Characterization of Interleukin-1 Receptor-associated Kinase in Normal and Endotoxin-tolerant Cells*

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Liuw Li, Sue Cousart, Jean Hu, and Charles E. McCall

From the Section of Infectious Diseases, Department of Medicine, Wake Forest University School of Medicine, Winston-Salem, North Carolina 27157

Interleukin-1 receptor-associated kinase (IRAK), a signal transducer for interleukin-1, has also been suggested to participate in the Toll-like receptor-mediated innate immune response to bacterial endotoxin lipopolysaccharide (LPS). Using the human promonocytic THP-1 cell line, we demonstrated that the endogenous IRAK is quickly activated in response to bacterial LPS stimulation, as measured by its in vitro kinase activity toward myelin basic protein. LPS also triggers the association of IRAK with MyD88, the adaptor protein linking IRAK to the Toll-like receptor/interleukin-1β receptor intracellular domain. Macrophages with prolonged LPS treatment become tolerant to additional dose of LPS and no longer express inflammatory cytokines. Endotoxin tolerance is a common phenomenon observed in blood from sepsis patients. We observed for the first time that the quantity of IRAK is greatly reduced in LPS-tolerant THP-1 cells, and its activity no longer responds to further LPS challenge. In addition, IRAK does not associate with MyD88 in the tolerant cells. Furthermore, application of AG126, a putative tyrosine kinase inhibitor, can substantially alleviate the LPS-induced cytokine gene expression and can also decrease IRAK level and activity. Our study indicates that IRAK is essential for LPS-mediated signaling and that cells may develop endotoxin tolerance by down-regulating IRAK.

Upon bacterial lipopolysaccharide (LPS) stimulation, normal macrophages produce various inflammatory mediators such as interleukin-1β (IL-1) and tumor necrosis factor (TNF) α (1). Although appropriate amounts of these mediators may be essential for augmenting microcidual activities of macrophages, excessive production caused by acute bacterial infection during sepsis results in severe inflammatory complications (2). Perhaps developed as a self-protective adaptive response, human neutrophils and monocytes isolated from sepsis patients become refractory to further LPS challenge and no longer produce inflammatory cytokines (3). This phenomenon, referred to as endotoxin tolerance, has also been demonstrated in animal models of endotoxemia (4) and can be induced in a variety of primary monocyte/macrophage cells and cell lines. Upon LPS treatment, the human promonocytic THP-1 cells mimic all the responses exhibited by human whole blood and blood neutrophils from sepsis patients (5). Both human leukocytes obtained from sepsis patients, and the THP-1 cells treated with LPS show an adaptation of innate immune response as reflected by the suppression of steady-state mRNAs of proinflammatory genes (5). The molecular mechanism underlying endotoxin tolerance is not known.

Recently, a family of Toll-like transmembrane receptor (TLR) proteins were identified in humans and mice that respond to LPS and other microbial products, thereby modulating the innate immune response (6). The TLR proteins possess leucine-rich extracellular repeats that may recognize the LPS-lipid-binding protein-CD14 complex (7). Interestingly, the TLR proteins possess intracellular domains resembling the IL-1β receptor-like intracellular domain and may utilize IL-1β signaling components including adaptor protein MyD88, interleukin-1 receptor-associated kinase (IRAK), and TNF receptor-activated factor 6 (TRAF6) (6, 8). Upon IL-1β stimulation, IRAK is recruited to the IL-1 receptor through the adaptor MyD88 (9). IRAK subsequently undergoes phosphorylation and relays the signal downstream by interacting with TRAF6 (9, 10). Genetic analyses indicate that IRAK is shared by the IL-1/LPS signaling pathway. Overexpression of a dominant negative IRAK in human THP-1 cells inhibits the LPS-induced NFκB activation (6, 11). Furthermore, overexpression of wild type IRAK in an IRAK-deficient 293 cell line renders the cell responsive to LPS stimulation as measured by NFκB promoter reporter assay (12). However, there has been no study regarding the endogenous IRAK response upon LPS stimulation.

The inability of cells to express inflammatory genes in response to LPS in endotoxin-tolerant cells reflects a state of altered innate immunity. Disruption of the molecular signaling events linking from LPS stimulation to inflammatory gene activation could contribute to the altered innate immune response. C3H/HeJ mice, which have defects in the gene encoding the TLR4 protein, are unresponsive to LPS stimulation (13–15). MyD88 knock-out mice are also unable to induce LPS-dependent gene expression (16). In addition, both the C3H/HeJ and MyD88 knock-out mice exhibit high resistance to LPS-induced shock. However, it is not known what molecular alterations are responsible for the development of endotoxin tolerance in wild type human macrophage. Examination of the molecular alteration(s) in endotoxin-tolerant cells may shed light on the mechanism of the innate adaptation developed in human septic blood cells.

In this study, we examined the endogenous IRAK protein level and kinase activity in fresh and endotoxin-tolerant THP-1 cells. We observed that endogenous IRAK in fresh THP-1 cells

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†To whom correspondence should be addressed: 3045 Hanes Bldg., Section of Infectious Diseases, Dept. of Medicine, Wake Forest University School of Medicine, Winston-Salem, NC 27157. Tel.: 336-716-6040; Fax: 336-716-3825.
‡The abbreviations used are: LPS, lipopolysaccharide; IL, interleukin; TNF, tumor necrosis factor; TLR, Toll-like receptor; IRAK, interleukin-1 receptor-associated kinase; TRAF6, TNF receptor-activated factor 6; MOPS, 4-morpholinepropanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; MBP, myelin basic protein.
is activated upon LPS stimulation. In contrast, IRAK level is consistently low in endotoxin-tolerant THP-1 cells and is no longer responsive to further LPS treatment. Furthermore, IRAK interacts with MyD88 upon LPS treatment, whereas such interaction is abolished in tolerant cells. Application of AG126, a putative kinase inhibitor, causes IRAK level decrease and inactivation and also decreases the cellular IL-1β expression upon LPS treatment. Our results suggest that defective IRAK may be responsible for the development of endotoxin tolerance during sepsis.

MATERIALS AND METHODS

Reagents—LPS (Escherichia coli 0111:B4) was obtained from Sigma. AG-126 was purchased from Biomol. IRAK antibody was from Upstate Biotechnology, Inc. MyD88 and TRAF6 antibodies were obtained from Santa Cruz Biotechnology, Inc.

Induction of Endotoxin Tolerance—We employed the undifferentiated human promonocytic THP-1 cells for our study. The advantages of that are 2-fold. First, our lab has previously completed extensive studies characterizing the tolerant phenotype of THP-1 cells (5), which is similar to the tolerant phenotype seen in leukocytes from humans with sepsis. Second, undifferentiated THP-1 cells do not express IL-1 type I receptor and therefore do not respond to IL-1 stimulation (17). The IRAK response we observed is solely due to LPS stimulation rather than IL-1 effect. We have found that the model of LPS tolerance in undifferentiated THP-1 cells is similar to that of vitamin D3 differentiated THP-1 cells (5). The LPS responses of both undifferentiated and differentiated THP-1 cells are CD14-dependent. Endotoxin tolerance in undifferentiated THP-1 cells can be induced by treating the cells with LPS ranging from 10 ng/ml to 1 µg/ml. THP-1 cells are rendered tolerant within 6 h of LPS treatment and remain tolerant up to 96 h after the initial dose of LPS (5). In our hands, intracellular events modulating LPS tolerance are optimally studied using higher concentrations of LPS and stimulating the cells with a second dose of LPS at 12–24 h after the initial dose of LPS.

In this investigation, undifferentiated THP-1 cells were cultured in RPMI medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine. Tol- erant THP-1 cells were obtained by treating the cells (5 × 10⁶ cells/ml) with 500 ng/ml endotoxin (E. coli 0111:B4; Sigma) for 16 h (5). Fresh as well as endotoxin-tolerant THP-1 cells were washed with serum-free RPMI medium, resuspended in fresh culture medium at a final concentration of 5 × 10⁵ cells/ml, and treated with 500 ng/ml endotoxin for various times as indicated in the figure legends.

RNA Isolation and Northern Analysis—Total RNA was isolated using the RNeasy B method (Tel-Test, Inc., Friendswood, TX). Routinely, 5 × 10⁶ THP-1 cells/condition were used. Total RNA (10 µg/lane) was fractionated on a 1% agarose, 6.6% formaldehyde gel in 1× MOPS buffer (0.02 M MOPS, 5 mM sodium acetate, and 1 mM EDTA), and capillary blotted onto nylon membrane filters. UV cross-linking was performed using a Stratalinker UV cross-linker (Stratagene, La Jolla, CA). Northern analysis was performed as described (5).

Immunoprecipitation, Western Blot, and in Vitro Kinase Assay—5 × 10⁶ normal and endotoxin-tolerant cells were collected at each time point, pelleted at 1000 × g for 10 min, and lysed on ice for 10 min in 1 ml of lysis buffer (50 mM HEPES, pH 7.6, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM benzamidine, 5 mM para-nitrophenolphosphate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1 mM mg/ml each of aprotinin, leupeptin, and pepstatin). Cell debris were pelleted by centrifugation for 2 min at maximum speed in a microcen- trifuge. The protein concentration in the supernatant was determined using Bio-Rad protein assay kit according to the protocol provided by the manufacturer. Extracts with equal amount of proteins were used for the immunoprecipitation. 5 µl of polyclonal anti-IRAK antibody (Upstate Biotechnology, Inc.) was added to 800 µl each of the isolated cell extracts and incubated at 4 °C for 3 h on a rotator. 50 µl of a 50% slurry of prewashed protein G-agarose beads were then added to each sample, followed by incubation for an additional 2 h at 4 °C. The samples were spun briefly in a microcentrifuge and washed four times in lysis buffer. Each sample was divided into two equal portions. One portion of each sample was solubilized by SDS sample buffer (80 µl Tris-HCl, pH 6.8,
THP-1 cells, in contrast to the reported abrupt IRAK activation caused by IL-1β (9, 10). IRAK kinase activity decreases to basal level after 90 min of LPS treatment, coinciding with the decrease in IRAK level. Because undifferentiated THP-1 cells do not express IL-1 type I receptor (17) and do not respond to IL-1 stimulation (Fig. 3A) (17, 18), we conclude that the observed IRAK response is caused directly by LPS rather than by the subsequent IL-1 production. Control experiments where THP-1 cells treated with recombinant IL-1α showed that IRAK protein levels and kinase activities are not affected (Fig. 3, B and C). Furthermore, IL-1β pretreated THP-1 cells can still respond to LPS stimulation, as measured by IL-1β message induction and IRAK kinase activation (Fig. 3, B and C).

Consistent with previous genetic analysis, our observation provides biochemical evidence indicating that LPS signals through endogenous IRAK in normal human monocye-like cells. Our results also indicate that LPS induces a distinct IRAK response compared with IL-1β. IL-1β causes rapid IRAK phosphorylation/activation and degradation (10, 19), whereas LPS causes a gradual activation of IRAK.

We then examined the IRAK protein and its kinase activity in the endotoxin-tolerant THP-1 cells. IRAK was immunoprecipitated from tolerant THP-1 cells and analyzed by Western blot. Strikingly, IRAK protein level is low but constant in the tolerant cells. In addition, the ratio of hyperphosphorylated versus hypophosphorylated IRAK remains unchanged upon second dose of LPS treatment (Fig. 4A). We also noticed that the residue hypophosphorylated IRAK runs as a doublet on the gel, and this pattern persists upon second dose of LPS treatment. The cause of the doublet is unknown. Furthermore, although IRAK protein level in tolerant cells persists at ~20% compared with the level in normal cells, no IRAK kinase activation is observed in the tolerant THP-1 cells upon second dose of LPS treatment (Fig. 4B). We performed four independent experiments and obtained consistent results. Our results indicate that there is a disruption of IRAK response in tolerant cells. More interestingly, It was shown that THP-1 cells develop endotoxin tolerance as early as 2–3 h after LPS treatment (5), coinciding with our observation that IRAK level and activity decrease in normal THP-1 cells after 90 min of LPS stimulation. The tolerant THP-1 cells remain tolerant even 9 h after the removal of LPS (5). Examination of IRAK showed that, 9 h after removal of LPS by washing the cells with endotoxin-free medium, the endotoxin-tolerant cells still maintain a low level of IRAK protein and low kinase activity (data not shown). Taken together, our results suggest that the inability of tolerant cells to express inflammatory cytokine is at least in part contributed by the disruption of IRAK.

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**Fig. 2.** IRAK is phosphorylated and activated upon LPS treatment in undifferentiated THP-1 cells. Undifferentiated THP-1 cells were treated with 500 ng/ml LPS for various amounts of time. IRAK proteins were immunoprecipitated using anti-IRAK antibody. A, the IRAK protein levels were analyzed by Western blot. B, IRAK kinase activities were determined by in vitro kinase assay using myelin basic protein as described under "Materials and Methods." IRAK-P, hyperphosphorylated form of IRAK; IRAK-MBP-P, phosphorylated MBP. C, relative IRAK kinase activity upon LPS stimulation. Radioactively labeled MBP was quantified using a PhosphorImager plate and imageQuant software. Fold of increase in radioactive MBP signal was plotted. This plot represents three independent experiments. Data were expressed as the mean values ± S.D.

**Fig. 3.** THP-1 cells do not respond to IL-1 stimulation. A, fresh THP-1 cells were treated with either 10 ng/ml IL-1β or 500 ng/ml LPS for 3 h, and total RNAs were isolated and analyzed by Northern blot. B, THP-1 cells were treated with 10 ng/ml IL-1β for various amount of time and subsequently subjected to 500 ng/ml LPS challenge for the indicated times. IRAK was immunoprecipitated (IP) and analyzed by Western blot. C, in vitro IRAK kinase assay using myelin basic protein.

**Fig. 4.** IRAK remains at low level and unresponsive to further LPS challenge in tolerant THP-1 cells. Endotoxin-tolerant THP-1 cells were washed with serum-free RPMI medium, resuspended in fresh RPMI medium, and subsequently treated with 500 ng/ml LPS for various amounts of time. A, the IRAK protein levels were analyzed by Western blot. B, IRAK kinase activities were determined by in vitro kinase assay using myelin basic protein as described under "Materials and Methods." IRAK-P, hyperphosphorylated form of IRAK; IRAK-MBP-P, phosphorylated MBP. IP, immunoprecipitated.
It has been reported that administration of LPS treatment in normal cells but only associates with TRAF6 by Western blot using anti-MyD88 and anti-IRAK antibodies, respectively. TRAF6 proteins were immunoprecipitated after various amount of time in normal THP-1 cells but not in endotoxin-tolerant cells. MyD88 after 5 min of LPS stimulation (Fig. 5A). Such interaction persists about 1 h and disappears afterward. Strikingly, we could not detect any association of IRAK with MyD88 in the tolerant THP-1 cells (Fig. 5A).

To examine the IRAK-TRAF6 interaction, TRAF6 protein was similarly immunoprecipitated using anti-TRAF6 antibody and analyzed by SDS-PAGE and Western blot. Like MyD88, we observed that TRAF6 is also a constitutively expressed protein with a steady level in both normal and tolerant cells (Fig. 5B). Upon probing the membrane with anti-IRAK antibody, we can only detect the upper phosphorylated form of IRAK (Fig. 5B). This is in consistent with the previous observation that IL-1β-activated IRAK undergoes phosphorylation and subsequent association with TRAF6 protein. Interestingly, the amount of phosphorylated IRAK that co-immunoprecipitated with TRAF6 remains constant in normal and tolerant THP-1 cells. However, we cannot exclude the possibility that TRAF6-bound IRAK may undergo turnover during LPS stimulation in normal and tolerant cells.

AG126 Alleviates the IL-1β Expression and Also Induces IRAK Inactivation. It has been reported that administration of tyrphostin AG126, a putative tyrosine kinase inhibitor, can decrease the IL-1β and TNFα production in mice macrophages upon subsequent LPS stimulation (21). AG-126 can also protect the mice from LPS-induced septic shock (21). Because TLR-Myx88-IRAK-mediated signaling is critical for cellular LPS response, we examined whether AG-126 can interfere with this signaling process. Consistent with the previous report, we find that pretreatment of the THP-1 cells with AG126 for 1 h can dramatically decrease the IL-1β production upon subsequent LPS stimulation (Fig. 6A). We next examined the effect of AG126 on IRAK protein level and kinase activity. Interestingly, treatment of THP-1 cells with AG126 for 1 h consistently causes a dramatic decrease in the IRAK protein level (Fig. 6B). In addition, there is no induction of IRAK kinase activity upon subsequent LPS treatment (Fig. 6B). The correlation between IRAK level as well as activity decrease and subsequent inhibition of LPS-induced cytokine gene expression further indicates that disruption of TLR-mediated signaling at the point of IRAK contributes to endotoxin tolerance (Fig. 7).

Because IRAK is a putative serine/threonine kinase, it is unlikely that AG-126 directly inhibits IRAK activation. AG126 may actually target some other kinases and phosphatases that in turn cause IRAK level decrease and inactivation. Further biochemical analysis of IRAK phosphorylation site(s) and identification of other critical modifying enzymes of IRAK will provide insight into the regulation of IRAK function during TLR-mediated signaling pathway in normal and tolerant cells.

DISCUSSION

Genetic analyses indicate that LPS signaling utilizes Toll-like-receptors and shares IL-1 signaling components, namely MyD88, IRAK, and TRAF6 (6). Using the undifferentiated human promonocytic THP-1 cells, which do not respond to IL-1 stimulation, we provided biochemical evidence confirming that LPS stimulation leads to the activation of endogenous IRAK kinase and its subsequent interaction with MyD88. Our study showed that LPS induces a distinct IRAK response compared with IL-1β. Previous studies using IL-1 responsive cell lines (such as MRC-5 fibroblast cells) showed that IL-1β causes rapid IRAK phosphorylation/activation and degradation (10, 19). In this report, we observed that LPS causes a gradual IRAK activation and no apparent decrease in IRAK level within the first 20 min of LPS stimulation (10). Furthermore, it was reported that the association of MyD88 and IRAK occurs within 5 min after IL-1β stimulation and quickly dissociates within 20 min of IL-1β treatment (19). In contrast, we found that LPS-induced MyD88-IRAK interaction persists until 1 h after LPS stimulation. Undifferentiated THP-1 cells express relatively low levels of CD14 and are therefore less responsive to LPS. The delayed IRAK response we observed might be caused by its low surface CD14 level. It might also result from unique phosphorylation/modification events incurred on IRAK by LPS/TLR-mediated signaling. So far, however, there is no study regarding the phosphorylation/modification status of IRAK. Further examination of the phosphorylation status of IRAK upon LPS and IL-1β stimulation will provide clues regarding the delicate regulation of IRAK activity and its involvement in these two distinct yet related signaling pathways.
IRAK may not be the only component that is disrupted and responsible for acquiring endotoxin tolerance. There are two other IRAK-related kinases, namely IRAK-2 and IRAK-M. Although their physiological functions are still unknown, overexpression of either of these two kinases can reconstitute the IL-1 and LPS response in a 293 mutant cell line lacking IRAK (12). In agreement with the above study, transgenic mice with IRAK gene deletion was shown to still retain some response to IL-1, although with a drastic decrease (24). Those studies suggest that either IRAK-2 or IRAK-M may play some compensatory roles in IL-1- and/or LPS-mediated signaling. However, under physiological condition, it is also possible that certain endogenous form(s) can only respond specifically to certain stimulus (IL-1 or LPS). Because there are no antibodies available for IRAK-2 and IRAK-M, there has yet been no study regarding these endogenous proteins. Once the antibodies are available, it would be necessary to examine the exact roles of these related kinases in IL-1- and LPS-mediated signaling. It would be also interesting to study whether cells have to alter IRAK-2 and/or IRAK-M to acquire endotoxin tolerance.

Disruption of components upstream of IRAK may also be needed and necessary for development of LPS tolerance. Recently, it was reported that the surface level of TLR4 receptor was reduced in LPS-tolerant murine macrophages (25). However, the mechanism of TLR4 down-regulation is not known. Because there is no significant change in TLR4 message level between normal and tolerant cells (25), receptor internalization and degradation might account for the decrease. Surprisingly, the mutated TLR4 receptor in macrophages from C3H/HeJ mice was also internalized upon LPS challenge (25). This suggests that other LPS recognition events are necessary for TLR4 down-regulation and development of endotoxin tolerance. In addition, the TLR4 level gradually decreases upon LPS challenge and was dramatically reduced after 24 h of LPS pretreatment (25). The fact that endotoxin tolerance can be induced with a minimum of 2–3 h of LPS pretreatment suggests that LPS tolerance does not solely occur because of suppression of the surface TLR4. It would not be surprising to find out in the future that a series of changes including LPS receptors and intracellular components jointly contribute to the development of endotoxin tolerance.

In summary, LPS stimulation of human promonocytic THP-1 cells leads to the activation of endogenous IRAK kinase and its interaction with MyD88. The decrease of IRAK level and its unresponsiveness to further LPS stimulation coincide with the THP-1 cells acquiring endotoxin tolerance. These data support the concepts that endogenous IRAK is involved in the LPS-TLR-mediated signaling and that disruption of IRAK is one mechanism by which cells may acquire endotoxin tolerance.

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