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Astrocytic TLR4 expression and LPS-induced nuclear translocation of STAT3 in the sensory circumventricular organs of adult mouse brain

Yousuke Nakano [a], Eriko Furube [a], Shoko Morita [b], Akio Wanaka [b], Toshihiro Nakashima [a], Seiji Miyata [a,*]

[a] Department of Applied Biology, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606-8585, Japan
[b] The Department of Anatomy and Neuroscience, Faculty of Medicine, Nara Medical University, Shijo-cho, Kashihara, Nara 634-8521, Japan

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ABSTRACT

The sensory circumventricular organs (CVOs) comprise the organum vasculosum of the lamina terminalis (OVLT), subfornical organ (SFO), and area postrema (AP) and lack the blood–brain barrier. The expression of Toll-like receptor 4 (TLR4) was seen at astrocytes throughout the sensory CVOs and at microglia in the AP and solitary nucleus around the central canal. The peripheral and central administration of lipopolysaccharide induced a similar pattern of nuclear translocation of STAT3. A microglia inhibitor minocycline largely suppressed lipopolysaccharide-induced astrocytic nuclear translocation of STAT3 in the OVLT and AP, but its effect was less in the SFO.

1. Introduction

The reaction to pyrogen lipopolysaccharide (LPS), a component of Gram-negative bacteria, is a most well-characterized example of innate recognition which leads to a robust inflammatory response and fever. The binding of LPS to Toll-like receptor 4 (TLR4) and CD14 leads to the activation of two distinct signaling pathways finally to activate nuclear factor-κB (NF-κB) and activator protein-1 (Takeda and Akira, 2000, 2004; Rivest, 2003). NF-κB translocates into the nucleus and induces the transcription of inflammatory genes such as cyclooxygenase-2 (COX-2), tumor necrosisfactor-α, interleukin-1β (IL-1β), and IL-6 (Brasier, 2010). Activator protein-1 is shown to control the expression of IL-3, -4, -5, and -9, and interferon-α and -γ (Adcock, 1997). IL-6 is shown to be increased in the blood circulation of febrile animals and humans (Cartmell et al., 2000; Nijsten et al., 1987) and IL-6-deficient mice show a reduced fever response against peripheral infection (Chai et al., 1996).

Microglia, the resident macrophages, are the main immune response and effector cells in the brains (Rivest, 2009). Microglia are shown to express TLR4 and release various proinflammatory cytokines by the stimulation of LPS (Olson and Miller, 2004). Cultured brain astrocytes have been reported to express low levels of TLRs under normal condition, but they are capable of expressing high levels of TLRs upon inflammatory stimulation (Kielian, 2009). The stimulation of LPS induces both the upregulation of *Tlr4* mRNA and secretion of IL-6 in cultured microglia (Bowman et al., 2003; Carpentier et al., 2005). LPS strongly promotes the expression of COX-2 and prostaglandin-E2 synthase-1 mediated by the MyD88-dependent NF-κB pathway in cultured astrocytes (Font-Nieves et al., 2012). Following inflammatory activation, astrocytes are endowed with the ability to secrete soluble mediators, such as C–X–C motif chemokine 10, chemokine C–C motif ligand 2, and IL-6 and B cell activating factor belonging to the tumor necrosis factor family, which have an impact on both innate and adaptive immune responses (Farina et al., 2007). The responses of astrocytes to LPS are completely dependent on the presence of functional microglia via microglia release of soluble mediators in vitro, indicating glial crosstalk in brain responses to inflammation stimulation (Holm et al., 2012).

The brain vasculature has the blood–brain barrier (BBB) that maintains the chemical composition of neuronal milieu for proper functioning of neuronal circuits by preventing free access of blood-derived substances. The dysfunction of the BBB results in the accumulation of neurotoxic molecules within parenchyma and subsequent serious neuronal damages (Zlokovic, 2011). Nonetheless, brain cells are equipped with the mechanisms to recognize blood-derived information at the sensory circumventricular organs (CVOs) that include the organum

Abbreviations: AP, area postrema; BBB, blood–brain barrier; Cox2, cyclooxygenase-2; CVOs, circumventricular organs; GFAP, glial fibrillar acidic protein; icv, intracerebroventricular; IL-1β, interleukin-1β; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; OVLT, organum vasculosum of the lamina terminalis; PBS, phosphate-buffered saline; PBST, PBS containing 0.3% Triton X-100; pSTAT3, phosphorylated STAT3; Sol, the nucleus of the solitary tract; STAT3, signal transducer and activator of transcription factor 3; TLR4, Toll-like receptor 4; SFO, subfornical organ; TNFα, tumor necrosisfactor-α.

* Corresponding author at: Department of Applied Biology, Kyoto Institute of Technology, Kyoto 606-8585, Japan.
E-mail address: smiyata@kit.ac.jp (S. Miyata).

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vasculosum of the lamina terminalis (OVLT), subfornical organ (SFO), and area postrema (AP) (Engelhardt, 2003; Sisó et al., 2010; Miyata and Morita, 2011; Morita and Miyata, 2012). For example, plasma Na^+ and osmotic levels are monitored in the sensory CVOs to control salt-intake and/or drinking behaviors (Hiyama et al., 2004) and the release of vasopressin from the neurohypophysis (Miyata and Hatton, 2002). Emetic agents are recognized at chemosensitive receptors in the AP and the nucleus of the solitary tract (Sol) to cause vomiting and nausea (Hornby, 2001).

Although it is not conclusively determined how peripheral LPS induces neuroinflammatory responses, a most probable mechanism is that circulating pathogens and/or cytokines directly stimulate parenchyma cells in the sensory CVOs and the information is transmitted to other brain regions to cause neuroinflammatory responses and fever. The electrolytic lesion of the SFO reduces fever response by the intravenous administration of LPS (Takahashi et al., 1997). The expression of *Tlr4* mRNA is shown in the CVOs and ventricular ependymal cells in mouse brains (Laflamme and Rivest, 2001; Chakravarty and Herkenham, 2005). The peripheral and intracerebroventricular (icv) administration of LPS causes a rapid upregulation of CD14 in the sensory CVOs (Lacroix et al., 1998; Nadeau and Rivest, 2000).

The peripheral administration of LPS increases the expression of nuclear factor IL-6 in a time-dependent manner within the sensory CVOs which sustains hypothalamic inflammatory target gene induction (Damm et al., 2011). The peripheral administration of LPS induces nuclear translocation of the signal transducer and activator of transcription factor 3 (STAT3) at astrocytes in the sensory CVOs (Harré et al., 2002, 2003; Gautron et al., 2002; Rummel et al., 2004, 2005). IL-6 is shown to be increased in the blood circulation of animals and humans after inflammatory stimulation (LeMay et al., 1990; Nijsten et al., 1987; Cartmell et al., 2000). Mice deficient for IL-6 gene reveal a reduced fever response after peripheral infection (Chai et al., 1996). IL-6 triggers a signaling pathway of JAK-signaling transducer and activation of STAT3 (Akira, 1997) and the pathway of COX-2 in the brains most likely via STAT3-dependent pathway (Rummel et al., 2006).

Until now no study has been reported about TLR4-expressing cellular phenotype in the sensory CVOs, although strong expression *Tlr4* mRNA is reported in these regions of normal adult animals (Laflamme and Rivest, 2001; Chakravarty and Herkenham, 2005). Moreover, it has not been examined whether glial crosstalk between microglia and astrocytes is responsible for neuroinflammatory cascades in the sensory CVOs. In the present study, therefore, we performed immunohistochemistry for TLR4 and the effects of a microglia inhibitor minocycline on the nuclear translocation of STAT3 at astrocytes in the sensory CVOs of adult mice. The expression of TLR4 was observed at astrocytes in the sensory CVOs, whereas its expression was also seen at microglia in the AP and Sol around the central canal. Both intraperitoneal administration and icv infusion of LPS caused a similar pattern of nuclear translocation of STAT3 in the OVLT, SFO, and AP. Pretreatment of a microglia inhibitor minocycline suppressed the nuclear translocation of STAT3 at astrocytes in the OVLT and AP after intraperitoneal administration and icv infusion of LPS, but its inhibitory effect was less in the SFO, indicating heterogeneous contribution of microglia in activating astrocytic STAT3 among the sensory CVOs.

2. Materials and methods

2.1. Animals

Adult male mice (ICR) in 70–84 days old were used in the present experiments. The animals were housed in a colony room, under a pathogen-free condition controlled temperature (25.0 ± 1.0 °C), with a 12-h light/12-h dark cycle and given ad libitum access to commercial chow and tap water. Animal care and experiments were in accordance with the Guidelines laid down by the NIH and the Guideline for Proper Conduct of Animal Experiments Science Council of Japan to minimize the number of animals used and the suffering. The experimental protocol was approved by the Animal Ethics Experimental Committee of the Kyoto Institute of Technology.

2.2. Administration of LPS and minocycline

LPS (*Escherichia coli*; 055:type B5) was purchased from Sigma-Aldrich Japan (Tokyo, Japan) and dissolved in pyrogen-free physiological saline (Otsuka Pharmaceutical Co. LTD., Tokushima, Japan) and stored at −80 °C. Animals received a single intraperitoneal administration of LPS at the dose of 50 μg/kg or 1 mg/kg. It is shown that the administration of low dose (50 μg/kg) LPS induces hyperthermia, but that of high dose (1 mg/kg) LPS causes hypothermia in mice (Oka et al., 2003).

For the icv infusion of LPS, a stainless steel cannula (25-gauge) was implanted in each mouse under general anesthesia (chloral hydrate, Wako Chemical, Tokyo, Japan; 300 mg/kg), so that its tip lays in the lateral cerebral ventricle (anteroposterior −0.3 mm, lateral +1.0 mm to bregma and dorsoventral −2.5 mm below the skull) by using the standard stereotaxic technique (Paxinos and Franklin, 2001) and kept for 3 weeks. Freely moving mice received icv infusion of LPS (3 μl, 0.5 μl/min) using a Model EP-1000E infusion pump (Melquest, Toyama, Japan) at the dose of 30 and 300 ng/kg. Mice were sacrificed 1, 2, and 4 h after intraperitoneal administration and icv infusion of LPS for immunohistochemistry.

For suppression of microglia activity, we used minocycline or a second-generation semisynthetic tetracycline antibiotic. Minocycline is a lipid soluble chemical that is able to cross the BBB into brain parenchyma and possesses anti-inflammatory properties independent of its antimicrobial effects (Stirling et al., 2005). Minocycline (Sigma-Aldrich, St. Louis, MO) was dissolved in pyrogen-free physiological saline and sonicated to ensure complete solubilization. Mice received an intraperitoneal administration of vehicle or minocycline (50 mg/kg) twice daily for two days, and on the 3rd day mice received intraperitoneal administration of a mixture of LPS (50 μg or 1 mg/kg) and minocycline (50 mg/kg) or both icv infusion of LPS (30 ng or 300 ng/kg) and intraperitoneal administration of minocycline (50 mg/kg). Animals were sacrificed 2 h after the administration of LPS for STAT3 immunohistochemistry in these experiments.

2.3. Antibody

The following primary antibodies were used: mouse IgG against CD45 (eBioscience, dilution 1:1000) and glial fibrillar acidic protein (GFAP: astrocyte marker; Sigma-Aldrich Japan, Tokyo, Japan, clone G-A-5, dilution 1:1000); guinea pig IgG against laminin (vascular basement membrane marker: YI-2008, dilution 1:200, Imamura et al., 2010) and GFAP (astrocyte marker: dilution 1:500, Taniguchi et al., 2011); rabbit IgG against Iba1 (microglia marker: Wako Pure Chemical, Osaka, Japan, dilution 1:500), phosphorylated STAT3 (pSTAT3: Cell Signaling, Danvers, MA, dilution 1:2000), STAT3 (Cell Signaling; dilution 1:2,00), and TLR4 (SPC-200D, StressMarq, Victoria, Canada; dilution 1:100); and Armenian hamster IgG against CD31 (endothelial cell marker, clone 2HB; DSHB; dilution 1:20).

The SPCC-200D anti-TLR4 antibody is developed against a synthetic peptide corresponding to amino acids 420–435 of human TLR4 and demonstrated to detect recombinant mouse TLR4 by Western blotting. The immunohistochemistry using SPC-200D anti-TLR4 antibody (Supplemental Fig. 1) showed that the distribution of TLR4 in the intestine and spleen was well coincided with the previous reports (Ortega-Cava et al., 2003; Wassef et al., 2004; Hwang et al., 2009).

2.4. Immunohistochemistry

After deep anesthesia with urethane, mice were perfused with phosphate-buffered saline (PBS, pH 7.4) containing 5 U/ml heparin followed by 4% PFA in 0.1 M PB (pH 7.4). Fixed brains, gut, and spleen