

Tyrosine Phosphorylation of Mitogen-activated Protein Kinases Is Necessary for Activation of Murine Macrophages by Natural and Synthetic Bacterial Products

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Summary

The purpose of these studies was to determine the intracellular signal transduction pathways of bacterial products in murine macrophages from lipopolysaccharide (LPS)-responder C₃H/HeN and LPS-nonresponder C₃H/HeJ mice. Both LPS and synthetic lipopeptide CGP 31362 (LPP) induced production of tumor necrosis factor α (TNF- α) in C₃H/HeN macrophages. In C₃H/HeJ macrophages, however, TNF- α was induced only by incubation with LPP. Both LPS and LPP induced tyrosine phosphorylation on proteins with apparent molecular masses of 39, 41, and 45 kD (p35, p41, and p45) in C₃H/HeN macrophages, whereas in C₃H/HeJ macrophages, tyrosine phosphorylation was induced only by LPP. 20-h incubation with LPS or LPP downregulated TNF- α production/secretion and tyrosine phosphorylation in C₃H/HeN macrophages induced by additional LPS or LPP. In C₃H/HeJ macrophages, however, the downregulation of TNF- α production and tyrosine phosphorylation were observed only with LPP. Protein kinase assays, Western blotting analyses, phenyl-Sepharose chromatography, and immunocomplex kinase assay suggested that p45 and p39 were similar or identical to mitogen-activated protein (MAP) kinase 1 and 2, respectively. Pretreatment of macrophages with LPS or LPP did not change the amount of kinase proteins but inhibited the stimulation of kinase activity by the agents. These data suggest that MAP kinases are among target proteins involved in the transduction of LPS and LPP signals that lead to activation of murine macrophages to produce/secrete TNF.

Natural and synthetic bacterial products, such as LPS and lipopeptide CGP 31362 (LPP)¹, a synthetic analogue of a fragment of a lipoprotein from the outer wall of gram-negative bacteria, are potent immunoregulatory agents (1). Either alone or in combination with IFN- γ or other cytokines, such agents activate macrophages to perform a variety of functions, such as lyse tumor cells, eliminate intracellular infections, and secrete a plethora of distinct biologically active products, such as TNF (2, 3). The biochemical mechanisms of macrophage activation induced by the bacterial products are still not fully understood. Recently, activation of macrophages by LPS has been coupled with stimulation of calcium- and calmodulin-dependent protein kinase (4), protein kinase C (PKC) (5), and pertussis toxin-sensitive guanine nucleotide-binding proteins (G protein) (6). However, inhibition of PKC in a macrophage cell line does not prevent LPS priming of the cells for enhanced arachidonic acid metabolism (7),

and many functions of LPS are not affected by treatment of macrophages with pertussis toxin (8). Moreover, downregulation of intracellular PKC does not prevent macrophages from responding to LPS by secreting TNF and lysing some tumor cells (9). Collectively, these data suggest that additional signal-transducing pathways must participate in the macrophage activation process.

In recent work from our laboratory, we concluded that protein tyrosine kinase (PTK) activity is necessary albeit not sufficient for LPS-induced tumoricidal activation of macrophages. Specifically, the data demonstrated that protein tyrosine phosphorylation is involved in this process (10). Activation of MAP kinases, a family of serine/threonine protein kinases, by a variety of extracellular stimuli plays a crucial role in cell differentiation and proliferation (11, 12). In this study, we determined whether phosphorylation-activation of MAP kinases is also involved in signaling pathways for macrophage activation by bacterial products, i.e., LPS and LPP. We tested this possibility by using macrophages from congenic LPS-responsive C₃H/HeN and LPS-nonresponsive C₃H/HeJ mice (13, 14). Tyrosine phosphorylation at several

¹ Abbreviations used in this paper: FBS, fetal bovine serum; LPP, lipopeptide CGP 31362; MAP kinase, mitogen-activated protein kinase; PEM, peritoneal exudate macrophage; PKC, protein kinase C; PTK, protein tyrosine kinase.

proteins, activation of MAP kinases, and production/secretion of TNF- α were observed in C₃H/HeN macrophages incubated with either LPS or LPP, whereas only LPP was active in C₃H/HeJ macrophages. Pretreatment of macrophages with LPS or LPP downregulated the phosphorylation, activation of MAP kinases, and production/secretion of TNF- α . These findings suggest that MAP kinase activity is involved in macrophage activation by bacterial products.

Materials and Methods

Reagents. Eagle's MEM, HBSS, and fetal bovine serum (FBS) were purchased from M. A. Bioproducts (Walkersville, MD). Phenol-extracted *Salmonella* LPS, phosphotyrosine, phosphothreonine, and phosphoserine were purchased from Sigma Chemicals (St. Louis, MO). Monoclonal antiphosphotyrosine (4G10), polyclonal rabbit anti-MAP kinase 1 and 2 antibody, and synthetic MAP kinase substrate containing nine amino acids were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-MAP kinase 1 and 2 mAb (Z003) was purchased from Zymed Laboratories, Inc. (South San Francisco, CA). [³H]TdR (sp act 2 Ci/mmol), BSA, and OVA were purchased from ICN Biomedicals, Inc. (Costa Mesa, CA). PANSORBIN was obtained from Calbiochem-Novabiochem (La Jolla, CA). Enhanced Chemiluminescence (ECL) Western blotting detection system, horseradish peroxidase conjugated goat anti-mouse and anti-rabbit IgG, and γ -[³²P]ATP (sp act >5000 Ci/mmol) were purchased from Amersham Corp. (Arlington Heights, IL). LPP, a synthetic analogue of a fragment of a lipoprotein from the outer wall of gram-negative bacteria, was the gift of Ciba-Geigy, Ltd. (Basel, Switzerland). Phenyl-Sepharose was purchased from Pharmacia LKB (Piscataway, NJ).

Mice. Specific pathogen-free female C₃H/HeN and C₃H/HeJ mice were purchased from the Animal Production Area, Frederick Cancer Research Facility (Frederick, MD). The mice were used when 8 wk of age. Animals were maintained in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the United States Department of Agriculture, the Department of Health and Human Services, and the National Institutes of Health.

Collection and Cultivation of Mouse Peritoneal Exudate Macrophages (PEM). PEM were collected by peritoneal lavage from mice given an intraperitoneal injection of 1.5 ml of thioglycollate broth (Baltimore Biological Laboratories, Cockeysville, MD) 4 d before harvest (15). The PEM were washed with Ca²⁺ and Mg²⁺ free PBS and resuspended in serum-free MEM, and 10⁵ cells in 0.2 ml MEM were plated into 38-mm² wells of 96-well flat-bottomed plates (Microtest III; Falcon Plastics, Oxnard, CA). After 90 min, the wells were washed with Eagle's MEM to remove nonadherent cells. The resultant macrophage monolayer was >98% pure according to morphologic and phagocytic criteria. These cultures were then given supplemented medium containing macrophage activators or other reagents as described below.

In vitro Activation of Macrophages and Production of TNF. Purified cultures of mouse PEM were incubated at 37°C for 8–10 h with medium containing 1 μ g/ml LPS or 0.01 μ g/ml LPP. For a negative control, we incubated macrophages in medium alone. After the incubation period, the culture supernatants were collected, filtered through a 0.2- μ m filter, and added onto [³H]TdR-labeled L929 cell monolayers (10⁴ cells/well). The cultures were washed and harvested 72 h later. TNF activity was calculated by the standard curves plotted with human recombinant TNF- α and expressed

as pg/10⁵ macrophages. Neither LPS nor LPP were toxic nor did they increase the cytotoxic activity of TNF against the target cells.

Determination of Tyrosine Phosphorylation by Western Blot Analysis. Macrophages were treated as indicated in the legends to the figures. Western blotting was performed as previously described (10, 16). The cells were washed with PBS containing 1 mM sodium orthovanadate, 5 mM EDTA, and scraped into lysis buffer A (1% Triton X-100, 20 mM Tris-HCl, pH 8.0, 137 mM NaCl, 10% glycerol, 1 mM Na₃VO₄, 2 mM EDTA, 1 mM PMSF, 20 μ M leupeptin, 0.15 U/ml aprotinin), and placed on ice for 20 min. Triton X-100-soluble protein was separated by centrifugation, diluted to 1 mg/ml in sample buffer (62.5 mM Tris-HCl, pH 6.8, 2.3% SDS, 100 mM DTT, and 0.005% bromphenol blue), and boiled. The proteins (15–20 μ g/lane) were resolved on 10% SDS-PAGE and transferred onto 0.45 μ m nitrocellulose membranes. The filters were blocked with 3% BSA, 1% OVA in TBS (20 mM Tris-HCl, pH 7.5, 150 mM NaCl), probed with phosphotyrosine-specific mAb 4G10 (0.2 μ g/ml), MAP kinase 1 and 2 specific mAb Z003 (0.1 μ g/ml), or rabbit anti-rat MAP kinase antibody (0.2 μ g/ml) in TTBS (0.1% Tween 20 in TBS), incubated with second antibodies in TTBS, and visualized by the ECL Western blotting detection system.

Immunoprecipitation of Macrophage Extracts. Macrophages at a density of 2 \times 10⁷ cells/100 mm plate were incubated in 5% FBS-Eagle's MEM or in 5% FBS-Eagle's MEM containing 1 μ g/ml of LPS for 15 min. The monolayers were rinsed three times with PBS and scraped into 1 ml lysis buffer A. The lysates were precleared with PANSORBIN and incubated with 20 μ g/ml of rabbit anti-MAP kinase antibody for 4 h or 20 μ g/ml antiphosphotyrosine mAb 4G10 for 3 h, and then with 20 μ g/ml rabbit anti-mouse IgG for another 1 h at 4°C. The immunocomplex was precipitated with PANSORBIN, washed with a lysis buffer A and kinase assay buffer, and then used for the MAP kinase assay.

Hydrophobic Chromatography of Macrophage Extracts. The methods used for hydrophobic chromatography have been described in detail (17). After stimulation with 1 μ g/ml LPS for 15 min, macrophages were washed once and scraped into lysis buffer B (25 mM Tris, pH 7.5, 25 mM NaCl, 40 mM *p*-nitrophenyl phosphate, 2 mM EGTA, 1 mM DTT, 0.2 mM PMSF), followed by brief sonication and centrifugation at 18,000 g for 30 min at 4°C. Ethylene glycol (final concentration 10%) was added to the supernatant fractions, and 1 ml of the extracts was immediately mixed with 300 μ l phenyl-Sepharose and placed on ice for 5 min. The suspensions were then centrifuged and the supernatants removed. The phenyl-Sepharose was then successively washed with 0.5 ml buffer B containing increasing concentrations of ethylene glycol (up to 60%). The supernatants were assayed for MAP kinase activity or used for Western blotting analyses.

MAP Kinase Assay. MAP kinase activity was determined by a published procedure (18) with some modifications. A synthetic peptide (APRTPGGRR) was phosphorylated in a final 20- μ l volume of kinase assay buffer (12.5 mM MOPS, pH 7.2, 12.5 mM β -glycerol phosphate, 7.5 mM MgCl₂, 0.5 mM EGTA, 0.05 mM NaF, 2 mM DTT, 0.5 mM sodium vanadate, and 25 μ g/ml BSA) with 2 mg/ml substrate and 50 μ M γ [³²P]ATP (1,000–2,000 cpm/pmol) for 15 min at 30°C. The reaction was stopped by the addition of 10 μ l 15% TCA. After a 60-min incubation at 4°C, the precipitates were removed by centrifugation, and a 20- μ l aliquot of the supernatant was spotted onto a phosphocellulose disc (model P81; Whatman Inc., Clifton, NJ) and washed. The radioactivity associated with the substrate peptide was monitored. MAP kinase activity was expressed as pmol/min/mg protein.

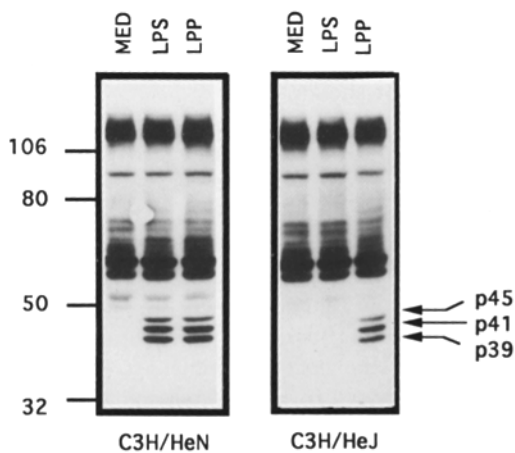


Figure 1. Induction of protein tyrosine phosphorylation in macrophages by LPS and LPP. Macrophages from C₃H/HeN or C₃H/HeJ mice were incubated in 5% FBS-Eagle's MEM (MED) or stimulated with LPS (1 μ g/ml) or LPP (0.01 μ g/ml) for 15 min. The treated cells were washed and lysed in buffer A. The lysate was centrifuged at 14,000 rpm for 15 min at 4°C. Triton X-100-soluble proteins (15 μ g/lane) were separated on 10% SDS-PAGE, transferred to nitrocellulose, and probed with antiphosphotyrosine mAb 4G10 (0.2 μ g/ml). The immunoreactive bands were detected by incubating the blots with horseradish peroxidase-conjugated goat anti-mouse IgG or anti-rabbit IgG, and visualized by the ECL Western blotting detection system. This is one representative experiment of three.

Results and Discussion

LPS and LPP Induce Protein Tyrosine Phosphorylation. A 15-min treatment of macrophages with LPS (1 μ g/ml) or LPP (0.01 μ g/ml) induced protein tyrosine phosphorylation at three bands of 39, 41, and 45 kD (p39, p41, and p45). In PEM, some proteins with apparent molecular masses of 50–170 kD were constitutively phosphorylated on tyrosine. The phosphorylation of these proteins was not significantly altered by LPS or LPP (Fig. 1). The signal detected by mAb 4G10 was phosphotyrosine specific since it could be blocked by 2 mM phosphotyrosine but not by phosphothreonine or phosphoserine (data not shown). We have also recently shown that the induction of tyrosine phosphorylation by LPS and LPP is dose dependent and transient and can be blocked by the PTK inhibitors genistein, herbimycin A, and tyrphostin (10 and our unpublished data).

The stimulus-specific induction of tyrosine phosphorylation on p39, p41, and p45 was also examined in LPS-unresponsive C₃H/HeJ macrophages. As shown in Fig. 1 *b*, the basic pattern of protein tyrosine phosphorylation in C₃H/HeJ macrophages was similar to that in C₃H/HeN macrophages. However, in C₃H/HeJ macrophages, tyrosine phosphorylation of p39, p41, and p45 was only induced by LPP (Fig. 1).

Pretreatment of C₃H/HeN macrophages for 20 h with either LPS (0.1 μ g/ml) or LPP (0.001 μ g/ml) significantly downregulated the tyrosine phosphorylation of p39, p41, and p45 normally induced by both LPS and LPP (Fig. 2). The downregulation was dose dependent and could be achieved by pretreatment with as little as 1 μ g/ml LPS and 0.0001

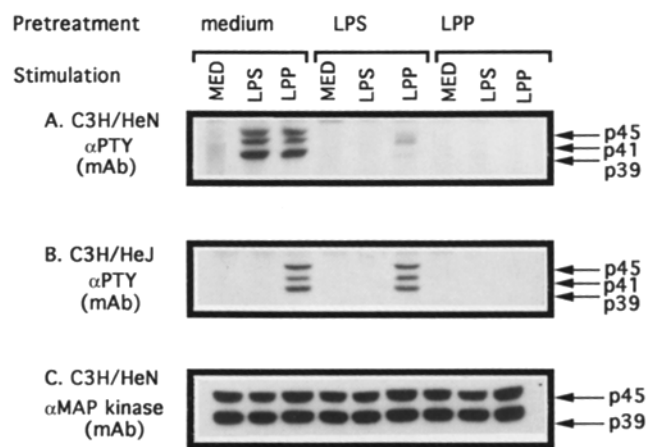


Figure 2. Downregulation of protein tyrosine phosphorylation by pretreatment of LPS or LPP. Macrophages from C₃H/HeN (A) or C₃H/HeJ (B) mice were incubated for 20 h in medium (lanes 1–3), or medium containing 0.1 μ g/ml LPS (lanes 4–6), or 0.001 μ g/ml LPP (lanes 7–9). The pretreated macrophages were then stimulated for 15 min with 1 μ g/ml LPS (lanes 2, 5, and 8) or 0.01 μ g/ml LPP (lanes 3, 6, and 9). The lysates were prepared and analyzed by Western blotting. Pretreatment of LPS or LPP did not alter the level of MAP kinase protein in macrophages (C). C₃H/HeN macrophages were incubated for 20 h in medium, 0.1 μ g/ml LPS, or 0.001 μ g/ml LPP. The treated cells were lysed in buffer A. Triton X-100-soluble proteins (15 μ g/lane) were separated on 10% SDS-PAGE, transferred onto nitrocellulose, and probed with mAb against MAP kinase 1 and 2. This is one representative experiment of three.

ng/ml LPP (data not shown). In C₃H/HeJ macrophages, this downregulation could only be induced by pretreatment with LPP (Fig. 2).

The p45 and p39 Are Similar or Identical to MAP Kinase 1 and 2. Since activation of MAP kinase requires tyrosine phosphorylation (12, 19) and LPS and LPP induce tyrosine phosphorylation of some proteins with molecular weights

Table 1. Effects of LPS or LPP Pretreatment on MAP Kinase Activation in C₃H/HeN and C₃H/HeJ Macrophages

Strain of mice	Pretreatment	Stimulation		
		Medium	LPS	LPP
C ₃ H/HeN	Medium*	3.6 \pm 0.7 [†]	54.8 \pm 12.4	42.9 \pm 0.2
	LPS	2.0 \pm 0.6	2.7 \pm 1.5	13.5 \pm 0.5
	LPP	1.1 \pm 0.5	1.6 \pm 0.5	3.8 \pm 0.3
C ₃ H/HeJ	Medium	4.8 \pm 0.5	5.4 \pm 0.2	58.2 \pm 4.2
	LPS	3.8 \pm 2.1	1.3 \pm 0.5	56.4 \pm 7.6
	LPP	3.0 \pm 1.0	1.0 \pm 0.5	2.0 \pm 0.3

* Macrophages were incubated in medium, 0.1 μ g/ml LPS, and 0.001 μ g/ml LPP for 20 h.

[†] 1 μ g/ml LPS or 0.01 μ g/ml LPP for 15 min. The stimulated macrophages were lysed in lysis buffer A. MAP kinase activity in the lysates was determined and expressed as pmol/min/mg protein \pm SD from duplicate assay tubes. This is one representative experiment of three.

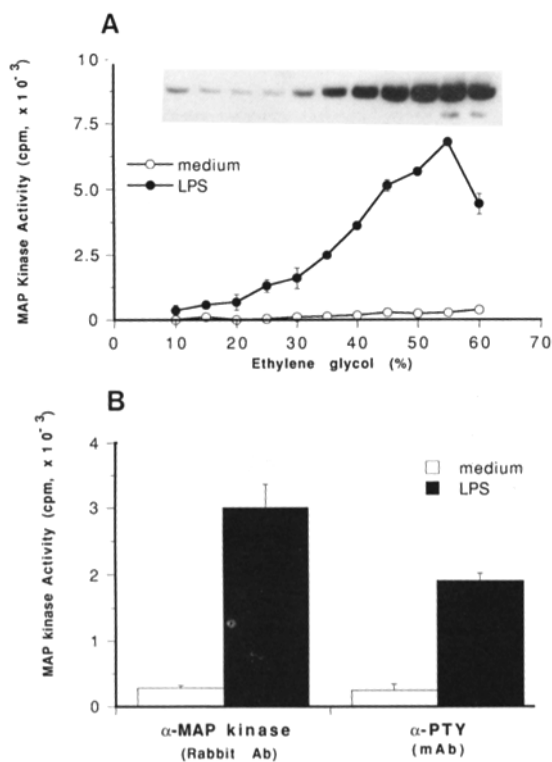


Figure 3. (A) Phenyl-Sepharose chromatography of MAP kinase. Macrophages (2×10^7 /plate) were stimulated with LPS ($1 \mu\text{g}/\text{ml}$) for 15 min, followed by washing and lysing in buffer B. Ethylene glycol was added to the extract at a final concentration of 10%. The extract (1 ml) was immediately mixed with 0.3 ml phenyl-Sepharose. After unbound materials were removed, the phenyl-Sepharose was washed with buffer B (0.5 ml each time) containing increasing concentrations of ethylene glycol (up to 60%). The supernatants were collected by centrifugation, saved for MAP kinase activity assay, and for Western blotting analysis with mAb against MAP kinase 1 and 2 (*inset*). This is one representative experiment of three. (B) Immunoprecipitation of MAP kinase activity. $\text{C}_3\text{H}/\text{HeN}$ macrophages were stimulated with $1 \mu\text{g}/\text{ml}$ LPS for 15 min and lysed in buffer A. The lysates were cleared with PANSORBIN and incubated with either $20 \mu\text{g}/\text{ml}$ of antiphosphotyrosine mAb (4G10) for 3 h at 4°C and then 1 h incubation with rabbit anti-mouse IgG, or with $20 \mu\text{g}/\text{ml}$ of rabbit anti-MAP kinase for 4 h. The immunocomplexes were precipitated with PANSORBIN and washed with lysis buffer A and kinase assay buffer. The MAP kinase activity in the immunocomplexes was determined as described in Materials and Methods. This is one representative experiment of two.

similar to those of MAP kinases, we tested whether LPS and LPP activated MAP kinases in macrophages. We determined MAP kinase activity by *in vitro* phosphorylation of a synthetic substrate peptide (APRTPGGRR) derived from bovine myelin basic protein, which contains a consensus sequence for phosphorylation by $\text{p}^{44\text{map}}$ kinase (Pro-X-[Ser/Thr]-Pro) (18). Control macrophages exhibited low levels of MAP kinase activity. Treatment with LPP resulted in a significant increase in MAP kinase activity in both $\text{C}_3\text{H}/\text{HeN}$ and $\text{C}_3\text{H}/\text{HeJ}$ macrophages, whereas LPS stimulated MAP kinase activity only in $\text{C}_3\text{H}/\text{HeN}$ macrophages (Table 1).

The possibility that the kinase activity detected in this system was derived from or was a result of contamination by PKC, cAMP- or Ca^{2+} -calmodulin-dependent protein kinases was excluded by demonstrating that 2 mM EGTA and

various concentrations (up to $1 \mu\text{M}$) of synthetic peptide inhibitor of cAMP-dependent protein kinase (20) did not alter kinase activity (data not shown).

We also partially purified kinase activity by phenyl-Sepharose chromatography (17, 20, 21). In the lysate extracted with lysis buffer B, MAP kinase activity was $3125 \pm 124 \text{ cpm}/9 \mu\text{l}$ lysate. The phenyl-Sepharose column bound all of the kinase activity at low ionic strengths, and the activity in the lysate decreased to $74 \pm 25 \text{ cpm}/9 \mu\text{l}$ sample. As shown in Fig. 3 A, the kinase activity was eluted by increasing concentrations of ethylene glycol. It appeared in the eluate of buffer B containing 25% ethylene glycol, maximal activity being eluted by 55% ethylene glycol. In direct correlation with chromatographic profile of the kinase activity, MAP kinase protein was also eluted with increasing concentrations of ethylene glycol as detected by Western blotting with anti-MAP kinase mAb (Fig. 3 a). Although a similar amount of MAP kinase protein was eluted in the lysate of untreated macrophages (data not shown), no MAP kinase activity was detected (Fig. 3 A). Finally, the MAP kinase activity in the extracts was immunoprecipitated by both anti-MAP kinases and antiphosphotyrosine antibodies (Fig. 3 B).

A significant increase in MAP kinase activity was detectable within 5 or 10 min after macrophages were stimulated with $0.01 \mu\text{g}/\text{ml}$ LPP or $1 \mu\text{g}/\text{ml}$ LPS, respectively (Fig. 4). Activity increased more than 10-fold over basal level at 10–15 min and decreased thereafter, returning to basal level at 60 min. LPP was more potent than LPS in activation of MAP kinase activity. An increase in the MAP kinase activity, determined after exposure for 15 min, could be detected by treatment with $10 \text{ ng}/\text{ml}$ of LPS and $1 \text{ ng}/\text{ml}$ LPP, respectively. Both time course and dose-related responses were strikingly similar to tyrosine phosphorylation induced by the same agents (data not shown). The delayed response of macrophages to LPS may reflect a difference in the time required for ligand binding to its putative receptors, perhaps because LPS does not bind directly to its receptor CD14 but rather forms a complex with a serum protein, LPS-binding protein (LBP), which in turn interacts with CD14 (22).

To determine whether expression of MAP kinase protein correlates with tyrosine phosphorylation of the kinase, we analyzed macrophage lysates extracted with buffer A by using antiphosphotyrosine mAb and antibodies against MAP kinase 1 and 2 in parallel (Fig. 5). Although tyrosyl phosphorylated p39, p41, and p45 were detectable only in LPS- and LPP-treated groups as described above, the same amount of proteins migrated at distances corresponding to MAP kinase 1 (p45) and MAP kinase 2 (p39) that were detected by both monoclonal and polyclonal anti-MAP kinase antibodies in the extracts from medium (control), LPS-, and LPP-treated macrophages (Fig. 5). These results indicate that MAP kinases are constitutively expressed in macrophages and that the regulation of kinase activity in macrophages most likely occurs at posttranscriptional levels, mainly at phosphorylation and/or dephosphorylation. This is consistent with our findings that blocking protein synthesis with cycloheximide does not affect tyrosine phosphorylation of p39, p41, and p45 or MAP kinase activity stimulated by LPS or LPP (data

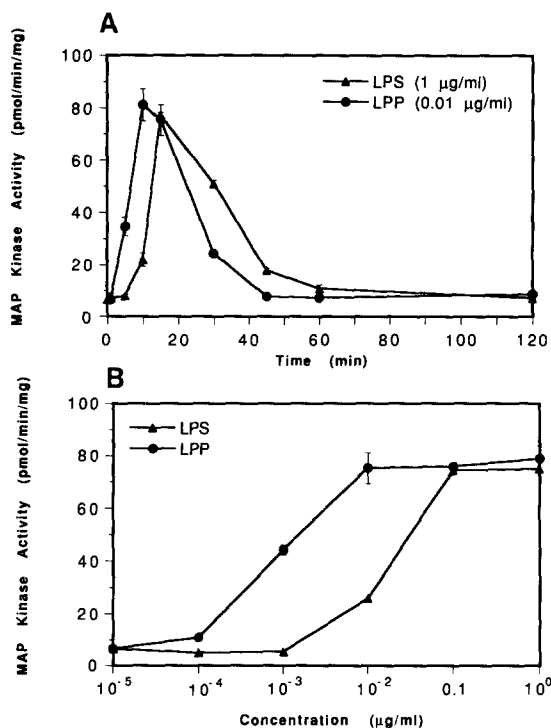


Figure 4. (A) Time course of MAP kinase activation in macrophages by LPS and LPP. C₃H/HeN macrophages were treated with 1 µg/ml LPS or 0.01 µg/ml LPP for different times. The treated cells were then lysed in buffer A. The MAP kinase activity in the extract was determined and expressed as pmol/min/mg protein ± SD from duplicate assay tubes. This is one representative experiment of two. (B) Dose-response of MAP kinase activation in macrophages by LPS and LPP. C₃H/HeN macrophages were treated for 15 min with various concentrations of LPS or LPP. The treated cells were lysed in buffer A. MAP kinase activity in the lysates was determined and expressed as pmol/min/mg protein ± SD from duplicate assay tubes. This is one representative experiment of two.

not shown). Moreover, pretreatment of macrophages with LPS or LPP blocked MAP kinase activation but had no effect on the expression of the MAP kinase proteins (see below). These results are in agreement with findings in the M phase of the *Xenopus* cell cycle (23). The p41 detected by antiphosphotyrosine mAb could not be probed by anti-MAP kinase antibodies (Fig. 5), and its identity is not clear. It might be another member of the MAP kinase family that cannot be detected by the antibodies we used or it might be a MAP kinase activator that regulates MAP kinase activity (24).

Consistent with the observations in tyrosine phosphorylation, a 20-h pretreatment of macrophages from both strains of mice with LPP (0.001 µg/ml) significantly downregulated MAP kinase activation by LPS or LPP. Pretreatment with LPS (0.1 µg/ml) also blocked MAP kinase activation induced by both stimuli but was only effective in C₃H/HeN macrophages (Table 1). Downregulation of kinase activation has been attributed to depletion of receptors by ligand binding (25, 26). However, if LPS and LPP shared a receptor, then pretreatment with LPS should have contributed to the reduction of MAP kinase activation in C₃H/HeJ macrophages. Although these macrophages do not respond to LPS, they

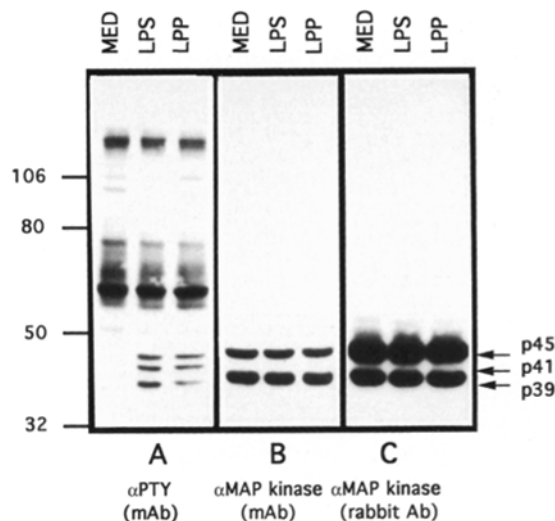


Figure 5. Detection of MAP kinase protein in murine macrophages by Western blot analysis. C₃H/HeN macrophages were incubated in medium (lane 1), stimulated for 15 min with 1 µg/ml of LPS (lane 2), or 0.01 µg/ml of LPP (lane 3). The Triton X-100-soluble proteins (15 µg/ml) were separated on 10% SDS-PAGE, transferred onto nitrocellulose, probed with 0.2 µg/ml antiphosphotyrosine mAb 4G10 (Upstate Biotechnology, Inc.) (A, αPTY), 0.1 µg/ml anti-MAP kinase mAb Z003 (Zymed Laboratories, Inc.) (B, αMAP kinase mAb), or 0.2 µg/ml polyclonal anti-MAP kinase Ab (Upstate Biotechnology, Inc.) (C, αMAP kinase [rabbit Ab]), and visualized by using the ECL Western blotting detection system. This is one representative experiment of two.

do express normal levels of the potential LPS receptor as demonstrated by LPS binding (27) and immunocytochemistry (28). The possibility that the downregulation is due to depletion of MAP kinase per se is also ruled out. In contrast with MAP kinase activity, neither the pretreatment nor the stimulation of LPS or LPP markedly changed MAP ki-

Table 2. Effects of LPS or LPP Pretreatment on TNF Production/Secretion by C₃H/HeN and C₃H/HeJ Macrophages

Strain of mice	Pretreatment	Stimulation		
		Medium	LPS	LPP
C ₃ H/HeN	Medium*	2 ± 4 [†]	145 ± 5	169 ± 25
	LPS	12 ± 1	6 ± 3	51 ± 18
	LPP	8 ± 4	11 ± 3	7 ± 3
C ₃ H/HeJ	Medium	3 ± 2	3 ± 0	153 ± 18
	LPS	5 ± 2	4 ± 1	173 ± 15
	LPP	4 ± 1	3 ± 0	4 ± 1

* Macrophages were incubated in medium, 0.1 µg/ml LPS, or 0.001 µg/ml LPP for 20 h.

[†] 1 µg/ml LPS or 0.01 µg/ml LPP for 8 h. The supernatants were added to [³H]TdR-labeled L929 cells at 1:2 dilution. Cytotoxicity was determined 72 h later. The data were expressed as pg/10⁵ macrophages ± SD from triplicate cultures. This is one representative experiment of four.

nase protein accumulation in either C₃H/HeN (Fig. 2 C) or C₃H/HeJ (data not shown) macrophages. Taken together, our data suggest that the downregulation occurs at stages between receptor binding and activation of MAP kinase (24).

Correlation between MAP Kinase Activation and Induction of TNF Production/Secretion. Macrophages from C₃H/HeN or C₃H/HeJ mice were incubated in medium, LPS, or LPP for 20 h (pretreatment) and then stimulated with the same agents at 10 times the dose for 8 h. TNF activity in the culture supernatants was measured by a bioassay with L929 as target cells (Table 2). Culture supernatants from control PEM contained low levels of TNF activity. After incubation with 1 µg/ml LPS or 0.01 µg/ml LPP, C₃H/HeN macrophages secreted/produced significantly higher amounts of TNF. In C₃H/HeJ macrophages, similar amounts of TNF secretion/production were induced only by LPP. In agreement

with tyrosine phosphorylation and MAP kinase activation, exposure of C₃H/HeN macrophages to LPS or LPP for 20 h downregulated TNF secretion/production induced by either LPS or LPP. Similarly, the response in C₃H/HeJ macrophages was downregulated only by pretreatment with LPP. These data indicate that activation of MAP kinase is directly correlated with induction of TNF production/secretion.

In summary, we provide evidence that MAP kinases are among the target proteins of bacterial products and that activation of MAP kinases play an important role in the interaction of macrophages with bacteria and/or their products. Our data therefore extend observations derived in a murine macrophage tumor cell line (29). Activation of MAP kinase by LPS and LPP may result from different mechanisms whose exact nature is still unclear. The system described here should be useful for such an investigation.

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