Published in final edited form as:

Biochem Biophys Res Commun. 2012 April 20; 420(4): 762–767. doi:10.1016/j.bbrc.2012.03.068.

TNF α and IL-1 β are mediated by both TLR4 and Nod1 pathways in the cultured HAPI cells stimulated by LPS

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Abstract

A growing body of evidence recently suggests that glial cell activation plays an important role in several neurodegenerative diseases and neuropathic pain. Microglia in the central nervous system express toll-like receptor 4 (TLR4) that is traditionally accepted as the primary receptor of lipopolysaccharide (LPS). LPS activates TLR4 signaling pathways to induce the production of proinflammatory molecules. In the present studies, we verified the LPS signaling pathways using cultured highly aggressively proliferating immortalized (HAPI) microglial cells. We found that HAPI cells treated with LPS upregulated the expression of TLR4, phospho-JNK (pJNK) and phospho-NF- κ B (pNF- κ B), TNF α and IL-1 β . Silencing TLR4 with siRNA reduced the expression of pJNK, TNF α and IL-1 β , but not pNF- κ B in the cells. Inhibition of JNK with SP600125 (a JNK inhibitor) decreased the expression of TNF α and IL-1 β . Unexpectedly, we found that inhibition of Nod1 with ML130 significantly reduced the expression of pNF- κ B. Inhibition of NF- κ B also reduced the expression of TNF α and IL-1 β . Nod1 ligand, DAP induced the upregulation of pNF- κ B which was blocked by Nod1 inhibitor. These data indicate that LPS-induced pJNK is TLR4-dependent, and that pNF- κ B is Nod1-dependent in HAPI cells treated with LPS. Either TLR4-JNK or Nod1-NF- κ B pathways is involved in the expression of TNF α and IL-1 β .

Keywords

microglia; TLR4; JNK; Nod1; NF- κ B; cytokines

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Conflict of interest: The authors declare no competing financial interests.

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1. Introduction

Microglia, one of the resident immune cells, are activated in most pathological conditions of the central nervous system (CNS) and participate in the regulation of innate and adaptive immune responses[1]. Prolonged activation of microglia can cause neuroinflammation due to increased production of proinflammatory mediators, eventually leading to neuronal death[2], which is a common characteristic found in several neurodegenerative diseases and neuropathic pain [3,4].

The bacterial lipopolysaccharide (LPS) is responsible for its toxic properties, as well as a core polysaccharide and O-specific polysaccharide[5]. LPS stimulation has long been considered the gold standard for glial activation. It mimics infection of Gram-negative bacteria leading to a view of microglia as neurotoxic and proinflammatory cells [1].

Toll-like receptors (TLRs) are membrane-bound pattern-recognition receptors that sense a variety of microbial-specific motifs to trigger an innate immune response [6,7]. TLR4 is traditionally accepted as the primary LPS receptor [7,8,9,10,11] and has been reported as critical for the glial response to LPS [12,13,14]. Upon stimulation with LPS, TLR4 initiates a series of signaling cascades that result in the activation of nuclear factor kappa B (NF- κ B) and mitogen-activated protein kinases (MAPKs) [15] to induce the release of proinflammatory cytokines. However, evidence shows that several other receptors have also been involved in the LPS-induced effects [16,17], and that LPS-induced superoxide production in microglia is independent of TLR4 [13].

Nucleotide-binding and oligomerization domain (Nod)-like receptors (NLRs) are a family of intracellular sensors that have key roles in innate immunity and inflammation. The Nod1 receptor is demonstrated to confer responsiveness to LPS in 293T cells [17]. Inohara *et al.* demonstrates that Nod1 binds to ^3H -LPS and activates NF- κ B in a TLR4-independent fashion [17]. These data raise the possibility that Nod1 may act as an intracellular receptor for invasive bacterial pathogens and LPS in microglial cells. In the present study, we investigated whether the TLR4 or Nod1 pathways are involved in the JNK or NF- κ B-mediated expression of proinflammatory cytokines in rat microglial cells activated by LPS.

2 Materials and Methods

2.1. Cell culture

Highly aggressively proliferating immortalized (HAPI) microglial cells derived from the neonatal brain of rats[18], and grown in Dulbecco's Modified Eagle's Medium supplemented with 5% fetal bovine serum (Fisher, Pittsburgh, PA) at 37°C in 5% CO₂, as previously described [19]. HAPI cells were seeded into 6-well plates at 2×10^5 cells/well 24 h before treatment with drugs. The cells were stimulated by 1 μ g/ml of LPS or 0.5 μ g/ml of Nod1 ligand, C12-iE-DAP (DAP) for 6 h.

2.2. Preparation and transfection of siRNA

TLR4 siRNA duplexes targeting rat TLR4 and mismatch RNA were synthesized by Invitrogen (Invitrogen, Camarillo, CA). TLR4 siRNA (forward 5' -

GGAUCUUUCUUAUAACUAUdTdT -3', reverse-5' - AUAGUUAUAAGAAAGAUCdTT -3') and mismatch RNA (forward-5' - GCCCGUAGAACACGUCGUAdTT -3', reverse-5' - UACGACGUGUUCUACGGGdTT -3') were used. Transient transfection procedures were performed according to Lipofectamine RNAiMAX reagent instructions (Invitrogen). Briefly, HAPI cells were incubated with 100 pmol of siRNA and 6 µl of Lipofectamine RNAiMAX complexes, in Opti-MEM I reduced serum medium (Invitrogen). Twenty four hours after siRNA application, the cells were stimulated by 1 µg/ml of LPS from *Escherichia coli* (Sigma, St. Louis, MO). Six hours after LPS, the cells were harvested.

2.3. RT-PCR analysis

Total RNA was isolated from HAPI cells via TRIzol (Invitrogen), treated with RNase-free DNase-I (Roche), and re-purified, and then quantified spectrophotometrically. First strand cDNA was produced with omniscrypt reverse transcriptase (Qiagen), then amplified using following primer sets: TLR-4-forward (5' - GACTCCATTCAAGCCCAAGCC -3') and TLR-4-reverse (5' -GCAGAGCCAACTGACCAAAGC -3'); TNF α -forward (5' - TGCCTCAGCCTCTTCTCATTC -3') and TNF α -reverse (5' - GGGCAGCCTTGTCCTTGAA -3'); IL-1 β -forward (5' - GACTTGGGCTGTCCAGATGAGA -3') and IL-1 β -reverse (5' - GGAGAATACCACTTGTGGCTTA -3'). The level of β -actin was determined by its specific primers: β -actin-forward (5' - CAGTTCGCCATGGATGACGATATC -3') and β -actin-reverse (5' - CACGCTCGGTCAGGATCTTCATG -3') for β -actin. All reactions involved initial denaturation at 95 °C for 15 min followed by 30 cycles for TLR4, 27 cycles for TNF α and IL-1 β , 24 cycles for β -actin (94°C for 30 s, 58°C for 30 s, 72°C for 1 min) and 1 cycle 72°C for 10 min using a DNA Engine PCR (Bio-Rad).

2.4. Western-Blot analysis

HAPI cells were lysed using protein lysis buffer (150 mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris) containing protease inhibitors and phosphatase inhibitors (Phosphatase Inhibitor Cocktail 1 and 2). After centrifugation at 18,000g for 20 minutes, the supernatant was harvested as the total protein extract. Equal amounts of protein were separated by 4-12% Tris-glycine SDS-PAGE gel electrophoresis, and electrophoretically transferred to PVDF membrane. The membrane was then blocked and then incubated with primary antibodies overnight at 4°C, including rabbit anti-pNF- κ B (1:2000, Cell Signaling, Danvers, MA), mouse anti-NF- κ B (1:2000, Millipore, Billerica, MA), mouse anti-pJNK (1:2000, Cell Signaling), rabbit anti-JNK (1:2000, Cell Signaling), rabbit anti-TNF α (1:1000, Millipore, Billerica, MA), rabbit anti-IL-1 β (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-TLR-4 (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), and mouse anti- β -actin (1:10000, Santa Cruz Biotechnology). The blots were incubated with a secondary antibody, developed in a chemiluminescence solution. Quantification of Western blots was done from the obtained chemiluminescence values (Bio-Rad ChemiDoc, Hercules, CA). Targeted protein bands were normalized using the amount of β -actin as a standard.

2.5. Drug treatments and data analysis

HAPI cells were pretreated for 1 h with 10 μ M of SP600125 (an antagonist of JNK, Sigma), 100 μ M of pyrrolidine dithiocarbamate (PDTC, an inhibitor of NF- κ B, Sigma), or for 14 hours with 30 μ M of ML130 (a new inhibitor of Nod1, ID# BAS07162070, ASINEX USA, Winston Salem, NC) and then the cells were stimulated by 1 μ g/ml of LPS in 5% FBS DMEM for 6 hours. After further cultivation for 6 h, cells were harvested. LPS and PDTC were dissolved in saline; SP600125 and ML130 dissolved in DMSO. For Nod1 experiment HAPI cells were pretreated for 14 hours with 30 μ M of ML130 and then the cells were stimulated by 500ng/ml of DAP (Invivogen, San Diego, CA) for 6 hours. DAP was dissolved in DMSO. Statistical significance of the difference between drug-treated and control cells was determined by *t* test. Data are expressed as mean $\pm$ sem, with *p*-value less than 0.05 considered significant.

3. Results

3.1 LPS induces phosphorylation of JNK and NF- κ B to release TNF α and IL-1 β in HAPI cells

In LPS-stimulated macrophage, proinflammatory cytokines are produced, and these effects are at least partially mediated through suppression of the MAPK and NF- κ B signaling pathways [20]. We have reported that HAPI cells treated with LPS release TNF α [21]. Microglia are activated by LPS to produce proinflammatory factors through TLR4 [13]. Here, HAPI cells were treated with LPS, and collected for Western blots. We observed that LPS significantly induced the upregulation of TLR4, phosphorylation of JNK (pJNK), phosphorylation of NF- κ B (pNF- κ B), TNF α and IL-1 β (Fig.1A-E), but total JNK and NF- κ B did not change in HAPI cells. In a similar set of experiments, HAPI cells were collected for RT-PCR. We found that LPS also induced upregulation of mRNA of TLR4, TNF α and IL-1 β (Fig.1F-I).

3.2. TLR4 knockdown with siRNA decreased pJNK, TNF α and IL-1 β in HAPI cells treated with LPS

Several newly discovered families of non-coding RNAs include microRNA, siRNA, are involved in gene expression regulation [22]. Small interfering RNA plays a variety of roles in biology. The most notable role of siRNA is its involvement in the RNA interference, where it interferes with the expression of a specific gene. We silenced TLR4 gene expression with siRNA in HAPI cells, and found that siRNA of TLR4 significantly reduced the synthesis of TLR4 protein compared to mismatch RNA (mmRNA) (Fig.2A). And, treatment with siRNA to TLR4 simultaneously decreased pJNK (Fig.2B), and downregulated TNF α and IL-1 β (Fig.2D-E). Unexpectedly, TLR4 knockdown did not reversed upregulation of pNF- κ B induced by LPS (Fig.2C). TLR4 siRNA did not change total JNK and NF- κ B. In a separate set of the studies, HAPI cells were collected for RT-PCR; TLR4 knockdown also suppressed the mRNA expression of TLR4, TNF α , and IL-1 β (Fig.2F-I). Thus, we think that JNK, TNF α , and IL-1 β were downstream signals of TLR4 in HAPI cells stimulated by LPS.

3.3. Inhibition of JNK reduced TNF α and IL-1 β in HAPI cells treated with LPS

In traditional downstream signaling pathways of TLR4, the overexpression of many of proinflammatory cytokines is likely dependent on MAPK-dependent transcription factors[6]. Previous studies have shown that LPS activates glial cells releasing inflammatory cytokines through JNK[23]. To determine the involvement of JNK in the LPS-induced increase in cytokines, we gave SP600125 (a selective JNK inhibitor) to HAPI cells 1 h prior to LPS exposure. SP600125 significantly inhibited the expression of TNF α and IL-1 β in the same samples (Fig.3A and B). Thus, our data suggest that JNK is the upstream signal for TNF α and IL-1 β . But SP600125 did not significantly change pNF- κ B (data not shown), so we believe that JNK activation does not regulate pNF- κ B. Overall these results underscored the important role of JNK in regulating the expression of proinflammatory genes after LPS.

3.4. Inhibition of Nod1 reduced NF- κ B induced by LPS

Recently, Nod1-like receptor has been identified as key mediator of inflammatory and immune responses[17]. Upon activation, Nod1 initiates a proinflammatory response largely dependent on NF- κ B activation [24,25], which might be important in HAPI cells treated with LPS. To understand whether Nod1 was involved in the expression of NF- κ B, we treated HAPI cells with ML130 (a selective Nod1 inhibitor) 14 hour prior to exposure of LPS. Unexpectedly, we found that ML130 significantly inhibited the expression of pNF- κ B (Fig. 3C). Meanwhile, ML130 markedly decreased the expression of TNF α and IL1 β (Fig.3D and E) either. But ML130 did not change pJNK in HAPI cells (Fig. 3F). We further treated HAPI cells with Nod1 ligand, DAP, and found that DAP significantly upregulated the expression of pNF- κ B which was blocked by ML130 (Fig.3G). These results indicated that pNF- κ B was dependent on Nod1 in HAPI cells activated by LPS.

3.5. Inhibition of NF- κ B reduced TNF α and IL-1 β induced by LPS

It has been reported that LPS induced cellular inflammatory factors through NF- κ B-target inflammatory genes in macrophages [26]. To understand whether NF- κ B was involved in the expression of TNF α and IL-1 β , we treated HAPI cells with PDTC (a selective NF- κ B inhibitor) 1 h before LPS exposure. PDTC significantly inhibited the expression of TNF α and IL-1 β in protein levels (Fig. 4A and B). In a separate set of studies, we found that PDTC reduced the mRNA expression of TNF α and IL-1 β (Fig.4C-E). These results indicated that pNF- κ B was also an upstream signaling of both TNF α and IL-1 β in HAPI cells activated by LPS.

4. Discussion

The current study showed 1) that HAPI cells treated with LPS, induced pJNK and pNF- κ B, and released TNF α and IL-1 β , 2) that pJNK induced by LPS was TLR4-dependent, and 3) that, unexpectedly, pNF- κ B induced by LPS, is dependent on Nod1, but not TLR4, 4) that both JNK and NF- κ B mediated the expression of TNF α and IL-1 β .

Microglial cells are the main source of proinflammatory cytokines within the CNS [27]. Increased cytokines in the CNS have been observed especially after injury or ischemia, in bacterial and viral infections (e.g. HIV), as well as in multiple sclerosis and Alzheimers's

disease; harmful outcomes of increased TNF α relate to its activity to promote inflammation and edema[28]. Microglial cytokines also seems to be critical in boosting chemokine-induced glutamate release from astrocytes [28].

TLRs signal via a common pathway leads to the expression of diverse inflammatory genes [6,7,29]. TLRs are expressed in various cell types in the CNS, including microglia, astrocytes, neurons, and cerebral vascular cells [30]. It has recently been discovered that TLR4 is capable of activation by endogenous ‘danger signal’ molecules released following cellular injury. Recent evidence supports that TLR4 is involved in neurodegenerative diseases[31].

TLR4 is traditionally accepted as the primary LPS receptor. LPS induced early activation of the transcription factor NF- κ B and expression of TNF α in cultured astrocytes through TLR4; MAPKs (e.g., JNK) were also involved in TLR4-cytokine signaling [32]. LPS activates several intracellular signaling pathways in human monocytes that include NF- κ B and MAPKs pathways. These signaling pathways in turn activate a variety of transcription factors that include NF- κ B (p50/p65) and AP-1, which coordinate the induction of many genes encoding inflammatory mediators[33]. In the current studies, silencing TLR4 with siRNA reduced pJNK and TNF α and IL-1 β induced by LPS, and inhibition of JNK reversed the release of TNF α and IL-1 β . Thus, we think that LPS induced the release of TNF α and IL-1 β through TLR4-JNK pathway, which is in line with previous report [32,33].

NF- κ B is usually sequestered in the cytoplasm in an inactive form by molecules of the inhibitor of NF- κ B family. Activation of NF- κ B involves the phosphorylation and proteolysis of the I κ B proteins and the concomitant release and nuclear translocation of the NF- κ B factors [34]. LPS/TLR4 signaling can activate NF- κ B by MyD88-dependent and MyD88-independent pathways, which mediate the activation of proinflammatory cytokine (see review[15]). However, studies shows other receptors may be involved in the LPS-induced effect either [16,17]. Certain LPS preparations exert TLR4-independent effects [35]. LPS-induced superoxide production in microglia is independent of TLR4 [13].

Nod1 plays a key role in innate immunity as a sensor of specific microbial components derived from bacterial peptidoglycans and induction of inflammatory responses [36,37]. Nod1 polymorphisms are associated with several human inflammatory disorders [38,39]. Recently, Nod1 has additionally been implicated in neuroinflammatory processes involved in the progression of multiple sclerosis [40]. Mutations in Nod proteins have been associated with various inflammatory diseases that affect NF- κ B activity [36,37]. Inohara *et al.* demonstrated that Nod1 binds to LPS and activates NF- κ B in a TLR4-independent fashion in HEK293T cells [17]. LPS can be internalized through an as yet unknown mechanism, and may bind to intracellular Nod1 and result in activation of NF- κ B[41]. In the present studies, TLR4 knockdown did not reduce pNF- κ B, which raises the possibility that Nod1 may act as a receptor for LPS. Nod1 inhibitor ML130 significantly reversed the pNF- κ B and expression of TNF α and IL-1 β induced by LPS in HAPI cells; Nod1 ligand induced pNF- κ B. Inhibition of NF- κ B decreased the expression of TNF α and IL-1 β . Thus, we think that Nod1- NF- κ B pathway may be involved in the production of cytokines in HAPI cells.

In summary, LPS is a common inflammagen to activate glia in animal models of inflammation-mediated neurodegeneration [42,43]. The current studies demonstrated that LPS activated cultured microglia to release proinflammatory cytokines through both TLR4---JNK and Nod1---NF- κ B pathways. Our findings provide novel insights into the mechanisms by which microglia release proinflammatory cytokines.

Acknowledgments

Supported by NIH DA26734 (S.H.), DA25527 (S.H.), and NS66792 (S.H.), Department of Anesthesiology, University of Miami (S.H.), China Scholarship Council (X.Z.), Jilin University, China (W.Z.). HAPI cells were kindly provided by Drs. David Fink and Marina Mata (Neurology, University of Michigan). We acknowledge the support from Dr. David Lubarsky (Chair of Anesthesiology, University of Miami, FL).

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Highlights

► LPS induces proinflammatory cytokine release in HAPI cells. ► JNK pathway is dependent on TLR4 signaling to release cytokines. ► NF- κ B pathway is dependent on Nod1 signaling to release cytokines

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Figure 1.

The neurochemical effects of LPS on HAPI cells. The cultured HAPI cells were treated with LPS (1μg/ml) for 6 h, and HAPI cells were collected for Western blots. LPS upregulated the expression of TLR4 (A), pJNK (B), pNF-κB (C), TNFα (D) and IL-1β (E), ** $p < 0.01$ vs vehicle, t test, $n = 3-4$. In a similar set of experiment, HAPI cells were collected for RT-PCR (F). LPS treatment increased the mRNA expression of TLR4 (G), TNFα (H), and IL-1β (I), ** $p < 0.01$ vs vehicle, t test, $n = 3-4$.

Figure 2.

The neurochemical effect of TLR4 knockdown with TLR4 siRNA on HAPI cells treated with LPS. Pretreatment with TLR4 siRNA, but not mismatch RNA (mmRNA) reversed the expression of TLR4 (A), pJNK (B), TNFα (D), and IL-1β (E), however, the treatment did not change pNF-κB (C) compared to mmRNA, ** $p < 0.01$ vs mmRNA, t test, $n=3-4$. In a similar set of experiment, the effects of TLR4 knockdown on the expression of mRNA of TLR4, TNFα and IL-1β were carried out (F). TLR4 siRNA, but not mmRNA reversed the expression of mRNA of TLR4 (G), TNFα (H), and IL-1β (I), ** $p < 0.01$ vs mmRNA, t test, $n=3-4$.

Figure 3.

The inhibition of JNK and Nod1 reduced the expression of TNFα and IL-1β in HAPI cells treated with LPS. HAPI cells were pretreated with SP600125 (SP, JNK inhibitor) for 1 h, then treated with LPS. SP600125 treatment reduced the expression of TNFα (A), and IL-1β (B), ** $p < 0.01$ vs vehicle, t test, $n = 3-4$. The effects of Nod1 on the pNF-κB in HAPI cells treated with LPS were examined (C-E). HAPI cells were treated with ML130 (a Nod1 inhibitor) for 14 hour, followed by LPS. HAPI cells were collected, and pNF-κB was examined using Western blots. ML130 reduced the expression of pNF-κB (C). In the same sample, the expression of TNFα (D) and IL-1β (E) were suppressed by ML130. * $p < 0.05$, ** $p < 0.01$ vs vehicle, t test, $n = 4$. However, ML130 did not affect the JNK (F). C12-iE-DAP (DAP, a Nod1 ligand) treated HAPI cells significantly induced upregulation of pNF-κB, which can be blocked by ML130 (G). ** $p < 0.01$ vs vehicle, ## $p < 0.01$ vs DAP, t test $n = 3-4$.

Figure 4.

The inhibition of NF- κ B on HAPI cells treated with LPS. HAPI cells were treated with PDTC (a NF- κ B inhibitor, 100 μ M) for 1 hour, followed by LPS. HAPI cells were collected and protein of TNF α (A) and IL-1 β (B) was examined using Western blots. PDTC decreased the expression of TNF α and IL-1 β . ** $p < 0.01$ vs vehicle, t test, $n = 3-4$. In a similar set of experiment, the inhibition of NF- κ B on mRNA expression of TNF α and IL-1 β on HAPI cells treated with LPS was examined (C-E). HAPI cells were collected and mRNA of TNF α (D) and IL-1 β (E) was examined using RT-PCR. PDTC reduced the mRNA expression of TNF α and IL-1 β . ** $p < 0.01$ vs vehicle, t test, $n = 3-4$. Schematic description of LPS signaling pathways (F). LPS binds to the TLR4 on the membrane of HAPI cells to activate pJNK to induce the release of TNF α and IL-1 β . LPS can be internalized through an as yet unknown mechanism, and may bind to intracellular Nod1 and result in activation of NF- κ B, and finally, induces the expression of TNF α /IL-1 β in HAPI cells.