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To identify the TLR4-initiated signaling events that couple to formyl peptide receptor (FPR)1 mRNA stabilization, macrophages were treated with LPS along with a selection of compounds targeting several known signaling pathways. Although inhibitors of protein tyrosine kinases, MAPKs, and stress-activated kinases had little or no effect on the response to LPS, LY294002 (LY2) and parthenolide (an IκB kinase inhibitor) were both potent inhibitors. LY2 but not parthenolide blocked the LPS-induced stabilization of FPR1 mRNA. Although both LY2 and wortmannin effectively blocked PI3K activity, wortmannin had little effect on FPR1 expression and did not modulate the decay of FPR1 mRNA. Moreover, although LY2 was demonstrated to be a potent inhibitor of PI3K activity, a structural analog of LY2, LY303511 (LY3), which did not inhibit PI3K, was equally effective at preventing LPS-stimulated FPR1 expression. The mammalian target of rapamycin activity (measured as phospho-p70S6 kinase) was activated by LPS but not significantly blocked by LY2. In addition, although rapamycin blocked mTOR activity, it did not inhibit FPR1 mRNA expression. Finally, the mechanisms involved in stabilization of FPR1 by LPS could be distinguished from those involved in stabilization of AU-rich mRNAs because the prolonged half-life of FPR1 mRNA was insensitive to the inhibition of p38 MAPK. These findings demonstrate that LY2/LY3 targets a novel TLR4-linked signaling pathway that selectively couples to the stabilization of FPR1 mRNA.

Inflammation is a critical response to injury and infection that coordinates the development and execution of antimicrobial activities, the elimination of damaged cells and tissue, and the restoration of normal tissue structure and function. It is, however, a process that exhibits significant potential for unnecessary tissue damage and hence there are multiple levels of regulation (1). In particular, the magnitude and character of an inflammatory response are influenced by controlling the number and type of inflammatory leukocytes that are recruited to a site of injury (2–4). Leukocyte trafficking itself is subject to regulation at many levels including the expression or function of chemoattractants and their cognate cell surface receptors (2–5).

N-terminal formyl-methionine-containing peptides serve as molecular signatures of prokaryotic microbes and provide a potent chemoattractant signal to leukocytes through their recognition by the formyl peptide receptor (FPR) (6, 7). The founding member, FPR1, encodes a G protein-coupled receptor that exhibits high affinity for formylated peptides such as fMLP and promotes not only chemotactic responses in leukocytes but also degranulation and superoxide production. The function of FPR1 can be regulated directly via covalent modifications of the protein as well as by altering the levels of FPR1 protein (6–9). Protein levels are modulated both in terms of their subcellular distribution and by regulating expression of the gene (8, 10, 11). We have recently reported that FPR1 expression is increased by the action of LPS and other ligands for TLRs on mononuclear phagocytes and neutrophils both by enhancing gene transcription and by prolonging the half-life of the mRNA (12).

LPS signals to cells through TLR4 and has been shown to use at least two pathways that can be distinguished by their dependence upon one of two TLR adaptor proteins (MyD88 and Toll/IL-1R domain-containing adaptor-inducing IFN-κB (TRIF)) (13–16). The TRIF pathway has been linked with induction and secretion of IFN-κB, and many of the endpoints of this pathway are the result of the intermediate paracrine or autocrine action of this cytokine (16–18). The induction of FPR1 in response to LPS, at least in part, upon the intermediate secretion of a protein, though neither type I nor type II IFNs were able to induce the expression of FPR1 (12). Using mice deficient in either MyD88 or TRIF, it was shown that LPS-mediated induction of FPR1 requires only MyD88 and not TRIF (19). Collectively, these observations suggested that this process might represent a distinct pattern of response to LPS.

In this study, we have explored the signaling events that are requisite to the action of LPS in promoting enhanced expression of FPR1 mRNA with a particular focus upon stabilization of its mRNA. By examining in detail the effects of compounds targeting several different signal transduction components, we now report that LPS-induced FPR1 expression is regulated through a distinct pathway that is defined by highly selective sensitivity to LY294002 (LY2). Furthermore, the LY2-sensitive pathway is linked to the stabilization of FPR1 mRNA. Although LY2 is known as an inhibitor of the PI3K, the PI3K does not appear to be involved in FPR1 induction by LPS. In addition, the LPS-induced stabilization of FPR1 mRNA is mechanistically distinct from the...
well-recognized TLR-dependent stabilization of AU-rich mRNAs as these processes show differential sensitivity to LY2 and the p38 kinase inhibitor SB203580. These findings demonstrate that LPS uses multiple distinct signaling events to control the posttranscriptional regulation of genes involved in the inflammatory response.

Materials and Methods

Reagents

RPMI 1640 and Dulbecco’s PBS were obtained from the Media Laboratory at the Lerner Research Institute (Cleveland Clinic Foundation, Cleveland, OH). Antibiotics, agarose, and Tris were purchased from Invitrogen Life Technologies. Formamide, dextran sulfate, salmon sperm DNA, actinomycin D (Act D), MOPS, MG132, and LPS (prepared from the Escherichia coli serotype O111:B4) were purchased from Sigma-Aldrich. RNase-free DNase and RNasin were obtained from Promega. Brewer’s thiglycolate broth was obtained from BD Biosciences. FBS was purchased from Bio-Southwest. DNase and RNasin were obtained from Promega. Brewer’s thiglycolate broth was obtained from BD Biosciences. FBS was purchased from Bio-Southwest. 

Preparation of whole cell lysate

Treated macrophages were washed twice with ice-cold PBS. Whole cell extracts were obtained from washed macrophages by using ice-cold lysis buffer (20 mM Tris-HCL, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1.5 mM MgCl2, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 50 mM NaF, 10 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 μM leupeptin, 1 mM PMSF, and protease inhibitor (Protease Inhibitor Cocktail Tablets; Roche)) for 30 min. After incubation the lysates were centrifuged at 16,000 × g for 15 min at 4°C. The protein concentration of each sample was assayed using a Bio-Rad protein assay kit standardized to bovine serum albumin.

Western blot analysis

Western analysis for the presence of specific proteins or for phosphorylated forms of proteins was performed on whole cell lysates from primary macrophages. Protein (20–40 μg) was mixed 1:1 with 2× sample buffer (20% glycerol, 0.5% SDS, 0.05% bromophenol blue, and 0.01% Triton X-100). The samples were heated at 95°C for 5 min and then electrophoresed by using a 10% SDS-PAGE gel. The separated proteins were transferred to a nitrocellulose transfer membrane and processed as described in Materials and Methods.

Preparation of RNA and Northern blot hybridization analysis

Total cellular RNA was extracted from primary peritoneal exudate macrophages by the guanidine thiocyanate-cesium chloride method (22). Equal amounts of RNA (20 μg) were analyzed by Northern hybridization as previously described (21). Autoradiographs were quantified by image analysis using the NIH Image software package. Specific mRNA levels were normalized to levels of GAPDH mRNA measured in the same RNA sample.

Preparation of peritoneal exudate macrophages

Specific pathogen-free, female C57BL/6 mice, 6–8 wk of age, were purchased from Charles River Breeding Laboratories. All animals were housed in microisolator cages with autoclaved food and bedding to minimize exposure to viral and microbial pathogens and all procedures were approved by the Institutional Animal Care and Use Committee. Thiglycolate broth-elicited peritoneal macrophages were prepared as previously described (20) and cultured in RPMI 1640 medium containing t-glutamine, penicillin, streptomycin, and 5% FBS. The macrophages were cultured overnight in RPMI 1640 at 37°C in an atmosphere of 5% CO2 and then treated with stimuli for the indicated times as described.

Preparation of plasmids

The plasmids containing FPR1, GAPDH, and KC cDNA fragments were as previously described (12, 21). Plasmids were prepared using kits from Qiagen according to the manufacturer’s instructions.

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Because levels of FPR1 are very low in untreated cells and both events are mechanistically linked with the alteration in mRNA levels, we next wished to determine whether either one or both LY2 (a PI3K-sensitive event) or parthenolide (a 26 S proteasome-sensitive event) that contribute to the elevation in FPR1 mRNA accumulation. Interestingly, the compound free of LPS (and other TLR ligands) is known to be potent stimuli of the activation of NF-κB. To begin to examine the pathways responsible, we evaluated the effects of several agents known to inhibit a selection of different protein kinase activities on the expression of FPR1 mRNA. In macrophages treated with LPS for 4 h, inhibitors of the p38, ERK, and JNK MAPKs (SB203580, PD589320, and SP600125, respectively) had little or no effect on the levels of FPR1 mRNA (Fig. 1). Likewise, although the broad spectrum protein tyrosine kinase inhibitor genistein has been reported to block the effects of several agents known to inhibit PI3K family members, LY2, was able to reduce LPS-induced levels of FPR1 mRNA by >95%. The expression levels of another well-described LPS-inducible mRNA that is also known to be regulated via posttranscriptional mRNA stabilization (CXCL1 or KC neutrophil chemoattractant) were unaffected by any of these compounds demonstrating the selective nature of the inhibitory action of LY2. Because LPS (and other TLR ligands) is known to be potent stimuli of the activation of NF-κB, we also examined the effects of inhibiting this pathway. Inclusion of parthenolide, a relatively specific inhibitor of the IκB kinases (IKKs) (24), produced, like LY2, a profound inhibition of FPR1 mRNA accumulation. Unlike LY2, however, parthenolide treatment inhibited the expression of both KC and FPR1 mRNAs equivalently. Similar results were obtained when macrophages were treated with an inhibitor of the 26 S proteasome, MG132, which is known to be involved in the NF-κB activation pathway by degrading phosphorylated and ubiquitinated IκBα (data not shown) (25).

We previously reported that LPS-mediated induction of FPR1 mRNA involves prolongation of mRNA decay (12). Because there appear to be two separate signaling pathways (NF-κB activation and an LY2-sensitive event) that contribute to the elevation in mRNA levels, we next wished to determine whether either one or both events are mechanistically linked with the alteration in mRNA stability. Because levels of FPR1 are very low in untreated cells and both LY2 and parthenolide treatments fully block the accumulation of message, we evaluated whether either compound could modulate FPR1 mRNA decay when added after LPS treatment has already begun. The membrane was then blocked with 5% nonfat milk in 1× TBST for 2 h, washed and then incubated with the primary Ab overnight at 4°C. The blots were washed four times with TBST and incubated for 1 h with HRP-conjugated anti-IgG. Bands were developed using a chemiluminescent substrate (ECL plus; PerkinElmer). Blots were examined by autoradiography with exposure times ranging from 10 s to 2 min.

Results

The induction of FPR1 mRNA in response to LPS appears to represent a distinct pattern of signaling response initiated through TLR4. To begin to examine the pathways responsible, we evaluated the effects of several agents known to inhibit a selection of different protein kinase activities on the expression of FPR1 mRNA. In macrophages treated with LPS for 4 h, inhibitors of the p38, ERK, and JNK MAPKs (SB203580, PD589320, and SP600125, respectively) had little or no effect on the levels of FPR1 mRNA (Fig. 1). Likewise, although the broad spectrum protein tyrosine kinase inhibitor genistein has been reported to block the effects of several agents known to inhibit PI3K family members, LY2, was able to reduce LPS-induced levels of FPR1 mRNA by >95%. The expression levels of another well-described LPS-inducible mRNA that is also known to be regulated via posttranscriptional mRNA stabilization (CXCL1 or KC neutrophil chemoattractant) were unaffected by any of these compounds demonstrating the selective nature of the inhibitory action of LY2. Because LPS (and other TLR ligands) is known to be potent stimuli of the activation of NF-κB, we also examined the effects of inhibiting this pathway. Inclusion of parthenolide, a relatively specific inhibitor of the IκB kinases (IKKs) (24), produced, like LY2, a profound inhibition of FPR1 mRNA accumulation. Unlike LY2, however, parthenolide treatment inhibited the expression of both KC and FPR1 mRNAs equivalently. Similar results were obtained when macrophages were treated with an inhibitor of the 26 S proteasome, MG132, which is known to be involved in the NF-κB activation pathway by degrading phosphorylated and ubiquitinated IκBα (data not shown) (25).

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Thioglycolate-elicited macrophages were stimulated with LPS (100 ng/ml) for the indicated time points before preparation of cell extracts and analysis by Western blot for total and phospho-p70S6 kinase. As a final experiment to assess the contribution of PI3K activity in the induction of FPR1 mRNA by LPS, we used LY3, which is a compound structurally related to LY2 that is reported to lack significant activity toward PI3K (28). In LPS-treated macrophages, LY3 exhibited little or no inhibitory effect on AKT phosphorylation, whereas LY2 fully blocked activity at 1 μM (Fig. 5B). Although FPR1 mRNA is stable in LPS-treated cells, the addition of LY2 caused a dramatic decrease in the half-life of the message confirming the findings presented in Fig. 2. Interestingly, wortmannin had no effect on decay, further supporting the conclusion that PI3K activation by LPS does not contribute to the stabilization of FPR1 mRNA.

As a final experiment to assess the contribution of PI3K activity in the induction of FPR1 mRNA by LPS, we used LY3, which is a compound structurally related to LY2 that is reported to lack significant activity toward PI3K (28). In LPS-treated macrophages, LY3 exhibited little or no inhibitory effect on AKT phosphorylation, whereas LY2 fully blocked activity at 1 μM (Fig. 5A). Interestingly, although these two compounds varied significantly with respect to their capacity to block PI3K activity as well as FPR1 mRNA accumulation. Both LY2 and wortmannin were highly efficient at blocking the phosphorylation of AKT even at the lowest dose applied (1 μM and 25 nM, respectively) (Fig. 4A). Under similar treatment conditions, LY2 at 1 μM fully blocked LPS-induced FPR1 mRNA accumulation, whereas wortmannin treatment only modestly reduced the levels of FPR1 mRNA at all concentrations tested (Fig. 4B). These results suggest that PI3K may not be the primary target of LY2 and is, at best, only partially involved in LPS-stimulated FPR1 expression.

To more fully explore the role of PI3K activity in LPS-induced FPR1 expression, we determined whether wortmannin, at concentrations that can fully block PI3K, could modulate the decay of FPR1 mRNA. Macrophages were treated with LPS for 4 h, and Act D was added to block further transcription of the FPR1 gene. In some cultures, either LY2 or wortmannin were added along with Act D and the levels of residual FPR1 mRNA were assessed by Northern blot analysis after 2 or 4 h of further incubation (Fig. 4C). Although FPR1 mRNA is stable in LPS-treated cells, the addition of LY2 caused a dramatic decrease in the half-life of the message confirming the findings presented in Fig. 2. Interestingly, wortmannin had no effect on decay, further supporting the conclusion that PI3K activation by LPS does not contribute to the stabilization of FPR1 mRNA.
LPS STABILIZES FPR1 mRNA

LY2 is reported to inhibit members of the PI3K-related kinase family that include the mammalian target of rapamycin (mTOR) kinase (29). Hence, we wished to determine whether this enzyme might be involved in LPS-induced expression of FPR1 mRNA. LPS was able to activate mTOR kinase as measured by the phosphorylation of p70S6 kinase (Fig. 6A). Interestingly, the time course for this function was distinct from that for LPS-mediated activation of AKT phosphorylation, suggesting that p70S6 kinase is not activated via AKT or downstream of PI3K. LPS-stimulated p70S6 kinase phosphorylation was fully blocked by rapamycin, indicating that this activation was mediated by mTOR. Consistent with the different kinetics of activation for p70S6 kinase and AKT, LY2 had very modest inhibitory effects on phosphorylation of p70S6 kinase (Fig. 6B). As expected, LY2 exhibited strong inhibition of LPS-stimulated FPR1 mRNA levels, whereas rapamycin caused little or no reduction (Fig. 6C). Thus, although mTOR is activated following LPS stimulation, this does not appear to be an essential part of the pathway through which LPS modulates FPR1 expression.

LPS treatment is known to stabilize unstable cytokine and chemokine mRNAs that contain AU-rich sequences in their 3′ untranslated regions, and this activity has been demonstrated to involve the activation of p38 MAPK (30, 31). To determine whether this pathway might also be involved in stabilization of FPR1 mRNA, peritoneal macrophages were treated with LPS followed by the addition of Act D alone or along with either LY2 or SB203580 (an inhibitor of p38 kinase). Although LY2 treatment results in a dramatic increase in decay of FPR1 mRNA, SB203580 had no effect (Fig. 7). When the same blot was hybridized with a probe for the mouse KC gene, SB203580 markedly reduced the half-life for this mRNA while LY2 treatment had no effect. These findings indicate that LPS can modulate mRNA stability through at least two distinct mechanisms.

Discussion

TLR4 is known to signal through two pathways defined by the distinct Toll/IL-1R domain-interacting adaptor proteins MyD88 and TRIF (16). Although MyD88 is known to be necessary for direct cytokine gene expression via the activation of NF-κB, the non-MyD88 pathway depends partially on the intermediate production of IFN-β (16–18, 32). LPS-induced FPR1 also depends at least partially upon the production of secreted factors but cannot be induced by IFN-β (12). Moreover, a recent report indicates that LPS-induced FPR1 expression is MyD88-dependent and TRIF-independent (19). These findings suggested that the induction of FPR1 might represent a novel pattern of response through TLR4 and prompted the present examination of signaling pathways that might be involved. Our findings support the conclusion that the TLR4-dependent elevation in FPR1 expression requires at least two separate signaling pathways: one appears to be the activation of NF-κB (which is also required for the expression of the chemokine KC) (33), whereas the other, although yet to be identified, is targeted selectively by the LY2/LY3 inhibitor family. Furthermore, these two pathways operate through different mechanisms. These conclusions are supported by the following findings: 1) although inhibitors of NF-κB activation are able to fully inhibit the LPS-stimulated expression of both KC and FPR1 mRNAs, LY2 only blocks FPR1; 2) the LY2-sensitive pathway does not appear to involve PI3K because wortmannin has little or no inhibitory activity for FPR1 expression despite effectively inhibiting PI3K; 3) moreover, LY3 does not inhibit PI3K but does block FPR1 mRNA expression; and 4) treatment of macrophages with LY2 but not wortmannin or parthenolide after stimulation with LPS rapidly reduces FPR1 mRNA levels by abrogation of the LPS-induced prolongation of mRNA half-life.

The experimental results presented are based exclusively on use of primary mouse macrophages, and the conclusions are heavily dependent upon the use of cell permeable small molecule inhibitors that exhibit, at best, only relative specificity for their assumed targets. Indeed, one of the major observations demonstrates the relative lack of specificity exhibited by the LY2 compound. Because of the extremely limited transfection sensitivity of primary macrophages, we have been unable to use genetic manipulation strategies to further explore the issues. The major conclusion, however, is the exclusion of signaling pathways including PI3K, which is strongly supported by the evidence provided. Furthermore, a number of additional experimental observations demonstrate the selective nature of the inhibitory activity of these compounds and thereby increase the rigor of the conclusions. First, the inhibition of FPR1 mRNA expression exhibits selective sensitivity to LY2 as compared with another LPS-inducible gene (KC). Second, the ability of LPS to activate the both PI3K and mTOR kinase and the efficacy of the selected compounds to inhibit these pathways has been demonstrated. Finally, the effects of individual inhibitors on LPS-mediated stabilization of FPR1 mRNA illustrated the mechanistic specificity for the action of the LY family compounds particularly as compared with other inhibitors of PI3K.
Although the class I PI3K enzymes are the most likely targets for activation by LPS, there are multiple members of the PI3K and PI3K-related (PIKK) kinases that are also reported to exhibit sensitivity to LY2 (34, 35). The latter includes the mTOR kinases as well as ATM family kinases that are known to participate in regulation of responses to genotoxic stress. The insensitivity of LPS-induced FPR1 mRNA expression to treatment with rapamycin (a specific inhibitor of mTOR) largely rules out the participation of this subset. Furthermore, class II and class III PI3K family members and other PI3K-related kinases remain to be evaluated, but because they have often been reported to shown sensitivity to wortmannin (which did not inhibit FPR1 mRNA stabilization), they appear unlikely candidates (29, 35). Finally, there are numerous studies demonstrating that PI3K plays a negative or inhibitory role in controlling proinflammatory gene expression in immune cell types (36, 37). Thus, it seems unlikely that other PI3K family members are participating in the TLR-dependent change in FPR1 mRNA stability.

We must also consider LY-sensitive targets that are not PI3K-related signaling events. On the basis of the compounds and the strategies used, the data argue against the involvement of ERK1/2, p38, JNK, and genistein-sensitive protein tyrosine kinases because the inhibition of these kinases has little or no inhibitory effect on FPR1 mRNA levels. Although panthelenol can efficiently inhibit the response to LPS when added simultaneously, this IKK inhibitor did not interfere with FPR1 mRNA stability. Interestingly, there are a number of prior reports documenting the differential sensitivity of stimulus-dependent responses to inhibitors of class I PI3K activity. For example, Salih et al. (38) reported that LPS-induced NO production is inhibited by LY2 and rapamycin but not by wortmannin and ascribed this differential behavior to posttranslational mechanisms. A separate study concluded that LY2- and LY3-mediated inhibition of inducible NO synthase expression was a result of the ability of these compounds to inhibit NF-κB (27), although our results do not support this interpretation because neither LY2 nor LY3 inhibited iNOS degradation when used at concentrations that were fully effective at blocking FPR1 mRNA expression. In addition, neither compound was able to block the expression of KC mRNA, an event known to be NF-κB-dependent. Several additional studies have identified LY-sensitive PI3K-independent functions, though the identity of the target remains to be identified (39, 40). Another known target of LY2 is casein kinase 2 (41). Preliminary experiments using a casein kinase 2 inhibitor TBB showed limited efficacy on the LPS-induced FPR1 mRNA levels, suggesting that this kinase is not involved (data not shown). LY2 inhibits PI3K activity through occupancy of the ATP binding site (42). Interestingly, the compound is able to inhibit other kinases through binding in the ATP site as well, although the specific residue interactions are quite different (43). Moreover, LY2 has also been reported to block to other proteins that do not exhibit protein kinase activity (44, 45). Because these interactions also exhibit insensitivity to wortmannin, such interactions would be attractive candidates for the functions identified in the present study.

LPS and other TLR ligands are known to be able to modulate the decay of mRNAs containing AU-rich sequence elements (46, 47). FPR1 mRNA has a very short (~100 nucleotide) 3’ untranslated region and does not appear to have any AU-rich sequence. Moreover, the stability of mRNAs containing such AU-rich elements is known to be sensitive to inhibitors of the p38 MAPK (30, 31). As shown in Fig. 7, SB203580 but not LY2 can destabilize KC mRNA, whereas LY2 reduces the stability of FPR1 mRNA but has no effect on the decay of KC. It should be noted that SB203580 is most effective in blocking mRNA stabilization when added after the initial stimulus, accounting for the lack of inhibitory effect seen when added simultaneously with LPS (48) (Fig. 1). These findings demonstrate that there are at least two mechanisms through which TLRs can modulate mRNA stability in macrophages.

Collectively, the results suggest that the control of FPR1 mRNA decay in response to stimulation with LPS depends upon MyD88-dependent events that have not been previously identified. Although MyD88 is essential for the response, this may reflect the apparent requirement for activation of NF-κB as indicated by the sensitivity to parthenolide. It is not clear whether the additional LY-sensitive signal pathway is also downstream of MyD88 or couples to TLR4 independently. The elucidation of the LY-sensitive target and determination of a mechanistic basis for linkage both upstream (TLR4) and downstream (mRNA stabilization) will require substantial further study.

Disclosures

The authors have no financial conflict of interest.

References


