Lipopolysaccharide Stimulates Proopiomelanocortin Gene Expression in AtT20 Corticotroph Cells

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Abstract. While lipopolysaccharides (LPS) are known to activate the hypothalamo-pituitary-adrenal axis, their direct effects on proopiomelanocortin (POMC) and adrenocorticotropin (ACTH) expression at the pituitary level through Toll-like receptors (TLRs) remain unclear. In this study, we examined the effects of LPS on ACTH secretion and the transcription of the POMC gene in the AtT20 mouse pituitary corticotroph cell line. RT-PCR analysis showed that TLR1-4 and 6 subtype mRNAs were expressed in AtT20 cells. When the cells were treated with LPS, a significant increase in the 5'-promoter activity of POMC gene was observed at 24 h, without any stimulatory effect on ACTH secretion. LPS also stimulated the expression of c-Fos gene and protein, and AP1-, but not NF-κB-, mediated transcription. Overall, our data show the expression of TLRs in the pituitary corticotroph cells, and suggest the direct stimulatory effect of LPS on POMC gene expression via TLR (probably TLR4), although the intracellular signaling pathways in the corticotroph may be different from those in immune cells.

Key words: Proopiomelanocortin, Lipopolysaccharide, Toll-like receptor, Innate immunity

IT is well recognized that infection/inflammation is a kind of stress that activates the hypothalamo-pituitary-adrenal (HPA) axis through the immune-neuroendocrine network [1]. Researchers have been recently realized the importance of innate immunity as an initial step in the response to infection and have identified the receptors called Toll-like receptors (TLRs) that recognize a variety of microorganisms [2, 3]. TLRs are reported to be expressed not only in immune but also in non-immune cells including normal pituitary cells [4, 5], although their biological role has yet to be determined. In this study, we examined the expression and function of TLRs in the pituitary cells to see if lipopolysaccharide (LPS), one of the ligands for TLRs, has a direct effect on the HPA axis at the pituitary level. We found that a variety of TLR mRNAs including TLR4, which recognizes LPS, is expressed in the pituitary corticotroph-derived AtT20 cells, and that LPS indeed stimulated proopiomelanocortin (POMC) gene expression possibly via the activation of the transcription factor AP1.

Materials and Methods

Materials

LPS (E. coli O55:B5 and Salmonella typhimurium) were obtained from Sigma (St. Louis, MO), and pAP1- and pNF-κB-luciferase reporter plasmids were from Stratagene (La Jolla, CA). Mouse c-fos gene promoter (~733/+77 bp, +1 designates the transcription start site)-luciferase plasmid was as described previously [6].
**Cell culture**

AtT20PL cells, a clone of mouse corticotroph AtT20 cells in which the POMC-luciferase-fusion gene was stably incorporated [7], or native AtT20 cells were used. Cells were maintained in a T_25_ culture flask with Dulbecco’s Modified Eagle’s Medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and antibiotics (50 U/ml penicillin and 50 µg/ml streptomycin; Invitrogen) under 5% CO\(_2\)/95% atmosphere at 37°C. Culture medium was changed twice a week, and the cells were subcultured once a week.

**Experiment**

AtT20PL cells were plated in 24 well plates at approximately 60% confluency, and each experiment was carried out 2 days later. On the day of the experiment, LPS solution in 1000× concentration (1 mg/ml), or solvent alone, was added directly into the culture medium of each dish, and the cells were incubated for 6 or 24 h. At the end of incubation, the culture medium was removed, and the cells were harvested for luciferase assay. In experiments in which ACTH secretion was studied, culture medium was changed to the serum-free medium immediately before the addition of the test reagent(s). After the cells were incubated for 4 or 24 h, culture medium was collected for ACTH assay. In transient transfection experiments, AtT20 cells were transfected with each reporter gene using FuGENE6 reagents (Roche, Indianapolis, IN) for 24 h. The culture medium was then changed, and the experiment was carried out in the same way as in AtT20PL cells.

**Reverse transcription-polymerase chain reaction (RT-PCR) procedure**

RNA was isolated from the AtT20PL cells using RNeasy RNA extraction kit (Qiagen, Hilden, Germany), and 5 µg of the total RNA was used for the reverse transcription reaction with MMLV reverse transcriptase (Superscript II, Invitrogen). The cDNA obtained was then amplified by PCR with Taq DNA polymerase (Takara Shuzo, Tokyo, Japan). The sequences of primer sets for amplifying each cDNA were as follows: mouse TLR1, sense, 5'-GGACCTCGAGAATGGTCT ATGG-3', antisense, 5'-GTGGGTGAAAACT GGAACCTGG-3'; TLR3, sense, 5'-TTGCAACCAT AATCTGGCCTG-3', antisense, 5'-GAAGCAGAAGT AAAACACTTTGC-3'; TLR4, sense, 5'-CTGTGTGC AGAAAAATGCCAGG-3', antisense, 5'-TCCCAAGA TCAACCGATGGAC-3'; TLR5, sense, 5'-TCCCATAGGATCAGTCA-3', antisense, 5'-AGGCT GTGAATCTGGTGGCCA-3'; TLR6, sense, 5'-GCT GTGAAGAATGTTAAAGTCC-3', antisense, 5'-ATT CTTCCCTGTCAATTCTCTC-3'; TLR7, sense, 5'-TCCACAGACCTCTTTGATTCC-3', antisense, 5'-TTGGAGAGGTCTAGTTCC-3'; TLR8, sense, 5'-TTTACCTCTCTTTGTCTATAGAC-3', antisense, 5'-TCCCTTTGAAAGTCTCTGAGTG-3'; and TLR9, sense, 5'-CCTCGGGAGAATCCTCCATC-3', antisense, 5'-CCCACATCAAGTACTCGAAGG-3'.

**Western blotting**

For TLR4 protein, AtT20PL cells were cultured in 3.5-cm-diameter dishes, and both membrane and cytoplasmic fractions of cellular proteins were extracted using Mem-PER and NE-PER eukaryotic protein extraction reagents (Pierce, Rockford, IL). The extracts were then applied for electrophoresis and incubated with antibodies to mouse TLR4 (Santa Cruz, Santa Cruz, CA), and with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (IgG) (Santa Cruz). Finally, the bands were detected by chemiluminescence using ECL plus Western blotting detection system (Amersham Pharmacia Biotech, Buckinghamshire, UK) following the manufacturer’s instructions.

For c-Fos protein, AtT20PL cells were treated without or with LPS (E. coli O55:B5, 1 µg/ml) for 0.5–2 h, and cellular protein was extracted using NE-PER reagents. The extracts were then applied for Western blotting analysis as above using anti-c-Fos antibody (BioLegend, San Diego, CA).

**Measurements and statistics**

Luciferase assay was performed as previously described [6]. ACTH in culture medium was measured by immunoradiometric assay (IRMA; ACTH IRMA-kit, Mitsubishi Chemical, Tokyo, Japan). All data were expressed as mean ± SE. When statistical analyses were performed, data were compared by one way analysis of variance with Duncan’s multiple range test, and p values below 0.05 were considered significant.
Results

**TLRs are expressed in AtT20 pituitary corticotroph cells**

To see whether TLRs are expressed in AtT20 cells, RT-PCR and Western blot analyses were carried out. We found that the amplified bands corresponded to the predicted lengths for TLR1-4 and 6 mRNAs (Fig. 1A). We also found the expression of TLR4 protein in membrane, but not cytoplasmic, fraction of AtT20 cells (Fig. 1B).

**LPS stimulates POMC gene expression**

We then treated the cells with LPS to see whether TLR2 or 4 is functional. We found that LPS (1 µg for 24 h, both *E. coli-* and *Salmonella* typhimurium-derived) significantly stimulated the 5′-promoter activity of POMC gene (Fig. 2). No effect was found when the cells were treated for 6 h.

**LPS does not stimulate ACTH secretion**

We also studied the effect of LPS on ACTH secretion. In contrast to the effect on POMC gene expression, LPS treatment (4 or 24 h) did not influence ACTH secretion (Fig. 3).

**LPS induces c-Fos and stimulates AP1-, but not NF-κB-, mediated gene expression**

Finally, to clarify the signal transduction stimulated by LPS, we transfected AtT20 cells transiently with reporter plasmids that reflect the activity of the two representative stress-responsive transcription factors NF-κB and AP1. When NF-κB-luciferase was used as a reporter, LPS showed no effect (Fig. 4A). In contrast,
TNFα had a significant positive effect on NF-κB-mediated transcription in the same experimental condition (Fig. 4B), indicating the validity of NF-κB signaling in AtT20 cells. When c-fos- and AP1-luciferase plasmids were used, LPS had a significant stimulatory effect on c-fos promoter activity at both 3 and 6 h (Fig. 5A) and on AP1-dependent transcription at 24 h (Fig. 5B). LPS also stimulated c-Fos protein expression (Fig. 5C). These results suggest that LPS induces c-Fos and activates AP1-mediated, but not NF-κB-mediated, gene transcription in AtT20 pituitary corticotroph cells.

**Discussion**

Infection and inflammation are known to exert positive effects through the so-called immune-neuroendocrine interaction [1]. The signal, mediated by various cytokines, is integrated at the hypothalamic level, and then the CRH/vasopressin-ACTH-cortisol pathway is activated [8, 9]. In addition, the effects of cytokines on pituitary cells have also been reported [10, 11]. In this study, we showed the expression of a variety of TLR subtype mRNAs in the AtT20 corticotroph cell line. Furthermore, we found a direct stimulatory effect of LPS on the transcriptional activity of POMC gene. Since LPS is known to be a specific ligand for TLR4 among the TLRs [12, 13], and both TLR4 mRNA and
protein were found to be expressed in AtT20 cells, it is likely that the effect of LPS we observed is mediated via TLR4.

Innate immunity had been recognized as a primitive immune system operating mainly in lower organisms until recently. However, since TLRs mediating the innate immunity is well preserved and expressed in higher organisms as well, the indispensable role of innate immunity in triggering the acquired immune system is now widely recognized [2, 3]. Our present data support the notion that innate immunity also involves the endocrine system including the HPA axis. The expression of TLR4 in normal human pituitary corticotroph cells and AtT20 cells has recently been reported [4, 5]. Furthermore, our laboratory showed the modulatory effect of LPS on the interleukin-1 receptor in AtT20 corticotroph cells [14], further suggesting the physiological role of the LPS/TLR system in the pituitary cells.

In our study, LPS clearly stimulated POMC gene transcription, while no effects were observed in a previous study [5]. This discrepancy may be caused by the culture condition of AtT20 cells employed: we previously observed a significant improvement in cellular responsiveness to regulatory molecules such as CRH following serum starvation for several days [6], and this may also be the case especially with less potent stimulatory factors like LPS. On the other hand, we observed no effect of LPS on ACTH secretion. Our preliminary experiments also showed that LPS did not stimulate ACTH secretion in primary culture of the rat pituitary cells (Takao T et al., unpublished). Thus, it is likely that, whereas LPS directly stimulates the basal activity of ACTH synthesis, the actual secretion of the hormone is still under the control of hypothalamic hypothalamic-pituitary hormones such as CRH and vasopressin.

It was also unexpected that LPS did not stimulate NF-κB signaling pathways, even though it did stimulate that of AP1. In many tissues LPS activates NF-κB, with the resultant transcriptional activation of immune/inflammation-related genes [15]. Our data show the validity of the NF-κB signaling pathway in AtT20 cells, because TNFα, if not LPS, potently stimulated the NF-κB-dependent transcription in this cell line. Thus, we assume that the LPS-mediated intracellular signaling system may be different from those in other tissues. LPS is known to activate AP1 as well as NF-κB in human monocytes [16]. Since AP1 is known to be involved in the transcriptional regulation of the POMC gene [17, 18], we hypothesize that LPS stimulates the expression of AP1 (Fos/Jun), which in turn enhances the POMC gene expression. The innate immunity system is an area of great interest among current researchers, and further studies may reveal the precise role of the system in endocrine organs.

References


