MyD88 plays a key role in LPS-induced Stat3 activation in the hypothalamus

Yosuke Yamawaki, Hitomi Kimura, Toru Hosoi, and Koichiro Ozawa

Department of Pharmacotherapy, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan

Submitted 9 July 2009; accepted in final form 30 November 2009

Yamawaki Y, Kimura H, Hosoi T, Ozawa K. MyD88 plays a key role in LPS-induced Stat3 activation in the hypothalamus. Am J Physiol Regul Integr Comp Physiol 298: R403–R410, 2010. First published December 2, 2009; doi:10.1152/ajpregu.00395.2009.—Infection causes the production of proinflammatory cytokines, which act on the central nervous system (CNS) and can result in fever, sleep disorders, depression-like behavior, and even anorexia, although precisely how cytokines regulate the functions of the CNS remain unclear. In the present study, we investigated the regulatory-molecular mechanisms by which cytokines affect hypothalamic function in a state of infection. The intraperitoneal administration of lipopolysaccharide (LPS), a ligand of Toll-like receptor 4 (TLR4), time-dependently (2–24 h) increased signal transducer and activator of transcription 3 (STAT3) phosphorylation in the hypothalamus and liver, which corresponded with anorexia observed within 24 h. Interestingly, the pattern of phosphorylation in response to LPS differed between the hypothalamus and liver. In the hypothalamus, LPS increased STAT3 phosphorylation from 2 h, with a peak at 4 h and a decline thereafter, whereas, in the liver, the peak activation of STAT3 persisted from 2 to 8 h. The time course of the LPS-induced expression of suppressor of cytokine signaling 3 (SOCS3), a STAT3-induced negative regulator of the Janus kinase-STAT-pathway, was similar to that of STAT3 phosphorylation. Using mice deficient in myeloid differentiation primary-response protein 88 (MyD88), an adapter protein of TLR4, we found that LPS-induced STAT3 phosphorylation and SOCS3 expression in the hypothalamus and liver were predominantly mediated through MyD88. Moreover, we observed that MyD88-deficient mice were resistant to LPS-induced anorexia. Taken together, our findings reveal a novel mechanism, i.e., MyD88 plays a key role in mediating STAT3 phosphorylation and anorexia in the CNS in a state of infection and inflammation.

inflammation; anorexia; central nervous system; liver; Toll-like receptor 4; myeloid differentiation primary-response protein 88; signal transducer and activator of transcription 3; lipopolysaccharide

There is evidence that the immune functions of peripheral tissues interact with those of the central nervous system (CNS) to combat infection and inflammation. During an infection, pro- or anti-inflammatory cytokines such as interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α and IL-10 are produced in the peripheral tissues and in the brain (7, 10, 21). Furthermore, inflammation can affect the CNS, leading to fever, sleep disorders, depression-like behavior and anorexia (3, 4, 9, 18). The precise molecular mechanisms of these pathophysiological effects remain unclear, but one of the factors responsible would be cytokines. For instance, the central administration of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α induced a febrile response (23, 28, 29). Moreover, the direct injection of IL-1β in the hypothalamus has been shown to inhibit food intake (19). Furthermore, increased levels of TNF-α and interferon (IFN)-γ were reported to be involved in reducing serotonin levels in the brain, which are associated with depression-like behavior (5).

Signal transducer and activator of transcription 3 (STAT3) is activated through the phosphorylation of tyrosine, mediated by receptor-associated kinases such as the Janus kinase (JAK) family. Furthermore, activated STAT3 induces the expression of suppressor of cytokine signaling 3 (SOCS3), a negative feedback regulator in the JAK-STAT signaling pathway (29). STAT3 is activated through various cytokines including IL-6, IL-10, leukemia inhibitory factor, oncostatin M, ciliary neurotrophic factor (CNTF), and leptin (1, 33). Thus levels of STAT3 activity may be a promising marker for the activation mediated by these cytokines. We previously reported that the peripheral administration of lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria, induces STAT3 phosphorylation in the hypothalamus (13). LPS-induced STAT3 activation is mediated by cytokines that are released after LPS stimulation. It was reported that an IL-6-specific antisera abolished LPS-induced fever and inhibited nuclear STAT3 translocation, which suggests that LPS-induced STAT3 phosphorylation plays an important role (31). However, the roles and mechanisms of STAT3 activation in the CNS at a state of infection and inflammation still remain unclear.

LPS activates Toll-like receptor 4 (TLR4) (27), which plays an important role in the innate immune system. Myeloid differentiation primary-response protein 88 (MyD88) is an adapter of the TLR4 signal transduction pathway. LPS activates two signal transduction pathways, i.e., MyD88-dependent and -independent pathways (6, 16, 25, 37). The MyD88-dependent pathway activates nuclear factor-kB, which results in the production of pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α. The MyD88-independent pathway activates interferon regulatory factor-3, resulting in the expression of IFN-β and IFN-inducible genes. However, the relationships between STAT3 and MyD88 in regulating CNS functions during inflammation are not clear.

We investigated the molecular mechanisms of CNS-mediated immune function by evaluating the link between MyD88 and STAT3 in a state of infection and inflammation using mice.

**MATERIALS AND METHODS**

**Animals.** Experiments were conducted using adult male MyD88-deficient (C57BL/6 background) and control C57BL/6 mice (body wt 25.9 ± 0.23 g). MyD88-deficient mice were kindly provided by Dr. Shizuo Akira (Department of Host Defense, Research Institute for Microbial Disease, Osaka University). C57BL/6 control mice were obtained from Japan SLC (Hamamatsu, Japan). Mice were maintained in a room at 22–24°C under a constant day-night rhythm and given food and water ad libitum. All animal experiments were carried out in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and approved by the animal care and use committee at Hiroshima University.
Injection of LPS and preparation of samples. LPS (100 μg/kg, 055:B5; Sigma) was administered intraperitoneally in a volume of 5 ml/kg. Mice were killed by decapitation, and the brain was quickly removed. Hypothalamus and liver were rapidly dissected on an ice-cold plate. The samples were snap-frozen in liquid nitrogen and stored at −80°C.

Western blot analysis. Time-course experiments of STAT3 phosphorylation in the hypothalamus and liver after the peripheral injection of LPS (100 μg/kg ip) were performed. Western blotting was performed as described previously (12). Tissue samples were homogenized with 100 revolutions in a ground glass homogenizer in a buffer containing 10 mM HEPES-NaOH (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM Na3VO4, 10 mM NaF, 10 mg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 1% Nonidet P-40. The samples were centrifuged at 20,630 g for 45 min at 4°C, and the supernatants were collected. Laemmli buffer was added to the samples and then boiled for 3 min. The samples were fractionated by SDS-PAGE (200 μg/lane) and transferred at 4°C to nitrocellulose membranes. The membranes were blocked and incubated with an anti-STAT3 (1:1,000; Santa Cruz Biotechnology) or anti-phospho-STAT3 (1:1,000; Cell Signaling Technology) antibody at 4°C. The membranes were washed and then incubated with an anti-horseradish peroxidase-linked antibody. Peroxidase was detected using an enhanced chemiluminescence system (GE Healthcare). The density of bands was measured using Image J 1.37v (Wayne Rasband, NIH) software. The amount of phosphorylated STAT3 is expressed as a ratio densitometric measurement of each sample of STAT3, an internal control.

Fig. 1. Lipopolysaccharide (LPS) increased the amount of phosphorylated signal transducer and activator of transcription 3 (STAT3) time-dependently, which corresponded with LPS-induced anorexia. A–D: graphs show the results of a peripheral injection of saline or LPS (100 μg/kg ip) in wild-type mice. Hypothalamus or liver samples were obtained after the injection of LPS at the indicated time points. Western blotting was performed, and the densitometric measurements for phospho-STAT3 were analyzed. Values are presented as means ± SE (n = 5 mice/group). *p < 0.05 (Dunnett’s test, statistically significant difference vs. control). E: simple schematic illustration of how food intake was measured. The arrows indicate the time point of the measurement. F: food intake was measured every 24 h after the peripheral injection of saline or LPS (100 μg/kg ip). Values are presented as means ± SE (n = 7/group). *p < 0.05 (Dunnett’s test, vs. control).
RT-PCR analysis. Time-course experiments of SOCS3 mRNA in the hypothalamus and liver after the peripheral injection of LPS (100 μg/kg ip) were performed. Tissue samples were homogenized at 10,000 rpm using a Polytron homogenizer in TriPure Isolation Reagent (Roche Diagnostics), and total RNA was isolated according to the manufacturer’s directions. cDNA was synthesized from 2 μg of total RNA by reverse transcription using 25 units of Superscript Reverse Transcriptase III (Invitrogen) and 0.25 μg of oligo(dT)12–18 primer (Invitrogen) in a 20-μl reaction mixture containing 1× First-Strand Buffer (Invitrogen), 1 mM dNTP mix, 10 mM dithiothreitol, and 20 U of RNaseOUT Recombinant Ribonuclease Inhibitor (Invitrogen). Total RNA and the oligo(dt)12–18 primer were preincubated at 70°C for 10 min before the reverse transcription. After incubation for 1.5 h at 46°C, the reaction was terminated by incubating for 15 min at 70°C.

For PCR amplification, 1.2 μl of cDNA was added to 10.8 μl of a reaction mix containing 0.2 μM of each primer, 0.2 mM of dNTP mix, 0.6 units of Taq polymerase, and 1× reaction buffer. PCR was performed in a DNA Thermal Cycler (PTC-220; MJ Research). The following primer sequences and cycle numbers were used: SOCS3 upstream, 5’-gtg gag cat cat act gat cc-3’ and downstream, 5’-agg ggc cat cca cag tct ctc-3’ (18 cycles for hypothalamus and liver); IL-6 upstream, 5’-gtc gcc gct ctc tca ccc ggc-3’ and downstream, 5’-ttg act cca ggt age tat ggc-3’ (29 or 26 cycles for hypothalamus and liver, respectively); GAPDH upstream, 5’-aaa ccc atc acc atc ttc cag-3’ and downstream, 5’-agg ggc cat cca cag tct ctc-3’ (18 cycles for hypothalamus and liver). The PCR products (10 μl) were resolved by electrophoresis in an 8% polyacrylamide gel in 1× TBE buffer. The gel was stained with ethidium bromide and photographed under ultraviolet light. The density of bands was measured using Image J 1.37v (Wayne Rasband, NIH) software. Because of differences in Tm and number of cycles, CDNA for IL-6, SOCS3, or glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was amplified, and each PCR was run separately. The amount of IL-6 or SOCS3 mRNA is expressed as a ratio densitometric measurement of each sample of GAPDH mRNA, an internal control.

Measuring food intake. Wild-type (WT) and MyD88-deficient mice were housed individually before experiment. The first day of their isolation was set as “day 0.” From day 4, saline (5 ml/kg) was injected intraperitoneally for 2 days (days 5 and 6), and then control experiments were performed with a saline injection on day 7 followed by an LPS injection (100 μg/kg ip) on day 8 (Fig. 1E). All treatments were started 30 min before the onset of the dark cycle. Cumulative food intake was measured by weighting of food at specific time points manually.

Statistics. Results were expressed as means ± SE. Statistical analyses were performed using Dunnett’s test or a two-way ANOVA with Tukey’s honestly significant difference post hoc comparison.

RESULTS

Peripheral injection of LPS increased STAT3 phosphorylation in the brain and liver. In this study, we chose the hypothalamus, central to the regulation of food intake, and the liver as representative of peripheral tissues. In a preliminarily study using other peripheral tissues (heart, spleen, and kidney tissue), LPS also induced STAT3 phosphorylation [Supplemental Fig. 1 (Supplemental material for this article can be found on the American Journal of Physiology: Regulatory, Integrative and Comparative Physiology website.)]. In addition, we confirmed that the injection of saline did not affect hypothalamic STAT3 phosphorylation at any time point (4, 8, or 24 h) (data not shown). Thus we used 4 h as control experiments of saline injection. LPS increased STAT3 phosphorylation from 2 h, with a peak at 4 h, and declined thereafter in the hypothalamus (Fig. 1, A and B). In the liver samples, there was an increase in STAT3 phosphorylation from 2 h, which persisted for up to 8 h, and a decline thereafter (Fig. 1, C and D). We did not observe any phosphorylated STAT3 at 30 min after the LPS injection in either tissue. We next measured the effect of LPS on food intake every 24 h after the injection of LPS (100 μg/kg ip). We observed a significant decrease in food intake on the first day (0–24 h) compared with saline-treated mice. However, on the second day (24–48 h) and third day (48–72 h), we did not observe any difference between the LPS- and saline-treated groups (Fig. 1F). Overall, these results suggest that the LPS-induced activation of STAT3 (2–24 h) corresponded with that of anorexia, which was observed within 24 h (see Fig. 1, A, B, and F).
Fig. 3. LPS induced interleukin (IL)-6 mRNA expression and activated a STAT3-SOCS3 pathway in the hypothalamus and liver mostly through myeloid differentiation primary-response protein 88 (MyD88). All graphs show results of a peripheral injection of saline or LPS (100 μg/kg ip, 4 h) in wild-type (WT) or MyD88-deficient [knockout (KO)] mice. Open bars, saline-treated mice; filled bars, LPS-treated mice. Hypothalamus or liver samples were obtained after the injection of LPS, and then an RT-PCR analysis or Western blotting was performed. A–D: RT-PCR analysis for IL-6 mRNA. E–H: Western blot analysis for phospho-STAT3. I–L: RT-PCR analysis for SOCS3 mRNA. The densitometric measurements were analyzed as a ratio of each internal control. Values are presented as means ± SE (n = 5–7/group). *p < 0.05, significant difference between indicated groups [2-way ANOVA with Tukey’s honestly significant difference (HSD) post hoc comparison].
Peripheral injection of LPS induced SOCS3 transcription in the hypothalamus and liver. SOCS3 is a negative regulator of STAT3 phosphorylation and is expressed in response to activated STAT3. Thus we investigated whether LPS induces SOCS3 mRNA expression in the hypothalamus and liver. As shown in Fig. 2, LPS increased SOCS3 expression both in the hypothalamus and in the liver. The time course of the LPS-induced SOCS3 mRNA expression was similar to that of the LPS-induced STAT3 phosphorylation in both tissues (Fig. 1 vs. Fig. 2), suggesting phosphorylated STAT3 to be functionally activated.

LPS induced STAT3 phosphorylation mostly through a MyD88-dependent mechanism. LPS is a ligand for TLR4 (27), and downstream signaling of TLR4 has two major pathways, one dependent on MyD88 and one not (6, 16, 25, 37). Thus we next investigated whether the LPS-induced phosphorylation of STAT3 is dependent on MyD88. First of all, we examined whether a peripheral injection of LPS could induce the expression of IL-6 through MyD88. LPS (100 μg/kg, 4 h) increased IL-6 mRNA levels in both the hypothalamus and liver of WT mice (Fig. 3, A–D). However, the LPS-induced IL-6 mRNA expression was significantly attenuated in MyD88-deficient mice compared with WT mice in both tissues (Fig. 3, A–D). These results suggest MyD88 signaling to play a role in inducing IL-6 expression in both the hypothalamus and liver. We next examined whether the LPS (100 μg/kg, ip, 4 h)-induced phosphorylation of STAT3 would be mediated through MyD88 in the hypothalamus and liver. We found the phosphorylation to be attenuated in MyD88-deficient mice (Fig. 3, E–H). Moreover, we found that LPS-induced SOCS3 mRNA expression was attenuated in MyD88-deficient mice (Fig. 3, I–L). This attenuation would correspond with the decrease in STAT3 phosphorylation after the administration of LPS in MyD88-deficient mice.

LPS-induced STAT3 phosphorylation was partially mediated through a MyD88-independent pathway. We found that LPS-induced STAT3 phosphorylation is mainly mediated through a MyD88-dependent pathway. However, it was not completely inhibited in the liver samples of MyD88-deficient mice (Fig. 3, G and H). These results suggest the phosphorylation to be mediated through a MyD88-independent pathway as well. To test these possibilities, we next examined the time course of LPS-induced STAT3 phosphorylation in the hypothalamus and liver of MyD88-deficient mice. As shown in Fig. 4, LPS induced the phosphorylation of STAT3 with a peak at 2 h and a decline thereafter in both the hypothalamus and liver. The results suggest that the phosphorylation is in part mediated through a MyD88-independent pathway in both tissues. Similarly, LPS induced SOCS3 mRNA expression in MyD88-deficient mice, which peaked at 2 h and declined thereafter in these tissues (Fig. 5). In addition, the time course of SOCS3 mRNA expression corresponded with that of STAT3 phosphorylation in MyD88-deficient mice (compare Figs. 4 and 5).

LPS-induced anorexia depended on MyD88. Ogimoto et al. (24) reported that MyD88-deficient mice are resistant to LPS-induced anorexia. However, while we used 100 μg/kg, Ogimoto et al. used 50 μg/kg for the LPS injection. To investigate whether MyD88-deficient mice are resistant to LPS-induced anorexia at a dose of 100 μg/kg, we measured the cumulative food intake in WT and MyD88-deficient [knockout (KO)] mice. First, saline was administered (ip), and cumulative food intake was measured for up to 24 h at specific time points (Fig. 6). Next, LPS was injected, and cumulative food intake was measured again. The results indicated that LPS abolished food intake in WT mice from 2 to 24 h (Fig. 6B). KO mice, however, were completely resistant to LPS-induced anorexia (Fig. 6B). These results suggest that MyD88-dependent signaling is a key pathway in LPS-induced anorexia. Considering that MyD88 is central to the LPS-induced activation of STAT3 in the hypothalamus, it is possible that LPS-induced anorexia is mediated through STAT3 activation via MyD88.

DISCUSSION

MyD88 is an adapter molecule of TLR4 and reported to play an important role in LPS-induced inflammation. However, its role in the functions of the CNS under pathophysiological conditions remains unclear. In the present study, we found that...
MyD88-dependent and/or -independent pathways would play a role in activating STAT3 in a state of LPS-induced infection. Thus we investigated the molecular mechanisms of these effects.

**Central role of MyD88 in LPS-induced STAT3 activation in the hypothalamus: Mechanistic insights.** Using MyD88-deficient mice, we found that LPS-induced activation of STAT3 and expression of IL-6 were dependent on MyD88 in the hypothalamus as well as in the liver. It was reported that the LPS-induced nuclear translocation of STAT3 was dependent on circulating IL-6 levels in rats (31), and significantly diminished in IL-6-deficient mice (30). Furthermore, IL-6-induced fever is dependent on STAT3 (31). Taken together, the decrease in LPS-induced STAT3 phosphorylation in MyD88-deficient mice might be because of the impaired production of IL-6, which supports the notion that LPS-induced IL-6 production is mediated through a MyD88-dependent pathway resulting in the activation of STAT3 and expression of SOCS3 in the hypothalamus. In addition to IL-6, the possible involvement of other factors should be considered. For instance, LPS (100 μg/kg ip) increased levels of leptin, an adipose-derived hormone known as a regulator of food intake and body weight (26, 36), at the periphery and in serum in fasted hamsters (8), and leptin induced STAT3 activation in the hypothalamus and brain stem (12, 14, 35). In addition, CNTF was also shown to activate STAT3 in the brain (20, 22). Thus these factors may also mediate the LPS-induced activation of STAT3.

Interestingly, we noted a slight but significant upregulation of IL-6 mRNA expression in the hypothalamus (data not shown) and the liver of LPS-injected MyD88-deficient mice.

![Fig. 5. LPS time-dependently induced SOCS3 mRNA expression in the hypothalamus and liver of MyD88-deficient mice. A–D: both graphs show the results of a peripheral injection of saline or LPS (100 μg/kg ip) in MyD88-deficient mice. Hypothalamus or liver samples were obtained after the injection. RT-PCR analysis was performed, and the densitometric measurements for SOCS3 mRNA were analyzed. Values are presented as means ± SE (n = 5/group). *p < 0.05 (Dunnett’s test, vs. control).](http://ajpregu.physiology.org/)

![Fig. 6. LPS-induced anorexia was impaired in MyD88-deficient mice. A: simple schematic of how food intake was measured. The arrows indicate the time point at which cumulative food intake was measured. B: graph shows the effect of a peripheral injection of saline or LPS (100 μg/kg ip) on cumulative food intake in WT or MyD88-deficient (KO) mice. White bars, saline-treated MyD88-deficient mice [KO-control (Cont)]; gray bars, saline-treated wild-type mice (WT-Cont); hatched bars, LPS-treated MyD88-deficient mice (KO-LPS); black bars, LPS-treated wild-type mice (WT-LPS). Values are presented as means ± SE (n = 7/group). *p < 0.05 vs. WT-Cont (2-way ANOVA with Tukey’s HSD post hoc comparison).](http://ajpregu.physiology.org/)
Elucidating the mechanisms of this upregulation through a MyD88-independent pathway is an important subject for future studies.

**Mechanisms behind the slight activation of STAT3 in MyD88-deficient mice.** We also noted that LPS slightly induced STAT3 phosphorylation and SOCS3 mRNA expression in MyD88-deficient mice. Thus a MyD88-independent (such as TRAM-mediated) pathway may be involved as a weak immune response. The simultaneous stimulation of MyD88-dependent and -independent pathways using several TLR agonists has been shown to have synergistic effects on the production of pro-inflammatory cytokines such as IL-6 and TNF-α (2). Because TLR4 has both MyD88-dependent and -independent pathways, it is speculated that LPS-induced phosphorylation of STAT3 mediated through TLR4 is required by both pathways. In other words, the weak response of the MyD88-independent pathway may provoke a synergistic response in the presence of the MyD88-dependent pathway. Further studies using TRAM-deficient mice are required.

**LPS-induced anorexia and STAT3 activation were less extensive in MyD88-deficient mice.** Consistent with a previous report [24], we observed that LPS-induced anorexia was absent in MyD88-deficient mice. The administration of leptin or CNTF, which activates STAT3, has been shown to induce anorexia [15, 26, 36]. Because LPS-induced STAT3 phosphorylation was impaired in MyD88-deficient mice, hypothalamic STAT3 activation through MyD88 may play a central role in mediating LPS-induced anorexia. Interestingly, we detected a slight decrease in the food intake of LPS (2 h)-treated MyD88-deficient mice (Fig. 6B). This slight decrease may correspond to the increased hypothalamic STAT3 phosphorylation in the MyD88-deficient mice treated with LPS. It would be interesting to investigate whether LPS-induced STAT3 activation is involved in anorexia by using KO mice.

**Perspectives and Significance**

The roles of MyD88 in regulating CNS functions remain unclear. Here, we demonstrated that MyD88 plays a key role in the LPS-induced phosphorylation of STAT3, and that phosphorylated STAT3 acting through MyD88 would be important to LPS-induced anorexia. In addition, we speculate that different systems regulate the JAK-STAT pathway in the CNS and periphery. Our findings should improve the general understanding of the complex mechanisms of communication between the immune systems and the brain. Moreover, they provide a basis for elucidating novel targets in the treatment of inflammatory diseases affecting the CNS as well as periphery.

**ACKNOWLEDGMENTS**

We thank Dr. Shizuo Akira (Department of Host Defense, Research Institute for Microbial Disease, Osaka University, Osaka, Japan) and Kiyoshi Takeda (Department of Molecular Genetics, Medical Institute of Bioregulation, Kyushu University, Kyushu, Japan) for kindly providing the MyD88-deficient mice.

**GRANTS**

This research was supported by the Institute of Laboratory Animal Science and by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan.

**DISCLOSURES**

The authors have nothing to disclose.

**REFERENCES**


