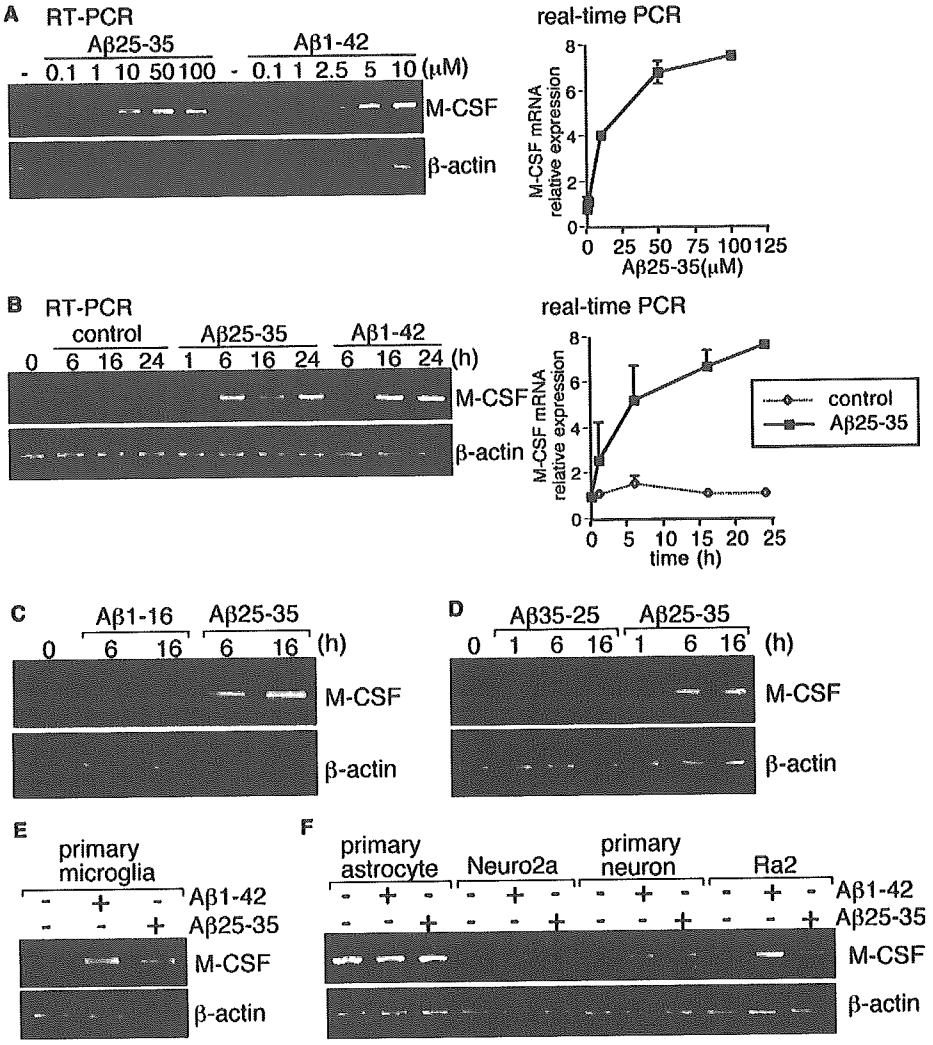

Fig. 2. Aβ stimulates M-CSF mRNA expression in microglia. (A and B) M-CSF and β-actin mRNA were determined by RT-PCR (left) and quantified by real-time PCR (right). Data represent means ± S.D. of three separate determinations. Ra2 cells were treated with Aβ25–35 or Aβ1–42 at indicated concentrations for 16 h (A). Time course of M-CSF relative expression of Ra2 cells treated with 50 μM Aβ25–35 and 10 μM Aβ1–42 (B). (C and D) Ra2 cells were treated with 50 μM Aβ1–16 (C) or 50 μM Aβ35–25 (D) for indicated times. (E and F) Primary microglia (E), primary neurons, primary astrocytes and neuroblastoma Neuro2a (F) were treated with 50 μM Aβ25–35 or 10 μM Aβ1–42 for 16 h.

did Aβ 35–25, which was a reverse sequence of Aβ25–35 (Fig. 2D). In primary microglia, as well as in Ra2 cells, Aβ25–35 and Aβ1–42 increased M-CSF mRNA expression (Fig. 2E). We also examined whether Aβ induced increases in M-CSF mRNA expression in primary astrocytes, primary neurons, and neuroblastoma cells Neuro2a. These cells constitutively expressed M-CSF mRNA, but Aβ25–35 and Aβ1–42 did not induce further expression of M-CSF mRNA (Fig. 2F). These results demonstrate that Aβ induced M-CSF mRNA expression in only microglia.

3.3. *Aβ induces M-CSF mRNA via Src family tyrosine kinase and PI3-kinase signal cascade*

Because our studies showed that Aβ25–35 had induced M-CSF expression in Ra2 cells, we examined signal cascades for Aβ-induced M-CSF mRNA expression by using several chemical inhibitors. The Src family tyrosine kinase is associated with CD36, which transduces signal cascades by Aβ [7,8,20]. In addition, Syk tyrosine kinase is activated by Aβ [21]. First, we examined if M-CSF mRNA expression was induced by Aβ via tyrosine kinase, Src family and Syk. A specific inhibitor of Src family kinase, PP1, prevented the increase in M-CSF mRNA induced by Aβ (Fig. 3A). A Syk-selective inhibitor, piceatannol, also blocked the increase in M-CSF mRNA expression (Fig. 3B). Next, to investigate whether the PI3-kinase pathway regulates Aβ-induced M-CSF expression, Ra2 cells were pretreated with the PI3-kinase inhibitors, wortmannin or LY294002. Wortmannin and LY294002 inhibited the increase in M-CSF mRNA expression dose-dependently (Fig. 3C and D). Fig. 3E shows the result of quantitative amounts of mRNA by real-time PCR. It has been reported that Aβ stimulates tyrosine kinase, PI3-kinase and Akt activation in neural and macrophage cells [21–24]. However, analysis of these signal transductions in microglia has not been reported. This is the first report that Aβ induces M-CSF expression through

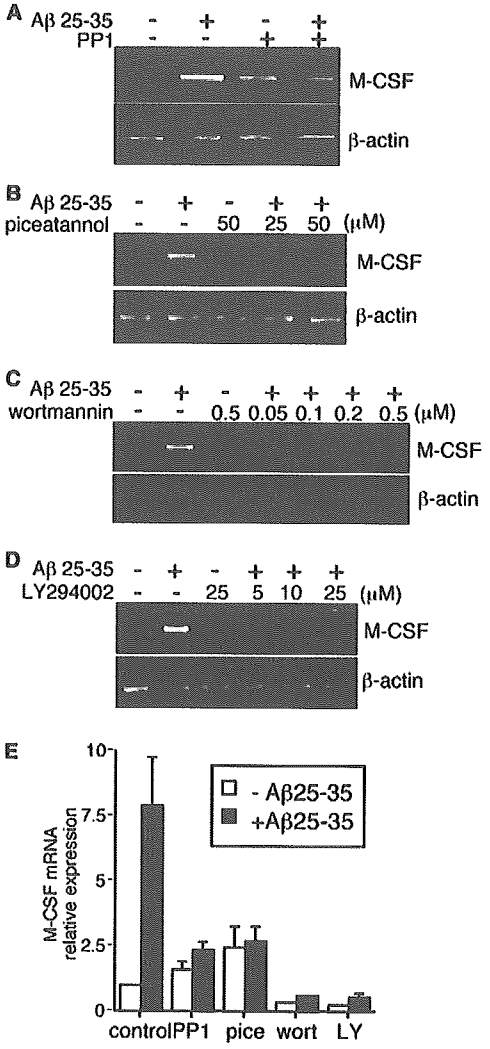

Fig. 3. Signal transduction for M-CSF mRNA expression induced by Aβ. (A–D) M-CSF and β-actin mRNA expression were determined by RT-PCR. Ra2 cells were preincubated with 10 μM PP1 (A), wortmannin (C), LY294002 (D) for 30 min or piceatannol (B) for 1 h before treatment of 50 μM Aβ25–35 for 6 h. Beacuse all inhibitors were dissolved in DMSO, control cells were treated with DMSO. (E) M-CSF mRNA expressions were measured by real-time PCR (pice, piceatannol; wort, wortmannin; LY, LY294002). Data represent means ± S.D. values of three separate determinations.

the Src family and Syk tyrosine kinases and the PI3-kinase in microglia.

3.4. Aβ activates Akt signaling pathway in microglia

We examined whether Akt was involved in the Aβ-induced M-CSF expression in Ra2 cells. Akt inhibitor blocked the increase of M-CSF mRNA expression (Fig. 4A). Immunoblotting analysis revealed that Akt was transiently phoshorylated at serine 473 by Aβ (Fig. 4B). LY294002 and PP1 suppressed the phosphorylation of Akt induced by Aβ (Fig. 4D). Piceatannol also blocked the phosphorylation of Akt (Fig. 4E). Because tyrosine kinases and PI3-kinase activate MEK/Erk/Elk [25,26], we examined whether these signal pathways were related to M-CSF mRNA expression induced by Aβ. Aβ induced MEK and Erk1/2 phosphorylation in Ra2 cells. However, specific inhibitors of MEK, U0126 and PD98059 did not inhibit Aβ-induced M-CSF mRNA expression (data not shown). These results indicate that the tyrosine kinases, Src family and Syk, and the PI3-kinase activate Akt for Aβ-induced M-CSF expression.

3.5. Aβ activates NF-κB via PI3-kinase signal cascade

Because NF-κB is a target of Akt [27], next we examined if IκBα phosphorylation was induced by Aβ. The phosphorylation of IκBα on serine 32 results in the release and nuclear translocation of active NF-κB [28]. IκBα was phosphorylated time-dependently, the phosphorylation peaked at 60 min and then declined (Fig. 4C). The phosphorylation was inhibited by LY294002 and PP1 (Fig. 4D). Piceatannol also blocked the phosphorylation of IκBα (Fig. 4E).

The M-CSF promoter region has a putative NF-κB binding site at −369–378 bp from the transcriptional start site [29]. To investigate whether this NF-κB binding site is associated with Aβ-induced M-CSF expression, EMSA was carried out with nuclear extracts prepared from untreated and Aβ-treated Ra2 cells. The amount of protein binding to the NF-κB probe was increased by Aβ-treatment (Fig. 5A, compare lanes 2 and 3). NF-κB binding activity was almost completely eliminated by adding an excess of the unlabeled NF-κB probe but not by the unlabeled AP1 probe (Fig. 5A, lanes 6 and 7). Anti-p50 antibody supershifted the complexes (Fig. 5A, lane 4) and anti-p65 antibody partially disrupted the DNA binding of the complexes (Fig. 5A, lane 5). To examine whether the Aβ-induced increase in nuclear NF-κB binding activity correlated with tyrosine kinase and PI3-kinase, Ra2 cells were preincubated with chemical inhibitors before treatment with Aβ. LY294002 reduced Aβ-induced binding to the NF-κB probe and piceatannol blocked the DNA-binding complex (Fig. 5B, lanes 4–7). These results indicate that Aβ enhances the binding of NF-κB to M-CSF promoter via the Syk tyrosine kinase and the PI3-kinase.

We have shown in the present study that Aβ proliferates microglia and induces M-CSF via the PI3-kinase/Akt/NFκB signal pathways. It has been reported that Aβ binds to CD36, which transduces signals via tyrosine kinase [6,20]. CD36 may participate in the initiation of intracellular signaling to M-CSF expression. RAGE also has been reported to induce NF-κB activation to M-CSF production [30]. Further works are needed to prove the receptors of Aβ, which induces PI3-kinase/Akt/NF-κB signal pathways to M-CSF mRNA expression. Aβ increases production of reactive oxygen species (ROS) and activates Akt in neural cells [23]. And in microglia CD36 mediates production of ROS in response to Aβ [31]. We found that antioxidants such as reduced glutathione and α-tocopherol slightly blocked M-CSF mRNA expression (data not shown). Also in microglia, ROS may partly participate in activating the signal cascade to M-CSF expression. It is important to reveal the relation among the receptors of Aβ, production of ROS and signal cascades.

Monsonego et al. [32] showed that activated microglia migrated outside the brain and could present Aβ peptide to T lymphocytes. Further analysis of microglial activation may reveal the immunological mechanism of AD, and may enhance the prospects of immune manipulation to prevent AD.

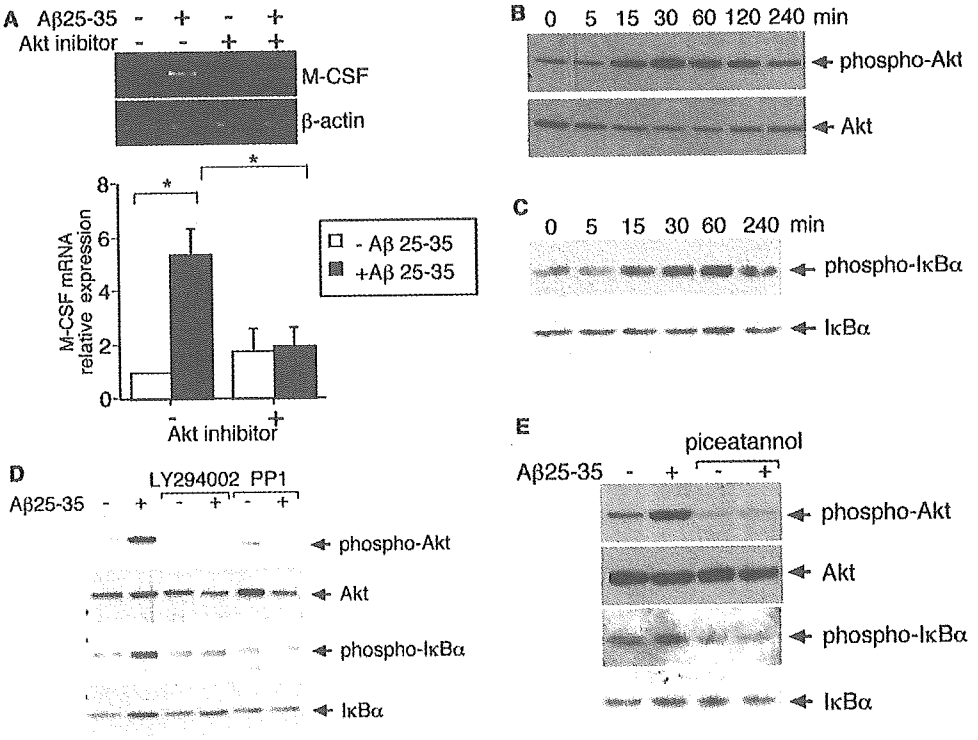

Fig. 4. Aβ-induced Akt and IκB phosphorylation through tyrosine kinase and PI3-kinase. (A) RT-PCR (top) and real-time PCR (bottom) of M-CSF mRNA. Ra2 cells were preincubated with or without 20 μM Akt inhibitor before treatment with 25 μM Aβ25–35 for 6 h. Data represent means ± S.D. values of three separate determinations. ($^*P < 0.01$) (B–E) Immunoblotting analysis using anti-phospho Akt (Ser 473) or anti-phospho IκBα (Ser 32) antibody. The same blots were reprobed with anti-Akt or anti-IκB antibody. Ra2 cells were treated with Aβ25–35 for indicated times (B and C). Ra2 cells were preincubated with 25 μM LY294002 or 10 μM PP1 for 30 min or 50 μM piceatannol for 1 h before treatment with 50 μM Aβ25–35 for 30 min (D and E).

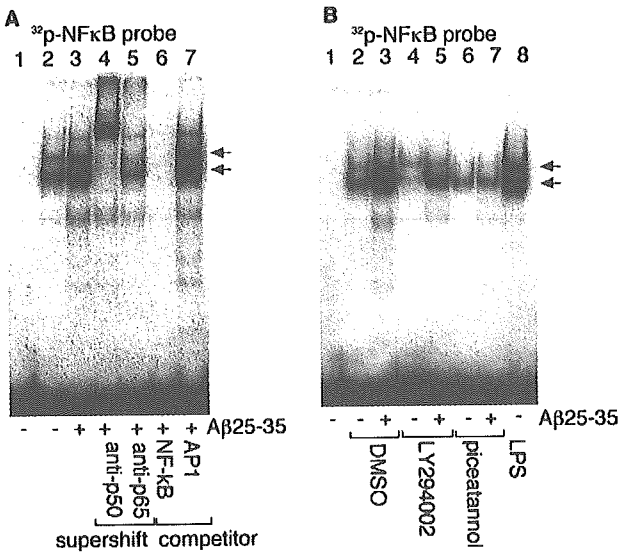

Fig. 5. NF-κB binding activity in EMSA. ^{32}P-labeled probe without nuclear extracts are showed in lane 1. (A) Ra2 cells were treated with 50 μM Aβ25–35 for 4 h (lanes 3–7). Anti-p50 or anti-p65 antibody was added to the extracts for supershift assay (lanes 4 and 5). Unlabeled competitor of NF-κB or AP1 probe was added to the extract (lanes 6 and 7). (B) Ra2 cells were preincubated with 25 μM LY294002 for 30 min (lanes 4 and 5) or 50 μM piceatannol for 1 h (lanes 6 and 7) before treatment with or without 50 μM Aβ25–35 for 4 h (lanes 2–7). Control cells were preincubated with DMSO (lanes 2 and 3). Nuclear extract of Ra2 cells treated with 1 μg/ml LPS for 4 h were used as a positive control (lane 8).

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Peripheral injection of lipopolysaccharide enhances expression of inflammatory cytokines in murine locus coeruleus: possible role of increased norepinephrine turnover

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Abstract

Cytokines and catecholamines are known to constitute a significant portion of the regulatory neuroimmune networks involved in maintaining homeostasis in the central nervous system (CNS). Although we have already reported an increase in norepinephrine (NE) turnover within the locus coeruleus (LC) at 2 and 4 h after the intraperitoneal (i.p.) injection of lipopolysaccharide (LPS), the implication of this increase remains unclear. In view of evidence that norepinephrine (NE) acts in an anti-inflammatory manner by way of negatively regulating pro-inflammatory cytokine expression, we examined the inflammatory cytokine expression levels in the LC of C3H/HeN mice (male, 8 weeks old) after an i.p. LPS injection. The mRNA expression levels of the genes encoding IL-1β and TNF-α within the LC increased during the first 2 h, and showed two peaks, the first at 4 h and the second lesser one at 15 h after the LPS injection. Microglia, which are one of the major cell types that produce pro-inflammatory cytokines in the CNS, were isolated from mouse neonate brains in order to clarify more precisely the relationship between the changes in NE content and the up-regulation of inflammatory cytokines in the LC. Simultaneous incubation of microglia with LPS and NE enhanced the expression of IL-1β at both mRNA and protein levels, but reduced the mRNA and protein levels of TNF-α. These data support the hypothesis that NE negatively regulates the expression of pro-inflammatory cytokine expression, at least in the case of TNF-α, which action could contribute to the observed anti-inflammatory properties of NE. This report, based on the results of both *in vivo* and *in vitro* experiments, is the first to suggest a relationship between NE content and cytokine expression levels in the CNS.

Keywords: inflammatory cytokine, lipopolysaccharide, locus coeruleus, norepinephrine.

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It is well established that lipopolysaccharide (LPS), an endotoxin released from the outer membranes of gram-negative bacteria, behaves as a stimulator of the nuclear factor (NF)-κB (Müller *et al.* 1993; Fischer *et al.* 1999; Fitzgerald *et al.* 2001) and as an activator of the immune system by stimulating the secretion of tumour necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 from macrophages through mitogen-activated protein (MAP) kinase signalling pathways (Zuckerman *et al.* 1989; Quan *et al.* 1994; Poltorak *et al.* 1998). About a decade ago, the results of an *in situ* hybridization study demonstrated that systemic administration of LPS to mice induced TNF-α mRNA expression in circumventricular organs and pericircumventricular nuclei, such as the arcuate nuclei of the hypothalamus and the nucleus of the solitary tract (Breder *et al.* 1994). Therefore, TNF-α produced by a cascade of events within the central nervous system (CNS) has been expected to participate in the complex autonomic, neuroendocrine, metabolic, and behavioural responses to infection and inflammation. Following this observation, numerous groups, including ours, began to

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Abbreviations used: CNS, central nervous system; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IκB, inhibitor of NF-κB; IL-1β, interleukin-1β; LC, locus coeruleus; LPS, lipopolysaccharide; MAP, mitogen-activated protein; MEM, minimal essential medium; NE, norepinephrine; NF-κB, nuclear factor-κB; TNF-α, tumour necrosis factor-α.

use systemic LPS administration as a model for infection and inflammation in the CNS.

Systemic LPS injection has been also reported to activate the hypothalamo–pituitary–adrenal (HPA) axis by way of triggering the transcription of the genes encoding corticotropin-releasing factor (CRF) and CRF type 1 receptor (CRFR1) in the paraventricular nucleus of the hypothalamus (Rivest and Laflamme 1995; Rivest *et al.* 1995). In addition, it was also reported that systemic LPS injection induced alterations in the biosynthesis and/or projection of catecholamines in areas such as the medial prefrontal cortex, paraventricular nucleus, arcuate nucleus, locus coeruleus (LC) and hippocampus (Lavicky and Dunn 1995; Lacosta *et al.* 1999).

Then, in light of the influence of peripheral LPS on CNS function, the effects of the intraperitoneal (i.p.) administration of LPS on GTP cyclohydrolase I (GCH) mRNA and protein expression levels in the LC in the mouse brain were investigated (Kaneko *et al.* 2001, 2003), basically for the following three reasons: (i) the murine *GCH* gene up to 681 nucleotides upstream of the initiation site reveals a putative NF-κB binding site located in a GC-rich region (Shimoji *et al.* 1999); (ii) GCH is a rate-limiting enzyme participating in the *de novo* biosynthetic cascade of tetrahydrobiopterin (BH4) (Nichol *et al.* 1985), which functions as a co-factor for nitric oxide synthase (Kwon *et al.* 1989; Tayeh and Marletta 1989) and tyrosine hydroxylase (Nagatsu *et al.* 1964), the latter being the rate-limiting enzyme in the catecholamine biosynthetic pathway; and (iii) LC contains the greatest number of NE cell bodies in the brainstem and has been repeatedly implicated in stress responses. Although the above studies revealed the fact that peripherally injected LPS enhanced norepinephrine (NE) turnover within 2–4 h in the LC, accompanied by an increase in GCH expression at the site, the physiological roles assigned to such a quick elevation of NE turnover in the LC in response to peripheral LPS injection were not precisely addressed.

In contrast, it is generally recognized that pro-inflammatory cytokines such as IL-1β, TNF-α, and IL-6, as well as reactive oxygen species such as nitric oxide (NO), can provoke damage in neurological diseases (del Zoppo *et al.* 2000; Rothwell and Luheshi 2000; Asensio and Campbell 2001; Benveniste *et al.* 2001; Feinstein *et al.* 2002) including multiple sclerosis (Cannella and Raine 1995), Parkinson's disease (Nagatsu *et al.* 2000), Alzheimer's disease (Akiyama *et al.* 2000), and AIDS (Tyor *et al.* 1992). Although pro-inflammatory cytokines such as TNF-α, IL-1, and IL-6 may play an important role against infection in the CNS, they can become harmful when produced excessively over a prolonged period of time. Thus, activation of the inflammatory response in the CNS must be tightly regulated in order to prevent the potentially cytotoxic mediators, including cytokines and reactive oxygen species, from accumulating in the CNS. In addition to blood-derived cells, microglia also participate in releasing cytokines and inflammatory mediators in the CNS (Lee *et al.* 1995; Lee and Brosnan 1997; Benveniste 1998).

NE is considered to be involved in the regulation of cytokine production in microglia for the following reasons. Microglia, which are major cells that produce pro-inflammatory cytokines in the CNS, possess functional β1- and β2-adrenergic receptors (Mori *et al.* 2002; Tanaka *et al.* 2002). Stimulation of β-adrenergic receptors of microglia inhibited the release of IL-12 from the cells (Prinz *et al.* 2001). Many laboratories have reported that NE can regulate nitric oxide synthase by binding to β-adrenergic receptors to trigger a cAMP and protein kinase A-dependent pathway in microglia and astrocytes (Feinstein *et al.* 1993; Feinstein 1998; Galea and Feinstein 1999; Cho *et al.* 2001; Gavrilyuk *et al.* 2002; Dello Russo *et al.* 2004). In addition, several reports indicate that a diminished NE level or perturbation of the NE-signalling system exacerbate the neuroinflammation that occurs in patients suffering from multiple sclerosis or Alzheimer's disease (Mann *et al.* 1983; De Keyser *et al.* 1999; Heneka *et al.* 2002, 2003).

In view of all available data taken altogether, we proposed the working hypothesis that the enhanced NE turnover in the LC caused by a peripheral injection of LPS protects the area surrounding the LC, as well as the areas to which the LC-derived NE neurons project, from the propagation of a cascade of inflammatory events elicited by the expression of pro-inflammatory cytokines. In order to test our working hypothesis, we analyzed the mRNA expression levels of pro-inflammatory cytokines and IκB in the LC obtained from C3H/HeN mice intraperitoneally (i.p.)-injected with LPS and the expression level of mRNA and protein in murine microglia in primary culture incubated with LPS and/or NE. Our findings are interpreted and discussed in this paper.

Materials and methods

Chemical reagents

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

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). The mice were kept under conditions of a 12-h light/12-h dark cycle (lights on from 08.00 to 20.00 h), constant temperature (23–25°C), and free access to food and water. 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.

Preparation of LC samples

Brain samples containing the LC (designated as 'LC sample') were prepared chronologically after i.p. injection with 5 μg of LPS or saline as already reported (Kaneko *et al.* 2001).

Microglia from primary cultures

Microglia were harvested from mixed populations of glial cells in primary cultures prepared from neonatal C3H/HeN mouse pups as previously reported (Suzumura *et al.* 1987; Sawada *et al.* 1990). In brief, after the meninges had been carefully removed, the neonatal brain was dissociated by pipetting. The cell suspension was then plated in 75-cm^2 culture flasks (Falcon 3024; Becton-Dickinson Japan, Tokyo, Japan) at the density of one brain per flask in 10 mL of Eagle's minimum essential medium (MEM) (Sigma) supplemented with 10% fetal bovine serum (FBS) (Sanko Jun-yaku Co., Chiba, Japan), 5 μg/mL bovine insulin (Sigma), and 0.2% glucose. Microglia were isolated on days 14–21 by the 'shaking off' method of Suzumura *et al.* (1987). The reverse transcriptase–polymerase chain reaction (RT–PCR) was performed to detect PLP/DM-20 expression, and the results revealed that the microglia population contained neither O2-A progenitors, oligodendrocytes nor astrocytes. Then, the microglia were incubated with graded doses of LPS or NE, or with the combination of LPS and NE for the appropriate periods.

Measurement of mRNA expression levels by using quantitative real-time PCR

Total RNA was extracted from the LC samples or from 5×10^5 microglia by using RNA isolation reagent (Isogen; Nippon Gene Co.; Tokyo, Japan) according to the manufacturer's instructions. Synthesis of complementary DNA (cDNA) was performed by using 1 μg of total RNA and oligo $(dT)_{12-18}$ primer in a reverse transcription reaction (Life Technologies; Tokyo, Japan). Each primer pair used for the quantitative real-time PCR (Table 1) generated a single band 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 sample comparable with 50 ng of cDNA was performed on an ABI 7700 PCR Instrument (Perkin Elmer Biosystems, Foster City, CA, USA) with SYBR Green (Qiagen, Hilden, Germany) according to the manufacture's instructions. Each sample was tested in triplicate by quantitative real-time PCR, and five mice and five dishes (6-cm diameter) of microglia for each group were used to calculate the means and SD. Calculated data were corrected by reference to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression level.

Measurement of cytokine protein levels in culture supernatant by using enzyme-linked immunosorbent assays (ELISA)

Prepared microglia (1×10^5 cells per well in a 24-well plate) were treated with 0.1 μg/mL LPS and/or 1 μM NE in the presence of fetal calf serum (Sanko Jun-yaku). The supernatants of the microglia cultures were collected at 6 h after LPS and/or NE stimulation, and the concentrations of IL-1β and TNF-α were measured by ELISA using monoclonal antibodies against the respective cytokines [no. MLB00 for IL-1β (BD Biosciences Pharmingen, San Diego, CA, USA); KMC0011 for IL-1β, KMC3011 for TNF-α (BioSource, Sunnyvale, CA, USA)]. ELISA was performed according to the procedure recommended by the supplier. The serum in the media neither affected the absorbance nor interfered with the assay.

Immunoprecipitation of secreted IL-1β and TNF-α protein in culture supernatant

The supernatants of the microglia cultures, which were prepared and treated as above, were collected at 6 h after LPS and/or NE stimulation. Five hundred microlitres of supernatant was incubated with the anti-IL-1β polyclonal antibody (sc-1251, 1 : 100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-TNF-α polyclonal antibody (sc-1351, 1 : 100 dilution; Santa Cruz Biotechnology) or normal goat serum (Santa Cruz Biotechology). The mixtures were gently agitated for 1 h at 4°C, after which 20 μL of Protein G agarose beads (sc-2002, Santa Cruz Biotechnology) was added; and then incubation was continued for 1 h at 4°C. The beads

Table 1 Primers for real-time PCR

Gene name	Genebank accession number	Primer orientation	Nucleotide sequence (from 5′ to 3′)	Starting sequence position	Size of the PCR amplicon (bp)
Mouse IL-1β	M15131	Forward	GAGCCCATCCTCTGTGACTCAT	298	83
		Reverse	AGCCTGTAGTGCAGCTGTCTAATG	380	
Mouse TNF-α	M38296	Forward	GACCCTCACACTCAGATCATCTT	228	77
		Reverse	CCACTTGGTGGTTTGCTACGA	304	
Mouse NF-κB p65	M61909	Forward	CAGACCCAGGAGTGTTCACAGA	1370	81
		Reverse	ACATGGACACACCCTGGTTCA	1450	
Mouse IκBα	U36277	Forward	GTGATCCTGAGCTCCGAGACTT	404	93
		Reverse	TCTGCGTCAAGACTGCTACACTG	496	
Mouse I IκBβ	NM_0109081	Forward	CCCACTCCATGTAGCTGTCATC	627	93
		Reverse	CACGTAGGCTCCGGTTTATTGA	719	
Mouse caspase-1	BC008152	Forward	GAATACAACCACTCGTACACGTCTTG	465	120
		Reverse	AGATCCTCCAGCAGCAACTTCA	584	
Mouse GADPH	NM_008084	Forward	ATGTGTCCGTCGTGGATCTGA	710	81
		Reverse	ATGCCTGCTTCACCACCTTCT	790	

were washed three times with IP-kinase buffer [50 mM HEPES (pH 8.0), 150 mM NaCl, 25 mM EGTA, 1 mM EDTA, 0.1% Tween 20, and 10% glycerol], and the proteins bound to them were released and dissolved in Laemmli's sample buffer. Then, the samples were immunoblotted as described in the next chapter.

Immunoblot analysis

Detection of IL-1β and TNF-α in culture supernatant and in microglia

Microglia prepared (1×10^6 cells in a 6-cm dish) were lysed in IP-kinase buffer containing a cocktail of protease inhibitors (P8340, Sigma). The immunoprecipitated samples and the cell lysate (10 μg of proteins) were separated by sodium dodceyl sulfate – polyacrylamide gel electrophoresis (SDS–PAGE) (15% acrylamide for immunoprecipitated samples, and 10% acrylamide for the cell lysate samples) and transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore, Billerica, MA, USA) by using the KS-8640™ blotting system (Marysol, Tokyo, Japan). The membranes were blocked with 3% skimmed milk in phosphate-buffered saline containing 0.5% Tween 20 (PBST) and then incubated with primary antibodies against mouse IL-1β (goat anti-mouse IL-1β polyclonal antibody, sc-1251, 1 : 1000 dilution; Santa Cruz Biotechnology) or those against mouse TNF-α (goat anti-mouse TNF-α polyclonal antibody sc-1351, 1 : 500 dilution; Santa Cruz Biotechnology). After the membrane had been washed thoroughly in PBST, horseradish peroxidase-conjugated secondary antibody (sc-2768, 1 : 5000 dilution; Santa Cruz Biotechnology) was applied to the membrane. Finally, the blots were developed by using an enhanced chemiluminescence detection kit (Amersham Biosciences, Uppsala, Sweden). The densitometric scanning of the corresponding band was performed and evaluated by using an Image Reader coupled with Image Gauge (Science Laboratory 98, Fujifilm, Tokyo, Japan).

Detection of caspase-1 precursor protein and its two subunits in microglia

Procedures to detect caspase-1 precursor protein and its p10 and p20 subunits in the cell lysates of microglia cultured with 0.1 μg/mL LPS and/or 1 μM NE for 6 h were the same as those described in the previous chapter. The primary antibodies used were rabbit anti-mouse caspase-1 p10 polyclonal antibody (sc-514, 1 : 500 dilution; Santa Cruz Biotechnology) and goat anti-mouse caspase-1 p20 polyclonal antibody (sc-1597, 1 : 500 dilution; Santa Cruz Biotechnology), respectively. Both of them are able to recognize caspase-1 precursor protein. The blotted membranes were also developed by using an enhanced chemiluminescence detection kit (Amersham Biosciences).

Immunohistochemistry to detect IL-1β and TNF-α in microglia

Microglia prepared as described above were treated with 0.1 μg/mL LPS and/or 1 μM NE for 6 h. The cells were harvested and incubated with anti-IL-1β antibody (sc-1251, 1 : 500 dilution; Santa Cruz Biotechnology) or anti-TNF-α antibody (sc-1351, 1 : 100 dilution: Santa Cruz Biotechnology) for 30 min at 25°C and then with 3% normal goat serum (Santa Cruz Biotechnology) for 10 min at 25°C. Next, they were incubated with biotinylated anti-goat IgG antibody (E1903, Santa Cruz Biotechnology) for 30 min at 25°C, and subsequently with avidin-biotin complex for 30 min at 25°C. Finally, the specimens were stained with 3,3′-diaminobenzidine-tetrahydrochloride and hydroperoxide.

Assay of enzymatic activity of caspase-1 in microglia

The assay for the enzymatic activity of caspase-1 using the Caspase-1/ICE Colorimetric Protease Assay Kit (Medical and Biological Laboratories, Nagoya, Japan) was performed according to the supplier's instruction. All buffers, dithiothreitol (DTT), and the substrate [tyrosine-valine-alanine-aspartate coupled with chromophore-*p*-nitroanilide (YVAD-*p*NA)] used in the assay were supplied in the kit. Briefly, cell pellets (2×10^6 cells) of microglia cultured with 0.1 μg/mL LPS and/or 1 μM NE for 6 h were re-suspended in 50 μL of chilled cell lysis buffer and kept on ice for 10 min. After centrifugation at 10 000 *g* for 1 min, the supernatants were adjusted to 50 μL containing 200 μg protein. They were mixed with 50 μL 2 × reaction buffer containing 10 mM dithiothreitol and incubated with 5 μL of 4 mM YVAD-*p*NA (final concentration of the substrate: 200 μM) for 2 h at 37°C. Then, the *p*NA light emission was quantified in a microtitre plate at a 405 nm wavelength.

Statistics

All numerical data were expressed as the mean ± SD. 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 Sheffe's *F*-test as a post-hoc test. For all statistical analyses, $p < 0.05$ was considered to be statistically significant.

Results

Time course of mRNA expression levels of the genes encoding pro-inflammatory cytokines, IκBα and NF-κB in the LC of C3H/HeN male mice

At first, the time course of the pro-inflammatory cytokine mRNA expression level in the LC of C3H/HeN male mice after 5 μg of LPS i.p. injection was investigated by using the quantitative real-time PCR method. IL-1β and TNF-α mRNA expression levels in the LC samples were measured at times ranging from 15 min to 5 days after the LPS or vehicle i.p. injection. The mRNA expression level of the gene encoding IL-1β in LPS-injected mice increased within 2 h and reached a maximum level at 4 h (Fig. 1a). The level in the LPS-injected group was 8.3-fold higher than that of the vehicle-injected one at 2 h and 13.1-fold higher at 4 h. Then, it decreased sharply at 6 h after the injection and approached the basal level. Surprisingly, the expression was again up-regulated to become 3.5-fold higher than that of vehicle-injected mice at 15 h after the i.p. injection (Fig. 1a). The mRNA expression level of the TNF-α gene was also up-regulated within 2 h and reached a maximum level between 2 and 4 h after the i.p. injection of LPS (Fig. 1b). At that time, the expression was about 4.5-fold higher than that of the vehicle-injected mice. Then, it decreased gradually, and again increased to become 1.9-fold higher than that of vehicle-injected mice at 15 h after the injection. The

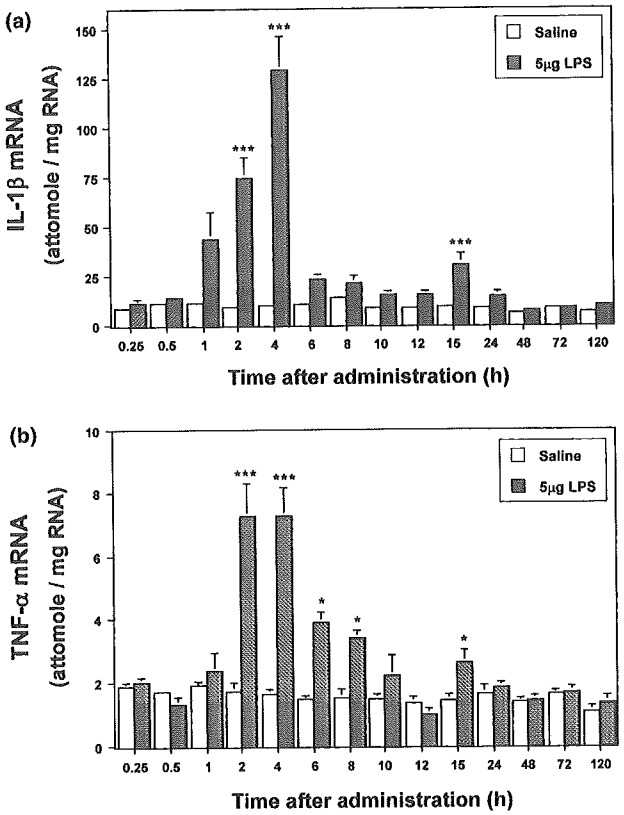

Fig. 1 Changes in mRNA expression levels of IL-1β (a) and TNF-α (b) in LC samples obtained from C3H/HeN mice injected i.p. with LPS. Mice (five mice per group) were i.p. injected with 5 μg of LPS or vehicle (saline), and decapitated at the indicated time points. Immediately after the decapitation, their brains were removed. Total RNA was extracted from LC samples and reverse transcribed to cDNA. Quantitative SYBR Green real-time PCR was performed by using the specific primers listed in Table 1 and cDNA as a template. The experiments were carried out in duplicate and repeated three times with similar results. Data are displayed as the mean (column) ± SD (bar) values. Each value marked with an asterisk was significantly greater than the corresponding one obtained from the vehicle-injected mice (control): $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$.

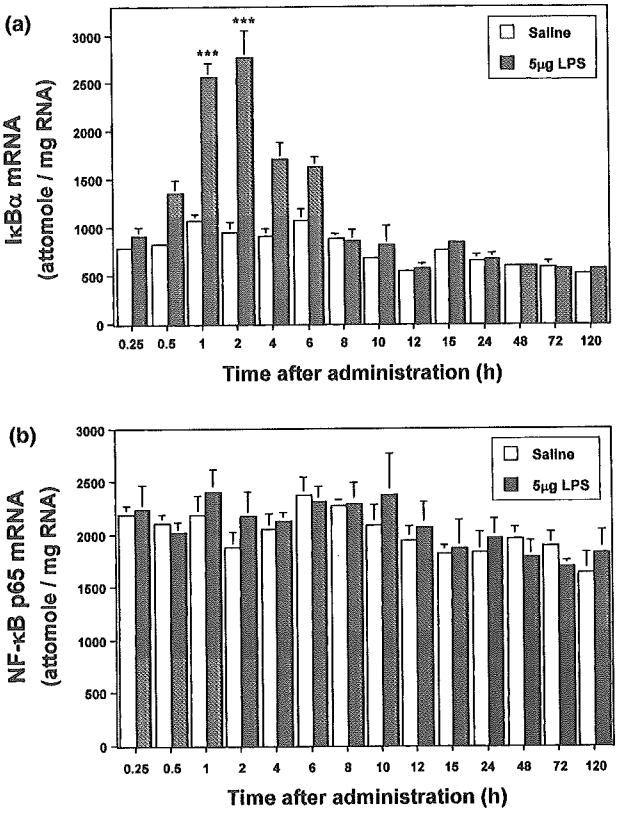

Fig. 2 Changes in mRNA expression levels of IκBα (a) and NF-κB (b) in LC samples obtained from C3H/HeN mice injected i.p. with LPS. Mice (five mice per group) were i.p. injected with 5 μg of LPS or vehicle (saline), and decapitated at the indicated time points. Immediately after the decapitation, their brains were removed. Total RNA was extracted from LC samples and reverse transcribed to cDNA. Quantitative SYBR Green real-time PCR was performed by using the specific primers listed in Table 1 and cDNA as a template. The experiments were carried out in duplicate and repeated three times with similar results. Data are displayed as the mean (column) ± SD (bar) values. Each value marked with an asterisk was significantly greater than the corresponding one obtained from the vehicle-injected mice (control): $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$.

time-course profile of mRNA expression levels of IL-1β and TNF-α was very similar in that they responded very quickly to the i.p. injection of LPS and the second peak appeared at 15 h after the peripheral LPS injection.

As a next step, we measured the mRNA expression level of the gene encoding IκBα in the LC samples. As shown in Fig. 2(a), the IκBα mRNA expression level increased to a statistically significant level within 1 h after the LPS injection and reached a maximum level at 2 h. Its expression level in LPS-injected mice became 2.4-fold higher at 1 h and 2.9-fold higher at 2 h than that of vehicle-injected mice, at the respective times (Fig. 2a). It then decreased at 4 h post-injection and declined to almost the same level found for the vehicle-injected mice at 8 h after the injection. Contrary to the expression levels of IL-1β and TNF-α mRNAs, the IκBα mRNA expression level did not show any difference from that of the vehicle-injected mice thereafter. Next, we measured the mRNA expression level of the gene encoding NF-κB p65. However, no significant enhancement of its mRNA expression level following the peripheral LPS injection was observed (Fig. 2b).

Effects of LPS and/or NE on expression levels of IL-1β mRNA and TNF-α mRNA in microglia

It has been reported that pro-inflammatory cytokine expression is negatively regulated by NE, which could contribute to the observed anti-inflammatory properties of NE. Therefore, we conducted experiments using a cell culture system in

order to elucidate the relationship between the increase in NE content and the up-regulation of pro-inflammatory cytokines in the LC at 2 and 4 h after LPS injection.

Incubation of primary-cultured microglia with 0.1 μg/mL LPS for 1 h enhanced the expression level of IL-1β mRNA, making it about 25-fold higher, and that of TNF-α mRNA about 160-fold higher compared with those levels for the control cells (Figs 3a and b). Although the incubation of microglia with 1 μM NE for 1 h enhanced the expression levels of IL-1β mRNA 4.8-fold higher compared with that of control cells (Fig. 3a), the increase did not attain a statistically significant difference. As described above, the expression level of TNF-α mRNA was not affected by the incubation with 1 μM NE for 1 h (Fig. 3b, closed column). Incubation of cells with a combination of 0.1 μg/mL LPS and 1 μM NE for 1 h magnified the IL-1β mRNA expression, causing the level to be 2.1-fold higher than the one obtained from the incubation with LPS alone (Fig. 3a). However,

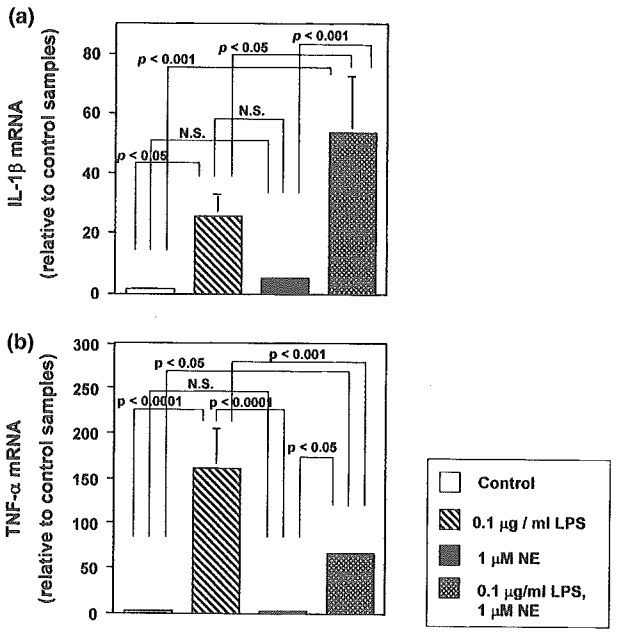

Fig. 3 The mRNA expression of IL-1β (a) and TNF-α (b) in microglia. Microglia (5×10^5 cells per 6-cm-diameter dish) prepared from primary cultures were incubated for 1 h with 0.1 μg/mL LPS and/or 1 μM NE in MEM containing 10% FBS. Total RNA was extracted from the treated microglia and reverse transcribed to cDNA. Quantitative real-time PCR was performed by using the specific primers listed in Table 1 and cDNA as a template. The amounts of IL-1β mRNA ((a) and TNF-α mRNA (b) were measured by using the SYBR Green real-time PCR method. The experiments were carried out in duplicate and repeated three times with similar results. Data shown in Fig. 3 were standardized by reference to GAPDH mRNA expression level and normalized to the standardized values obtained from control samples. They are displayed as the mean (column) ± SD (bar) values. All *p*-values for the comparisons performed are shown in the figures.

TNF-α mRNA expression level enhanced by the incubation with 0.1 μg/mL LPS alone was suppressed to 40% by the addition of 1 μM NE to the incubation medium (Fig. 3b).

Effects of LPS and/or NE on expression levels of IL-1β and TNF-α proteins in microglia

ELISA method

The protein amounts of pro-inflammatory cytokines released into the medium of microglia cultures were measured by using the ELISA method. Although the amounts of IL-1β protein released into the culture medium were measured by using two kinds of kits (see Materials and methods), their values were at or around the detection limits of the kits (data not shown).

The incubation of the cells with 0.1 μg/mL LPS for 6 h dramatically enhanced the amount of TNF-α protein released into the medium to a value about 240-fold higher than that for the control (Fig. 4a). The incubation of the cells with 1 μM NE for 6 h did not affect the amount of secreted TNF-α protein at all (Fig. 4a). The enhanced release of TNF-α protein by the incubation of the cells with 0.1 μg/mL LPS alone was suppressed to 29% by the addition of 1 μM NE to the incubation medium (Fig. 4a)

Immunoblot analysis

Because the amount of IL-1β protein released into the culture medium could not be measured by the ELISA method, it was evaluated by a method combining immunoprecipitation and immunoblotting. Culture media of microglia that were treated with LPS and/or NE were incubated with anti-mouse IL-1β antibody or anti-mouse TNF-α antibody to immunoprecipitate secreted proteins, respectively. Immunoprecipitated proteins were analyzed by immunoblotting (Fig. 4). Secreted IL-1β protein was not detected in the supernatant of microglia stimulated with LPS or LPS/NE (Fig. 4b, left panel). By contrast, the secreted TNF-α protein was detected in the supernatant of the cultures stimulated with LPS, and the amount of the protein was reduced by the treatment with NE (Fig. 4b, right panel) whose intensity scanned by the Image Reader coupled with Image Gauge was 30% of the one of the supernatant of the cells treated with LPS alone. The molecular weight of secreted TNF-α protein was about 17 kDa, which coincides with the mature form of mouse TNF-α.

As shown by the immunoblot results in Fig. 5, incubation of the cells with 0.1 μg/mL LPS for 6 h enhanced the intracellular amounts of both IL-1β protein and TNF-α protein. Intracellular IL-1β or intracellular TNF-α protein was scarcely detected when the cells were incubated with 1 μM NE for 6 h (Fig. 5). Incubation of the cells with a combination of 0.1 μg/mL LPS and 1 μM NE for 6 h resulted in a band of IL-1β protein of greater intensity to the 1.2-fold increase than that obtained from the incubation with

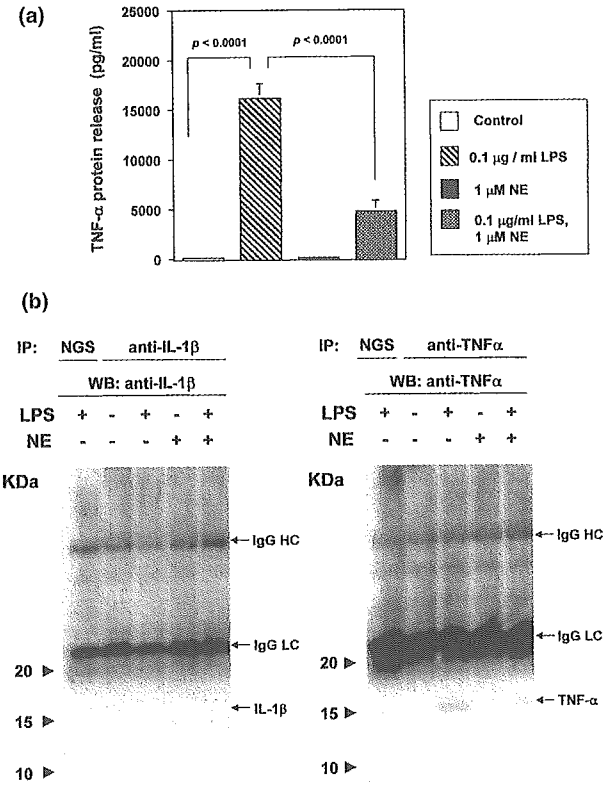

Fig. 4 The release of pro-inflammatory cytokines from microglia into culture supernatant. Microglia (1×10^5 cells per well in a 24-well plate) were incubated for 6 h with 0.1 μg/mL LPS and/or 1 μM NE in MEM containing 10% FBS. The amounts of IL-1β and TNF-α protein in the supernatants were measured by using the ELISA method described in Materials and methods. Data on the release of TNF-α into culture supernatant are displayed as the mean (column) ± SD (bar) values in (a). The serum added in the medium did not affect cytokine production. The experiments were carried out in duplicate and repeated three times with similar results. Contrary to TNF-α, IL-1β protein in culture supernatant was not detected by ELISA method. *p*-values not displayed in (a) are as follows: control versus NE-treated group, non-significant; control versus LPS/NE-treated group, $p < 0.0001$; LPS-treated group versus NE-treated group, $p < 0.0001$; NE-treated group versus LPS/NE-treated group, $p < 0.0001$. The amounts of IL-1β and TNF-α protein secreted into culture media were evaluated by the combination of immunoprecipitation and immunoblotting (b). The experiments were repeated three times with similar results, and representative data from one of them are shown. The densities of corresponding bands were scanned by Image Reader coupled with Image Gauge (see text). IgG HC, immunoglobulin heavy chain; IgG LC, immunoglobulin light chain; NGS, normal goat serum.

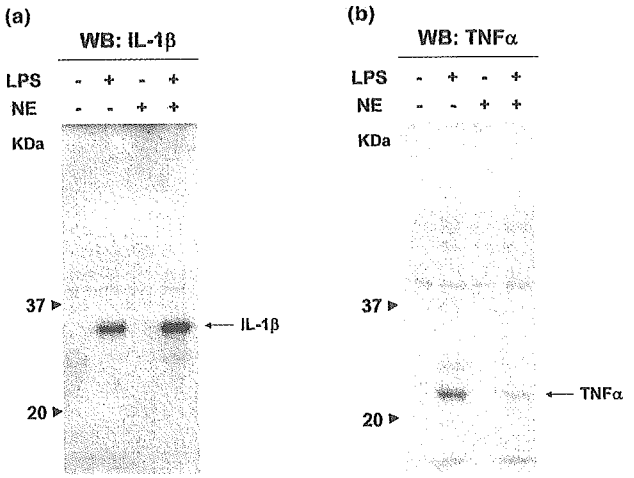

Fig. 5 Immunoblots of cell extracts prepared from microglia by using specific antibodies raised against IL-1β and TNF-α. Cell extracts prepared from microglia (1×10^6 cells) treated with or not with 0.1 μg/mL LPS and/or 1 μM NE for 6 h were subjected to immunoblot analysis as described in Materials and methods. The experiments were repeated three times with similar results. Representative data are shown in IL-1β (a) and TNF-α (b). The densities of corresponding bands were scanned by Image Reader coupled with Image Gauge (see text).

0.1 μg/mL LPS alone (Fig. 5a), whereas the band of TNF-α protein was reduced to 40% in its intensity under the same conditions (Fig. 5b). It should be noted that, in Fig. 5, the molecular weight of intracellular IL-1β protein was about 31 kDa, and that of TNF-α was about 25 kDa. These values coincide with those of the immature forms of the proteins of mouse IL-1β and mouse TNF-α. Bands corresponding to the processed forms of IL-1β (17 kDa) and TNF-α (17 kDa) proteins were not detected in the immunoblot analysis with using the cell lysates of microglia stimulated with LPS and/or NE (Fig. 5).

Immunohistochemical analysis

The data obtained by the immunoblot method were confirmed by using an immunohistochemical method (Fig. 6). The presence of both IL-1β and TNF-α proteins in microglia stimulated with 0.1 μg/mL LPS for 6 h was observed (Figs 6b and f). The cells incubated with 1 μM NE for 6 h expressed a little higher amount of IL-1β protein than the control cells (Fig. 6c), but did not produce any more TNF-α protein than the control ones (Fig. 6g). Six-hour incubation of the cells with 0.1 μg/mL LPS plus 1 μM NE increased the intracellular amount of IL-1β protein (Fig. 6d) and reduced that of TNF-α protein (Fig. 6h) compared with the levels found with LPS treatment only.

Effects of LPS and/or NE stimulation of microglia on caspase-1 in the cells

On mRNA expression level

We measured caspase-1 mRNA expression levels in microglia by using real-time PCR amplification to investigate whether caspase-1 mRNA expression levels in microglia were affected by LPS and/or NE stimulation. The pair of the primers used in the amplification is shown in Table 1. mRNA

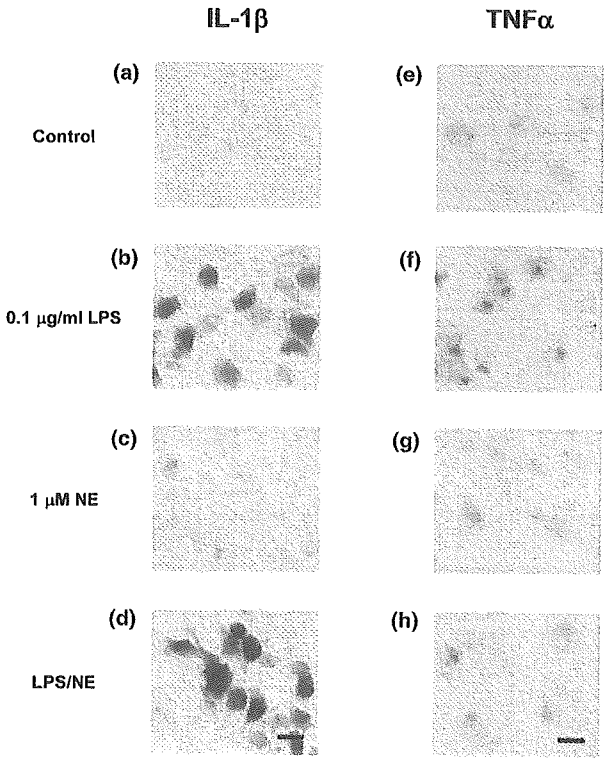

Fig. 6 Immunohistochemical staining of IL-1β and TNF-α proteins in microglia. Microglia (5×10^4 cells per cover glass in a 6-well plate) were incubated for 6 h with 0.1 μg/mL LPS and/or 1 μM NE in MEM containing 10% FBS. The cells were stained with anti-IL-1β antibody (a–d) or anti-TNF-α antibody (e–h) as described in Materials and methods. (a, e) Control cells, (b, f) cells incubated with LPS, (c, g) cells incubated with NE and (d, h) cells incubated with a combination of LPS and NE. The experiments were repeated three times with similar results and representative data are shown. Scale bar = 50 μm.

was extracted from microglia cultured with 0.1 μg/mL LPS and/or 1 μM NE for 1 h. Caspase-1 mRNA was constitutively expressed in microglia and its expression level was not affected at all by the stimulation of LPS and/or NE (data not shown).

On protein expression level

On the immunoblot analysis, the expression level of 45 kDa caspase-1 precursor protein in microglia cultured with 0.1 μg/mL LPS and/or 1 μM NE for 6 h was not affected by LPS and/or NE treatment. Furthermore, 45 kDa caspase-1 precursor protein in microglia under LPS and/or NE treatment was not cleaved to produce 10 kDa and 20 kDa subunits (data not shown).

On enzymatic activity

The enzymatic activity of caspase-1 in microglia cultured with 0.1 μg/mL LPS and/or 1 μM NE for 6 h was below the detection limit of the assay.

Effects of LPS and/or NE on mRNA expression levels of the genes encoding IκBα, IκBβ, and NF-κB in microglia

Finally, the effects of NE on the mRNA expression levels of the genes encoding IκBα, IκBβ and NF-κB in primary-cultured microglia were investigated by using the semiquantitative real-time PCR method (Fig. 7). IκBα mRNA expression in the cells incubated with 0.1 μg/mL LPS for

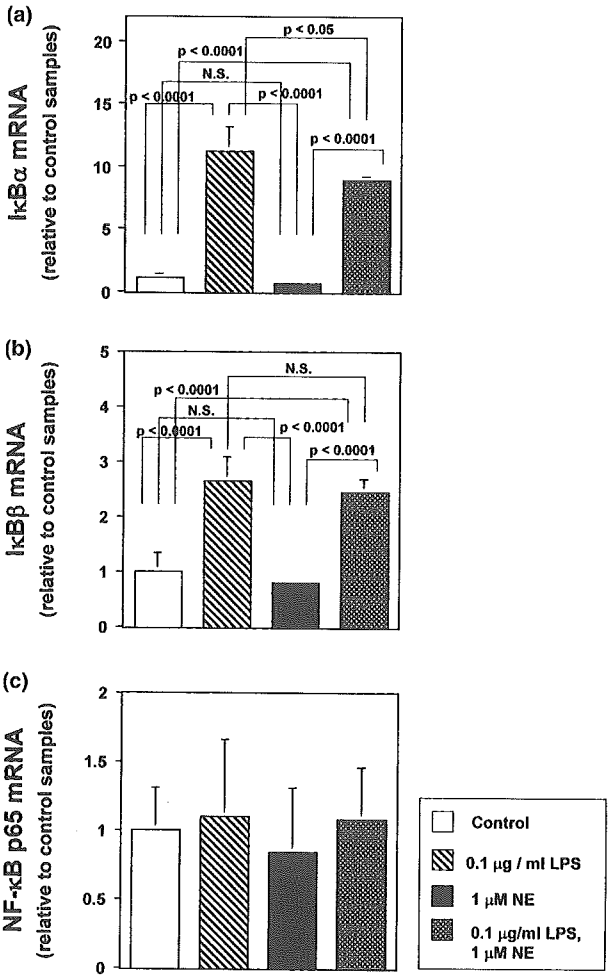

Fig. 7 The mRNA expression of transcription factors IκBα (a), IκBβ (b) and NF-κB p65 (c) in microglia. Microglia (5×10^5 cells per 6 cm-diameter dish) were incubated for 1 h with 0.1 μg/mL LPS and/or 1 μM NE in MEM containing 10% FBS. Total RNA was extracted from the treated microglia and reverse transcribed to cDNA. Quantitative real-time PCR was performed by using the specific primers listed in Table 1 and cDNA as a template. The expression levels of IκBα mRNA (a), IκBβ mRNA (b) and NF-κB p65 mRNA (c) were measured by using the SYBR Green real-time PCR method as described in Materials and methods. The experiments were carried out in duplicate and repeated three times with similar results. Data shown in the Fig. 7 were standardized by reference to GAPDH mRNA expression level and normalized to the standardized values obtained from control samples. They are displayed as the mean (column) ± SD (bar) values. All *p*-values for the comparisons performed are shown in the figures.

1 h was up-regulated to become 11.2-fold higher than that in the control cells (Fig. 7a). However, the incubation of the cells with 1 μM NE alone did not affect IκBα mRNA expression at all (Fig. 7a). One-hour incubation of the cells with a cocktail of 0.1 μg/mL LPS and 1 μM NE suppressed the enhanced IκBα mRNA expression caused by the incubation with 0.1 μg/mL LPS alone to 80% of its value (Fig. 7a). The responses of IκBβ mRNA in the cells to LPS and/or NE were almost the same as those of IκBα mRNA, except for the fact that the incubation of the cells with the combination of LPS and NE did not suppress the enhanced IκBβ mRNA expression caused by LPS alone (Fig. 7b). The NF-κB mRNA expression levels in the cells were not affected at all by the 1-h incubation with LPS and/or NE (Fig. 7c).

Discussion

As the first step of a series of experiments, the expression levels of pro-inflammatory cytokines in the LC samples were examined. Under normal conditions, the expression levels of IL-1β and TNF-α mRNAs in the LC were very low (Fig. 1). An i.p. injection of LPS dramatically increased their respective expression levels to values more than 10-fold higher and 4-fold higher than those obtained with the vehicle i.p. injection at 4 h post-injection (Fig. 1). Kaneko *et al.* (2001) previously reported that NE turnover in the LC was enhanced to a statistically significant level within 2–4 h after an i.p. injection of LPS and reached its maximum level at 4 h after the injection. It should be noted that the time profile of NE production in the LC in that study was synchronous with those profiles of IL-1β and TNF-α mRNA expression at the site in our present study. We thought that this increased NE might act to down-regulate the mRNA expression of IL-1β and TNF-α.

Microglia comprise up to 20% of the total glial cell population in the brain, and these cells can be readily transformed to an activated state in response to a wide range of stimuli (Nakamura *et al.* 1999). It is well known that microglia are activated prior to astrocytes when an infection occurs in the brain (Kreutzberg 1998). Because the increases in expression of IL-1β and TNF-α mRNAs in the LC happened very quickly after the LPS injection (Fig. 1), we suspected that microglia were activated in the LC. Therefore, microglia from mouse neonate brains were prepared in order to elucidate whether there is a close correlation between the increase in NE turnover and the up-regulation of pro-inflammatory cytokines. The mRNA and protein levels of IL-1β and TNF-α in microglia were vigorously elevated by the incubation with LPS (Figs 3–6).

NF-κB is one of the transcription factors involved in the transcription of the *IL-1β* gene (Cogswell *et al.* 1994) and *TNF-α* gene (Collart *et al.* 1990). The activation of NF-κB can be estimated by the mRNA induction level of IκB, which functions as an inhibitor of NF-κB in living cells, because the IκB mRNA expression level itself is regulated by NF-κB (Sen and Baltimore 1986; Sun *et al.* 1993). As shown in Fig. 2(a) and Fig. 7(a), IκBα mRNA expression levels were enhanced by LPS stimulation both *in vivo* and *in vitro*. Hence, our results suggest that the enhanced level of IκBα mRNA reflects NF-κB activation, and that the enhanced mRNA expression of IL-1β and TNF-α in microglia incubated with LPS was caused by NF-κB activation.

As shown in Figs 3–6, the effects of NE on the upward expression of mRNA and protein of IL-1β and TNF-α in microglia caused by the incubation with LPS were different between these two pro-inflammatory cytokines. NE enhanced the expression of IL-1β synergistically with LPS, whereas it suppressed the expression of TNF-α that was enhanced by the incubation with LPS. The regulation mechanisms for IL-1β and TNF-α must be explained by different scenarios.

The synergistic effect of NE and LPS on IL-1β expression may be explained by the scenarios in which the binding of NE to β adrenergic receptors on microglia is followed by an enhanced activation of the MAP kinase via cAMP-protein kinase A-dependent pathway (Tanaka *et al.* 2002; Woo *et al.* 2003). Because transcription factor NF-κB regulates both consensus and non-consensus cAMP response element sites in the promoter region of the *IL-1β* gene, NF-κB plays multiple roles in the induction of IL-1β transcription (Cogswell *et al.* 1994).

It is also known that cAMP-elevating agents such as prostaglandin E_2 suppress TNF-α expression in microglia (Aloisi *et al.* 1999; Petrova *et al.* 1999; Kim *et al.* 2000; Facchinetti *et al.* 2003). However, it is reported that protein kinase A-independent pathways may have the major role in the regulation of TNF-α expression in microglia (Woo *et al.* 2003). According to Woo *et al.* (2003), dibutyryl cAMP suppressed NF-κB-mediated transcription of the *TNF-α* gene in microglia. As shown in Fig. 7(a), the addition of NE suppressed the LPS-induced augmentation of IκBα mRNA expression in cultured microglia. Taken together, these data support the view that NE exerts a protective effect against the insults to nerve cells by inhibiting NF-κB activation in microglia.

Because mature IL-1β protein was not released into culture media by LPS treatment, we investigated the expression level and the activity of caspase-1 in primary cultured microglia under LPS and/or NE treatment. The expression level of caspase-1 mRNA in microglia was not affected by LPS and/or NE treatment (data not shown). On the immunoblot, the expression level of 45 kDa caspase-1 precursor protein was not affected by LPS and/or NE treatment. Furthermore, 45 kDa caspase-1 precursor protein in microglia under LPS treatment was not cleaved to produce 10 and 20 kDa subunits (data not shown). In addition, the enzymatic activity of caspase-1 in microglia could not be detected. These data suggest that the activation of caspase-1

in microglia was not to a level that allows the precursor of IL-1β protein to be converted into the secreted form. These data coincided well with the observation that, in spite of the upward expression of IL-1β protein in microglia stimulated by LPS and/or NE, IL-1β protein was not secreted into culture supernatant. The release of processed IL-1β from isolated macrophages was also relatively inefficient, although endotoxin induced the formation of large intracellular stores of pro-IL-1β (Wewers *et al.* 1984). Extracellular ATP has emerged as an inducer of rapid processing and massive release of endotoxin-induced IL-1β in macrophages and microglia (Hogquist *et al.* 1991; Walz *et al.* 1993; Perregaux and Gabel 1994; Griffiths *et al.* 1995; Andrei *et al.* 2004; Kahlenberg and Dubyak 2004; Suzuki *et al.* 2004). Therefore, a sufficient amount of ATP might have to be supplied to the purinergic receptors on microglia that gather in the vicinity of injured nerve cells.

As already described briefly in the Introduction, the noradrenergic regulation of IκBα expression and inflammatory gene expression is relevant to some clinical aspects of Alzheimer's disease, such as the damage to or loss of NE neurons in the LC (Mann *et al.* 1983), to the protection of animal models from experimental autoimmune encephalomyelitis by treatment with a β adrenergic receptor agonist (Chelmicka-Schorr *et al.* 1989; Wiegmann *et al.* 1995), to the decreased levels of β2 adrenergic receptors in astrocytes in multiple sclerosis patients (Zeinstra *et al.* 2000). In this study, we characterized the plausible mechanisms for the regulation of pro-inflammatory cytokines at the LC. Our data suggest that NE exerts neuroprotective and anti-inflammatory actions, mainly by way of the inhibition of TNF-α production in microglia. These findings will be useful for constructing new tactics to treat neurodegenerative disorders in which microglial activation and inflammatory responses are directly linked to their pathological phenomena.

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Inflammatory Process in Parkinson's Disease: Role for Cytokines

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Abstract: Parkinson's disease (PD) is a movement disorder caused by degeneration of the nigrostriatal dopamine (DA) neurons in the substantia nigra pars compacta and the resultant deficiency in the neurotransmitter DA at the nerve terminals in the striatum. We and other investigators found increased levels of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-alpha, interleukin (IL)-1beta, and IL-6, and decreased levels of neurotrophins such as brain-derived neurotrophic factor (BDNF) in the nigrostriatal region of postmortem brains and/or in the ventricular or lumbar cerebrospinal fluid (CSF) from patients with sporadic PD, and in animal models, such as 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP)- and 6-hydroxydopamine (6-OHDA)-induced PD. These changes in cytokine and neurotrophin levels may be initiated by activated microglia, which may then promote apoptotic cell death and subsequent phagocytosis of DA neurons. Cytokines as pleiotropic factors, promote signals that either lead to cell death or exert neuroprotective effects. The discovery of toxic changes in trophic microglia by M. Sawada and co-workers is important to this point. Ultimately, microglial cells may regulate cellular changes that cause either harm or benefit by producing cytokines or neurotrophins depending upon the primary cause and the circumstances during the inflammatory process of PD.

Key Words: Parkinson's disease, inflammation, microglia, astroglia, cytokines, neurotrophins, apoptosis

1. INTRODUCTION

Parkinson's disease (PD), named after the English physician, James Parkinson, who defined it in 1817, is characterized by specific degeneration of the dopamine (DA) neurons in the substantia nigra pars compacta and the resulting loss of the nerve terminals, which is accompanied by a deficiency in the neurotransmitter DA, in the striatum. This DA deficiency is responsible for most of the movement disturbances called parkinsonism, i.e., muscle rigidity, akinesia, and resting tremor. Familial PD (PARK1-PARK10, NR4A2), for which the causative genes and their chromosomal locations have been identified, constitutes a small percentage of PD cases (approximately 5~10%); and most PD is sporadic without hereditary history. Sporadic PD is also called "idiopathic," because its pathogenesis remains unknown. PD is the second most common aging-related neurodegenerative disease after Alzheimer's disease (AD). Sporadic PD is characterized by the presence of intracellular eosinophilic inclusion bodies called Lewy bodies, which were named after the German physician who described them in 1913.The pathogenesis of sporadic PD is still enigmatic; and many factors are speculated to operate in the mechanism of cell death of the nigrostriatal DA neurons in PD, e.g., oxidative stress and cytotoxicity of reactive oxygen species (ROS), disturbance of intracellular calcium homeostasis regulated by the excitatory neurotransmitter glutamate *via* NMDA receptors, and endogenous or exogenous neurotoxins [1-3].

In the case of familial PD, possible causative factors have recently been defined: *alpha-synuclein* in autosomal-dominant PARK 1 [4]; *parkin*, which encodes a ubiquitin ligase E3, in autosomal-recessive juvenile (early-onset) PARK 2 [5, 6]; *UCH-L1* (ubiquitin C-terminal hydrolase) in autosomal-recessive PARK 5 [7]; *PINK1* in autosomal-recessive PARK 6 [8]; DJ-1 in autosomal-recessive PARK 7 [9]; and *Nurr1* (nuclear receptor-related 1) in autosomal-dominant NR4A2 [10]. The main component of PD-associated intracellular inclusions, Lewy bodies, is alpha-synuclein. The intracellular accumulation of abnormal "unfolded" or "misfolded" proteins consequential to dysfunction of the ubiquitin proteasome system in familial PD produces endoplasmic reticulum (ER) stress, which might subsequently lead to oxidative stress and finally presumed apoptotic cell death (programmed cell death). These findings from studies on familial PD may also give important clues for elucidating the signaling pathway, initiated by possible initiation factors such as oxidative stress, which triggers presumed apoptotic neuronal death in sporadic PD.

However, most cases of sporadic PD start in the elderly in their sixties or seventies and slowly progress over a long period of 10 to 20 years. This characteristic suggests that some continuous pathological process may exist in PD. The presence of activated glial cells, especially reactive microglia, in the substantia nigra in PD, suggests that inflammation *via* reactive glial cells may be an important process that promotes the progressive neurodegeneration of the DA neurons. The inflammatory response in PD, called "neuroinflammation," is different from classical inflammation, which is the defense reaction of living tissues to injury. "Neuroinflammation" is assumed not to be accompanied by dilatation of blood vessels and emigration of leukocytes from the blood to the brain, which is characteristic of the classical inflammation. In the peripheray, the typical initial reaction of classical inflammation consists primarily of changes in the blood vessels, the escape of cells and fluid from the blood

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into the tissues, and subsequent changes in the tissue; but thereafter, chronic inflammation ensues, characterized by exudation of lymphocytes and macrophages with increasing fibrosis [11]. In PD, the blood vessels are assumed to be intact with normal blood-brain barrier functions, although this is a controversial issue. Also controversial, is the exact mode of cell death in PD. At least in certain cases the DA neuronal death in PD appears to be related to the process of apoptosis (programmed cell death). Apoptosis affects isolated cells scattered in normal or diseased tissues and at first elicits no classical inflammatory reaction.

The concept of neuroinflammation and the elucidation of the changes in the glia-neuron network and interaction of cytokines and neural growth factors, i.e., neurotrophins, in PD gained much attention during the 1990s [16], and neuroinflammation is now considered to be fundamental to at least the progression if not the pathogenesis of PD. Thus, elucidation of the mechanisms underlying neuroinflammation in PD may contribute to new and effective therapies [18, 19]. As the first features of inflammation in PD, McGeer and the collaborators reported an increased numbers of major histocompatibility complex (MHC) class II antigen [human leukocyte antigen-DR (HLA-DR)]-positive microglial cells in the substantia nigra [12, 13]. In agreement with this finding, we also found that the level of beta 2-microglobulin, the light chain of MHC class I molecules, was higher in the striatum of PD patients than in that of control subjects [14]. High expression of mRNAs of MHC class I heavy chain and beta2-microglobulin were observed in nigral DA neurons and brainstem motoneurons in adult rats, and these neurons also displayed interferon (IFN)-gamma receptor mRNA [15]. The MHC initiates and propagates the immune response. MHC class I molecules present antigens to CD8 cytotoxic T cells (CTL), whereas MHC class II do so to CD4 positive helper T cells (Th1 and Th2). Not only microglia, but also astrocytes supposedly play a role in the loss of DA neurons, although a lesser one than microglia [16, 17].

Recent findings support the hypothesis that the process of DA cell death either in sporadic PD or in familial PD might be programmed cell death, i.e., apoptosis [20-23]; although this is still a controversial issue [24, 25]. We and other researchers have found changes in the levels of pro-inflammatory cytokines, anti-apoptotic neurotrophins and factors related to programmed cell death, specifically, in the nigrostriatal region of postmortem brain and/or in the ventricular or lumbar cerebrospinal fluid (CSF) from patients with sporadic PD or from animal models of PD such as mice with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced PD or rats with 6-hydroxy-DA (6-OHDA)-induced PD.

The findings concerning familial PD (effects of causative genes) and sporadic PD (activated glial cells that may produce pro-inflammatory cytokines, anti-apoptotic neurotrophins and apoptosis-related factors) may be mutually related, and consequently may provide a clue as to the effect of neuroinflammatory responses on neuronal death not only in PD, but also in other neurodegenerative diseases such as Alzheimer's disease (AD). Both of these diseases are speculated to be "protein conformational diseases" caused by the accumulation of "unfolded or misfolded proteins" with abnormal protein conformations, possibly as the result of a compromised ubiquitin proteasome system.

2. IMMUNE RESPONSE AND CYTOKINE CHANGES IN CEREBROSPINAL FLUID IN PARKINSON'S DISEASE

The brain is generally considered to be an "immune privileged" site, i.e., one free from immune reactions, since it is protected behind the blood-brain-barrier. However, accumulating findings have revealed that immune responses may occur in the brain, especially due to activation of the microglia that are known to produce pro-inflammatory cytokines. If a significant immune response and changes in cytokine levels can be detected in the CSF during the course of PD, cytokines would become valuable clinical markers. However, since the immune response-mediated inflammatory changes, if present, may be localized mainly in the nigrostriatal region of the brain in PD, representative detection of cytokines in the CSF would be difficult.

Wachter's group first reported that neopterin(N) is released from macrophages during immune responses and is a sensitive index of immune reactions [26]. Activated microglia in the brain also produce this molecule. Neopterin is a metabolite formed *via* D-erythro-6, 7-dihydroneopterin (NH2) from D-erythro-6, 7-dihydroneopterin triphosphate (NH2P3), which is synthesized as the main pteridine in microglia. It is also an intermediate in the biosynthesis of (6R)-L-erythro-5,6,7,8-tetrahydrobiopterin (BH4), the cofactor of pteridine-dependent monooxygenases (phenylalanine hydroxylase [PAH] for phenylalanine degradation in the liver [27], tyrosine hydroxylase [TH] for catechloamine biosynthesis [28], and tryptophan hydroxylase [TPH] for indoleamine biosynthesis [29]. Likewise, neopterin is also an intermediate of nitric oxide synthase (NOS) for NO biosynthesis [30, 31]) in neurons having catecholamines [DA, noradrenaline/norepinephrine, adrenaline/epinephrine], indoleamines [serotonin (5-HT), melatonin] and nitric oxide (NO) as neurotransmitters. The reaction mechanism of TH, TPH, and PAH coupled with BH4 as a cofactor is essentially similar, but that of NOS seems to be more complex (Fig. **1**). More precise reactions of TH coupled with BH4 are shown in Fig. (**2**). BH4 is synthesized from guanosine triphosphate (GTP) *via* 3 enzymes, GTP cyclohydrolase I (GCH1), 6-pyruvoyl-tetrahydropterin synthase, and sepiapterin reductase. Catecholamines (DA, noradrenaline/norepinephrine, adrenaline/epinephrine) are synthesized from tyrosine. TH is the key enzyme for DA biosynthesis. TH in nonprimate animals is generally a single protein encoded by a single TH gene consisting of 13 exons. Only the human TH gene has an extra exon 2, giving it 14 exons; and this TH produces 4 isoform proteins (human TH type 1 [hTH1] to type 4 [hTH4]) [32, 33]. In contrast, the monkey TH gene, lacking the extra exon 2 of humans, has 2 isoform proteins (TH1 and TH2)[34], arising by alternative mRNA splicing. An interesting question is whether the multiple isoforms of TH in humans and monkeys bear any relation with the fact that humans and monkeys are highly sensitive to the PD-producing neurotoxin MPTP. BH4 formed in catecholamine neurons is metabolized *via* quinonoid BH4 (formed in the TH reaction) and 7, 8-dihydrobiopterin (BH2) to biopterin

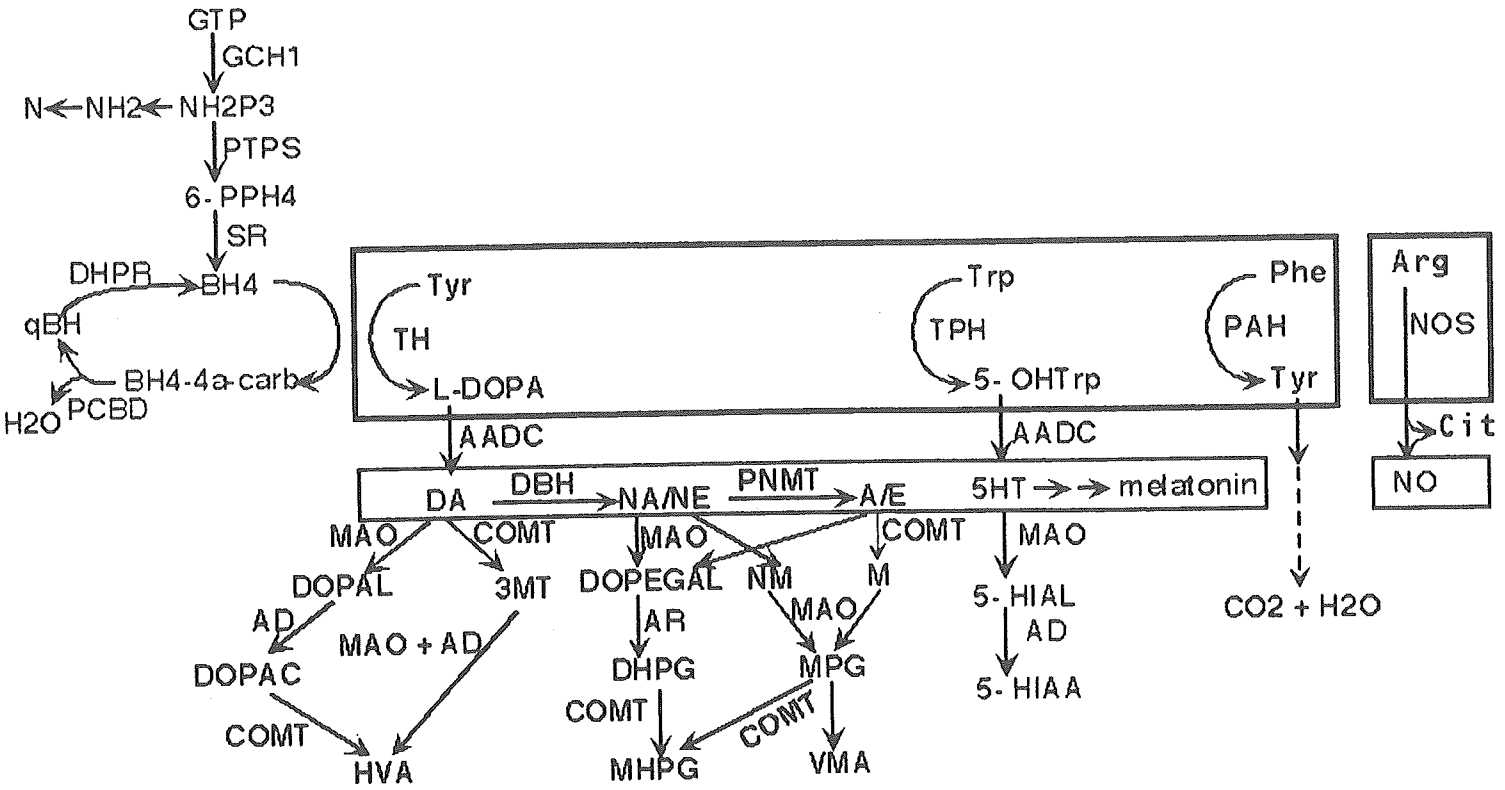

Fig. (1). Metabolism of catecholamines (dopamine, noradrenaline, adrenaline), indoleamines (serotonin, melatonin), phenylalanine, and nitric oxide in relation to tetrahydrobiopterin metabolism by pteridine-dependent monooxygenases (tyrosine hydroxylase, tryptophan hydroxylase, phenylalanine hydroxylase, and nitric oxide synthase).

A/E: adrenaline/epinephrine, AADC: aromatic L-amino acid decarboxylase, ALD: aldehyde dehydrogenase, ALR: aldehyde reductase, BH4: tetrahydrobiopterin, BH4-4a-carb: tetrahydrobiopterin-4a-carbinolamine, qBH2: quinonoid dihydrobioptein, Cit: citrulline, COMT: catechol O-methyltransferase, DA: dopamine, DHPG: 3, 4-dihydroxyphenylglycol, DHPR: dihydropteridine reductase, DOPA: 3, 4-dihydroxyphenylalanine, DOPAC: 3, 4-dihydroxyphenylacetic acid, DOPAL: 3, 4-dihydroxyphenylacetaldehyde, DOPGAL: 3, 4-dihydroxyphenylglycolaldehyde, E: epinephrine, GCH1: GTP cyclohydrolase I, GTP: guanosine triphosphate, 5HIAA: 5-hydroxyindoleacetic acid, 5HIAL: 5-hydroxyindoleacetaldehyde, 5HT: 5-hydroxytryptamine, serotonin, HVA: homovanillic acid, M: metanephrine, MAO: monoamine oxidase , MHPG: 3-methoxy-4-hydroxyphenylglycol, MOPGAL: 3-methoxy-4-hydroxyphenylglycolaldehyde, 3MT: 3-methoxytyramine, NA/NE: noradrenaline/norepinephrine, NM: normetanephrine, PAH: phenylalanine hydroxylase, PCBD: pterin-4a-carbinolamine dehydratase, Phe: phenylalanine, 6-PPH4: 6-pyruvoyltetrahydropterin, PTPS: 6-pyruvoyltetrahydropterin synthase, SR: sepiapterin reductase, TH: tyrosine hydroxylase, TPH: tryptophan hydroxylase, Trp: tryptophan, Tyr: tyrosine, VMA: vanillylmandelic acid.

(B) (Fig. 2) [35]. The high contents of TH, BH4, GCH1, and sepiapterin reductase (SPR, the last enzyme acting in BH4 synthesis) in the nigrostriatum of the human brain suggest that the nigrostriatal DA neurons produce the largest amounts of BH4 and thus biopterin in the brain [36-38].

In our earlier studies, we found that, during the progression of PD, the ratio of total neopterin (NH2P3+NH2+N) to total biopterin (BH4+BH2+B) in the lumbar CSF gradually increased, even though the absolute amounts of total biopterin and total neopterin were significantly decreased as compared with those of control subjects. During the progression of PD the contents of total biopterin and total neopterin seen in CSF may be decreased due to the loss of nigrostriatal DA neurons. However, activated microglia, if present, may release neopterin, resulting in the increased ratio of total neopterin to total biopterin seen in the CSF [39]. Activation and production of neopterin from microglia in the PD brain are thought to occur in the nigrostriatal region. We found that the activity of GCH1, the first and rate-limiting enzyme for BH4 biosynthesis, in DA neurons was decreased specifically in that region [40]. Since GCH1 activity is high in the nigrostriatal DA neurons, large amounts of BH4 and the intermediate neopterin are produced there; and their concentrations in the nigrostriatum may be decreased due to the degeneration of the DA neurons. Thus, the increase in the ratio of total neopterin to total biopterin in the CSF during the progression of PD may reflect neuroinflammation accompanied by microglial activation and neopterin release in the nigrostriatal region. This concept agrees with the fact that, in patients with autosomal-dominant GCH1 deficiency/DOPA responsive dystonia (DRD) without DA cell death, both biopterin and neopterin concentrations in the CSF are decreased in parallel without a change in the neopterin / biopterin ratio [41]. This condition is also called "Segawa's disease, " which was first described by Segawa in 1971 as "hereditary progressive dystonia with marked diurnal fluctuation (HPD)" [42, 43].

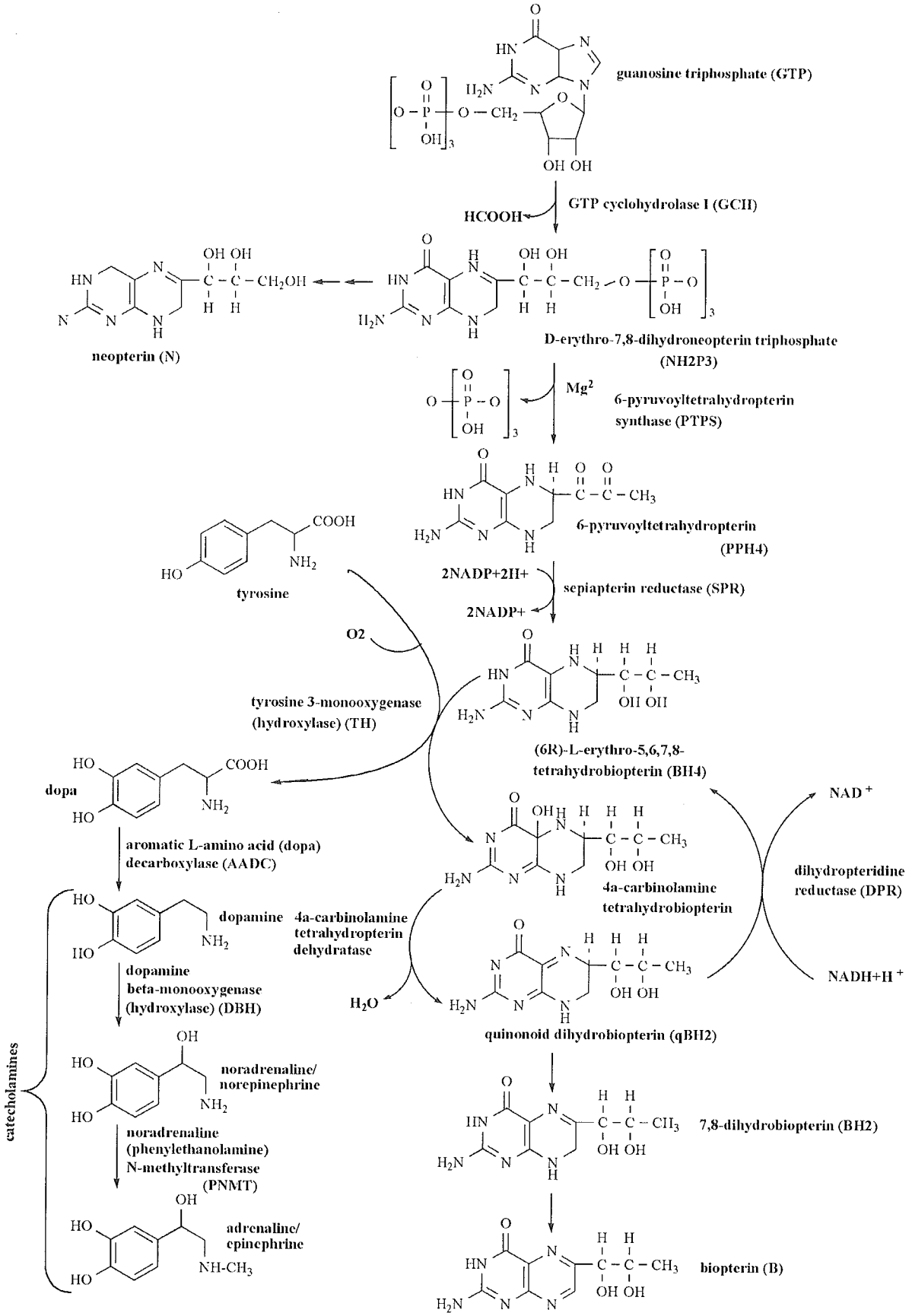

Fig. (2). Biosynthesis of catecholamines (dopamine, noradrenaline, adrenaline) in relation to biosynthesis of tetrahydrobiopterin, the cofactor of tyrosine hydroxylase.