EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 42, No. 12, 823-832, December 2010

Systemic LPS administration induces brain inflammation but not dopaminergic neuronal death in the substantia nigra

Hey-Kyeong Jeong[1,2], Ilo Jou[1,2,3] and Eun-hye Joe[1,2,3,4,5]

[1]Neuroscience Graduate Program
[2]Department of Pharmacology
[3]Chronic Inflammatory Disease Research Center
[4]Brain Disease Research Center
Ajou University School of Medicine
Suwon 442-721, Korea
[5]Corresponding author: Tel, 82-31-219-5062;
Fax, 82-31-219-5069; E-mail, ehjoe@ajou.ac.kr
DOI 10.3858/emm.2010.42.12.085

Accepted 18 October 2010
Available Online 19 October 2010

Abbreviations: CD68, cluster of differentiation 68; DHEA, dehydroepiandrosterone; GFAP, glial fibrillary acidic protein; Iba-1, ionized calcium binding adaptor molecule 1; MPO, myeloperoxidase; NSAID, nonsteroidal anti-inflammatory drug; PD, Parkinson's disease; Q-PCR, quantitative polymerase chain reaction; SI, systemic inflammation; SN, substantia nigra; TH, tyrosine hydroxylase

Abstract

It has been suggested that brain inflammation is important in aggravation of brain damage and/or that inflammation causes neurodegenerative diseases including Parkinson's disease (PD). Recently, systemic inflammation has also emerged as a risk factor for PD. In the present study, we evaluated how systemic inflammation induced by intravenous (iv) lipopolysaccharides (LPS) injection affected brain inflammation and neuronal damage in the rat. Interestingly, almost all brain inflammatory responses, including morphological activation of microglia, neutrophil infiltration, and mRNA/protein expression of inflammatory mediators, appeared within 4-8 h, and subsided within 1-3 days, in the substantia nigra (SN), where dopaminergic neurons are located. More importantly, however, dopaminergic neuronal loss was not detectable for up to 8 d after iv LPS injection. Together, these results indicate that acute induction of systemic inflammation causes brain inflammation, but this is not sufficiently toxic to induce neuronal injury.

Keywords: brain inflammation; neuronal damage; systemic inflammation

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder and is characterized by degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc), resulting in movement disorder (Gelb *et al*., 1999). However, as most (90-95%) cases of PD are sporadic in nature, it remains unclear how PD occurs and progresses. Mutations in genes such as those encoding α-synuclein, parkin, PINK1, DJ-1, LRRK2, and Htra2 are found in familial PD patients (Klein *et al*., 2005; Klein and Schlossmacher, 2006; Thomas and Beal, 2007). However, animals carrying mutations in or knock-outs of these PD-related genes rarely show PD-like symptoms or dopaminergic neuronal loss (Rockenstein *et al*., 2002; Goldberg *et al*., 2003; Itier *et al*., 2003; Von Coelln *et al*., 2004; Chen *et al*., 2005b; Zhu *et al*., 2007). These findings indicate that abnormal functioning of PD-related genes is not enough to cause PD. Thus, certain microenvironmental factors such as brain inflammation have been suggested as important in causation and/or aggravation of neurodegenerative diseases includeing PD (Herrera *et al*., 2000; Gao *et al*., 2002). In PD brains, the expression levels of proinflammatory cytokines are higher than in control brains (Mogi *et al*., 1994; Blum-Degen *et al*., 1995; Muller *et al*., 1998; Knott *et al*., 2000; Nagatsu and Sawada, 2005; Whitton, 2007). Microglia (brain macrophages) are activated in post-mortem PD brains (McGeer *et al*., 1988; Croisier *et al*., 2005) and many experimental models of PD (Liberatore *et al*., 1999; Dehmer *et al*., 2000; Cicchetti *et al*., 2002; Wu *et al*., 2002; Sriram *et al*., 2006; Vijitruth *et al*., 2006). It has been suggested that nonsteroidal anti-inflammatory drug (NSAID) use reduces the incidence of PD (Chen *et al*., 2003; Esposito *et al*., 2007; Samii *et al*., 2009), although this contention remains controversial (Chen *et al*., 2005a; Etminan *et al*., 2008).

Brain damage induces brain inflammation. Microglia, known to be the principal inflammatory cells in the brain, continuously survey the environment of the normal brain and rapidly respond to damage, producing inflammatory mediators (Kreutzberg, 1996; Raivich *et al*., 1999; Min *et al*.,

2004; Davalos *et al*., 2005; Hanisch and Kettenmann, 2007). Systemic inflammation may also induce brain inflammation. Systemically administered LPS may act on endothelial cells in the brain, in turn affecting adjacent microglia (Cao *et al*., 1999; Inoue *et al*., 2002; Rummel *et al*., 2008; Saper, 2010). In addition, inflammatory cells of the bloodstream, activated by systemically administered LPS, may enter the brain and participate in inflammation (Bohatschek *et al*., 2001; Cunningham *et al*., 2005; Qin *et al*., 2007). However, it is not clear whether systemic inflammation induces a level of brain inflammation sufficiently intense to cause neuronal damage. Cultured microglia challenged with LPS and interferon-γ produce neurotoxic inflammatory mediators including inducible nitric oxide synthase (iNOS) (Liu *et al*., 2000; Possel *et al*., 2000; Min *et al*., 2006), whereas microglia activated *in vivo* by brain injury and/or LPS injection synthesize very little iNOS and are not neurotoxic (Ji *et al*., 2007; Matsumoto *et al*., 2007). In the present study, we investigated how systemic inflammation affected brain inflammation, and whether such inflammation caused neuronal toxicity.

Results

Behavior of microglia and astrocytes in response to systemic administration of LPS

To investigate how systemic inflammation (SI) influenced the brain, we first examined the behavior of microglia and astrocytes after systemic administration of LPS. We focused on the SN region because inflammation in this area has been suggested as a risk factor for degeneration of dopaminergic neurons, resulting in PD. When 100 and 500 μg amounts of LPS in 250 μl PBS were intravenously (iv) injected into rats weighing 230-250 g, the TNF-α level in plasma increased sharply within 1 h, but decreased rapidly to the basal level by 3 h, as previously described (Liaudet *et al*., 2002; Chow *et al*., 2005; Qin *et al*., 2007). There was no significant difference in tumor necrosis factor-alpha (TNF-α) levels after injection of 100 and 500 μg LPS (Supplemental Data Figure S1), and we thus used 250 or 500 μg LPS in various experiments. In PBS-treated control animals, ionized calcium binding adaptor molecule 1-immunopositive (Iba-1$^+$) microglia showed a ramified morphology (Figure 1A). Microglial cell density in the substantia nigra reticulate (SNr), where dopaminergic neuronal processes are located, was higher than that in the SNpc, as previously reported (Ji *et al*., 2007). The processes of Iba-1$^+$ microglia became slightly shorter and thicker 8 h after iv LPS injection, and these features were more prominent in the SNpc than in the SNr. By 24 h post-injection, morphology had returned to normal (Figure 1A).

Astrocyte behavior was also examined in rat brains after induction of SI. Astrocyte density in the SNpc was much lower than in the SNr (Figure 1B). In contrast to what was noted when microglia were studied, there was no dramatic change in either morphology or astrocyte number in either region after iv LPS injection (Figure 1B). These results indicate that systemic LPS administration rapidly (within 8 h) induces brain inflammation, particularly microglial responses.

Neutrophils infiltrate the brain in response to systemic administration of LPS

Neutrophils are recruited to LPS-injected, traumatic, and ischemic brains, and the inflammatory responses are neurotoxic (Ji *et al*., 2007; Matsumoto *et al*., 2007). Thus, we examined the neutrophil infiltration pattern of the brain in response to

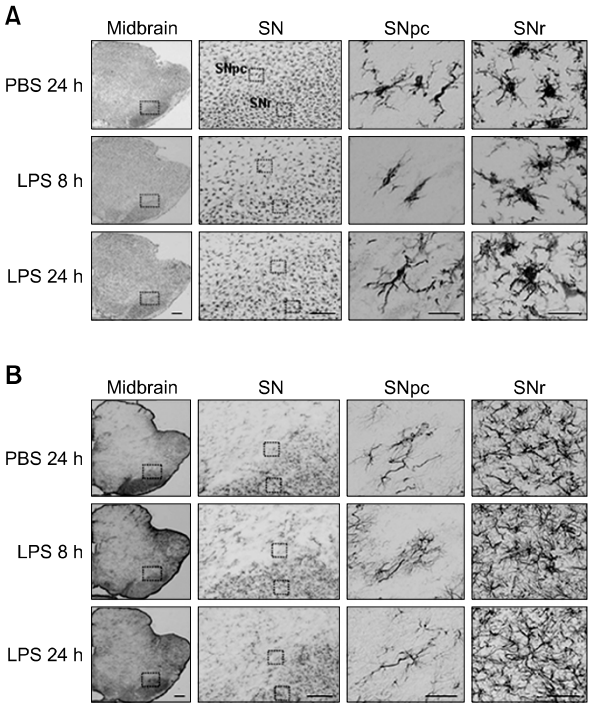

Figure 1. Behavior of microglia and astrocytes in response to iv LPS administration. Rats were injected iv with LPS (250 μg) dissolved in 250 μl PBS, or with PBS alone. At the indicated times after injection, brains were removed and prepared for immunohistochemistry as described in Methods. Midbrain sections (30 μm in thickness) were stained with anti-Iba-1 (A) or anti-GFAP antibody (B), and expression of Iba-1 or GFAP was visualized using peroxidase-conjugated secondary antibodies. Scale bars: 200 μm in the two left columns; 20 μm in the two right columns.

iv LPS injection. To this end, brain sections were stained for a marker of neutrophils, myeloperoxidase (MPO). In the SN, MPO^+ cells (arrows) were barely detectable within 4 h of LPS injection, increased in number at 8 h, and decreased in level at 16-24 h (Figure 2A). However, fewer neutrophils infiltrated the brain after iv LPS injection compared with the numbers seen after direct intranigral infusion of LPS (Figure 2A). We also investigated whether the SN was more permeable to neutrophils than were other brain regions, and found that neutrophils appeared to infiltrate the SN and the cortex to similar extents (Figure 2B). These results indicate that the SN is not particularly prone to infiltration of neutrophils during systemic inflammation.

Expression of proinflammatory mediators in response to systemic administration of LPS

Next, in the SN, we examined expression of mRNAs encoding proinflammatory mediators such as interleukin-1 beta (IL-1β, TNF-α, and interleukin-6 (IL-6), in response to systemic administration of LPS, using real-time quantitative polymerase chain reaction (Q-PCR). All tested mRNA levels increased within 4 h after iv LPS injection, peaked at 8 h, and then to close to (or slightly greater than) basal levels (Figure 3A).

Upon immunohistochemical analysis, IL-1β expression was barely detectable 4 h after LPS administration, increased at 8-16 h, decreased at 24 h, and completely disappeared by 3 d (Figure 3B). We also investigated the expression of iNOS catalyzing nitric oxide production (Bogdan, 2001; Aktan, 2004), and cluster of differentiation 68 (CD68) representing phagocytic status (da Silva and Gordon, 1999; Nerlich *et al*., 2002; Rezaie *et al*., 2005). In contrast to what was seen when IL-1β expression was studied, only a limited number of cells expressed iNOS and CD68 (Figure 3B). Interestingly, IL-1β was detected in Iba-1^+ but not in MPO^+ cells, whereas iNOS and CD68 were detected in MPO^+ but not in Iba-1^+ cells (Figure 3C). Together, these results indicate that systemic inflammation induced transient expression of proinflammatory mediators, and that microglia and neutrophils synthesized different subsets of such mediators.

Effect of systemic administration of LPS on dopaminergic neuronal viability

Although iv LPS injection induced brain inflammation, such inflammation was transient and relatively mild compared with that seen after direct injection of LPS into the brain (Ji *et al*., 2007). Thus, we examined whether systemic inflammation induced dopaminergic neuronal injury. In PBS-injected rats, dopaminergic neurons was unaffected (Figure 4A). In LPS-injected rats, dopaminergic neuronal loss was not detectable for up to 8 d after LPS administration (Figure 4A). Upon stereological counting, the numbers of dopaminergic neurons did not differ significantly after injection of PBS (15,100 ± 1,754 cells, mean ± SEM) or LPS (15,900 ± 2,587 cells, mean ± SEM) (Figure 4B, $P > 0.05$). Together, these results indicate that,

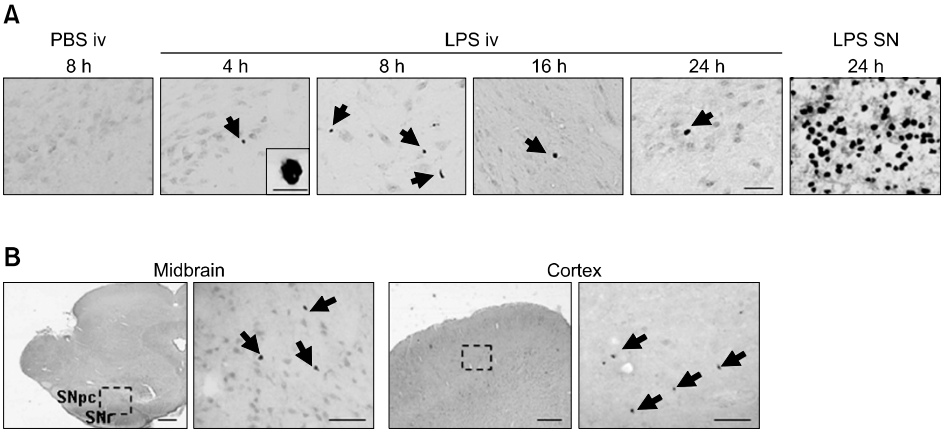

Figure 2. Neutrophils infiltrate the brain in response to iv LPS administration. (A) Sections were obtained from the midbrain at the indicated times after LPS injection (LPS iv), or 24 h after direct LPS injection into the SN (LPS SNpc), and stained with an anti-MPO antibody. PBS-injected brain sections were used as positive controls. (B) Brain sections were obtained from the midbrain and the cortex 8 h after iv LPS injection, stained with an anti-MPO antibody, and MPOexpression was visualized using a peroxidase-conjugated secondary antibody. Scale bars: 200 μm (left panels in B); 50 μm (A and right panel of each region in B); and 10 μm (inset in A).

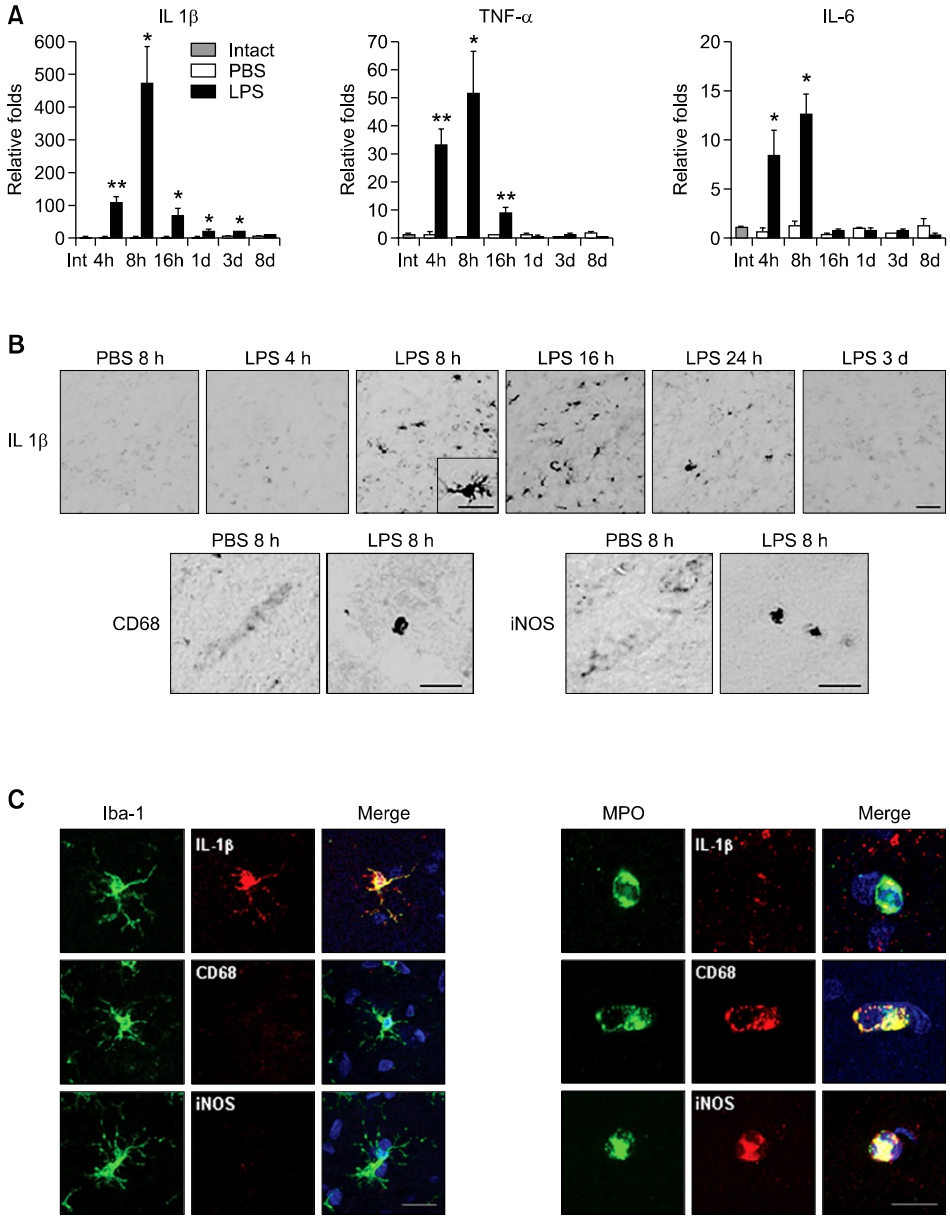

Figure 3. Expression of proinflammatory mediators in the brain in response to iv LPS injection. (A) mRNA was isolated from the midbrain regions of rats at the indicated times after iv LPS injection, and the levels of mRNAs encoding IL-1β, TNF-α, and IL-6 were analyzed by Q-PCR. Values are means ± SEMs of data from 3-5 animals. *$P < 0.05$; **$P < 0.01$, compared to intact brain. (B, C) Sections were obtained from the midbrain at the indicated times after LPS or PBS injection, and stained with antibodies detecting IL-1β, CD68, or iNOS; peroxidase-conjugated secondary antibodies (B) or double-labeled antibody combinations (C) were used to visualize binding. Midbrain sections of brains from animals injected iv with LPS were obtained at the indicated times and stained with the combinations of antibodies shown. Scale bars: 50 μm (upper panels in B); 20 μm (inset in the upper panel, and the lower panels in B); and 10 μm (C).

although acute systemic inflammation indeed induces brain inflammation, the effect is mild and does not cause neuronal injury.

Discussion

It has been suggested that systemic inflammation may affect both the onset and progression of chronic brain diseases including PD and Alzheimer's disease, by induction of brain inflammation (Breitner, 1996; in 't Veld *et al.*, 2002; Chen *et al.*,

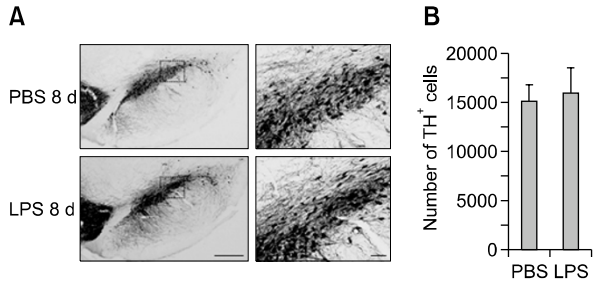

Figure 4. LPS administration iv does not influence dopaminergic neuronal viability. (A) Midbrain sections of animals receiving LPS or PBS iv were obtained at 8 d, and stained with an anti-TH antibody. Scale bars: 200 μm (left panels) and 50 μm (right panels). (B) By stereology, the total numbers of TH^+ neurons were estimated in the SNpc of LPS- or PBS-injected rats, as described in Methods. Values are means ± SEMs of data from three animals.

2003; Klegeris and McGeer, 2005; Esposito *et al*., 2007; Perry *et al*., 2007; Samii *et al*., 2009). The results of the present study show that iv LPS injection indeed induced brain inflammation. Microglia became morphologically activated; neutrophils infiltrated the brain; and inflammatory mediators, including IL-1β, TNF-α, IL-6, iNOS, and CD68, were synthesized (Figure 1-3). However, no neuronal death was evident (Figure 4). These results indicate that systemic inflammation indeed causes brain inflammation, but to only a mild extent and does not result in neuronal death.

The extent of neutrophil infiltration may significantly influence the level of neuronal death in an injured brain. Neutrophils are a major source of proinflammatory mediators including iNOS, Cyclooxygenase-2, Monocyte chemotactic protein-1, which play important roles in neuronal death within LPS-injected and/or ischemic brains (Ji *et al*., 2007; Matsumoto *et al*., 2007). Previously, we found that direct injection of LPS recruited more neutrophils to the SN than to the cortex or hippocampus, which suggests that susceptibility to LPS neurotoxicity may differ among these regions (Figure 2B LPS SN; Ji *et al*., 2008). LPS iv injection recruited fewer neutrophils than did direct LPS injection (Figure 2B), and induced only transient expression of inflammatory mediators, lasting for up to 1-3 d (Figure 1-3). Previously, it was reported that a single intraperitoneal LPS injection increased TNF-α mRNA and protein expression levels in the brain for more than 10 months (Qin *et al*., 2007). Although we did not analyze the expression levels of inflammatory mediators at such late timepoints, it may be difficult to re-stimulate inflammatory responses after they subside, in the absence of a challenge, for several reasons. First, cerebrospinal fluid is renewed about 11 times daily in adult rats and maintains homeostasis of the brain environment (Johanson *et al*., 2008). Second, neutrophils of the brain, the major source of proinflammatory mediators, die 1-5 days after LPS intranigral injection, reflecting an early response to damage (Ji *et al*., 2007). Third, The suppressor of cytokine signaling family proteins and antioxidant enzymes that resolve inflammation processes are activated to prevent long-lasting inflammation (Camhi *et al*., 1995; Ji *et al*., 2004). Therefore, acute brain inflammation may not last for months in the absence of a fresh trigger.

Intriguingly, microglia studied *in vivo* expressed very low levels (if any) of neurotoxic inflammatory mediators including iNOS (Ji *et al*., 2007; Matsumoto *et al*., 2007), although microglia in pure culture synthesized high amounts of iNOS and TNF-α (Chao *et al*., 1992; Meda *et al*., 1995). This difference in inflammatory response *in vitro* and *in vivo* may reflect an influence of astrocytes, which inhibit microglial activities (Vincent *et al*., 1997; Pyo *et al*., 2003; Min *et al*., 2006). Dehydroepiandrosterone (DHEA) and prostaglandins are candidate anti-inflammatory factors secreted by astrocytes (Zwain and Yen, 1999; Molina-Holgado *et al*., 2000; Taniura *et al*., 2002). DHEA inhibits the activation of nuclear factor-kappaB (NF-κB) induced by TNF-α (Altman *et al*., 2008) whereas prostaglandin E2 decreases Akt activation and the nuclear translocation of NF-κB (Shi *et al*., 2010). Astrocytes remained healthy in the brains of animals injected iv with LPS (Figure 1B), and possibly secreted anti-inflammatory factors suppressing microglial expression of inflammatory mediators. In addition, activated microglia produce neurotrophic factors such as transforming growth factor-β1, neurotrophin-3, and brain-derived neurotrophic factor (Elkabes *et al*., 1996; Lehrmann *et al*., 1998; Batchelor *et al*., 1999; Streit, 2002; Streit, 2005). Furthermore, not all inflammatory mediators produced in the brain are neurotoxic. For example, the effect of IL-1β on neuronal viability is controversial; the material has been suggested to be neurotoxic (Patel *et al*., 2003; Viviani *et al*., 2003; Thornton *et al*., 2006) or neurotrophic/neuroprotective (Strijbos and Rothwell, 1995).

Although systemic inflammation alone does not induce dopaminergic neuronal injury in the SNpc, we cannot exclude the possibility that systemic inflammation may yet be a risk factor for development of neurodegenerative disease. Systemic inflammation may convert a subtoxic insult to toxicity, and/or mild toxicity to potent toxicity. Although parkin-deficient mice do not display degeneration of dopaminergic neurons in the SNpc (Goldberg *et al*., 2003; Itier *et al*., 2003; Von Coelln *et al*., 2004;

Zhu *et al*., 2007), long-term systemic treatment with low-dose LPS triggered persistent neuroinflammation and selective loss of dopaminergic neurons (Frank-Cannon *et al*., 2008). In a murine prion disease model, systemic LPS administration aggravated the extent of neuronal death (Cunningham *et al*., 2005). In support of this possibility, it has been reported that long-term use of NSAIDs (that penetrate lipid membranes only poorly) reduces the risk of neurodegenerative disease development (Chen *et al*., 2003; Esposito *et al*., 2007).

In summary, systemic inflammation alone may not be sufficiently toxic to induce neuronal death, even in the SN. However, we cannot exclude the possibility that such inflammation may interact with other insults to enhance neuronal damage Therefore, inhibition of systemic inflammation may reduce the likelihood of brain inflammation, thus helping to protect against neuronal damage and development of neurodegenerative disease.

Methods

Systemic administration of LPS

All experiments were performed in accordance with approved animal protocols and guidelines established by the Ajou University School of Medicine Ethics Review Committee for animal experiments. Male Sprague-Dawley (SD) rats (230-250 g in weight, 7 weeks of age) were anesthetized by intraperitoneal injection of ketamine (40-80 mg/kg) and xylazine (5-10 mg/kg). LPS (100-500 μg in 250 μl aliquots of sterile PBS; Sigma, St. Louis, MO) was administered iv through the tail vein. PBS-injected animals were used as controls.

Tissue preparation

Rats were anesthetized and transcardially perfused with saline solution containing 0.5% (w/v) sodium nitrate and heparin (10 U/ml), followed by 4% (v/v) paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, to achieve tissue fixation. Brains were obtained and post-fixed overnight at 4°C in 4% (v/v) paraformaldehyde. Fixed brains were added, at 4°C, to 30% (w/v) sucrose solution until the brain segments sank. Six separate series of coronal brain slices, with each slice being 30 μm in thickness, were obtained using a sliding microtome (Microm, Walldorf, Germany). For RNA preparation, rats were anesthetized and transcardially perfused with saline solution without paraformaldehyde. Brains were sliced using a Rat Brain Slicer Matrix (1.0 mm slice intervals; RBM-4000C; ASI Instruments, Warren, MI) and a razor blade. The slice including the needle injection spot was selected, and tissue blocks (2 × 2 × 2 mm^3) just below the needle tip were prepared and stored at -70°C until use.

Immunohistochemistry

Prior to 3, 3'-diaminobenzidine (DAB) staining, serial sections were rinsed three times with PBS, treated with 3% (v/v) H_2O_2 for 5 min, and rinsed with PBS containing 0.2% (v/v) Triton X-100 (PBST). Non-specific binding was blocked by addition of 1% (w/v) BSA in PBST. Sections were incubated for 2 h at room temperature with primary antibodies directed against Iba-1 (1:1,000; Wako Pure Chemical Industries, Osaka, Japan), GFAP (1:300; Sigma, St. Louis, MO), MPO (1:1,000; Dako, Glostrup, Denmark), IL-1β (1:200; R&D Systems, Minneapolis, MN), iNOS (1:200; Abcam, Cambridge, UK), CD68 (1:200; AbD Serotec, Oxford, UK), or tyrosine hydroxylase (TH; 1:2,000; Pelfreeze Biologicals, Rogers, AR). Following rinsing with PBST, sections were incubated with biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA) and the avidin/biotin system (Vector Laboratories), and bands were visualized using 0.05% (w/v) DAB and 0.003% (v/v) hydrogen peroxide in 0.1 M PB. Next, sections were mounted on gelatin-coated slides and examined under a bright-field microscope (Olympus Optical BX51, Tokyo, Japan). Bright-field images were stored using PictureFrame Application 2.3 software. For immunofluorescence staining, sections were washed twice in PBS, treated with 1% (w/v) BSA, and incubated with combinations of antibodies against Iba-1, MPO, iNOS, interleukin-1β, and CD68. Visualization was achieved using Alexa Fluor488- or Alexa Fluor555-conjugated secondary antibodies (1:600 dilution; Invitrogen, Eugene, OR). DAPI (Vector Laboratories) was employed to detect nuclei. Sections were analyzed by confocal microscopy (Carl Zeiss, Jena, Germany) using 40 × water and 63 × oil immersion objectives at 20°C, and images were captured using Zeiss LSM510 confocal software (Carl Zeiss, Jena, Germany).

Real-time quantitative polymerase chain reaction (Q-PCR)

Total RNA was isolated using an Easy-BLUE RNA Extraction Kit (iNtRON, Sungnam, Korea), and cDNA was prepared using Reverse Transcription Master Premix (ELPisbio, Taejeon, Korea), according to the manufacturers' instructions. For Q-PCR, approximately 50 ng cDNA was analyzed using a KAPA SYBR FAST qPCR Kit (KAPA Biosystems, Boston, MA) and a Corbett Rotor-Gene 6000 real-time rotary analyzer (Corbett Research, Mortlake, NSW, Australia). Specific primers amplifying mRNAs encoding IL-1β, TNF-α, IL-6, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and used in Q-PCR, are shown in Supplemental Data Table S1. Q-PCR conditions were as follows: 95°C for 30 s; followed by 40 cycles of 95°C for 3 s (melting), 55°C for 20 s (annealing), and 72°C for 3 s (elongation). For TNF-α, the annealing conditions were modified to include a 63-59°C touch-down protocol, thus decreasing the temperature by 0.5°C per cycle over the first eight cycles. To confirm that single products were amplified under the conditions used, a melting curve analysis was performed for each primer pair, using a melt ramp of 72-95°C and raising the temperature by 1°C at each step (5 s/step). Amplified products were also viewed after electrophoresis on 1.5% (w/v) agarose gels, using ethidium bromide staining. The results were normalized to

GAPDH levels.

Stereological analysis of dopaminergic neurons

The number of TH^+ neurons in every sixth section of the entire SNpc was counted under a bright-field microscope (Olympus Optical BX51, Tokyo, Japan), using Stereo Investigator software (MBF Bioscience, Williston, VT). Counting frames (100×100 μm) were randomly placed over SNpc sections, and TH^+ neurons were counted using a $40 \times$ objective.

Statistical analysis

Statistical data are expressed as means ± SEMs. The significance of between-group differences was analyzed using the one-way ANOVA tool in the Statistical Package for Social Sciences, version 12 (SPSS, Chicago, IL).

Supplemental data

Supplemental Data include a figure and a table and can be found with this article online at http://e-emm.or.kr/article/article_files/SP-42-12-4.pdf.

Acknowledgements

This work was supported by a KOSEF NRL Program grant funded by the Korean government (MEST) (no. 2-2008025-0), a grant (no. M103KV010011-06K2201-01110) from the Brain Research Center of the 21st Century Frontier Research Program of the Ministry of Science and Technology, Republic of Korea, and a grant (no. R13-2003-019) from KOSEF through the Chronic Inflammatory Disease Research Center (CIDRC) at Ajou University; all to E. Joe.

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