Systemic LPS Causes Chronic Neuroinflammation and Progressive Neurodegeneration

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KEY WORDS
TNFα; LPS; substantia nigra; microglia; neurodegeneration; neuroinflammation

ABSTRACT
Inflammation is implicated in the progressive nature of neurodegenerative diseases, such as Parkinson's disease, but the mechanisms are poorly understood. A single systemic lipopolysaccharide (LPS, 5 mg/kg, i.p.) or tumor necrosis factor alpha (TNFα, 0.25 mg/kg, i.p.) injection was administered in adult wild-type mice and in mice lacking TNFα receptors (TNF R1/R2−/−) to discern the mechanisms of inflammation transfer from the periphery to the brain and the neurodegenerative consequences. Systemic LPS administration resulted in rapid brain TNFα increase that remained elevated for 10 months, while peripheral TNFα (serum and liver) had subsided by 9 h (serum) and 1 week (liver). Systemic TNFα and LPS administration activated microglia and increased expression of brain pro-inflammatory factors (i.e., TNFα, MCP-1, IL-1β, and NF-κB p65) in wild-type mice, but not in TNF R1/R2−/− mice. Further, LPS reduced the number of tyrosine hydroxylase-immunoreactive neurons in the substantia nigra (SN) by 23% at 7-months post-treatment, which progressed to 47% at 10 months. Together, these data demonstrate that through TNFα, peripheral inflammation in adult animals can: (1) activate brain microglia to produce chronically elevated pro-inflammatory factors; (2) induce delayed and progressive loss of DA neurons in the SN. These findings provide valuable insight into the potential pathogenesis and self-propelling nature of Parkinson’s disease.

INTRODUCTION
Inflammation and microglial activation is a common component of the pathogenesis for multiple neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease (PD), Huntington's disease, Multiple sclerosis, and Amyotrophic lateral sclerosis (Nguyen et al., 2002). Microglia, the resident innate immune cells in the brain, actively monitor their environment and can become over-activated in response to diverse cues to produce cytotoxic factors, such as superoxide (Colton and Gilbert, 1987), nitric oxide (Liu et al., 2002; Moss and Bates, 2001), and tumor necrosis factor alpha (TNFα) (Lee et al., 1993; Sawada et al., 1989). While microglial activation is necessary and critical for host defense, over-activation of microglia is neurotoxic (McGeer et al., 2005; Polazzi and Contestabile, 2002). At this time, the mechanisms initiating deleterious neuroinflammation in neurodegenerative disease are poorly understood. Lipopolysaccharide (LPS), endotoxin from gram-negative bacteria, damages DA neurons only in the presence of microglia (Gao et al., 2002; Gibbons and Dragunow, 2006). Further, LPS activation of microglia both in vivo and in vitro causes the progressive and cumulative loss of DA neurons over time (Gao et al., 2002; Ling et al., 2006). During critical periods of embryonic development (E 10.5), maternal exposure to low concentrations of LPS in mice impacts microglial activation and DA neuron survival in offspring that persists into adulthood (Carvey et al., 2003; Ling et al., 2006). Several of these reports indicate that due to immunological perturbation during critical periods of development (Ling et al., 2006) or aging and senience (Godbout et al., 2005), microglia can become primed, where additional stimuli result in an exaggerated and prolonged pro-inflammatory response that enhances neuron damage. Thus, not only can microglia induce neuron damage, they can become persistently activated to produce continuous and uncontrolled neurotoxicity that fails to reside after the instigating stimulus has dissipated.

Several lines of evidence suggest that adult microglia can also become chronically activated from a single stimulus in the absence of any predispositioning event or aging. For example, chronic microglial activation continues years after brief MPTP exposure in humans (Langston et al., 1999) and primates (McGeer et al., 2003) through a process called reactive microgliosis. Reactive microgliosis is defined by the microglial activation that occurs in response to neuronal damage. This microglial activation is then toxic to neighboring neurons, which...

Grant sponsors: National Institute of Health, National Institute of Environmental Health Sciences, National Institute on Alcohol Abuse and Alcoholism.

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Received 5 September 2006; Revised 23 October 2006; Accepted 15 November 2006

DOI 10.1002/glia.20467

Published online 3 January 2007 in Wiley InterScience (www.interscience.wiley.com).
causes further microglial activation and a self-propelling progressive cycle of inflammation and neuron damage. Regardless of the class of instigating stimuli (pro-inflammatory toxin or neuronal death) the microglial pro-inflammatory response to neuron damage can drive the progressive inflammation and cumulative loss of neurons over time (Block and Hong, 2005). However, neuroinflammation and microglial activation is not always deleterious (Glezer et al., 2006; Neumann et al., 2006; Simard and Rivest, 2006; Simard et al., 2006; Sriram et al., 2006). Thus, the identification of the stimuli and mechanisms responsible for progressive microglial activation and associated continuous neuron damage is essential to understand the etiology and pathology of neurodegenerative diseases.

Here, we are the first to test whether a single peripheral LPS exposure in adult mice results in: (1) microglial activation and neuroinflammation that persists long after peripheral events have abated; (2) transfer of peripheral inflammation to the brain through TNFα; (3) delayed and progressive loss of DA neurons in the substantia nigra (SN), similar to PD. The results from these studies indicate that a single occurrence of sepsis and peripheral inflammation may result in self-propelling neuroinflammation and progressive DA neurodegeneration through TNFα.

MATERIALS AND METHODS

Animals

Eight-week-old male (20–22 g) B6; 129S-Tnfrsf1a<sup>tm1Imx</sup> Tnfrsf1b<sup>tm1Imx</sup>/J (TNFR1<sup>−/−</sup>, KO) and B6; 129SF2/J (TNFR1<sup>−/+</sup>, WT), and C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME). B6; 129S-Tnfrsf1a<sup>tm1Imx</sup> Tnfrsf1b<sup>tm1Imx</sup>/J (TNFR1<sup>−/−</sup>) mice were lacking both the p55 and p75 TNF receptors and were maintained in B6; 129SF2/J background. Therefore, B6; 129SF2/J (TNFR1<sup>−/+</sup>) mice were used as strain controls of TNFR1<sup>−/−</sup> mice.

Reagents

Lipopolysaccharide (LPS, strain O111:B4) was purchased from Calbiochem (San Diego, CA). The polyclonal antibody against tyrosine hydroxylase (TH) was a kind gift from Dr. John Reinhard of Glaxo Wellcome (Research Triangle Park, NC). Rat monoclonal antibody raised against F4/80 antigen was purchased from Serotec (Washington, DC). Rabbit anti-Iba1 antibody was purchased from Wako Pure Chemical Industries (1–2Doshomachi 3-Chome Chuo-ku Osaka 540–8605, Japan). Tumor necrosis factor alpha (TNFα) and MCP-1 ELISA kits were purchased from R&D Systems (Minneapolis, MN). All other reagents came from Sigma Chemical Co. (St. Louis, MO).

Drug Treatment

Male C57BL/6J, TNFR1<sup>−/−</sup>, and TNFR1<sup>−/+</sup> mice were intraperitoneally injected with a single dose of LPS (5 mg/kg), TNFα (0.25 mg/kg), or vehicle (0.9% saline). The dosage of LPS used (5 mg/kg, i.p.) was based on our previous study of endotoxic shock (Li et al., 2005). However, pilot studies using lower doses of LPS (1–4 mg/kg) showed significant increases in brain TNFα mRNA and protein levels in a graded response (data not shown). Mice were sacrificed at selected time points and tissues (serum, brain, and liver) were used for mRNA, protein, or morphological analyses. Procedures using laboratory animals were in accordance with the National Institutes of Health guidelines for the use of live animals and approved by IUCAC boards.

Analysis of Neurotoxicity

The loss of dopaminergic neurons was assessed by counting the number of TH-immunoreactive (TH-IR) neurons following immunostaining of brain sections. Twenty-four consecutive brain slices (35 μm thickness), which encompassed the entire SNc, were collected. A normal distribution of the number of TH-IR neurons in the SNc was constructed based on the counts of 24 slices from C57BL/6J mice. Eight evenly spaced brain slices from saline or LPS-injected animals were immunostained with an antibody against TH and counted. The distribution of the cell numbers from each animal was matched with the normal distribution curve to correct for errors resulting from the cutting. Samples were counted in a double-blind manner with stereology equipment, three individuals, and the CAST system. Conclusions were drawn only when the difference was within 5%.

Immunohistochemistry

Brains were fixed in 4% paraformaldehyde and processed for immunostaining as described previously (Qin et al., 2004). Dopaminergic neurons were detected with the polyclonal antibody against tyrosine hydroxylase (TH). Microglia was stained with rat monoclonal antibody raised against F4/80 antigen and rabbit anti-Iba1 antibody, respectively. Immunostaining was visualized by using 3,3'-diaminobenzidine and urea-hydrogen peroxide tablets from Sigma.

Real-Time RT-PCR Analysis

Total RNA was extracted from the mouse brain samples treated with LPS, TNFα, or saline at the selected time points and used for reverse transcription PCR analysis as described previously (Qin et al., 2004). The primer sequences used in this study were as follows: for mouse TNFα, 5'-GAC CCT CAC ACT CAG ATC TTC T3' (forward) and 5'-CCT CCA CTT GGT GGT TGG CT3' (reverse); for mouse IL-1β, 5'-CTG GTG TGT GAC TGT CCC ATT A3' (forward) and 5'-CCG ACA GCA CGA GGC TTT-3' (reverse); for mouse NF-κB p65 (essential modulator), 5'-GGG GGC ACG TTT TAC TCT TT3' (forward) and 5'-CCG TCT CCA GGA GGT TAA TGC-3' (reverse); for
mouse β-actin, 5′-GTA TGA CTC CAC TCA CGG CA A-3′ (forward) and 5′-GTT CTC GCT CCT GGA AGA TG-3′ (reverse). The SYBR green DNA PCR kit (Applied Biosystems, Foster City, CA) was used for real-time PCR analysis. The relative differences in expression between groups were expressed using cycle time (Ct) values normalized with β-actin, and relative differences between control and treatment groups were calculated and expressed as relative increases setting control as 100%.

**TNFα and MCP-1 Assay**

Frozen livers and brains were homogenized in 100 mg tissue/mL cold lysis buffer (20 mM Tris, 0.25 M sucrose, 2 mM EDTA, 10 mM EGTA, 1% Triton X-100) and one tablet of Complete Mini protease inhibitor cocktail tablets/10 mL (Roche Diagnostics, Indianapolis, IN). Samples were centrifuged at 100,000g for 40 min. Supernatant was collected for protein assay using the BCA protein assay reagent kit (PIERCE, Milwaukee, WI). The levels of TNFα in livers, sera, and brains and MCP-1 in brains were measured with TNFα and mouse JE/MCP-1 commercial enzyme-linked immunosorbent assay (ELISA) kits from R&D Systems (Minneapolis, MN), as described previously (Gu et al., 1998).

**Statistical Analysis**

The data are expressed as the mean ± SEM and statistical significance was assessed with an ANOVA followed by Bonferroni’s t test. A value of P < 0.05 was considered statistically significant.

**RESULTS**

**TNFα Response to Systemic LPS Administration**

To better understand the effects of systemic LPS on brain inflammation, we administered a single dose of LPS (5 mg/kg) i.p. to male C57BL/6 mice and followed liver, serum, and brain TNFα expression over time. LPS increased liver TNFα mRNA 140-fold that peaked within 30 min. This was followed by liver TNFα protein increase of ~29-fold that peaked at 60 min. Serum TNFα protein increased from 0 to ~6.452 ng/mL at 60 min after LPS treatment (see Fig. 1). Brain showed an increase in TNFα mRNA (7,336%) and protein levels (653%) that both peaked at 60 min. Thus, while TNFα protein levels in response to systemic LPS peaked in the liver, serum, and brain at 1 h, brain TNFα mRNA synthesis occurred shortly after liver TNFα mRNA synthesis, very likely after the TNFα protein was able to enter the brain. These results support that liver-derived TNFα was the predominant source of the immediate TNFα response to systemic LPS (1-h post treatment) in the liver, serum, and brain, which then initiated the synthesis of TNFα in the brain.

We also examined the long-term effects of systemic LPS injection from several hours until 10-months post treatment. Interestingly, serum TNFα declined to saline control levels by 9 h (Figs. 1B, 2A). In liver, TNFα protein levels declined to 18% of peak values by 9 h after LPS (Figs. 1A, 2A). Surprisingly, TNFα protein remained elevated in brain. At 1 week after LPS treatment TNFα levels were elevated in brain to ~319 ±20 pg/mg protein compared to control values of 65 ±5 pg/mg protein, a 490% increase similar to the 1-h peak induction. TNFα protein remained elevated at 14 days, 21 days, and even 10 months after LPS treatment (Fig. 2B). In addition, 3 h after LPS treatment staining of microglia in cortex, hip-
receptors are critical for transport of the TNFα protein from the periphery, across the blood brain barrier, and into the brain (Pan and Kastin, 2002). To test the role of systemic TNFα on inflammation in the brain, we administered a single dose of LPS (5 mg/kg) i.p. or TNFα (0.25 mg/kg) i.p. to male wild type B6:129SF2J mice (WT) or male mice deficient in the TNF Type 1 and Type 2 receptors (TNFR-KO). TNFα expression (brain, liver, and serum) and the up regulation of other pro-inflammatory genes (IL-1β, NFκB, and MCP-1) in the brain were determined.

Systemic LPS treatment increased TNFα mRNA and protein in the liver of both WT and TNFR-KO as expected, as LPS triggers an immune response independent of TNFα receptors (Fig. 4A). LPS increased serum levels of TNFα in both WT and TNFR-KO mice. However, brain TNFα mRNA and protein increased only in the WT, but not in TNFR-KO mice, indicating that TNFα receptors are necessary for peripheral LPS to cause brain TNFα mRNA and protein production (Fig. 4C). In a separate experiment, similar to Fig. 3, WT mice showed increased Iba1 (a specific marker for microglia) staining in several brain regions, such as SN, hippocampus, and cortex (images not shown) at 2-h post LPS-treatment. Iba1-IR cells showed increased cell size, irregular shape, and intensified Iba1 staining consistent with morphological changes in activated microglia. Iba1-IR cells in saline controls in WT mice showed resting microglial morphology. However, TNFR-KO animals did not show increased TNFα mRNA or protein synthesis in the brain (Fig. 4C). These findings indicate that TNFα receptors are essential for systemic LPS administration to cause activation of microglia and TNFα synthesis within brain.

To confirm the role of systemic TNFα in brain inflammation, WT and TNFR-KO mice were treated with TNFα (0.25 mg/kg, i.p.). TNFα treatment resulted in a 132-fold increase in liver TNFα mRNA and a 27-fold increase in liver TNFα protein in WT, but not in TNFR-KO mice (Fig. 5A). The injection of TNFα increased serum levels of TNFα in both WT and TNFR-KO (Fig. 5B). Importantly, systemic TNFα injection induced brain TNFα mRNA and protein in WT, but not in TNFR-KO mice (Fig. 5B). Further, TNFα induced brain synthesis of other pro-inflammatory factors, such as monocyte chemotactic protein 1 (MCP-1) as well as NF-κB p65 subunit, interleukin-1β (IL-1β) in WT mice. However, TNFα failed to induce production of the pro-inflammatory factors in TNFR-KO mice (see Fig. 6). Thus, systemic TNFα induces pro-inflammatory cytokine production in brain, serum, and liver, as well as induces brain microglial activation.

A comparison of TNFα and LPS indicates these two inflammatory activators are remarkably similar. Our findings suggest that systemic LPS acts by inducing TNFα synthesis in the liver to increase serum TNFα, which then activates brain microglia to induce TNFα, other cytokines, and neuroinflammatory protein synthesis. Taken together, these findings suggest that an acute systemic injection of LPS or TNFα activates brain microglia through TNFα receptors that initiate sustained activation of brain cytokine synthesis and neuroinflammation.
Peripheral Inflammation Induces a Delayed and Progressive Loss of Dopaminergic Neurons in the Substantia Nigra

The substantia nigra (SN) is densely populated with microglia (Kim et al., 2000; Lawson et al., 1990) and it is hypothesized that this brain region is particularly susceptible to inflammatory insult. To discern the effects of chronic neuroinflammation (sustained increase in brain TNFα for at least 10 months) on neuron survival in the SN, we measured the number of dopamine neurons, as indicated by the number of tyrosine hydroxylase immunoreactive (TH-IR) cells within the SN, after a single LPS i.p. treatment. Previous reports using intranigral injection of LPS demonstrated that the LPS-induced decline of TH-IR cells is due to cell loss, as evidenced by a decline in the over-all number of neurons in the SN (Gao et al., 2002). As shown in Fig. 7, LPS injection caused a delayed and progressive loss of nigral TH-IR neurons. Comparison of the number of nigral TH-IR neurons between the LPS-treated and saline-treated mice showed no significant loss of TH-IR neurons within the first 4 months after LPS injection (Fig. 7A). However, significant loss of TH-IR neurons started to manifest between 7 and 10 months after LPS treatment. At 7 months, a 23% loss was observed. The LPS-induced loss of nigral TH-IR neurons further progressed over time and a 47% loss was shown at 10 months (Fig. 7A). Using double labeled (TH-IR and NeuN-IR) staining, we have previously reported that inflammation-induced loss of TH-IR cells in the nigra represents neuronal death, rather a down regulation of TH expression (Gao et al., 2002). Changes in TH-IR cells were more pronounced in SN than in the adjacent ventral tegmental area (VTA) DA neurons (Fig. 7B). Interestingly, staining for microglia using F4/80 antibody indicated that the SN contains much more microglia than the adjacent VTA (Fig. 7C). Cell loss in other brain regions was not determined. Thus, the SN may be inherently susceptible to inflammatory insult when compared to other regions of the basal ganglia due to the presence of increased microglia. These studies support the hypothesis that an acute increase in systemic TNFα activates neu-
DISCUSSION

The mechanisms responsible for the progressive loss of DA neurons in PD are poorly understood. Here, we are the first to show that a single exposure to systemic inflammation transfers to the brain and initiates self-propelling neuroinflammation that persists for 10 months, which results in the progressive loss of DA neurons in the SN (see Fig. 8). Specifically, we investigated three aspects of peripheral LPS administration: the role of TNFα in the...
Peripheral Inflammation Causes Chronic Neuroinflammation Through TNFα

In the current study, we demonstrate that a single intraperitoneal LPS injection results in TNFα production in the periphery, which initiates brain TNFα production (mRNA and protein) in the brain that continues for months after the systemic source of TNFα has abated. It was previously reported that liver is the major source for the circulating TNFα after a single systemic LPS injection, due to activation of kupffer cells, the resident macrophage-like cells in the liver (Kumins et al., 1996). These cytokines are then released into the blood and induce inflammation throughout the body (Crews et al., 2006). Here, we found that liver and serum TNFα were elevated at early time points, but declined over time to return to basal levels by 1 week and 9 h, respectively. Remarkably, TNFα levels remained elevated in the brain for up to 10 months, long after liver and serum levels had subsided. Further, immunohistochemical analysis of brain sections showed that microglia was activated in several brain regions, such as the SN, hippocampus, and cortex. Previous reports have shown that a single exposure to MPTP, a toxin selective for DA neurons, in humans (Langston et al., 1999) and monkeys (McGeer et al., 2003) resulted in microglial activation that persisted years after the brief exposure. Earlier studies investigating the long-term effects of systemic LPS exposure in utero has shown that low levels of systemic inflammation must occur during a critical window of development in order for the long lasting, pro-inflammatory changes in the brain to occur (Carvey et al., 2003; Ling et al., 2002). Our current work indicates that a single pro-inflammatory event in the periphery can induce chronic, self-propelling neuroinflammation outside of the embryonic critical period and in the adult brain.

It is well-documented that in the normal condition, very little peripheral LPS enters the brain due to the poor passage through the blood brain barrier (Nadeau and Rivest, 1999). Thus, the LPS-induced neuroinflammation is likely an indirect effect. In an effort to understand how chronic neuroinflammation was transferred from the periphery to the brain, we administered TNFα peripherally and found that both systemic TNFα and LPS administration increased the expression of brain pro-inflammatory factors (TNFα, MCP-1, IL-1β, and NF-κB p65) in a similar pattern. Further, TNFR1/R2−/− mice failed to show brain neuroinflammation in response to systemic LPS or systemic TNFα, supporting that TNFα was critical for the transfer of inflammation from the periphery to the CNS. This is consistent with other studies demonstrating that systemic cytokines mediate the effects of peripheral inflammation on the brain. For example, TNFα and other systemic cytokines are reported to mimic several behavioral effects of systemic LPS (Berkenbosch et al., 1987; Bernardini et al., 1990; Sapolsky et al., 1987; Sharp et al., 1989). Thus, systemic cytokines are critical for CNS effects in response to peripheral immune activation.

Here, we are the first to show chronic neuroinflammation and progressive neurodegeneration a single injection of LPS. Currently, the detailed mechanisms of this progressive neuroinflammation are unknown. However, we speculate that the initial entry of the pro-inflammatory factors, such as TNFα to the brain will cause the activation of microglia to produce more inflammatory factors, which may cause neuronal death. Upon exposure to TNFα, microglia are well known to synthesize and secrete TNFα (Rivest et al., 2000). Thus, this initial damage may then result in reactive microgliosis, causing the release of additional pro-inflammatory factors to begin a self-propelling and a vicious cycle of neuronal damage. We propose that reactive microgliosis may underlie the progressive and long-lasting neuroinflammation and neurodegeneration of numerous neurodegenerative diseases (Block and Hong, 2005).
**Peripheral Inflammation Causes Progressive Dopaminergic Neurotoxicity**

Inflammation is implicated as a neurodegenerative stimulus (Maurizi, 2002) and DA neurons in the SN are thought to be more susceptible to inflammation when compared to other neuronal subtypes and other brain regions (Block and Hong, 2005; Carvey et al., 2003; Gao et al., 2002; Ling et al., 2002). Microglial activation has been shown to induce the selective loss of DA neurons in response to multiple stimuli (Block et al., 2004; Wu et al., 2005; Zhang et al., 2005), including LPS (Gao et al., 2002; Ling et al., 2002, 2004a,b, 2006). Additionally, the SN contains 4.5 times as many microglia when compared to other brain regions (Kim et al., 2000), making this region particularly susceptible to inflammatory insult. In the current study, we have stained the SN for microglia and document that there are visibly more microglia in the SN, when compared to the VTA and other brain regions (see Fig. 7), helping to explain why global neuroinflammation can result in selective damage. Previously, it has been shown that a single injection of the selective dopaminergic neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), results in chronic neuroinflammation and progressive neurotoxicity in humans (Langston et al., 1999) and monkeys (McGeer et al., 2003), where the mechanisms of microglial activation and progressive toxicity are poorly defined. Here, we show that a single systemic administration of LPS results in significant loss of DA neurons beginning at 7-months post treatment (23% loss of TH-IR neurons) and increasing in severity with time to a 47% loss at 10-months post treatment. Thus, we are the first to show that a single pro-inflammatory stimulus from the periphery in the adult mouse results in delayed and progressive loss of DA neurons in the SN (over months), similar to what occurs in PD. This is consistent with a previous report of a single human case study where accidental peripheral administration of LPS was associated with later onset of PD (Niehaus, 2003). The delayed onset, progressive nature, and long-lasting DA neuronal degeneration after a single injection of LPS shown in this study suggests a clinical implication for the link between peripheral inflammation and neuroinflammation. Thus, it would be interesting to deter-
receptors to induce the synthesis of additional TNFα and other pro-inflammatory factors, creating a persistent and self-propelling neuroinflammation that induces delayed and progressive loss of DA neurons in SN of adult animals. This work offers strong support for the premise that inflammation is both an initiating stimulus and a self-propelling mechanism of progressive neurodegeneration.

ACKNOWLEDGEMENTS

The authors thank Drs. Jenny Ting, John Gilmore, and Richard Mailman for their scholarly comments and Melissa Mann for manuscript preparation.

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