of LPS (Nolan et al., 2000), *in situ* hybridization histochemistry method performed in this study did not reveal the peripheral LPS-induced alterations in the mRNA expression levels of all the enzymes involved in BH<sub>4</sub> de novo biosynthesis in mouse dorsal raphe nucleus (data not shown). The steady state concentration of BH<sub>4</sub> as a cofactor for TH within monoaminergic neurons in the brain does not allow the saturation of the TH enzyme (Nagatsu, 1981). It has also been reported that BH<sub>4</sub> is at subsaturating level *in vivo* as a cofactor for TPH, and that, therefore, the concentration of BH<sub>4</sub> may play an important role in the regulation of TPH activity *in vivo* (Sawada et al., 1986). Collectively, increased and/or activated GCH may enhance NE and 5-HT synthesis by promoting the amount of BH<sub>4</sub> supplied to the TH and TPH protein (Thöny et al., 2000; Kaneko et al., 2001a).

The series of events that might occur after LPS administration are schematized in Fig. 3. It is still unclear how the signals generated by the peripheral administration of LPS are transmitted into the CNS. Because it is unlikely that LPS can penetrate easily the blood-brain barrier, some cytokines such as IL-1 $\beta$  and TNF- $\alpha$ , which can be induced

by peripherally administered LPS, would be the major candidates for the mediators (Nadeau and Rivest, 2000; Quan et al., 1994; Zucherman et al., 1989). These earlier studies demonstrated that peripherally administered LPS could induce those cytokines in various brain regions as well as in peripheral tissues and plasma within 4 h after LPS administration. Those reports suggest the presence of some other pathway and/or mediator to transmit signals to the CNS, because the GCH mRNA increased within 2 h after the LPS administration (Fig. 1). Therefore, our next study is to identify the mediator(s) that transmits the peripheral signal to the LC, and such a research is currently in progress in our laboratory.

## CONCLUSION

Intraperitoneal administration of LPS increased GCH mRNA expression level at the LC in the mouse brain. In contrast, the mRNA expression level of TH, PTPS and SR was not affected by LPS treatment. We concluded that GCH is the key molecule that stimulates NE biosynthesis in the LC in response to the i.p. administration of LPS.

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