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Bacterial lipopolysaccharide stimulates protein tyrosine phosphorylation in macrophages

(arachidonic acid/herbimycin A/endotoxin)

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ABSTRACT **Lipopolysaccharide (LPS), a membrane component of Gram-negative bacteria, stimulates immune responses by activating macrophages, B lymphocytes, and other cells of the immune system. The mechanisms by which LPS activates these cells are poorly characterized. Since protein tyrosine phosphorylation appears to be a major intracellular signaling event that mediates cellular responses, we examined whether LPS alters tyrosine phosphorylation in macrophages. We found that *Escherichia coli* K235 LPS increased tyrosine phosphorylation of several proteins in the RAW 264.7 murine macrophage cell line and in resident peritoneal macrophages from C3H/HeSNJ mice. Changes in tyrosine phosphorylation were detectable by 4–5 min, reached a maximum by 15 min, and declined after 30–60 min. Protein tyrosine phosphorylation increased following stimulation with LPS at 100 pg/ml and was maximal with 10 ng/ml. Similar changes in tyrosine phosphorylation were induced by *Salmonella minnesota* R595 LPS and by the biologically active domain of LPS, lipid A, but not by the inactive lipid A derivative N^2-monoacylglucosamine 1-phosphate. Phorbol 12-myristate 13-acetate also stimulated protein tyrosine phosphorylation, but some of the modulated proteins were different than those phosphorylated by LPS. Treatment of RAW 264.7 cells with a tyrosine kinase inhibitor, herbimycin A, inhibited both LPS-stimulated tyrosine phosphorylation and LPS-stimulated release of arachidonic acid metabolites. Thus, increased protein tyrosine phosphorylation is a rapid LPS-activated signaling event that may mediate release of arachidonic acid metabolites in RAW 264.7 cells.**

Bacterial lipopolysaccharide (LPS) is a potent activator of the immune system that induces local inflammation, antibody production, and, in severe infections, septic shock (1). Macrophages play a central role in the host defense against bacterial infection and are major cellular targets for LPS action. LPS has multiple effects on macrophages, including the induction of secretion of inflammatory mediators such as interleukin 1, tumor necrosis factor, and arachidonic acid (20:4) metabolites, as well as the stimulation of bactericidal activity (2, 3).

Despite the importance of the LPS–macrophage interaction, the mechanism by which LPS activates macrophages and other cells is poorly understood. Recently, two different cell surface molecules have been implicated as possible LPS receptors (4, 5). The contributions of these molecules to LPS cellular activation remains to be determined. Even less is known about the early intracellular events that mediate LPS responses. Several investigators have reported that LPS activates a pertussis toxin-sensitive guanine nucleotide-binding protein (G protein) (6–9). However, some LPS actions in cells are unaffected by pertussis toxin treatment (refs. 8 and 10; M.R.G., S. J. Estey, S.L.W., and A.L.D., unpublished data), suggesting that LPS responses do not occur solely through a G protein-dependent signaling mechanism. In terms of second-messenger systems, LPS-stimulated inositolphospholipid hydrolysis has been observed in peritoneal macrophages (11). However, LPS does not stimulate detectable inositolphospholipid breakdown in many LPS-responsive cell types (9, 12, 13), including several murine macrophage cell lines (M.R.G., S. J. Estey, J. P. Jakway, and A.L.D., unpublished data). Thus, inositolphospholipid hydrolysis does not appear to be obligatory for many cellular responses to LPS.

Many receptors stimulate protein tyrosine phosphorylation following ligand binding, and this event is thought to be part of the signal-transduction mechanism that mediates later cellular responses (14). In this report, we show that LPS treatment rapidly increases tyrosine phosphorylation of several proteins in the RAW 264.7 macrophage cell line and in resident peritoneal macrophages. In RAW 264.7 cells, inhibition of LPS-stimulated tyrosine phosphorylation was accompanied by inhibition of LPS-induced release of 20:4 metabolites. Thus, tyrosine phosphorylation appears to be an early signaling event that mediates LPS-stimulated 20:4 metabolite release in RAW 264.7 cells.

MATERIALS AND METHODS

RAW 264.7 Cell Culture and Stimulation. RAW 264.7, an Abelson virus-transformed murine macrophage cell line (American Type Culture Collection), was cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, and 2 mM glutamine (growth medium). For cell stimulation, 1.5×10^6 RAW 264.7 cells per well were grown in six-well plates (Costar) in 1.5 ml of growth medium for 18 hr to allow the cell number to approximately double. The growth medium was replaced and the indicated stimulus was added. In some experiments, prior to the addition of stimulators, cells were pretreated for 4 hr with herbimycin A (obtained from N. R. Lomax, Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute). LPS preparations and lipid A were purchased from List Biological Laboratories (Campbell, CA), synthetic lipid A (diphosphoryl, *Escherichia coli* type) was from ICN, N^2-monoacylglucosamine 1-phosphate was from Lipidex (Middleton, WI), and phorbol 12-myristate 13-acetate (PMA) was from Sigma.

Macrophage Preparation and Stimulation. Resident peritoneal macrophages were isolated from female C3H/HeSNJ mice (The Jackson Laboratory) by peritoneal lavage. The cells (6×10^6 per well) were incubated in six-well plates in 2 ml of alpha modified minimal essential medium (GIBCO) containing 10% heat-inactivated fetal bovine serum and gen-

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Abbreviations: LPS, lipopolysaccharide; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; 20:4, arachidonic acid.

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tamicin (medium) for 2 hr. Wells were washed with phosphate-buffered saline (PBS) to remove nonadherent cells and incubated overnight in 2 ml of fresh medium. Macrophages were then washed *in situ* with PBS and incubated with 1.5 ml of fresh medium containing the indicated stimulus for 15 min.

Preparation of Cell Lysates and Anti-Phosphotyrosine Immunoblotting. After stimulation, cells were washed *in situ* with ice-cold PBS containing 1 mM Na_3VO_4, then lysed in 0.25 ml of lysis buffer [20 mM Tris Cl, pH 8/137 mM NaCl/10% (wt/vol) glycerol/1% (wt/vol) Triton X-100/1 mM Na_3VO_4/2 mM EDTA/1 mM phenylmethanesulfonyl fluoride/20 μM leupeptin containing aprotinin at 0.15 units/ml] for 20 min at 4°C. These conditions have been shown to block *in vitro* phosphorylation and dephosphorylation following cell lysis (15). Detergent-insoluble material was pelleted by centrifugation (10,000 × *g*, 15 min, 4°C). The solubilized proteins were separated on 12-cm SDS/12% polyacrylamide gels run at 20 mA. To improve the separation of the relevant proteins, electrophoresis was continued for 1 hr after the bromophenol blue dye front had run off the gel. The separated proteins were transferred to nitrocellulose (4 hr at 0.5 A) and immunoblotted as described (16) with the 4G10 monoclonal anti-phosphotyrosine antibody (17). Blots were then incubated with goat anti-mouse immunoglobulin alkaline phosphatase conjugate and immunoreactive proteins were visualized colorimetrically. In Fig. 4*a*, the anti-phosphotyrosine immunoblot was developed with ^{125}I-labeled protein A and autoradiography. After the 4G10 incubation and washing, the blot was incubated with 2 μCi ^{125}I-protein A (specific activity, >30 μCi/μg; ICN; 1 μCi = 37 kBq) diluted in Tris-buffered saline containing 0.05% Tween 20 for 1 hr at 25°C. The blot was washed and exposed to preflashed film at −70°C.

Release of [^{3}H]20:4 Metabolites from RAW 264.7 Cells. Cells (1.5 × 10^6 per well in six-well plates) were labeled for 18 hr in 1.5 ml of growth medium containing 0.5 μCi of [5,6,8,9,11,12,14,15(n)-^{3}H]20:4 (specific activity, 60–100 Ci/mmol; DuPont/NEN). After washing *in situ* with PBS and addition of fresh growth medium, the cells were pretreated for 4 hr with the indicated concentration of herbimycin A. Prior to stimulation, the wells were washed with PBS and 1.5 ml of fresh growth medium containing herbimycin A was added. Cells were stimulated with LPS (1 μg/ml) from *E. coli* K235 or 100 nM PMA for 1 hr. The culture medium was collected, loose cells were removed by centrifugation (10,000 × *g*, 30 sec), and 1 ml of the supernatant fraction was taken for liquid scintillation counting in 10 ml of Universol ES (ICN).

RESULTS

LPS-Induced Tyrosine Phosphorylation in RAW 264.7 Cells. Using anti-phosphotyrosine immunoblotting, we examined whether LPS affects protein tyrosine phosphorylation in RAW 264.7 cells, a murine monocyte–macrophage cell line. *E. coli* K235 LPS rapidly increased tyrosine phosphorylation of Triton X-100-soluble proteins with apparent molecular masses of 41, 42, 43.5, 44, 52, 74, 77, 110, 123, and 142 kDa (Fig. 1*a*). Increased tyrosine phosphorylation was detectable by 4–5 min, reached a maximum by 15 min, and declined after 30–60 min. The tyrosine phosphorylation response was detectable following stimulation with LPS at 100 pg/ml and was maximal at 10 ng/ml (Fig. 1*b*), doses comparable to those needed to induce biological responses in these cells (refs. 18–20 and data not shown). No changes in tyrosine phosphorylation of Triton X-100-insoluble proteins were observed following LPS treatment (data not shown). Thus, protein translocation from the Triton X-100-insoluble fraction to the detergent-soluble fraction was not responsible for the observed increase in tyrosine phosphorylation of the polypeptides described above. Moreover, LPS-induced tyrosine phosphorylation occurred in cells pretreated for 30 min with actinomycin D (20 μg/ml) or cycloheximide (10 μg/ml), conditions which completely inhibited transcription or translation in RAW 264.7 cells (data not shown). Thus, increased tyrosine phosphorylation was not due to increased synthesis of constitutively phosphorylated proteins.

Stimulus Specificity of Induced Tyrosine Phosphorylation. Wild-type LPS consists of two structural domains: a carbohydrate region, which is variable among different bacterial strains, and a conserved lipid region, called lipid A. The predominant biological activity of the LPS molecule is contained in the lipid A domain (1, 21). Therefore, we tested whether different forms of LPS could stimulate increased tyrosine phosphorylation. Purified bacterial lipid A, synthetic lipid A, or LPS from *Salmonella minnesota* R595, which lacks most of the carbohydrate domain, stimulated changes in tyrosine phosphorylation similarly to wild-type LPS from *E. coli* K235 (Fig. 2 and data not shown). In contrast, a biologically inactive derivative of lipid A, N^2-monoacylglucosamine 1-phosphate (22), did not increase tyrosine phosphorylation. Thus, the capacity of different forms of LPS to stimulate tyrosine phosphorylation paral-

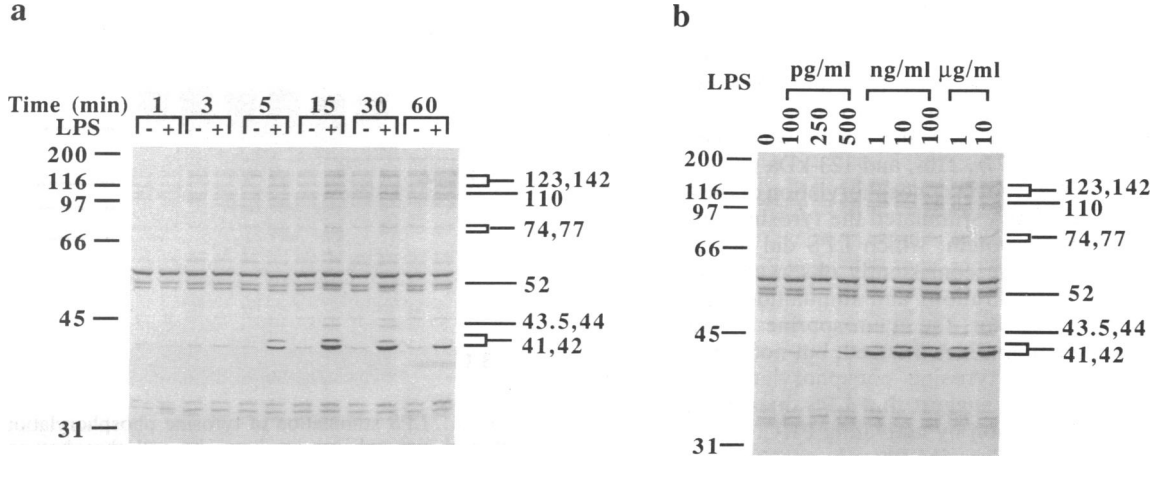

FIG. 1. LPS-induced tyrosine phosphorylation in RAW 264.7 cells as shown by anti-phosphotyrosine immunoblots of Triton X-100-soluble proteins. Cells were incubated with (+) or without (−) *E. coli* K235 LPS (1 μg/ml) for the indicated time (*a*) or with the indicated concentrations of *E. coli* K235 LPS for 15 min (*b*). Approximate molecular masses (kDa) of the induced proteins are indicated to the right of each blot. They were estimated using molecular size standards that are indicated to the left of each blot.

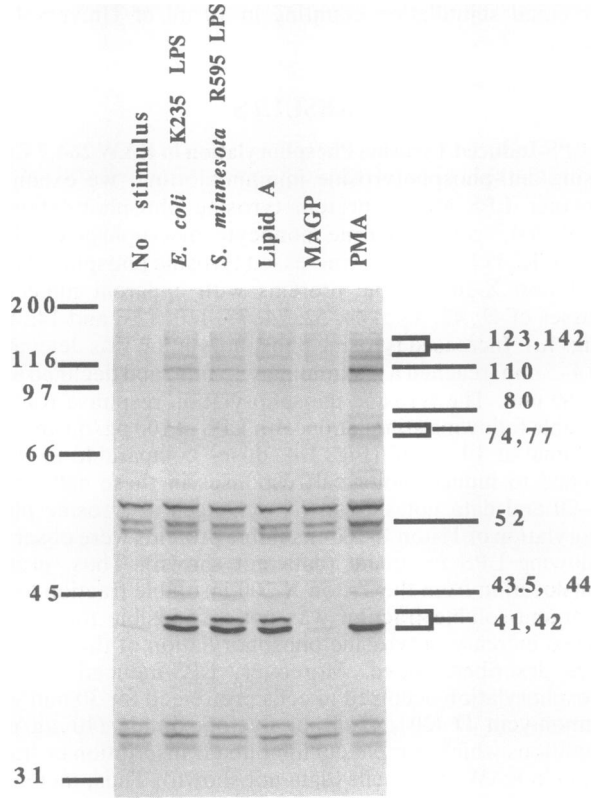

FIG. 2. Stimulus specificity of induced tyrosine phosphorylation in RAW 264.7 cells as shown by anti-phosphotyrosine immunoblot analysis of Triton X-100-soluble proteins. Cells were stimulated for 15 min with *E. coli* K235 LPS (1 μg/ml), *S. minnesota* R595 LPS (1 μg/ml), lipid A (1 μg/ml), N^2-monoacyglucosamine 1-phosphate (MAGP, 1 μg/ml), or PMA (100 nM).

leled their biological activity. Moreover, these results indicate that contaminants in the preparations of purified bacterial LPS and lipid A are not responsible for the enhanced tyrosine phosphorylation and that lipid A alone is sufficient to stimulate tyrosine phosphorylation.

Some LPS-induced biological responses in macrophages are also elicited by activators of protein kinase C (PKC) such as phorbol esters, suggesting that LPS signal transduction may involve activation of PKC (23–25). Since PKC is a serine/threonine kinase, its role in the stimulation of tyrosine phosphorylation would presumably be indirect. We found that PMA increased tyrosine phosphorylation of several proteins and that some of them appeared to be the same as LPS-modulated proteins (Fig. 2). However, the pattern of modulated proteins induced by LPS and PMA differed in several respects: PMA only marginally increased the tyrosine phosphorylation of the 42-kDa protein but was a more potent stimulator of the 74-, 77-, 110-, and 123-kDa proteins; PMA did not increase the tyrosine phosphorylation of the 43.5-kDa protein at all; and PMA stimulated the tyrosine phosphorylation of an 80-kDa protein, which LPS did not modulate. These differences were consistently observed over a wide range of PMA concentrations and stimulation times. Additionally, a PKC inhibitor, the staurosporine analogue compound 3 (26), inhibited PMA-induced, but not LPS-induced, increases in protein tyrosine phosphorylation (data not shown). These results suggest that LPS does not stimulate tyrosine phosphorylation by activating PKC.

Stimulated Protein Tyrosine Phosphorylation in Peritoneal Macrophages. LPS, lipid A, and PMA also increased tyrosine phosphorylation in resident peritoneal macrophages obtained from LPS-responsive, C3H/HeSNJ mice. The biologically inactive N^2-monoacylglucosamine 1-phosphate did not increase tyrosine phosphorylation in these cells (Fig. 3). Thus, the stimulus specificity for induced tyrosine phosphorylation in peritoneal macrophages and RAW 264.7 cells was similar. Moreover, the induced phosphorylated proteins in peritoneal macrophages and RAW 264.7 cells had similar molecular masses, suggesting that the same proteins were modulated in nontransformed macrophages and in the cell line. In peritoneal macrophages, however, LPS did not increase the tyrosine phosphorylation of the 43.5-, 123-, or 142-kDa proteins, and PMA did not induce phosphorylation of the 52- or 142-kDa proteins. These differences could reflect differences in the activation/differentiation states of resident macrophages and RAW 264.7 cells.

Some additional experiments were done with macrophages from LPS-hyporesponsive, C3H/HeJ mice. We found that lipid A, which does not elicit responses from these macrophages (27), did not stimulate tyrosine phosphorylation (data not shown). Thus, the defect in C3H/HeJ macrophages that makes these cells unresponsive to lipid A also interferes with the induction of protein tyrosine phosphorylation.

Effects of Herbimycin A on LPS- and PMA-Induced Tyrosine Phosphorylation and Release of 20:4 Metabolites. The rapid induction of tyrosine phosphorylation by LPS and the close correlation between the doses required for induced tyrosine phosphorylation and for release of 20:4 metabolites in RAW 264.7 cells (ref. 28; M.R.G. and A.L.D., unpublished data) suggested that LPS-induced tyrosine phosphorylation may be required for 20:4 metabolite release. Therefore, we examined whether the protein-tyrosine kinase inhibitor herbimycin A (29–31) could inhibit LPS-stimulated release of 20:4 metabolites. Herbimycin A inhibited both LPS-induced tyrosine phosphorylation and 20:4 metabolite release in RAW

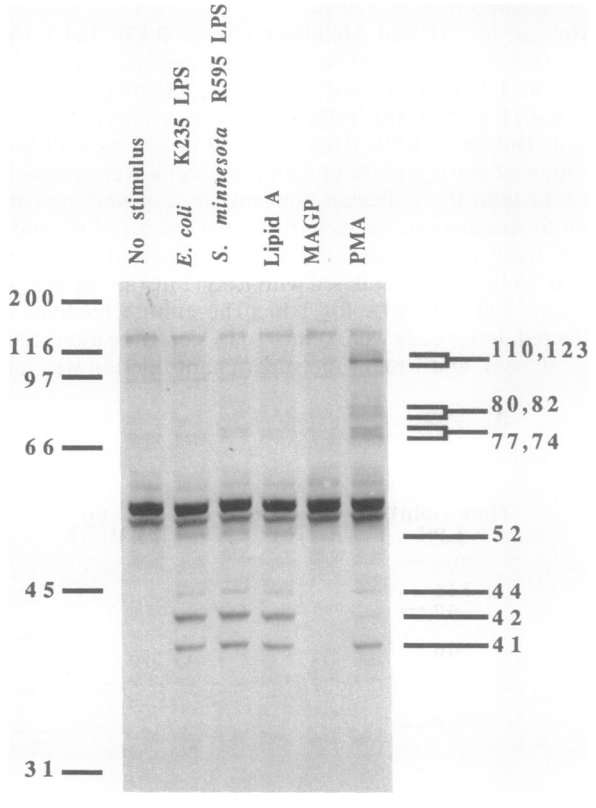

FIG. 3. LPS stimulation of tyrosine phosphorylation in murine peritoneal macrophages as shown by anti-phosphotyrosine immunoblot of Triton X-100-soluble proteins from murine peritoneal macrophages. Macrophages were treated for 15 min with the indicated stimulus at the same concentration used in Fig. 2. None of the stimuli altered tyrosine phosphorylation in the Triton X-100-insoluble fraction (data not shown).

264.7 cells (Fig. 4). Inhibition of both responses was dose-dependent, with detectable inhibition occurring with herbimycin A at 0.1 μg/ml, 50% inhibition at 0.5–1.0 μg/ml, and nearly complete inhibition at 10 μg/ml. In contrast to the results with LPS, PMA-induced tyrosine phosphorylation and release of 20:4 metabolites were only weakly inhibited by herbimycin A. Thus, for both LPS and PMA stimulation, there was a good correlation between inhibition of tyrosine phosphorylation and inhibition of 20:4 metabolite release.

The inhibition of LPS-stimulated 20:4 metabolite release was not due to cellular toxicity. Herbimycin A had little effect on cellular metabolism [MTT assay (32)] or cell viability (trypan blue exclusion) (data not shown). However, herbimycin A (1–10 μg/ml) did reduce the incorporation of [^{35}S]methionine into proteins by 20–40% (data not shown). This inhibition of protein synthesis could be a nonspecific effect of the drug or it could be caused by inhibition of tyrosine phosphorylation. Since LPS- or PMA-induced 20:4 metabolite release from RAW 264.7 cells requires protein synthesis (S. J. Estey, S.L.W., and A.L.D., unpublished data), we examined whether the partial inhibition of protein synthesis caused by herbimycin A could account for the inhibition of 20:4 metabolite release. This appeared not to be the case, as greater inhibition of [^{35}S]methionine incorporation following treatment with cycloheximide at 0.1 μg/ml (75% inhibition) had little effect on 20:4 metabolite release (data not shown). Thus, the amount of protein synthesis inhibition caused by herbimycin A cannot by itself account for the inhibition of 20:4 metabolite release. Moreover, the calcium-activated activity of phospholipase A_2, which cleaves 20:4 from membrane phospholipids, was unaffected by herbimycin A treatment (data not shown). We cannot, however, exclude the possibility that herbimycin A preferentially blocked the synthesis of some other protein required for the release of 20:4 metabolites. Nonetheless, these results are consistent with the hypothesis that LPS-induced tyrosine phosphorylation is involved in regulating 20:4 metabolite release in RAW 264.7 cells.

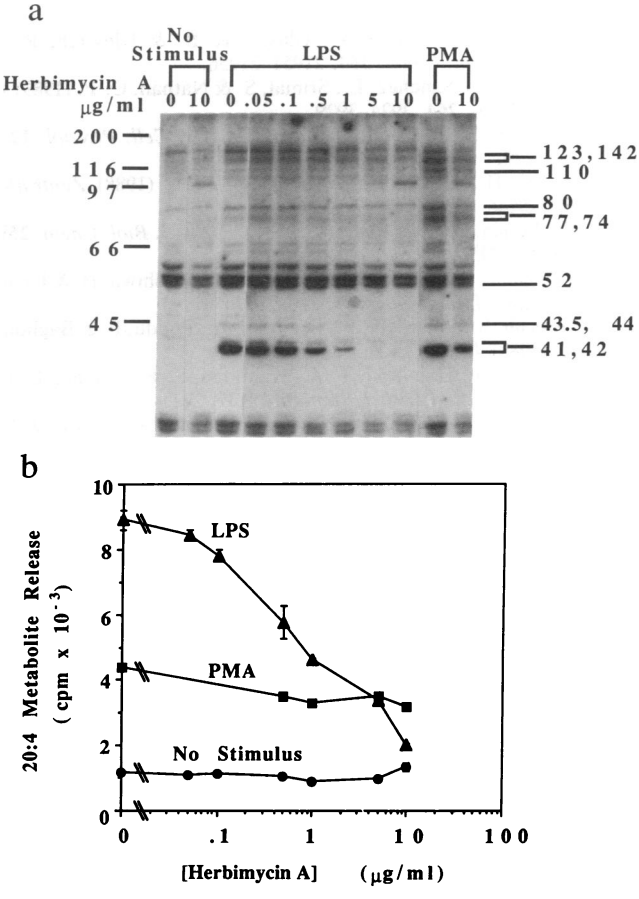

FIG. 4. Herbimycin A inhibition of LPS-stimulated tyrosine phosphorylation and release of 20:4 metabolites in RAW 264.7 cells. (*a*) Anti-phosphotyrosine immunoblot of Triton X-100-soluble proteins from RAW 264.7 cells treated with herbimycin A. Cells were pretreated for 4 hr with the indicated concentration of herbimycin A and then stimulated for 15 min with *E. coli* K235 LPS (1 μg/ml) or PMA (100 nM). Immunoreactive proteins were detected with the 4G10 monoclonal antibody followed by ^{125}I-protein A and autoradiography for 15 days. (*b*) Release of [^{3}H]20:4 metabolites from RAW 264.7 cells. Cells were labeled with [^{3}H]20:4 overnight and then treated with herbimycin A for 4 hr. The cells were stimulated for 1 hr with LPS or PMA. The culture medium was collected for determination of released [^{3}H]20:4 metabolites by liquid scintillation counting. Each data point represents the mean and SE of triplicate cultures. Herbimycin A treatment had no effect on cellular uptake of [^{3}H]20:4 (data not shown).

DISCUSSION

In this report, we have shown that LPS or the biologically active moiety of it, lipid A, increases protein tyrosine phosphorylation in the RAW 264.7 macrophage cell line and in resident peritoneal macrophages. This response did not require protein synthesis and did not involve translocation of tyrosine phosphorylated proteins. Thus, the changes in protein tyrosine phosphorylation observed in LPS-treated cells represent modifications to preexisting proteins. Whether LPS-induced tyrosine phosphorylation results from altered activity of protein-tyrosine kinases or protein-tyrosine-phosphatases remains to be determined.

LPS-induced tyrosine phosphorylation was detectable within 4–5 min and is, therefore, one of the fastest intracellular responses to LPS identified thus far. Nonetheless, LPS-induced tyrosine phosphorylation is less rapid than other ligand-mediated tyrosine phosphorylation responses. One possible explanation for the delayed response to LPS is provided by recent studies of the putative LPS receptor, CD14. Wright *et al.* (5) have suggested that LPS does not directly bind to CD14 on macrophages. Instead, LPS binds to a serum protein, LPS-binding protein, resulting in complexes which then interact with CD14. Additionally, CD14, which is a phosphatidylinositol-glycan-linked membrane protein, may require interaction with transmembrane molecules that provide signaling activity. Thus, LPS receptor activation may be a multistep process requiring several minutes to initiate intracellular events such as tyrosine phosphorylation.

Some LPS-induced cellular responses are also stimulated by activators of PKC, suggesting that LPS signal transduction involves PKC activation. Our data, however, indicate that LPS-induced tyrosine phosphorylation involves a PKC-independent mechanism. Activation of PKC by PMA did not reproduce the LPS-induced pattern of tyrosine phosphorylated proteins, and inhibition of PKC did not diminish LPS-induced tyrosine phosphorylation. Additionally, LPS-stimulated tyrosine phosphorylation was completely inhibited by treatment with herbimycin A at 10 μg/ml, whereas the PMA-induced response was only weakly inhibited. Since herbimycin A is thought to inhibit tyrosine kinases, this observation suggests that LPS- and PMA-induced tyrosine phosphorylation involve different tyrosine kinases.

Increased protein tyrosine phosphorylation following receptor activation is believed to be an important signaling event that leads to cellular responses. To test whether LPS-induced tyrosine phosphorylation mediated later cellular responses, we inhibited LPS-stimulated tyrosine phosphorylation in RAW 264.7 cells and examined the effect on 20:4 metabolite release. We found that herbimycin A treatment inhibited both LPS-stimulated tyrosine phosphoryla-

tion and 20:4 metabolite release. In contrast, disruption of other cellular processes by herbimycin A was minimal. For example, PMA-induced tyrosine phosphorylation and release of 20:4 metabolites were not greatly impaired. This result suggests that herbimycin A did not inhibit serine/threonine kinases such as PKC or any of the PKC-dependent signaling steps that mediate 20:4 metabolite release. Similarly, June *et al.* (30) found that herbimycin did not inhibit PKC-mediated cellular responses or the activity of another serine/threonine kinase, c-raf, in human T cells. Therefore, the most straightforward interpretation of our results is that herbimycin A inhibited a tyrosine kinase that is required for LPS-stimulated tyrosine phosphorylation, and this action prevented further signaling and release of 20:4 metabolites. Thus, induced tyrosine phosphorylation may be a necessary intermediate leading to LPS-stimulated release of 20:4 metabolites.

While tyrosine phosphorylation is likely to mediate some LPS responses in macrophages, other signaling pathways are probably also involved. LPS has been shown to stimulate inositolphospholipid hydrolysis in peritoneal macrophages (11), and this signaling pathway may mediate some LPS effects. In the $P388D_1$ macrophage cell line, LPS priming was reported to be relatively insensitive to treatment with genistein, a tyrosine kinase inhibitor (33). This result suggests that signaling events other than tyrosine phosphorylation mediate this LPS response. In that study, however, it was not determined whether LPS induced tyrosine phosphorylation or whether the inhibitor effectively blocked such events. In fact, genistein was not an effective inhibitor of LPS-induced tyrosine phosphorylation in RAW 264.7 cells (data not shown). Tyrosine phosphorylation also does not appear to mediate LPS action in B cells (34), suggesting that this signaling pathway is activated by LPS in only some LPS-responsive cell types. Although the mechanisms by which cells respond to LPS remain incompletely understood, our results indicate that protein tyrosine phosphorylation is a rapid and important signaling event induced by LPS in macrophages.

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