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IL-1 Receptor-Associated Kinase and Low Molecular Weight GTPase RhoA Signal Molecules Are Required for Bacterial Lipopolysaccharide-Induced Cytokine Gene Transcription

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Proinflammatory cytokines such as IL-1, TNF, IL-6, and IL-8 are produced by leukocytes in response to bacteria or bacterial components. A great deal has been learned during the past few years about the synthesis and release of proinflammatory cytokines by leukocytes; however, relatively little is known about the intracellular events that lead to leukocyte proinflammatory cytokine gene transcription. This study examined the signal transduction pathway of IL-8 induction by bacterial LPS. Stimulation of monocytes with LPS rapidly activated RhoA, and pretreatment of monocytes with a RhoA inhibitor, C3 transferase exoenzyme, effectively blocked LPS-induced IL-8 gene expression. Overexpression of dominant negative RhoA (T19N) or IL-1R-associated kinase completely inhibited LPS-stimulated reporter gene expression. Induction of IL-8 was also inhibited by dominant negative IκB kinase and myeloid differentiation protein (MyD88). These results indicate that RhoA and IL-1R-associated kinase are novel signal transducers for LPS-induced Toll-like receptor 4-mediated proinflammatory cytokine synthesis in human monocytes. The Journal of Immunology, 2002, 169: 3934–3939.

Bacterial LPS can activate a variety of mammalian cell types and is a powerful activator of the innate immune system. LPS stimulates the synthesis and release of proinflammatory cytokines such as IL-1, TNF, IL-1, IL-6, and IL-8 from monocytes and macrophages. These cytokines can further activate monocytes, neutrophils, and lymphocytes, initiating cellular injury and tissue damage (1, 2).

Recently, the signal transducing receptor for LPS has been identified and has been shown to be member of the Toll-like receptor (TLR)3 family. Toll is a transmembrane receptor in Drosophila involved in the induction of the anti-fungal response (3). Activation of the Toll receptor results in the stimulation of several signaling molecules that are homologous to proteins involved in the NF-κB response in mammalian cells (4). The cloning of a family of human receptors structurally related to Drosophila Toll revealed cytoplasmic domains with sequence homology to the intracellular portion of the IL-1R (5). Beutler and colleagues (6) found that mutational inactivation of TLR4, occurring in mice of the C3H/HeJ and C57BL/10ScCr strains, completely abolishes LPS signal transduction. These results first documented that Toll-like receptor 4 (TLR4) was the cellular LPS receptor. There is increasing evidence that TLR4 mediates LPS-induced signaling events, including activation of ERK, c-Jun N-terminal kinase, p38 mitogen-activated protein kinase phosphorylation, and NF-κB (7–9).

GTPases of the Rho family exist in both GDP-bound inactive (GDP-Rho) and GTP-bound active (GTP-Rho) forms. When cells are stimulated with different ligands, GDP-Rho is converted to GTP-Rho, which binds to specific targets and then exerts its biological functions. Low m.w. G proteins of the Rho family (consisting of Cdc42, Rac, and RhoA) have been shown to regulate actin cytoskeletons, focal adhesion complex formation, cell aggregation, and cell motility (10–12). The function of these small G proteins in leukocyte cytokine gene transcription, however, has not been previously addressed.

We now report that LPS, acting through TLR4, stimulates IL-8 gene expression in human peripheral blood monocytes and that this is accompanied by enhanced RhoA GTPase activity. Furthermore, LPS-induced IL-8 synthesis is completely inhibited by a RhoA inhibitor, C3 transferase exoenzyme as well as by overexpression of dominant negative RhoA (T19N) and dominant negative IL-1R-associated kinase (IRAK). These results show that LPS stimulates proinflammatory cytokine synthesis via TLR4, and that IRAK and RhoA activities are necessary for this effect.

Materials and Methods

Reagents

LPS was isolated from Salmonella minnesota Re595 bacteria as previously described (13). Recombinant murine TNF-α was provided by V. Kravchenko (The Scripps Research Institute, La Jolla, CA). Recombinant human IL-1β was purchased from Calbiochem (San Diego, CA). An mAb against RhoA was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Oligonucleotides and their complementary strands for EMSAs were purchased from Promega (Madison, WI). [γ-32P]ATP (>5000 Ci/mmol) was obtained from Amersham (Arlington Heights, IL). The RhoA dominant negative (T19N) and constitutively active (Q63L) pCMV plasmids and recombinant Clostridium botulinum C3 transferase exoenzyme were obtained as previously described (14). The following eukaryotic expression plasmids (pcDNA3) expressing dominant negative mutant proteins were used for transfections: the dominant negative mutant of MyD881–298 was a gift from Dr. R. Medzhitov (Yale University, New Haven, CT), the dominant negative mutant of IRAK1–286 was prepared as described previously.

Abbreviations used in this paper: TLR, Toll-like receptor; CAT, chloramphenicol acetyltransferase; IRAK, IL-1-associated kinase; IκB, IκB kinase; RBD, Rho binding domain; WT, wild type.
Preparation of monocytes from peripheral blood and cell lines

Heparinized human peripheral blood from healthy donors was fractionated on Percoll (Pharmacia, Piscataway, NJ) density gradients. Monocytes were prepared from the mononuclear cell population as previously described (17). The purity of monocytes was >95% as determined by staining with the anti-CD14 mAb (CD14-PE; BD Pharmingen, San Diego, CA). Cell viability was >98% as measured by trypan blue exclusion. Monocytes were resuspended in RPMI 1640 medium (Irvine Scientific, Santa Ana, CA) with 10% (v/v) heat-inactivated FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), and l-glutamine (2 mM; Irvine Scientific). The monocyte cell line THP-1 cells stably expressing CD14 was kindly provided by Dr. R. Ulevitch (The Scripps Research Institute) (18) and was cultured in RPMI 1640 (Irvine Scientific) with 10% (v/v) heat-inactivated FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), l-glutamine (2 mM; Irvine Scientific), and 2-ME (complete medium).

Detection of cellular GTP-Rho

When activated, Rho undergoes GDP-GTP exchange, and activated Rho can thus be detected by analyzing GTP-bound Rho. RhoA activity was detected by the method recently described by Ren et al. (19). This assay uses the Rho binding domain (RBD) from the effector protein rhotekin as a probe to specifically isolate the active forms of RhoA. Human peripheral blood monocytes (5 × 10^6) were stimulated with LPS or control medium and then lysed (lysis buffer: 50 mM Tris-HCl (pH 7.5), 10 mM MgCl_2, 500 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, and 10 μg/ml each of leupeptin and aprotinin). Equal volumes of lysates were incubated with GST-RBD (20 μg) beads at 4°C for 45 min. The beads were washed three times with Tris buffer containing 1% Triton X-100, 10 mM MgCl_2, 150 mM NaCl, 1 mM PMSF, and 10 μg/ml each of leupeptin and aprotinin. Bound Rho proteins were detected by Western blotting using a mAb against RhoA (Santa Cruz Biotechnology).

EMSA

Nuclear extracts were prepared from human peripheral blood monocytes using a modified method described by Dignam et al. (20), and EMSA were performed using 2.5 μg nuclear extract as described previously (21).

Detection of immunoreactive IL-8

Monocytes were stimulated with LPS at a concentration of 10 ng/ml for various times up to 8 h. The conditioned media were collected, and secreted IL-8 was measured by ELISA using a commercial kit (Genzyme, Cambridge, MA) according to the manufacturer’s recommended protocol. The quantities of secreted IL-8 in the test samples were determined using a standard curve generated with purified recombinant human IL-8 provided with the kit.

Luciferase activity assay

The plasmid pIL-8(κB)LUC (wild-type (WT)-IL-8-LUC) contains a κB site from the promoter region of the IL-8 gene, and a separate plasmid pIL-8(κB)LUC (κB-L8-LUC) has a nonfunctional mutant κB site. Both constructs were provided by Dr. N. Mackman (The Scripps Research Institute) (22). The plasmid pCMVβ (Clontech Laboratories, Palo Alto, CA) was used as a control for monitoring the transfection efficiency by the expression of β-galactosidase. THP1 cells were transiently transfected using DEAE-dextran (23) and were cultivated for 48 h before a 4-h stimulation with medium or LPS (10 ng/ml). Luciferase activity was determined using the luciferase assay kit (Promega) and the Monolight 2010 luminometer (Analytical Luminescence, San Diego, CA).

Isolation of transfected cells

Transfected THP1 cells were specifically isolated using the Capture-TecTM pHookTM-2 system (Invitrogen, San Diego, CA) according to the manufacturer’s protocol. Briefly, 4 × 10^6 THP1 cells were transfected with the pHookTM-2 plasmid that directs the synthesis of a fusion protein containing the platelet-derived growth factor receptor transmembrane domain fused to a single-stranded cell surface Ab recognizing the hepten 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (used in isolation) and were then recovered by incubating the cells in suspension for 30 min at 37°C with 2 × 10^6 magnetic beads coupled to 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one, followed by magnetic separation.

Results

LPS stimulates RhoA activity in human monocytes

To assess the ability of LPS to activate low m.w. GTPase Rho in monocytes, human peripheral blood monocytes were stimulated with LPS (100 nM) for varying times, total cell lysates were incubated with GST or GST-RBD beads, and Western blotting of the bound proteins was performed as described in Materials and Methods. Fig. 1 shows that LPS induced an increase in RhoA activity in a time-dependent fashion. Compared with the medium-alone control (Fig. 1A, lane 3), LPS induced RhoA activity within 5 min (lane 5), and RhoA activity peaked at 10 min (lane 7). In contrast, no RhoA was detected bound to beads with GST alone (lane 1). RhoA activity was also increased 5- to 9-fold after 10-min stimulation of cells with TNF-α (data not shown). Activation of RhoA by LPS in monocytes suggests (but does not prove) that RhoA plays a role in downstream signaling. The next logical step was to determine whether RhoA activation was necessary for proinflammatory cytokine gene expression. This question was addressed by using a Rho inhibitor as well as the dominant negative RhoA mutant plasmid.

RhoA activity is required for LPS-induced cytokine gene expression

Unstimulated human peripheral blood monocytes produced little IL-8. Addition of LPS (100 ng/ml), IL-1β (100 pg/ml), or TNF-α (40 ng/ml) resulted in production of IL-8 as measured by ELISA (Fig. 2A). We pretreated human peripheral blood monocytes with Clostridium difficile toxin B, an inhibitor of Rho, Rac, and Cdc42 proteins (24). C. difficile toxin B (40 ng/ml for 2 h) completely inhibited LPS-induced IL-8 synthesis (data not shown). We then tested whether a specific Rho inhibitor would also inhibit LPS-induced IL-8 synthesis. C3 transferase exoenzyme is an exotoxin...
produced by C. botulinum that specifically inhibits the Rho small GTP binding proteins (RhoA, -B, and -C), but does not inhibit Rac or Cdc42 (24). Preincubation of monocytes with 10 μg/ml recombinant C3 transverse exoenzyme completely abolished LPS- and IL-1β-stimulated IL-8 synthesis (Fig. 2A), but only marginally affected TNF-α-induced IL-8 synthesis (Fig. 2A, lane 7 vs lane 4). NF-κB is known to stimulate the transcription of many inflammatory genes, including a large number of cytokines (25). Preincubation with recombinant C3 transverse exoenzyme also inhibited LPS- and IL-1β-stimulated NF-κB activation in monocytes (Fig. 2B, lanes 5 and 6 vs lanes 2 and 3). Consistent with its effects on IL-8 synthesis, recombinant C3 transverse exoenzyme did not significantly affect TNF-α-induced NF-κB activation (lane 7 vs lane 4).

To further confirm the requirement for RhoA in LPS-induced IL-8 synthesis, monocytic THP1 cells were cotransfected with an expression vector encoding a dominant negative form of RhoA (RhoA-T19N) and a plasmid containing the promoter region of the IL-8 gene fused to the luciferase reporter gene (WT-IL-8-LUC) before LPS stimulation. Fig. 3 shows the effect of the dominant negative RhoA plasmid on LPS-stimulated luciferase activity in THP1 cells transfected with the chimeric IL-8 luciferase plasmid. LPS stimulated increased luciferase activity in THP1 cells transfected with the WT-IL-8-LUC plasmid (Fig. 3A, lane 2). Cotransfection of the dominant negative RhoA plasmid (2 μg) with the WT-IL-8-LUC plasmid completely blocked LPS-stimulated luciferase activity (Fig. 3A, lane 4). Fig. 3B shows that the degree of RhoA-T19N-mediated inhibition of LPS-stimulated luciferase activity was progressively lowered as the amount of transfected RhoA-T19N was decreased (Fig. 3B, lanes 3–7). Thus, RhoA-T19N blocked LPS-induced IL-8 synthesis in a dose-dependent manner. These results confirm that RhoA activity is required for LPS-induced NF-κB activation and cytokine gene expression.

The relationship between RhoA activity and subsequent IL-8 gene expression was further explored using RhoA-Q63L, a constitutively active form of RhoA. Transfection of THP1 cells with RhoA-Q63L resulted in increased luciferase activity when the cells were cotransfected with a WT-IL-8-LUC reporter plasmid containing functional κB site from the promoter region of the IL-8 gene (Fig. 3C, lane 2). When the THP1 cells were cotransfected with a separate plasmid pIL-8(μLUC) (which has a nonfunctional mutant κB site), the constitutively active RhoA did not stimulate luciferase activity (Fig. 3C, lane 3). Thus, we have shown that RhoA activity alone is sufficient to induce NF-κB activity. Taken together with the previous results, this strongly suggests that LPS-mediated IL-8 synthesis results from RhoA-dependent NF-κB activation.

LPS stimulates RhoA activity and cytokine gene transcription through TLR4

To determine whether TLR4 mediates LPS-induced RhoA activity and subsequent activation of NF-κB, HEK 293 cells were transiently cotransfected with TLR4 cDNA or an empty vector
(pcDNA3). After a 48-h incubation in a normal culture medium, cells were stimulated with the medium alone or LPS (100 ng/ml) and then harvested. RhoA activity was detected by cellular GTP-Rho assay (Fig. 4A), NF-κB activity was monitored by EMSA (Fig. 4B), and IL-8 synthesis was measured by ELISA using the manufacturer’s recommended protocol as described in Materials and Methods (Fig. 4C). In HEK293 cells overexpressing TLR4 (but not control cells, which do not express TLR4), LPS induced RhoA activity 3-fold (Fig. 4A, right panel, lane 2 vs lane 1), activated NF-κB 3.5-fold (Fig. 4B, right panel, lane 2 vs lane 1), and stimulated IL-8 protein 5.5-fold (Fig. 4C, right panel, lane 2 vs lane 1) compared with medium controls. These results suggest that LPS, acting through TLR4, stimulates RhoA activity and thereby induces NF-κB activation and IL-8 gene expression.

**LPS-induced RhoA activation is mediated by IRAK and MyD88 in THP1 cells**

In the IL-1 signaling pathway, activation of NF-κB and transcription of cytokine gene are mediated through the signal transduction molecules MyD88 and IRAK. Because the cytoplasmic domain of TLR4 is homologous to the IL-1R, we tested whether LPS-induced RhoA activity was also mediated through these signal transduction molecules. Monocytic THP-1 cells stably expressing CD14 were transiently cotransfected with dominant negative forms of IRAK, MyD88, or IKK-2 and chimeric IL-8 luciferase plasmid (WT-IL-8-LUC). Fig. 5A shows that cotransfection of the dominant negative IRAK, MyD88, or IKK-2 plasmids with the WT-IL-8-LUC plasmid completely blocked IL-1-stimulated luciferase activity, while dominant negative MyD88 also significantly inhibited LPS-induced luciferase activity (Fig. 5B).

Further demonstration of the role of these molecules in LPS-induced RhoA activity was obtained by overexpressing dominant negative forms of IRAK, MyD88, or IKK-2 in THP1 cells, then the transfected cells were selected by the capture-TecTM pHookTM-2 system. Transfected cells were stimulated with LPS (100 ng/ml) or IL-1β (100 pg/ml) and harvested. RhoA activity was detected by cellular GTP-Rho assay (Fig. 6A), NF-κB activity was monitored by EMSA (Fig. 6B), and IL-8 synthesis was measured by ELISA (Fig. 6C). LPS- and IL-1β-induced RhoA activity, NF-κB translocation, and IL-8 production were completely inhibited by transfection of dominant negative IRAK. Dominant negative MyD88 effectively blocked IL-1β-induced responses and significantly inhibited LPS-induced activation. Dominant negative IKK-2 completely abolished both LPS- and IL-1β-induced NF-κB activation and IL-8 production, but only marginally affected RhoA activity induced by LPS and IL-1β. These data indicate that IRAK and MyD88 are required for both LPS- and IL-1-induced responses, while IKK-2 is required for NF-κB and IL-8 transcription, but not for RhoA activity in THP1 cells.

**Discussion**

Low m.w. G proteins of the Rho family have been shown to play an important role in numerous aspects of cytoskeletal rearrangements in fibroblasts. The function of these small G proteins in...
NF-κB activity (14). Despite the fact that the small G protein RhoA has been shown to regulate actin cytoskeletons, focal adhesion complex formation, cell aggregation, and cell motility (10–12), no studies other than those cited above have specifically addressed the nature and importance of RhoA signaling in chemokine gene expression in leukocytes. We demonstrated that LPS induced a time-dependent increase in RhoA activity, suggesting that RhoA plays a role in downstream signaling. These results were supported by the ability of C3 transferase, an inhibitor of Rho, to abolish LPS-induced NF-κB activation and IL-8 production. The role of Rho in LPS-induced cytokine gene transcription was then confirmed by cotransfecting THP1 cells with a dominant negative RhoA plasmid as well as the chimeric IL-8–LUC reporter plasmid. The dominant negative RhoA plasmid significantly abrogated the LPS-stimulated luciferase activity. These results suggest that LPS-induced IL-8 gene transcription require activation of RhoA. The relationship between RhoA activity and subsequent IL-8 gene transcription was further explored using a constitutively active form of RhoA (RhoA-Q63L), which increased IL-8 gene transcription in the absence of additional stimulation. Taken together with previous results, these data suggest that LPS-induced IL-8 gene transcription results from RhoA activity. These results are in agreement with a recent report that constitutively active Rho proteins could activate NF-κB in NIH-3T3 cells (27). In addition, Rawadi et al. (32) found that mycoplasma fermentans lipoprotein-induced secretion of TNF-α was also dependent on RhoA. We found, however, that TNF-α-induced NF-κB activation in monocytes was not affected by inhibition of RhoA. The intracellular signaling pathways linking RhoA and cytokine gene transcription need to be further defined. The Rho family of small G proteins has been shown to activate several serine/threonine kinases, which can mediate downstream effects, particularly on the actin cytoskeleton. PAK1 is the primary kinase activated by both Cdc42 and Rac1, but not by RhoA (33). Several closely related serine/threonine kinases appear to be activated by RhoA, including p120 protein kinase N, p160 Rho-associated coiled coil-containing protein kinase, p164 Rho kinase, and p140 RSK2 kinase (34). Several lines of evidence support a potential role for Rho kinase as a signal molecule involved in gene expression. Chihara et al. (35) found that the constitutively active form of Rho kinase (chloramphenicol acetyltransferase (CAT)) strongly activated the transcription activity of the c-Fos serum response element. In addition, a dominant negative mutant of Rho kinase (Coil-KD) inhibited activation of the serum response element by either CAT or a constitutively active form of RhoA (35). These results suggest that Rho kinase, which is a downstream target of RhoA GTase, may be involved in activation of gene transcription.

A major advance in our understanding of LPS-mediated cytokine gene transcription was the discovery that TLR4 encodes the LPS receptor and transduces the effect of LPS stimulation. To assess whether TLR4 mediates LPS-induced RhoA activity and subsequent activation of NF-κB, HEK 293 cells were transiently cotransfected with TLR4 cDNA or an empty vector. LPS stimulated RhoA activity, NF-κB activation, and IL-8 production in the cells overexpressing TLR4, not in the cells transfected with the empty vector. These results suggest that LPS, acting through TLR4, stimulates RhoA activity and thereby induces NF-κB activation and IL-8 gene expression. The mechanism for RhoA activity mediated by TLR4 is currently unknown, although TLR-mediated NF-κB activation has been proposed to occur via a signaling pathway that is also used by IL-1 (15, 36). This pathway is activated by the interaction between myeloid differentiation protein (MyD88) with the receptor, followed by stimulation of IRAK. To test the possibility that LPS-induced RhoA activity involves MyD88 and IRAK, THP1 cells were transiently transfected with
dominant negative forms of MyD88, IRAK, or IKK-2. Our data demonstrated that dominant negative IRAK1−/−, which may block all four IRAKs, completely blocked both LPS- and IL-1-induced cytokine gene expression. Dominant negative MyD88 completely blocked IL-1-induced responses and had a 60% inhibitory effect on LPS-induced activation. These results, therefore, suggest that LPS-induced NF-κB activation may use a signaling pathway that is more complex than the signaling pathway used by IL-1. Recently, Medzhitov et al. (37) suggested that TLR4 is coupled to a novel signal molecule, TIR domain-containing adapter protein, that is independent of MyD88 in TLR4-mediated NF-κB signaling. Medzhitov et al. (37) suggested that TLR4 is coupled to a novel signal molecule, TIR domain-containing adapter protein, that is independent of MyD88 in TLR4-mediated NF-κB signaling. Medzhitov et al. (37) suggested that TLR4 is coupled to a novel signal molecule, TIR domain-containing adapter protein, that is independent of MyD88 in TLR4-mediated NF-κB signaling.

In summary, we have shown that LPS, acting through TLR4, rapidly activates RhoA GTPase and subsequently increases transcription of IL-8 in human peripheral blood monocytes. We have also shown that LPS-induced IL-8 synthesis requires activation of RhoA, and that RhoA alone is sufficient stimulus to transcribe IL-8 in THP1 cells. Furthermore, we have shown that IRAK is required for LPS-induced RhoA activity and IL-8 production. These findings provide the first evidence that RhoA and IRAK are novel signal transducers for LPS-induced TLR4-mediated proinflammatory cytokine synthesis in human monocytes. Additional experiments are needed to define the signaling steps both upstream and downstream of RhoA and the relationship between RhoA and IRAK that is necessary for proinflammatory cytokine gene expression induced by LPS.

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