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Pure Lipopolysaccharide or Synthetic Lipid A Induces Activation of p21Ras in Primary Macrophages through a Pathway Dependent on Src Family Kinases and PI3K

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Recognition of bacterial LPS by macrophages plays a critical role in host defense against infection by Gram-negative bacteria. However, when not tightly regulated, the macrophage’s response to LPS can induce severe disease and septic shock. Although LPS triggers the activation of multiple signaling pathways in macrophages, it was unclear whether these include activation of the p21Ras GTPases. We report that p21Ras is rapidly and transiently activated in murine primary macrophages stimulated with an ultra-pure preparation of LPS or with synthetic lipid A. The molecular basis of this activation was investigated using a pharmacological approach. LPS-induced activation of p21Ras was inhibited in the presence of PP2, LY294002, or wortmannin, suggesting that it depends on the activity of one or more members of the Src kinase family and the subsequent activation of PI3K.

In that pharmacological inhibitors of PI3K inhibited LPS-induced activation of p21Ras, but not activation of ERK, we concluded that LPS-induced activation of ERK occurs through a pathway that is not dependent on the activation of p21Ras. The Journal of Immunology, 2005, 175: 8236–8241.

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3 Abbreviations used in this paper: GEF, guanine-nucleotide exchange factor; 7-AAD, 7-aminoactinomycin D; BM, bone marrow; PKB, protein kinase B; PMA, phorbol myristate acetate; RBD, Ras-binding domain.

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these assays were performed several hours after stimulation by LPS and thus may measure not only the direct actions of LPS, but also effects secondary to the LPS-induced secretion of autocrine factors such as TNF or IL-1.

We report in this study that both ultra-pure LPS and synthetic lipid A, the component of LPS responsible for its ability to stimulate the innate immune system, induce rapid activation of endogenous p21Ras in primary macrophages. Experiments using pharmacological inhibitors of the signaling pathways leading to this activation indicate that p21Ras was not essential for the LPS-induced activation of ERK.

Materials and Methods

Reagents

LPS from Escherichia coli 015 (Calbiotech) and ultra-pure LPS from E. coli K12 (InvivoGen) were used at 5 μg/ml, unless otherwise indicated. Synthetic Lipid A (compound 506) was purchased from the Peptide Institute and was used at 1 μg/ml. CpG oligonucleotides 5′-tecagctgctctgagc3′ (also commonly referred to as ODN 1826) were purchased from Sigma-Aldrich. The inhibitors PP2, LY294002, and wortmannin were purchased from Calbiochem. Polymyxin B sulfate was obtained from Sigma-Aldrich.

Generation of primary macrophages

Bone marrow (BM) cells were isolated from C57BL/6, C57BL/10, C57BL/10scN (TLR-4−/−), or CBA/CaJ mice that had been purchased from The Jackson Laboratory and were cultured for 7 days in RPMI 1640 medium supplemented with 10% FCS and 20% L929-cell conditioned medium as a source of CSF-1. Before stimulation, the adherent macrophages were washed thoroughly with RPMI 1640 medium supplemented with 10% FCS and cultured for 1–2 h without CSF-1. Where indicated, cells were pre-treated for 1 h with PP2 (5 μM), LY294002 (50 μM), or wortmannin (50 nM), then stimulated with LPS. Where indicated, LPS was incubated with polymyxin B (50 μg/ml) for 30 min before use.

Immunoblotting

Primary macrophages were lysed in a buffer containing Triton X-100 (0.5%), Tris (pH 7.5; 50 mM), sodium chloride (150 mM), sodium fluoride (50 mM), sodium pyrophosphate (10 mM), sodium vanadate (1 mM), EDTA (5 mM), PMSF (1 mM), and a mixture of protease inhibitors (Roche). Lysates were clarified by centrifugation at 15,000 × g for 20 min at 4°C, and equivalent amounts of protein were resolved by SDS-PAGE. After transfer onto nitrocellulose membranes, the presence of specific proteins was assessed by immunoblotting using anti-phospho-ERK (no. 9101) antibody as described above. As shown in Fig. 1A, treatment of BM-derived macrophages from CBA/CaJ mice with J5 LPS increased the level of activated p21Ras. Comparison with parallel immunoblots of aliquots of whole cell lysates indicated that 5% of the total p21Ras proteins expressed in these cells was activated in response to J5 LPS, with a maximal response induced by 0.5 μg/ml J5 LPS. A similar dose-response relationship was observed for J5 LPS-induced activation of ERK (Fig. 1B). Activation of p21Ras was detectable as rapidly as 2 min after stimulation with J5 LPS (Fig. 1C) and had decreased below background levels by 10 min. Similar results were observed in primary macrophages from C57BL/6, C3H/OuJ, and C57BL/10 mice (data not shown), with minor differences in the kinetics or dose-response relationships observed among the different strains of mice. In that this preparation of LPS activated p21Ras with rapid kinetics, we concluded that this effect was direct and not secondary to the induction of cytokine expression.

Our findings contradicted those of Buscher et al. (9), who reported that LPS failed to activate Ras in the macrophage cell line BAC-1.2 F5, although ERK was activated efficiently. Because we repeatedly saw p21Ras activation in response to J5 LPS in primary macrophages from a variety of mouse strains, it was tempting to postulate that the lack of activation of Ras by LPS in BAC-1.2 F5 cells reflected an idiosyncrasy of this cell line or the possibility that the intracellular signals triggered by LPS differ depending on the type of macrophage used (13, 14). To address this issue, we treated BAC-1.2 F5 cells with J5 LPS. We observed a clear increase in the level of activated p21Ras (Fig. 1D).

It was possible that the preparation of LPS we used was contaminated with TLR agonists other than LPS, and that these could participate in or be solely responsible for the activation of p21Ras seen in response to J5 LPS. To investigate this possibility, we assessed the effects of pretreating J5 LPS with polymyxin B (PMB), which specifically binds and neutralizes LPS. Pretreatment with PMB reproducibly and significantly decreased the activation of p21Ras triggered by J5 LPS, with a mean ± SEM reduction of 81 ± 4.1% (n = 11). However, the residual activation of p21Ras by PMB-treated J5 LPS suggested that the J5 LPS preparation was contaminated with other TLR agonists that were capable of inducing the activation of p21Ras. Indeed, it has been reported that agonists of both TLR-2 and TLR-9 induce activation of Ras in macrophages (15, 16). These data also raise the possibility that
LPS synergizes with this contaminant in inducing the activation of p21Ras and that, in the absence of such contaminants, LPS itself might be unable to trigger this response.

Both ultra-pure LPS and synthetic lipid A induce activation of p21Ras in BM-derived macrophages

We obtained two commercially available preparations of TLR-4 agonists putatively free from contamination with other bacterial products, an “ultra-pure” K12 LPS and a chemically synthesized analog of lipid A. Previously, it had been reported that agonists of TLR-2/-1, TLR-2/-6, TLR-4, TLR-7, and TLR-9 induce blastogenesis of B lymphocytes (17, 18). Therefore, to assess whether these preparations were indeed free of agonists for TLRs other than TLR-4, we investigated their ability to stimulate blastogenesis of B lymphocytes from either C57BL/10 mice or the TLR-4-deﬁcient C57BL/10ScN mice. As shown in Fig. 2A, ultra-pure K12 LPS (at 5 μg/ml) and synthetic lipid A (at 1 μg/ml) were able to induce blastogenesis of splenocytes from wild-type, C57BL/10 mice. However, even at these high concentrations, neither the ultra-pure K12 LPS nor the synthetic lipid A induced blastogenesis.

**FIGURE 1.** LPS induces rapid activation of p21Ras in BM-derived macrophages from CBA/CaJ mice. A, Whole cell lysates (WCL) of primary macrophages that had been stimulated for 3 min with J5 LPS (doses as indicated) were incubated with GST-RBD proteins coupled to glutathione Sepharose beads to precipitate GTP-bound, activated p21Ras. Proteins that coprecipitated with GST-RBD were eluted, resolved on SDS-PAGE, and immunoblotted using an Ab speciﬁc for p21Ras. Fractions of p21Ras activated in LPS-treated macrophages were assessed by comparison of the levels of p21Ras present in eluates (PD/GST-RBD) and in an aliquot of 10% of the WCL. B, Activation of ERK in BM-derived macrophages stimulated for 3 min with the indicated doses of LPS. C, Pull down with GST-RBD of activated p21Ras from BM-derived macrophages stimulated with 5 μg/ml LPS for the indicated time periods. D, Pull down with GST-RBD of activated p21Ras from BAC 1.2F5 macrophages stimulated with 5 μg/ml LPS for the indicated time periods.

**FIGURE 2.** Ultra-pure preparations of LPS and lipid A that are devoid of contaminants induce activation of p21Ras. A, Splenocytes from wild-type (C57BL/10) or TLR-4-null (C57BL/10ScN) mice were cultured for 48 h in the presence of 5 μg/ml ultra-pure K12 LPS, 1 μg/ml lipid A, or 1 μM CpG or were left untreated. Flow cytometry was performed to assess cell size (forward scatter) and viability (exclusion of 7-AAD). Numbers represent the percentage of cells in each panel. The ability of ultra-pure K12 LPS and lipid A to sustain cell survival/proliferation and to induce a lymphoblastic phenotype was found to be strictly dependent on the expression of TLR-4. B, BM-derived macrophages from wild-type (○) or TLR-4-null (■) mice were cultured for 6 h in the presence of 5 μg/ml ultra-pure LPS from K12 E. coli, 1 μg/ml synthetic lipid A, or 1 μM CpG or were left untreated. The concentration of TNF-α in the supernatants was assessed by ELISA. Results are expressed as the mean ± SEM of three experiments. C, Solutions of 5 μg/ml ultra-pure K12 LPS or 1 μg/ml lipid A, with or without 50 μg/ml polymyxin B, or of polymyxin B alone were incubated at room temperature for 30 min before stimulation of BM-derived macrophages for 3 min. Activation of p21Ras was assessed by pull-down experiments using GST-RBD as bait.
in splenocytes from TLR-null, C57BL/10ScN mice. Indeed, TLR-4-null splenocytes treated with these compounds were indistinguishable from untreated cells with respect to their viability/proliferation or morphology. As a positive control, we showed that these TLR-4-null splenocytes responded as well to CpG as their wild-type counterparts.

We also tested the ultra-pure K12 LPS and lipid A preparations for the presence of contaminants using another highly sensitive biological assay for TLR ligands, the induction of secretion of TNF-α by macrophages. We found that the ability of even high doses of ultra-pure K12 LPS (5 μg/ml) or lipid A (1 μg/ml) to induce TNF-α secretion by BM-derived macrophages was strictly dependent on expression of TLR-4 (Fig. 2). Again, in that low concentrations of ligands of TLR-2/-1, TLR-2/-6, TLR-3, TLR-4, TLR-7, and TLR-9 also trigger TNF-α secretion by macrophages (17, 19), these data indicate that these preparations of ultra-pure K12 LPS and lipid A were not contaminated by biologically significant amounts of other TLR agonists.

We found that the levels of activated p21Ras in BM-derived macrophages from C57BL/6 mice were increased in response to either ultra-pure K12 LPS or lipid A (Fig. 2C); the amplitude and rapid kinetics of this activation were similar to those we observed with the J5 LPS (data not shown). Moreover, treatment with polyymixin B totally abrogated the activation of p21Ras triggered by ultra-pure K12 LPS (101.5 ± 4.5% inhibition; n = 5) and lipid A (100.5 ± 1.7% inhibition; n = 3), as shown in Fig. 2C. Taken together, these results indicate that the preparations of ultra-pure K12 LPS and synthetic lipid A, that were devoid of agonists of TLRs other than TLR-4, induced activation of p21Ras in BM-derived macrophages. Similar effects were observed using the BAC-1.2 F5 macrophage line (data not shown). LPS-induced p21Ras activation requires the activities of kinases of the Src and PI3K families

Pharmacological inhibitors were used to investigate the molecular mechanisms involved in activation of p21Ras in response to LPS in primary macrophages. As shown in Fig. 3A, PP2, an inhibitor of kinases of the Src family, abolished the activation of p21Ras induced in response to LPS (110.8 ± 5.6% inhibition). As shown in Fig. 3A, LY294002 and wortmannin, two compounds that specifically inhibit PI3K through distinct mechanisms, also abrogated LPS-induced activation of p21Ras (95.5 ± 0.9 and 75 ± 1.5% inhibitions, respectively).

To assess the overall impact of these inhibitors on primary macrophages, and to determine whether Src and PI3K were functioning sequentially or in parallel pathways, we next assessed the effects of PP2, LY294002, and wortmannin on the phosphorylation of PKB, an index of PI3K activity (Fig. 3B). As expected, both LY294002 and wortmannin abrogated LPS-induced phosphorylation of PKB, indicating that these compounds were effective in inhibiting PI3K, one of the upstream enzymes required for its activation. Treatment with PP2 also inhibited the LPS-induced phosphorylation of PKB, suggesting that one (or several) member(s) of the Src kinases family is upstream of PI3K in the signaling pathway that leads to activation of p21Ras in response to LPS. However, none of these three inhibitors, PP2, LY294002, or wortmannin, blocked the LPS-induced activation of p38 MAPK (Fig. 3B, lower panels), indicating that the inhibitory effects of these compounds on LPS-induced activation of p21Ras were not artifacts due to general toxicity. Although additional studies will be required to completely delineate the molecular mechanisms responsible for LPS-induced activation of p21Ras, the results of our experiments with pharmacological inhibitors strongly suggested that the activity of the Src and PI3K were required.

**LPS-induced ERK activation is independent of activation of p21Ras**

As activation of p21Ras usually results in activation of ERK (4), we took advantage of the abilities of PP2, LY294002, and wortmannin to inhibit LPS-induced activation of p21Ras to investigate whether this decrease in the activation of p21Ras correlated with decreased activation of ERK. We noted first that treatment of primary macrophages with PP2 inhibited LPS-induced activation of ERK (Fig. 4A) and p21Ras, suggesting that Src activity was required for the activation of both ERK and p21Ras. In contrast, LY294002 and wortmannin, although efficiently inhibiting LPS-induced activation of p21Ras, had only a minor effect, if any, on LPS-induced activation of ERK (Fig. 4B). The fact that LY294002 or wortmannin uncoupled LPS-induced activation of p21Ras from activation of ERK formally demonstrates that LPS can activate ERK through a pathway that does not depend on activation of p21Ras.

![Figure 3](http://www.jimmunol.org/)

**FIGURE 3.** Activation of p21Ras is dependent on the Src/PI3K pathway. BM-derived macrophages were treated for 1 h with DMSO, 5 μM PP2 (Src inhibitor), 50 μM LY294002, or 50 nM wortmannin (PI3K inhibitors), as indicated, before stimulation with J5 LPS for 3 min. Whole-cell lysates were subjected to pull down with GST-RBD to measure levels of activated p21Ras (A) or were analyzed by immunoblotting with anti-phospho-Ser172 PKB and anti-phospho-p38 MAPK Abs (B).
Discussion

In the present study we have demonstrated that ultra-pure preparations of LPS or lipid A induce activation of p21Ras in primary macrophages. This activation was rapid and transient, ruling out the possibility that it resulted from an indirect, autocrine mechanism. The difficulty of obtaining preparations of LPS that are free of contamination by other bacterial products has greatly complicated analysis of the biological effects of LPS. Therefore, we carefully addressed the question of whether the effects of our LPS preparations on p21Ras activation were due to LPS itself rather than potential contaminants. We initially addressed this issue by showing that treatment of the preparation of LPS from E. coli J5 with polymyxin B greatly reduced the activation of p21Ras. However, although suggesting that LPS itself activated p21Ras, these experiments also demonstrated that contaminants that were not affected by polymyxin B also induced activation of p21Ras. This raised the formal possibility that LPS itself might only facilitate the activation of p21Ras induced by other contaminating bacterial products, rather than triggering this response on its own. Therefore, we screened preparations of LPS or lipid A to identify those lacking contaminants active in the absence of TLR-4. We used two assays that have been shown to detect low levels of TLR agonists, namely, blastogenesis of B lymphocytes and TNF-α secretion by primary macrophages (17–19), to compare the effects of different preparations of LPS on wild-type vs TLR-4-null cells. Such contaminants present in preparations of LPS would be revealed by their biological activities on cells lacking TLR-4. We used two primary macrophages (17–19), to compare the effects of different preparations of LPS or lipid A to identify those preferentially expressed in macrophages, are rapidly activated in response to LPS (20, 21). Moreover, stimulation with LPS induced the association of PI3K with Lyn (22). Interpretation of our data demonstrating that the two PI3K inhibitors, LY294002 and wortmannin, inhibited the LPS-induced activation of p21Ras are subject to the usual caveats about the lack of absolute specificity of pharmacological inhibitors. However, the fact that these inhibitors are mechanistically distinct increases the likelihood that their observed inhibitory effects on activation of p21Ras indeed reflect their inhibition of PI3K activity.

It is not clear why our results with BAC-1.2 F5 cells differ from those of Buscher et al. (9), who reported that LPS failed to induce activation of p21Ras. It is possible that the subline of BAC-1.2 F5 cells they used lacked a critical component of the signaling machinery upstream of p21Ras. Regardless of its mechanistic basis, their observation that LPS-induced activation of ERK occurred in the absence of activation of p21Ras is consistent with our conclusions from experiments with inhibitors of PI3K activity that demonstrated uncoupling of LPS-induced activation of p21Ras and ERK.

The present data on LPS, together with published data on peptidoglycan (15), indicate that the main components of the walls of Gram-negative and -positive bacteria, respectively, both stimulate activation of p21Ras in macrophages. Moreover, the binding of CpG-containing bacterial DNA to intracellular TLR-9 also results in activation of Ras (16). However, it is interesting that the molecular mechanisms that lead to Ras activation in response to these three bacterial products appear to differ. Thus, peptidoglycan induces the interaction of TLR-2 with the p85 subunit of PI3K, possibly via a conserved YXXM motif that is present in its intracellular domain, leading to the recruitment of Ras to the receptor complex (15). CpG also stimulates the recruitment of p21Ras to its cognate TLR, TLR-9, although in this case it lacks a consensus binding motif for p85 (16). The consequences of activation of p21Ras by the different bacterial products also appear to differ. The activation of ERK induced by peptidoglycan has been demonstrated to be dependent on activation of p21Ras (15). Likewise, in the case of stimulation with CpG, activation of ERK was dependent on activation of p21Ras, which was also required for formation of the canonical IL-1R-associated kinase-1/TNFR-associated factor-6 complex (16). In contrast, we show in this study that activation of p21Ras was not required for LPS-induced activation of ERK. Thus, it seems that three bacterial products, LPS, peptidoglycan, and CpG, all induce activation of p21Ras and ERK in mammalian cells, albeit through distinct mechanisms. Moreover, in insects, bacterial products also activate p21Ras, which appears to be involved in phagocytosis by hemocytes (23, 24). Thus, throughout evolution, the p21Ras pathway appears to have been repeatedly coupled with host recognition of bacterial products. The fact that these bacterial components activate different signaling cascades that converge upon activation of p21Ras suggests that activation of Ras is important for resistance against both Gram-positive and -negative pathogens. The multiple effector paths downstream of activated Ras control many aspects of cellular function, including activation of gene expression, cellular proliferation and viability, vesicle trafficking, morphology, adhesion, and motility; thus it is likely that LPS-induced activation of p21Ras will contribute to many facets of host defense.

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Disclosures
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