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Role of protein tyrosine phosphorylation in the response of macrophages to lipopolysaccharide

by

Steven L. Weinstein

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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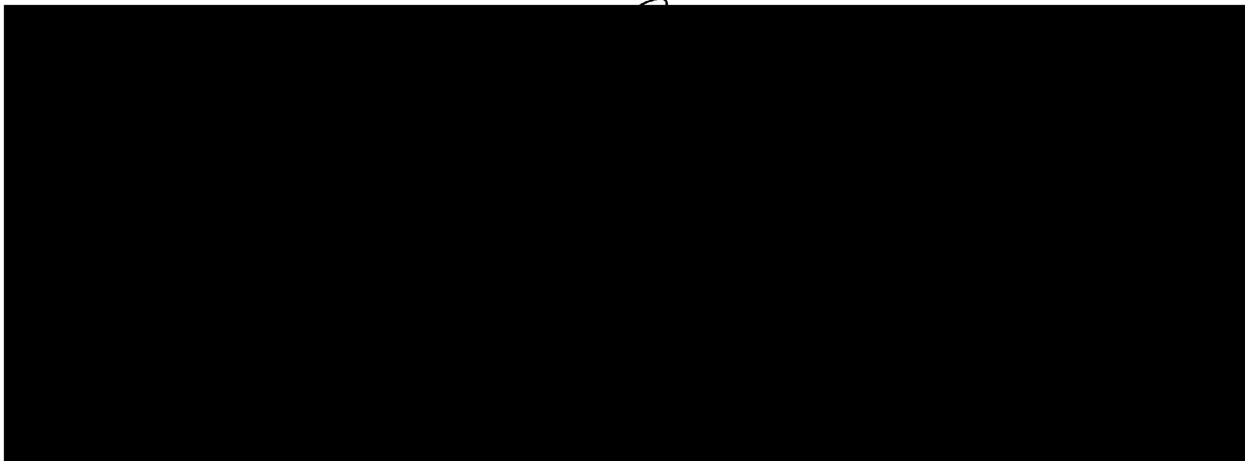

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This dissertation is dedicated

to my Grandfather, Morris Weinstein, who inspired my hands,

to my parents, Etta and Arnold, who inspired my mind,

and to Ruth Globus, who inspired my heart

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The work described in Chapter Two was done in collaboration with Carl June at Naval Medical Research Institute in Bethesda, Maryland. Carl June made a major contribution to this project by providing the human monocytes used in these studies. I also wish to thank Nancy Craighead who assisted Dr. June in preparing the monocytes.

The text of Chapter Three is a reprint of material currently *In press* for publication in the Journal of Biological Chemistry. This material appears here with the permission of the American Society for Biochemistry and Molecular Biology. These studies were co-authored with Steven Pelech at the Biomedical Research Center, University of British Columbia, Vancouver, British Columbia, and two members of Steve's lab, Jasbinder Sanghera and Krista Lemke. Jasbinder Sanghera, assisted by Krista Lemke, performed the MonoQ chromatography and analyzed the column fractions for MAP kinase activity, MAP kinase protein and tyrosine phosphoproteins.

In addition, Steve Pelech, his lab and collaborators generated several anti-MAP kinase polyclonal antibodies which were very useful in these studies.

Role of protein tyrosine phosphorylation in the response of macrophages to lipopolysaccharide

Steven Weinstein

Anthony DeFranco - Advisor

Abstract

Lipopolysaccharide (LPS), a major component of the outer membrane of Gram negative bacteria, is a potent activator of the immune system. LPS stimulates macrophages to secrete immune mediators and, in conjunction with other stimuli, to develop enhanced bactericidal capability. The biochemical events that mediate these responses are not understood. Since protein tyrosine phosphorylation is a common signaling reaction stimulated in response to many extracellular stimuli, I investigated whether LPS induces tyrosine phosphorylation in macrophages. As assessed by antiphosphotyrosine immunoblotting, LPS increased the tyrosine phosphorylation of several proteins in human and murine macrophages and macrophage cell lines. This response was elicited by both rough and smooth LPS, and by the lipid A subdomain of LPS , at concentrations of these molecules known to stimulate functional responses by macrophages. In contrast, the biologically inactive lipid A derivative, N^2-monoacylglucosamine 1-phosphate did not stimulate the response. At low doses of LPS, the putative LPS receptor, CD14, mediated the induction of tyrosine phosphorylation. However, the response was largely CD14-independent at higher LPS doses, as was previously reported for the secretion of tumor necrosis factor. Thus, induced tyrosine phosphorylation appeared to be coupled to both CD14-dependent and CD14-independent recognition mechanisms for LPS and correlated with the biological response. Moreover, a protein tyrosine kinase inhibitor prevented both the

phosphorylation and the release of eicosanoids mediators. Thus, at least some of the biological responses to LPS are dependent on the tyrosine phosphorylation response. Two of the tyrosine phosphoproteins induced by LPS were identified as 41-kDa and 44-kDa isoforms of MAP kinase. LPS increased both the tyrosine phosphorylation of these kinases and their enzymatic activity. Since MAP kinases appear to regulate cellular processes such as transcription and translation, MAP kinases may be critical targets of LPS action in macrophages. Finally, none of these effects were dependent on protein kinase C. Combined, these results indicate that LPS-induced tyrosine phosphorylation has many of the hallmarks of a receptor-coupled signaling reaction. Thus, the induction of protein tyrosine phosphorylation by LPS may connect the excellular recognition of LPS to the expression of functional responses by macrophages.

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Introduction

Everyday we come in contact with lots of bacteria in the air we breathe, in the food and water we ingest and when we cut our skin. Since many of these bacteria are pathogenic, infection by these organisms would clearly be harmful. Yet, most of the time, serious infection does not occur, which attests to the effectiveness of the immune system. Many different cells act in concert to provide this immunity including macrophages, neutrophils, lymphocytes and endothelial cells. To perform their anti-bacterial functions, these cells must recognize and respond to bacterial molecules. Through evolution, multicellular organisms have developed the capacity to respond to a diverse group of bacterial surface molecules. The best studied of these microbial components is lipopolysaccharide (LPS), a major element of the outer leaflet of the outer membrane of Gram negative bacteria (Fig. 1)(1). LPS or its lipid A subdomain can, by themselves, elicit the complete spectrum of anti-microbial responses that are activated during a Gram negative infection (1, 2). Since the lipid A moiety is structurally conserved among most strains of Gram negative bacteria (3), the recognition of this single determinant by host cells facilitates making effective immune responses to nearly all Gram negative infections.

LPS is interpreted by the body as very bad news and the presence of this bacterial molecule triggers aa extensive cellular response. For example, LPS, in combination with other stimuli, increases the microbicidal capacity of macrophages (4) and neutrophils (5, 6). LPS also activates B cells, leading to cellular proliferation and antibody production (7, 8). In addition, macrophages are induced by LPS to secrete a host of different molecules including interleukins 1, 6 and 8, tumor necrosis factor, interferons α and β,

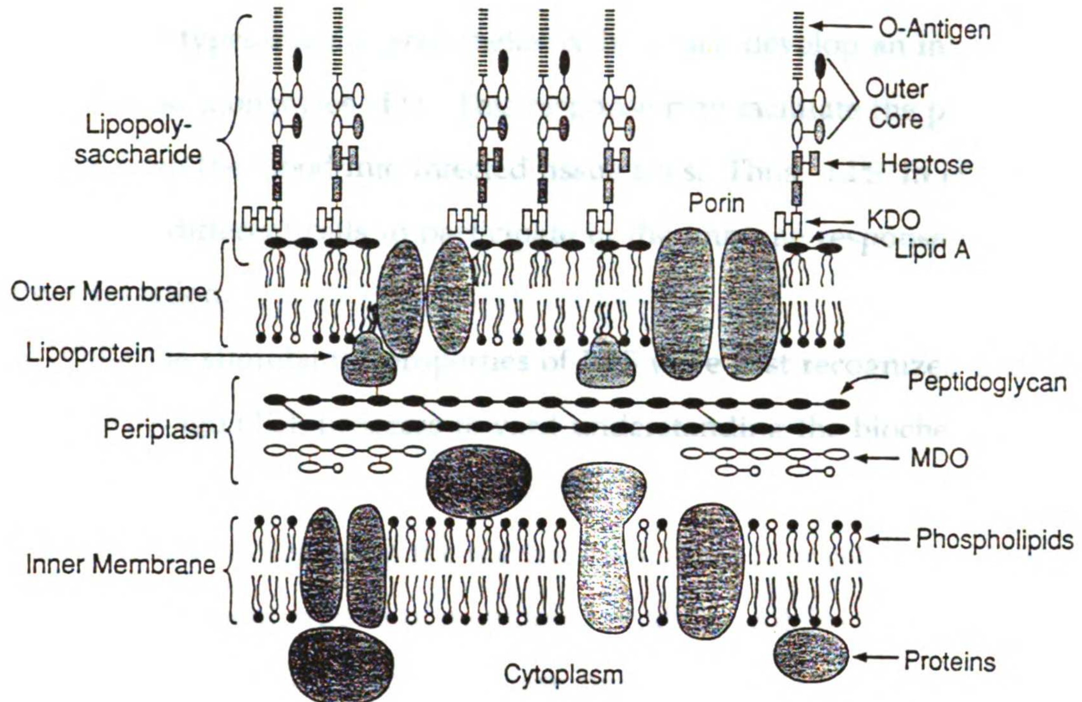

Figure 1 Schematic molecular representation of a Gram negative bacterial envelope. Ovals and rectangles depict sugar residues. Circles represent the polar headgroups of phospholipids. MDO are membrane-derived oligosaccharides, and KDO is 3-deoxy-D-*manno*-octulosonic acid. KDO and heptose make up the inner core of LPS. This figure is reproduced with the permission of C.R.H. Raetz and originally appeared in the 1990 *Annual Review of Biochemistry,* volume 59, page 132.

platelet activating factor and eicosanoid mediators (9,10). These molecules expand the response to LPS by promoting the anti-bacterial functions of cells which may not be directly responsive to LPS. Finally, LPS has effects on non-circulating cell types such as endothelial cells, which develop an increased capacity to bind monocytes (11). This response may facilitate the passage of leukocytes from the blood into infected tissue sites. Thus, LPS, in effect, recruits many different cells to participate in the immune response to Gram negative infection.

While the stimulatory properties of LPS were first recognized one hundred years ago (12), progress toward understanding the biochemical events that mediate cellular responses to LPS has been slow. This statement is certainly true with regard to the mechanisms utilized by cells to recognize LPS. Despite frequent reports of membrane binding proteins for LPS over the years (13-18), virtually none of these proteins were ever shown to mediate functional responses. The failure to identify bonafide receptor molecules, coupled with the known ability of LPS to directly intercalate into lipid bilayers (19), led to the belief by some that LPS initiated cellular responses by inserting into the lipid of the plasma membrane as opposed to binding to a membrane protein (13, 20, 21). However, in the last three years considerable evidence has been obtained that supports the existence of specific proteinaceous receptors. In fact, two different membrane proteins have been identified that appear to mediate biological responses to LPS. One of these proteins has a molecular mass of 80-kD and is expressed on macrophages and B lymphocytes (22). Monoclonal antibodies raised against p80 appear to mimic at least some of the effects of LPS on cells. The other putative receptor is CD14 (23, 24), a 55-kD GPI-linked protein that is expressed by macrophages and neutrophils, but not by B cells (25-27).

Interestingly, LPS does not directly bind to CD14; instead, CD14 recognizes LPS that is complexed with LPS binding protein, a component of serum (28). Antibodies to CD14 prevent LPS-LBP complexes from binding to CD14 and inhibit the secretion of tumor necrosis factor stimulated by low doses of LPS (23). However, responses of macrophages to high doses of LPS are not blocked by anti-CD14 antibodies. Thus, macrophages possess both CD14-dependent and CD14-independent mechanisms of LPS recognition. Whether the low affinity LPS receptor on macrophages corresponds to p80 is not yet clear. However, it is interesting that B cells which express p80, but not CD14, respond only to high concentrations of LPS. Thus, the low affinity receptor on B cells and macrophages may, in fact, be p80. An additional question concerning these receptors is how CD14, a GPI-linked protein that lacks an intracellular domain, transduces signals to the cell interior. One attractive possibility is that CD14 and the low affinity LPS receptor form a receptor complex, with the latter protein performing the signal transduction function. Clearly, further investigation will be needed to clarify the role of p80 and CD14 as receptors for LPS.

A great number of questions also remain to be answered concerning the intracellular events that connect the extracellular recognition of LPS to the expression of anti-bacterial functions. A major stumbling block in the development of a comprehensive model of LPS-activated signal transduction has been the complex nature of the intracellular response to LPS. One aspect of this complexity is the apparent coupling of different signaling pathways to different functional responses. For example, several investigators have shown in a variety of LPS-responsive cell types that pertussis toxin (PT) inhibits several cellular responses to LPS (29-32). Since PT ADP-ribosylates some G proteins and thereby inactivates them, the

receptors for LPS appear to be coupled to one or more G proteins. However, many functional responses to LPS are not PT-sensitive (31, 33, unpublished data, M. Gold, S. Estey, S. Weinstein, A. DeFranco), suggesting that not all of the effects of LPS are mediated by PT-sensitive G proteins.

An additional question concerning the role of a G protein in LPS signaling is the identity of the effector molecule(s) that communicate with the G protein. Since the particular G protein activated by LPS appears to be Gi_2 (29), an obvious candidate effector is adenylate cyclase (34). The activity of this enzyme is charcteristically inhibited by the interaction with Gi, resulting in the reduction of intracellular cAMP levels. However, Okonogi *et. al.* have reported that LPS treatment does not alter adenylate cyclase activity in peritoneal macrophages (35). Thus, the LPS-stimulated responses of macrophages that are Gi-mediated do not appear to involve adenylate cyclase.

Another possible effector that is or can be activated by G proteins is phospholipase C (PLC) (36). This enzyme catalyzes the hydrolysis of the minor membrane phospholipid, phosphatidylinositol 4,5 bisphosphate (PIP_2), leading to the production of inositol 1, 4, 5-trisphosphate (IP_3) and diacylglycerol (DAG) (37). These molecules, respectively, trigger the elevation of intracellular calcium and the activation of protein kinase C, two events thought to have critical roles in cellular signaling (38). Thus, the possible activation of PLC by Gi would be a key signal transduction event in LPS-stimulated cells. In fact, LPS reportedly stimulates some PIP_2 hydrolysis in macrophages (39) and in the 70Z/3 pre-B cell line (40). Countering these data, however, is a large number of investigations that have failed to observe any inositol phospholipid hydrolysis (20, 41, 42, unpublished data, M.

Gold, A. DeFranco). Moreover, in the LPS-responsive, immature B cell line, WEHI 231, LPS treatment not only does not stimulate PIP_2 hydrolysis, but actually blocks the biological consequences of PI breakdown (43, 44). Thus, the evidence to date suggests that PIP_2 hydrolysis does not have a pervasive role in LPS-activated signal transduction, although it is possible that some functional responses to LPS are mediated by the products of this reaction.

Despite the lack of solid evidence for LPS-stimulated PIP_2 hydrolysis, many cellular responses to LPS exposure appear to be calcium- and/or PKC-dependent. For example, preloading cells with chelators to clamp the intracellular calcium concentration, prevents LPS-stimulated interleukin 1 secretion (45) and tumoricidal function in macrophages (46) as well as inhibits LPS-priming for superoxide release from neutrophils (33). In addition, Letari and co-workers have shown that LPS treatment elevates intracellular calcium in macrophages (47). Thus, LPS alters the intracellular calcium concentration in at least some LPS-responsive cells and this event appears to have downstream effects. Similarly, PKC may be involved in mediating some responses to LPS. Many, but not all, LPS-induced cellular effects can be mimicked by phorbol ester treatment or can be inhibited by H-7, an inhibitor of PKC (33, 45, 48-52). Thus, PKC, like intracellular calcium elevation, may have a role in LPS-activated signaling. However, the precise nature of the role played by these molecules remains unknown as do the mechanisms by which LPS stimulation elevates calcium and activates PKC.

Another intracellular effect stimulated by LPS is increased Na^+/H^+ exchange, resulting in cytosolic alkalinization (40, 53, 54). This response is triggered within 5-10 minutes of LPS exposure by the activation of the

Na^+/H^+ antiporter in the plasma membrane. Inhibiting Na^+/H^+ exchange with amiloride has been shown to abrogate LPS-induced B cell proliferation (53) and transcription of the tumor necrosis factor gene in macrophages (54). Thus, Na^+/H^+ exchange appears to be required for LPS signaling. However, the activation of Na^+/H^+ exchange and the resultant cytosolic pH increase, may not actually be signaling steps in the sense of directly mediating downstream events. In fact, the role of increased Na^+/H^+ exchange in signal transduction is not clear in any cell, even though, this is a common cellular response to extracellular ligands (55). An alternative explanation for the role of Na^+/H^+ exchange in stimulated cells is to remove excess H^+ ions produced as a consequence of increased metabolic activity. Thus, the amiloride data may simply indicate that acidic intracellular pH is not permissive for LPS signaling reactions.

Interestingly, the activation of the Na^+/H^+ exchanger in some cells appears to involve protein phosphorylation (56, 57). Whether LPS stimulates phosphorylation of the Na^+/H^+ exchanger is not known, although several reports indicate that LPS alters protein phosphorylation in macrophages (39, 58, 59). Additionally, as noted above, inhibitors of protein kinases such as H-7 and staurosporine block many the responses induced by LPS (45, 49-52). Thus, one or more protein kinases, whose identities are not currently known, seem to have a role in LPS-activated signal transduction. However, a determination of the actual significance of protein phosphorylation in LPS signaling awaits identification and analysis of the phosphorylated substrates.

It is intriguing, nonetheless, that the NF-κB (60, 61), AP-1 (62) and LR1 (63) transcription factors, which are believed to regulate LPS-inducible genes, appear to be targets for protein phosphorylation (63-65). Moreover,

phosphorylation of these proteins regulates their DNA binding capacity and, thus, their ability to influence gene transcription. Since most of the anti-bacterial responses stimulated by LPS are transcription-dependent (62, 66, 67) protein phosphorylation could clearly be a critical event in the expression of LPS cellular effects.

A growing list of extracellular ligands rapidly increase intracellular protein tyrosine phosphorylation and this modification appears to regulate the function of these proteins (68). While only a small number of cellular proteins are targets for this modification, tyrosine phosphoproteins appear to have key roles in proliferation and differentiation. Given the emerging picture that tyrosine phosphorylation is a common and important event in signal transduction, as well as the apparent significance of protein phosphorylation in LPS-stimulated cells, I chose for my dissertation project to examine whether LPS induces protein tyrosine phosphorylation in LPS-treated macrophages and, if so, to further investigate the role of this reaction in LPS-activated signal transduction. I focused these studies on macrophages because these cells coordinate the immune response to bacterial infection and they are major targets of LPS action in animals. Chapters 1-3 describe this investigation in detail. Chapter one describes the initial examination of LPS-stimulated protein tyrosine phosphorylation in the RAW 264.7 murine macrophage cell line and in murine peritoneal macrophages. In addition, the relationship between induced tyrosine phosphorylation and the release of arachidonic acid metabolites, a response characteristic of LPS-stimulated macrophages was investigated. Chapter two describes experiments that examined whether the intracellular tyrosine phosphorylation induced by LPS is coupled to the putative LPS receptor, CD14. Finally, chapter three describes experiments that probed the correspondance between two of the

induced tyrosine phosphoproteins and mitogen-activated protein kinases. Together, the results of these experiments support the hypothesis that protein tyrosine phosphorylation is part of the LPS-activated signal transduction pathway in macrophages and begin to delineate the molecular events occurring.

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Chapter One:

Bacterial Lipopolysaccharide Stimulates Protein Tyrosine Phosphorylation in Macrophages

Summary

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 100 pg/ml LPS 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 the tyrosine kinase inhibitor, herbimycin A, inhibited both LPS-stimulated tyrosine phosphorylation and LPS-stimulated arachidonic acid metabolite release. Thus, increased protein tyrosine phosphorylation is a rapid LPS-activated signaling event that may mediate arachidonic acid metabolite release in RAW 264.7 cells.

Introduction

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 inducing secretion of inflammatory mediators such as interleukin-1, tumor necrosis factor, and arachidonic acid (20:4) metabolites, as well as stimulating 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 which mediate LPS responses. Several investigators have reported that LPS activates a pertussis toxin-sensitive G protein (6-9). However, some LPS actions in cells are unaffected by pertussis toxin treatment (8, 10 and unpublished data, M.R.Gold, S.J. Estey, S.L.Weinstein, A.L.DeFranco) suggesting that LPS responses do not occur solely through a G protein-dependent signaling mechanism. In terms of second messenger systems, LPS-stimulated inositol phospholipid hydrolysis has been observed in peritoneal macrophages (11). However, LPS does not stimulate detectable phosphoinositide breakdown in many LPS-responsive cell types (9, 12, 13), including several murine macrophage cell lines (unpublished data, M.R.Gold, S.J. Estey, J.P. Jakway, A.L.DeFranco). Thus,

phosphoinositide 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 which 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.

Methods

RAW 264.7 cell culture and stimulation. RAW 264.7, an Abelson-transformed murine macrophage cell line (ATCC, Rockville, MD), was cultured in Dulbecco's modified Eagle medium containing 10% heat-inactivated fetal calf serum, 1 mM sodium pyruvate and 2 mM glutamine (growth medium). For cell stimulation, $1.5x10^6$ RAW 264.7 cells/well were grown in 6-well plates (Costar, Cambridge, MA) in 1.5 ml growth medium for 18 h 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 h with herbimycin A (obtained from N.R. Lomax, Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, NCI). LPS preparations and lipid A were purchased from List Biological Laboratories (Campbell, CA), synthetic lipid A, diphosphoryl, *E. coli* type from ICN (Irvine, CA), N^2-monoacylglucosamine 1-phosphate from Lipidex (Middleton, WI) and phorbol 12-myristate 13-acetate (PMA) from Sigma (St. Louis, MO).

Macrophage preparation and stimulation. Resident peritoneal macrophages were isolated from female C3H/HeSNJ mice (Jackson Labs, Bar Harbor, ME) by peritoneal lavage. $6x10^6$ peritoneal cells/well were incubated in 6-well plates in 2 ml of alpha modified minimal essential medium (GIBCO, Grand Island, NY) containing 10% heat-inactivated fetal calf serum and gentamicin (medium) for 2 h. Wells were washed with phosphate-buffered saline (PBS) to remove nonadherent cells and incubated overnight in 2 ml fresh medium. Macrophages were then washed *in situ* with PBS and incubated with 1.5 ml fresh medium containing the indicated stimulus for 15 min.

Preparation of cell lysates and anti-phosphotyrosine immunoblotting. Following stimulation, cells were washed *in situ* with ice-cold PBS containing 1 mM Na_3VO_4, then lysed in 0.25 ml lysis buffer (20 mM TrisCl, pH 8, 137 mM NaCl, 10% w/v glycerol, 1% w/v Triton X-100, 1 mM Na_3VO_4, 2 mM EDTA, 1 mM PMSF, 20 μM leupeptin, 0.15 U/ml aprotinin) for 20 min at 4^oC. 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 x g, 15 min, 4^oC). The solubilized proteins were separated on 12 cm 12% SDS-PAGE gels run at 20mA. To improve the separation of the relevant proteins, electrophoresis was continued for 1 h after the bromophenol blue dye-front had run off the gel. The separated proteins were transferred to nitrocellulose (4 h at 0.5 A) and immunoblotted as described in (16) with the 4G10 monoclonal anti-phosphotyrosine antibody (17). Blots were then incubated with goat anti-mouse Ig-alkaline phosphatase and immunoreactive proteins were visualized colorimetrically. In Figure 4a, the anti-phosphotyrosine immunoblot was developed with ^{125}I protein A and autoradiography. Following the 4G10 incubation and washing, the blot was incubated with 2 μCi ^{125}I protein A, (specific activity >30 μCi/mg, ICN, Irvine, CA) diluted in tris-buffered saline containing 0.05% Tween 20 for 1 h at 25 ^oC. The blot was washed and exposed to preflashed film at -70 ^oC

Release of [^3H] 20:4 metabolites from RAW 264.7 cells. Cells ($1.5x10^6$/well in 6-well plates) were labeled for 18 h in 1.5 ml growth medium containing 0.5 μCi [5,6,8,9,11,12,14,15-^3H(N)]-arachidonic acid (specific activity 60-100 Ci/mmole, NEN, Wilmington, DE). Following washing *in situ* with PBS and addition of fresh growth medium, the cells were pretreated for 4 h with the indicated concentration of herbimycin A. Prior to stimulation, the wells

were washed with PBS and 1.5 ml fresh growth medium containing herbimycin A was added. Cells were stimulated with 1 mg/ml LPS from *E. coli* K235 or 100 nM PMA for 1 h. The culture medium was collected, loose cells were removed by centrifugation (10, 000 x g, 30 s) and 1 ml of the supernatant fraction was counted by liquid scintillation counting in 10 ml Universol ES (ICN).

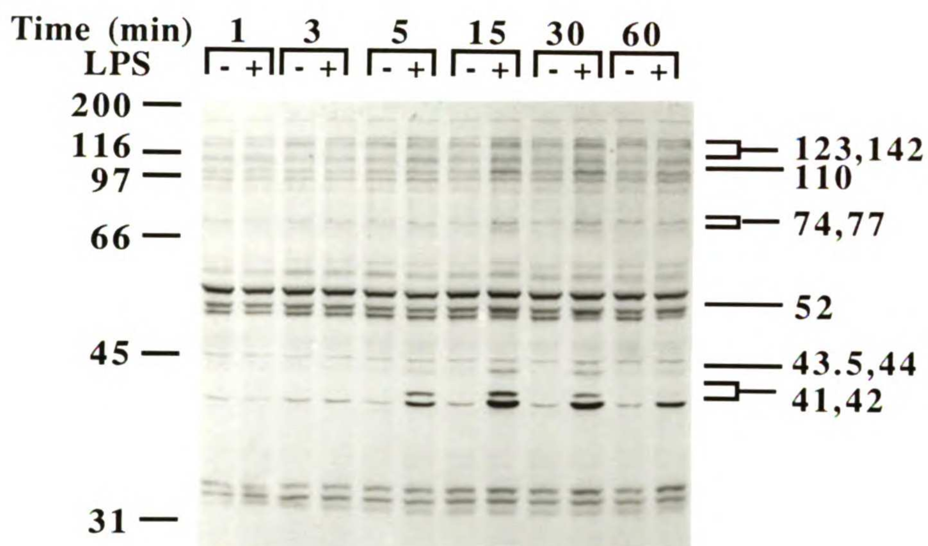
Results

LPS-induced tyrosine phosphorylation in RAW 264.7 cells.

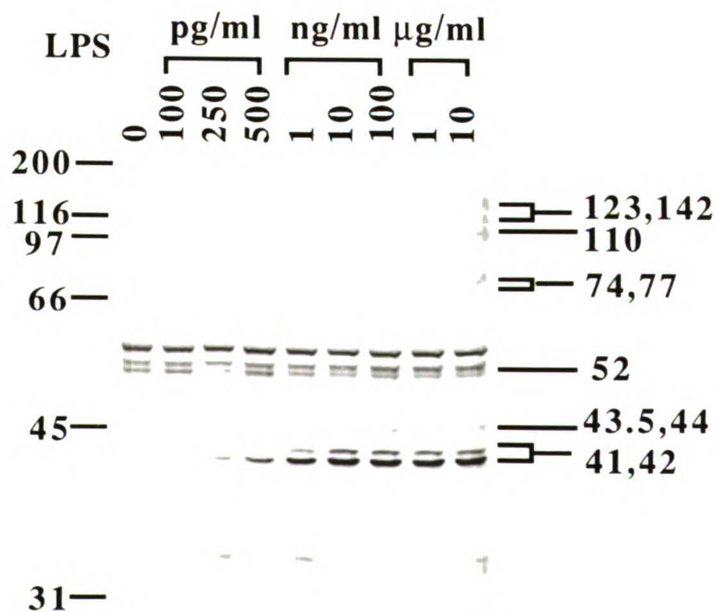
Using anti-phosphotyrosine immunoblotting, we examined whether LPS affects protein tyrosine phosphorylation in RAW 264.7 cells, a murine monocyte-macrophage cell line. *Escherichia coli* K235 LPS rapidly increased tyrosine phosphorylation of Triton X-100-soluble proteins with relative molecular weights of 41, 42, 43.5, 44, 52, 74, 77, 110, 123 and 142 kilodaltons (kDa) (Fig. 1a). 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 100 pg/ml LPS and was maximal at 10 ng/ml (Fig. 1b), doses comparable to those needed to induce biological responses in these cells (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 20 µg/ml actinomycin D or 10 µg/ml cycloheximide, 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.

Figure 1 LPS-induced tyrosine phosphorylation in RAW 264.7 cells. Anti-phosphotyrosine immunoblot of RAW 264.7 cell Triton X-100-soluble proteins. Cells were incubated with or without 1 μg/ml *Escherichia coli* K235 LPS for the indicated time (a); or, with the indicated concentrations of *E. coli* K235 LPS for 15 min (b). Approximate M_r in kDa of the induced proteins are indicated to the right of each blot. They were estimated using M_r standards that are indicated to the left of each blot.

a



b



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 similar 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 (MAGP)(22), did not increase tyrosine phosphorylation. Thus, the capacity of different forms of LPS to stimulate tyrosine phosphorylation paralleled 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 phorbol 12-myristate 13-acetate (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

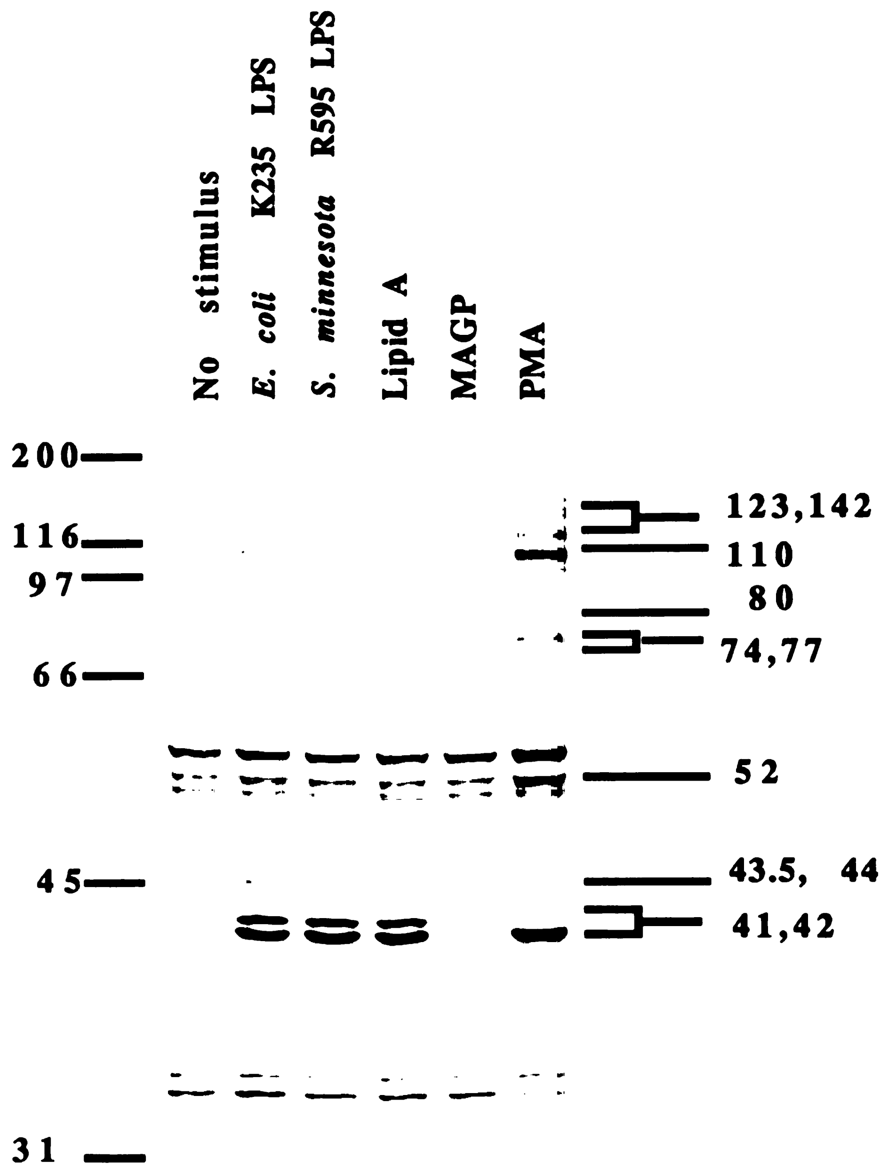


Figure 2 Stimulus specificity of induced tyrosine phosphorylation in RAW 264.7 cells. Anti-phosphotyrosine immunoblot analysis of RAW 264.7 cell Triton X-100-soluble proteins. Cells were stimulated for 15 min with 1 µg/ml *E.coli* K235 LPS, 1 µg/ml *Salmonella minnesota* R595 LPS, 1 µg/ml lipid A, 1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

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-, 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 MAGP 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 M_r, suggesting that the same proteins were modulated in non-transformed 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

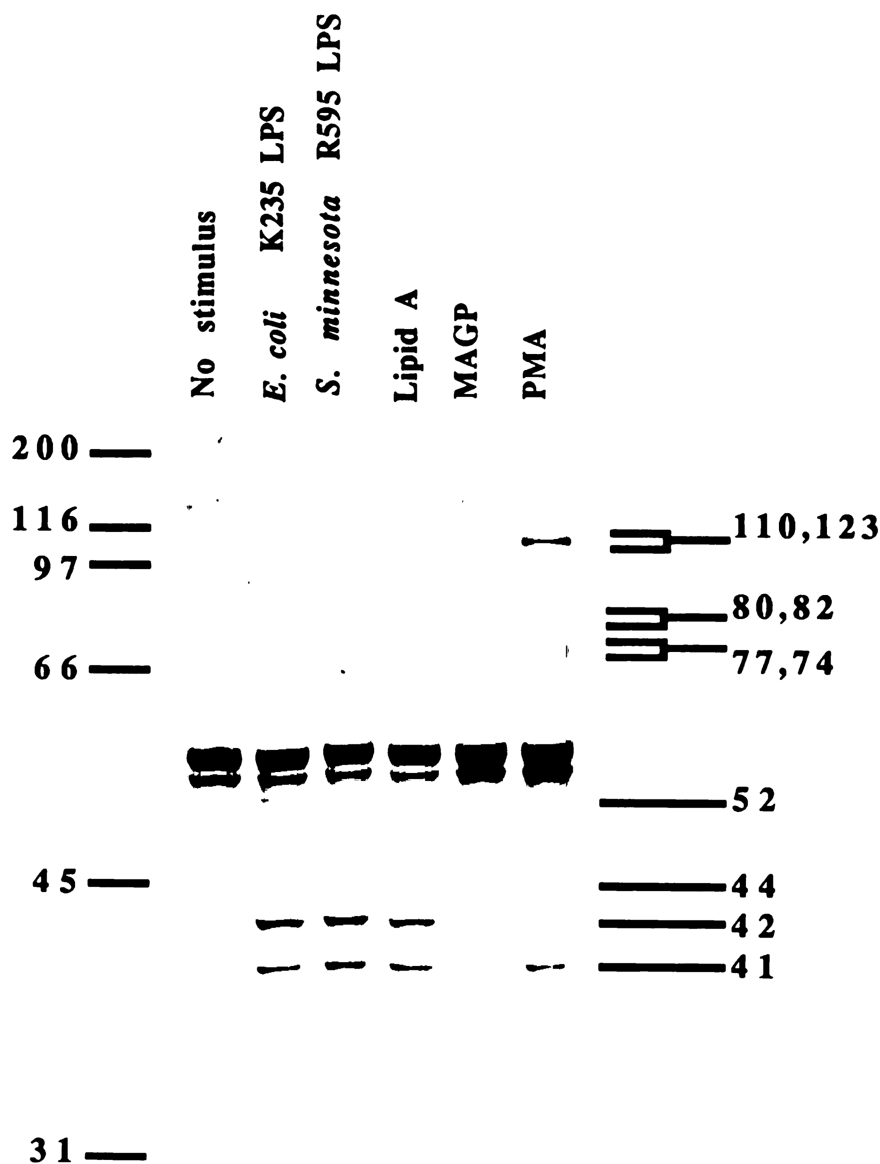


Figure 3 LPS stimulation of tyrosine phosphorylation in murine peritoneal macrophages. Anti-phosphotyrosine immunoblot of Triton X-100-soluble proteins from murine peritoneal macrophages. Macrophages were treated for 15 min with the indicated stimulus at the same concentration used in Fig. 2 . None of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

macrophages which makes these cells unresponsive to lipid A also interfers with the induction of protein tyrosine phosphorylation.

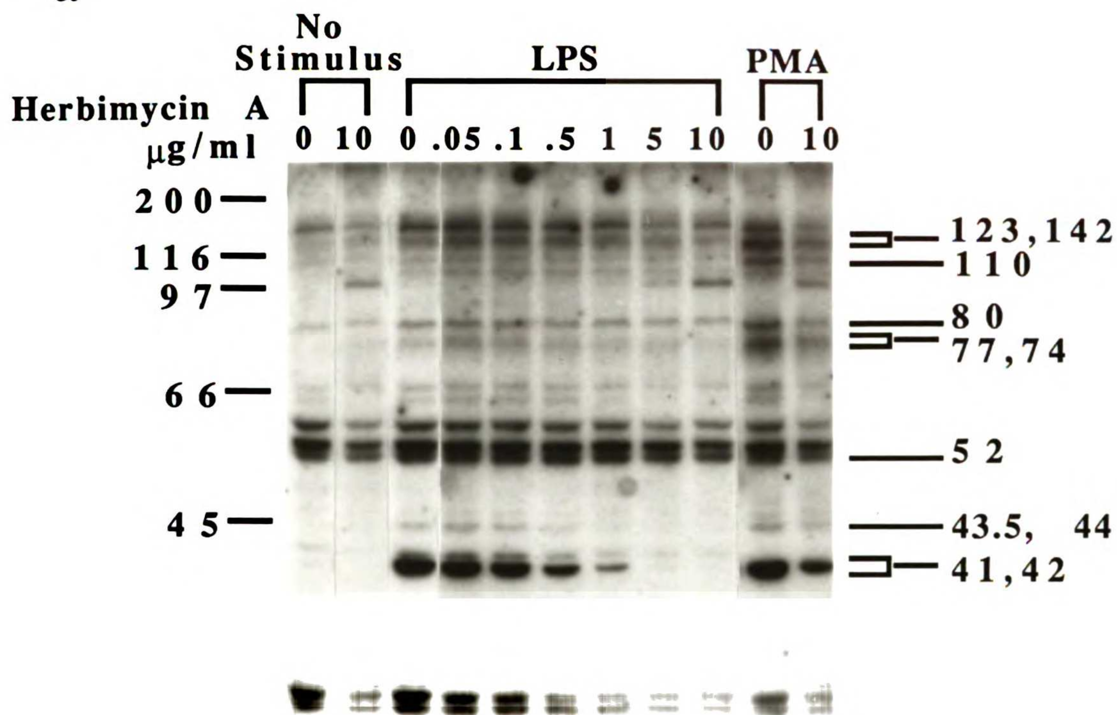
Effects of Herbimycin A on LPS- and PMA-induced Tyrosine Phosphorylation and Release of 20:4 Metabolites

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 (28 and unpublished data, M.R.Gold., A.L.DeFranco) 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 264.7 cells (Fig. 4). Inhibition of both responses was dose-dependent, with detectable inhibition occurring at 0.1 µg/ml herbimycin A, 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 non-specific effect of the drug or it could be caused by inhibition of tyrosine phosphorylation. Since LPS- and PMA-

Figure 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 h with the indicated concentration of herbimycin A and then stimulated for 15 min with 1 µg/ml *E. coli* K235 LPS or 100 nM PMA. Immunoreactive proteins were detected with the 4G10 monoclonal antibody followed by ^{125}I protein A and autoradiography for 15 d. (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 h. The cells were stimulated for 1 h with LPS or PMA. The culture medium was collected and counted for determination of released [^{3}H] 20:4 metabolites. 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).

a



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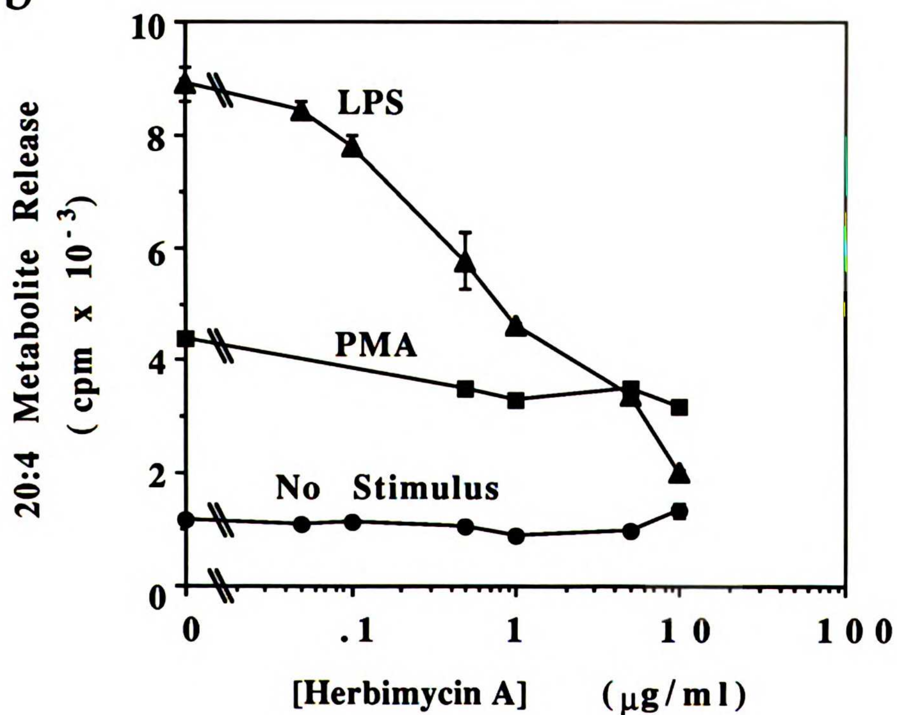

induced 20:4 metabolite release from RAW 264.7 cells require protein synthesis (unpublished data, S.J. Estey, S.L.Weinstein., A.L.DeFranco), we examined whether the partial inhibition of protein synthesis caused by herbimycin A could account for the inhibition of 20:4 metabolite release. This appeared not to be the case, as greater inhibition of ^{35}S-methionine incorporation following treatment with 0.1 μg/ml cycloheximide (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.

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 pre-existing 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) suggest that LPS does not directly bind to CD 14 on macrophages. Instead, LPS binds to a serum protein, LPS binding protein, resulting in complexes which then interact with CD 14. Additionally, CD14, which is a phosphatidylinositol glycan-linked membrane protein, may require interaction with transmembrane molecules which provide signaling activity. Thus, LPS receptor activation may be a multi-step 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 inhibiting PKC did not diminish LPS-induced tyrosine phosphorylation. Additionally, LPS-stimulated tyrosine phosphorylation was completely inhibited by 10 μg/ml herbimycin A treatment, 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 which 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 phosphorylation 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) have 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 phosphoinositide hydrolysis in peritoneal macrophages (11), and this signaling pathway may mediate some LPS effects. In the P388D1 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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Chapter Two:

Lipopolysaccharide-induced Protein Tyrosine Phosphorylation in Human Macrophages is Mediated by CD14

Summary

Induced protein tyrosine phosphorylation is an early intracellular response in lipopolysaccharide (LPS)-stimulated murine macrophages that appears to play a role in signal transduction in these cells. We have now demonstrated that LPS also stimulates protein tyrosine phosphorylation in human macrophages. This response was rapidly induced by biologically active forms of LPS or lipid A, at concentrations of these bacterial components which stimulate anti-bacterial responses by human macrophages. Thus, the characteristics of the tyrosine phosphorylation response were very similar to those previously observed in murine macrophages. CD14, which is thought to be a receptor for LPS, appeared to mediate induced protein tyrosine phosphorylation in human macrophages. Blocking CD14 with antibodies, specifically inhibited the induction of tyrosine phosphorylation by low concentrations (≤ 1 ng/ml) of LPS. At higher concentrations of LPS, however, anti-CD14 antibodies did not block the induction of protein tyrosine phosphorylation, indicating that a CD14-independent pathway also mediates the tyrosine phosphorylation response. Since both the CD14-dependent and CD14-independent LPS recognition mechanisms lead to similar functional responses by macrophages, induced protein tyrosine phosphorylation may be part of a shared intracellular signaling pathway.

Introduction

Macrophages play a central role in mobilizing the host defense against bacterial infection (1, 2). Following exposure to foreign microbes, macrophages develop an increased capacity to kill bacteria and secrete a number of immune mediators that stimulate anti-bacterial responses by other cells (3, 4). To perform these functions, macrophages must recognize and respond to bacterial molecules. One example of a bacterial component that can trigger macrophage responses is lipopolysaccharide (LPS), a major constituent of the outer membrane of Gram negative bacteria. LPS or its lipid A subdomain can elicit most of the macrophage responses seen during infection by Gram negative bacteria. Since the lipid A portion of LPS is structurally conserved in different strains of Gram negative bacteria (5), the recognition of this determinant by macrophages facilitates making effective immune responses to almost all Gram negative infections.

Recently progress has been made toward understanding the biochemical events that mediate macrophage responses to LPS. In the last few years, several groups have identified membrane molecules which may be receptors for LPS (6-11). Perhaps the most promising candidate receptor is CD14, a 55-kD glycoprotein that is attached to the macrophage membrane by a glycosyl-phosphatidylinositol (GPI) linkage (12-14). Interestingly, LPS, by itself, does not bind to CD14; instead, LPS first forms a complex with a serum protein, LPS-binding protein (LBP) (15) and the LPS-LBP complex serves as a ligand for CD14 (6). Activation of CD14 by LPS-LBP stimulates the release of tumor necrosis factor-alpha (TNF-α) from macrophages. Thus, CD14 appears to mediate at least some of the macrophage responses to LPS.

A second area of investigation which has also produced new insight concerns the early intracellular events induced by LPS. Recently, LPS was shown to rapidly increase the tyrosine phosphorylation of several proteins in murine macrophages and this response appears to have downstream cellular effects (16; chapter 1). For example, inhibiting the induction of protein tyrosine phosphorylation with the tyrosine kinase inhibitor, herbimycin A, prevents the release of eicosanoid mediators from LPS-stimulated RAW 264.7 macrophages. In addition, two of the LPS-induced tyrosine phosphoproteins are isozymes of mitogen-activated protein (MAP) kinase and the increased phosphorylation of these proteins leads to their enzymatic activation (17; chapter 3). Since MAP kinases are implicated in the regulation of a broad range of cellular processes (18, 19), the activation of these kinases in LPS-stimulated macrophages may be a critical step leading to anti-bacterial responses. Thus, the induction of protein tyrosine phosphorylation by LPS appears to be an important signal tranduction reaction in macrophages.

In this report, we show that LPS increases protein tyrosine phosphorylation in human macrophages and the features of this response are similar to those previously observed in murine macrophages. Moreover, antibodies to CD14, which are reported to prevent LPS-LBP complexes from binding to CD14, inhibit the induction of protein tyrosine phosphorylation by LPS. These results suggest that LPS-LBP binding to CD14 triggers intracellular signaling events such as protein tyrosine phosphorylation in macrophages.

Methods

Reagents LPS preparations were purchased from List Biological Labs (Campbell, CA) with the exceptions of *Escherichia coli.* type synthetic lipid A obtained from ICN Biomedicals (Cleveland, OH) and N^2-monoacylglucosamine 1-phosphate purchased from Lipidex (Madison, WI). Human AB serum was procured from Gemini (Calabasas, CA). Fetal bovine serum was purchased from Irvine Scientific (Irvine, CA). Hybridoma cell lines secreting the 3C10, anti-CD14 monoclonal antibody (TIB 228), the 63D3, anti-CD14 monoclonal antibody (HB 44) and the OKM1, anti-CD11b monoclonal antibody (CRL 8026) were obtained from the American Type Culture Collection (Rockville, MD). Ascites containing the 26ic anti-CD14 monoclonal antibody (20) was a gift from Dr. R. Todd (Univ. Michigan) and the 4G10 hybridoma cell line (21) that secretes an anti-phosphotyrosine antibody was a gift from Dr. D. Morrison (Frederick Cancer Research Center). Goat anti-mouse IgG-alkaline phosphatase conjugated antibodies were purchased from Bio-Rad (Richmond, CA). Phosphatidylinositol-specific phospholipase C was a gift from Dr. M. Low (Columbia Univ.). The BCA protein assay kit was obtained from Pierce Chemical Co. (Rockford, IL). The PVDF membranes were procured from Millipore (Bedford, MA). Ficoll-Hypaque and Percoll were purchased from Pharmacia (Piscataway, NJ). The ACK lysing reagent was purchased from Biofluids (Rockville, MD). All other chemicals were obtained from Sigma.

Preparation and culture of human monocytes Human peripheral blood mononuclear cells were purified from leucopaks by Ficoll-Hypaque gradient centrifugation (22). Residual erythrocytes in the mononuclear cell fraction were lysed using the ACK reagent and then the monocytes were isolated by

Percoll density gradient sedimentation (23). The resulting cell fraction contained greater than 85% monocytes ($CD14^+$) as assessed by flow cytometry. Monocytes (1 $x10^6$/ml) were cultured in RPMI 1640, 10% heat-inactivated human AB serum, 5% heat-inactivated fetal bovine serum, 50 µM 2-mercaptoethanol, 2mM glutamine and 50 µg/ml gentamicin (medium) in 400 ml teflon jars (Savillex, Minneapolis, MN) (100-150 ml cell suspension/jar) and were maintained in a 5% CO2/air atmosphere at 37 ^oC. The cultures were incubated 7-10 days prior to experimentation to allow the monocytes to differentiate into macrophages.

Macrophage stimulation and lysis For each experimental sample, 1 $x10^6$ macrophages were resuspended in 1 ml of medium supplemented with 20 mM Hepes, pH 7.4 in a sterile 1.5 ml polypropylene microcentrifuge tube (Brinkmann Instruments, Westbury, NY). Each tube was incubated at 37^oC and then the macrophages were stimulated by the addition of LPS or PMA as indicated. All experiments were conducted in the presence of serum which provided a source of LBP. In some experiments, the macrophages were incubated with affinity-purified anti-CD14 antibodies or affinity-purified control antibodies for 15 min prior to stimulation. Following stimulation, the macrophages were pelleted by centrifugation (10,000 xg for 20 s), the supernatant fraction was removed by aspiration and the cell pellet was washed with 1 ml of ice-cold phosphate-buffered saline containing 1 mM Na_3VO_4. The cells were pelleted again and lysed in 80 µl of lysis buffer (20 mM Tris-HCl, pH 8, 1% w/v Triton X-100, 137 mM NaCl, 2 mM EDTA, 1mM Na_3VO_4, 10% w/v glycerol, 1 mM PMSF, 10 µg/ml leupeptin amd 1 µg/ml aprotinin). The lysates were incubated on ice for 10 min and the insoluble material was removed by centrifugation (10,000 xg for 15 min at 4^oC). The

supernatant fraction was collected, analyzed for protein concentation using the BCA protein assay and then stored at -80°C prior to analysis.

Electrophoresis and anti-phosphotyrosine immunoblotting Equivalent amounts of macrophage lysate protein were resolved by SDS-PAGE on 1.5 mm thick, 12% polyacrylamide gels run at constant current (40 mA/gel). To improve the separation of the proteins of interest, electrophoresis was continued for 1 h after the bromophenol blue dye-front had run off the gel. The proteins were then electrotransferred from the gel to PVDF membranes at 0.5 A for 14 h at 4°C. Following transfer, the membranes were blocked with 2% w/v bovine serum albumin in Tris-buffered saline for 1 h at 25°C, washed two times with Tris-buffered saline containing 0.05% Tween 20 (TBST) and then incubated with the 4G10 anti-phosphotyrosine monoclonal antibody (hybridoma culture supernatant diluted 1:3 with TBST) overnight at 4°C. The next day the membranes were washed three times with TBST and then incubated with a 1:700 dilution of goat anti-mouse Ig-alkaline phosphatase conjugate in TBST for 1 h at 25°C. The membranes were washed three times with TBST and the phosphotyrosine-containing proteins were detected by incubating the membranes in 0.5 mg/ml 5-bromo-4-chloro-3-indolyl phosphate and 0.25 mg/ml nitro blue tetrazolium in 0.1 M $NaHCO_3$, 10 mM $MgCl_2$, pH 9.8. Color development was continued for 15-30 min to produce the desired darkness and the reaction was terminated by rinsing the membrane in water.

Results

LPS induces protein tyrosine phosphorylation in human macrophages

LPS rapidly increases the tyrosine phosphorylation of several proteins in murine macrophages and this intracellular effect appears to have some functional consequences in these cells (16; chapter 1). To further investigate the role of induced protein tyrosine phosphorylation in macrophages, we examined whether LPS elicits this response from human macrophages derived from peripheral blood monocytes. As detected by anti-phosphotyrosine immunoblotting, LPS (1 μg/ml) increased protein tyrosine phosphorylation in human macrophages with major induced species of: 41-, 42-, 43-, 74- and 82-kDa (Fig. 1). This response was detectable by 2.5 min of LPS treatment, maximal stimulation occurred by 10-15 min, and then the response declined with longer periods of LPS exposure. Concentrations of LPS as low as 0.1 ng/ml of LPS stimulated the response and doses of LPS between 10-100 ng/ml produced maximal induction (see below). Similar increases in protein tyrosine phosphorylation were stimulated by both smooth (wild type) and rough forms of LPS and by a synthetic preparation of the lipid A moiety of the LPS molecule (Fig. 2). Note that, induced tyrosine phosphorylation of the 74-kD and 82-kD proteins following synthetic lipid A treatment was more obvious in a duplicate experiment. In contrast to these results with biologically active forms of LPS, N^2-monoacylglucosamine 1-phosphate, an inactive lipid A derivative (24), did not induce any detectable changes in protein tyrosine phosphorylation. Thus, only biologically active LPS preparations, at concentrations known to trigger functional responses by human macrophages (25-27), increased protein tyrosine phosporylation. PMA also increased protein tyrosine phosphorylation in human

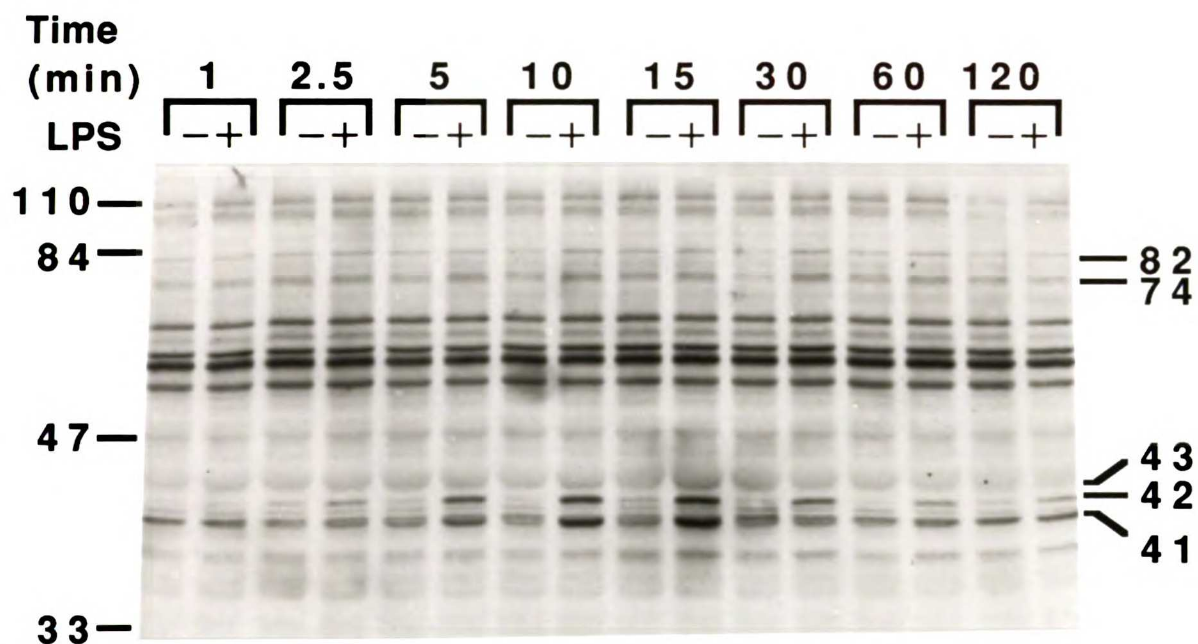


Figure 1 Kinetics of LPS-induced protein tyrosine phosphorylation in human macrophages. Macrophages were stimulated with 1 μg/ml of *Salmonella minnesota* Re595 LPS for the indicated time. The cells were lysed in Triton X-100 and the detergent-soluble proteins were separated by SDS-PAGE and analyzed by anti-phosphotyrosine immunoblotting. Induced tyrosine phosphoproteins were not observed in the Triton X-100 insoluble fraction. The relative molecular masses of the induced tyrosine phosphoproteins are indicated to the right of the blot. They were estimated from the Mr standards indicated to the left of the blot. Similar results were obtained in four experiments.

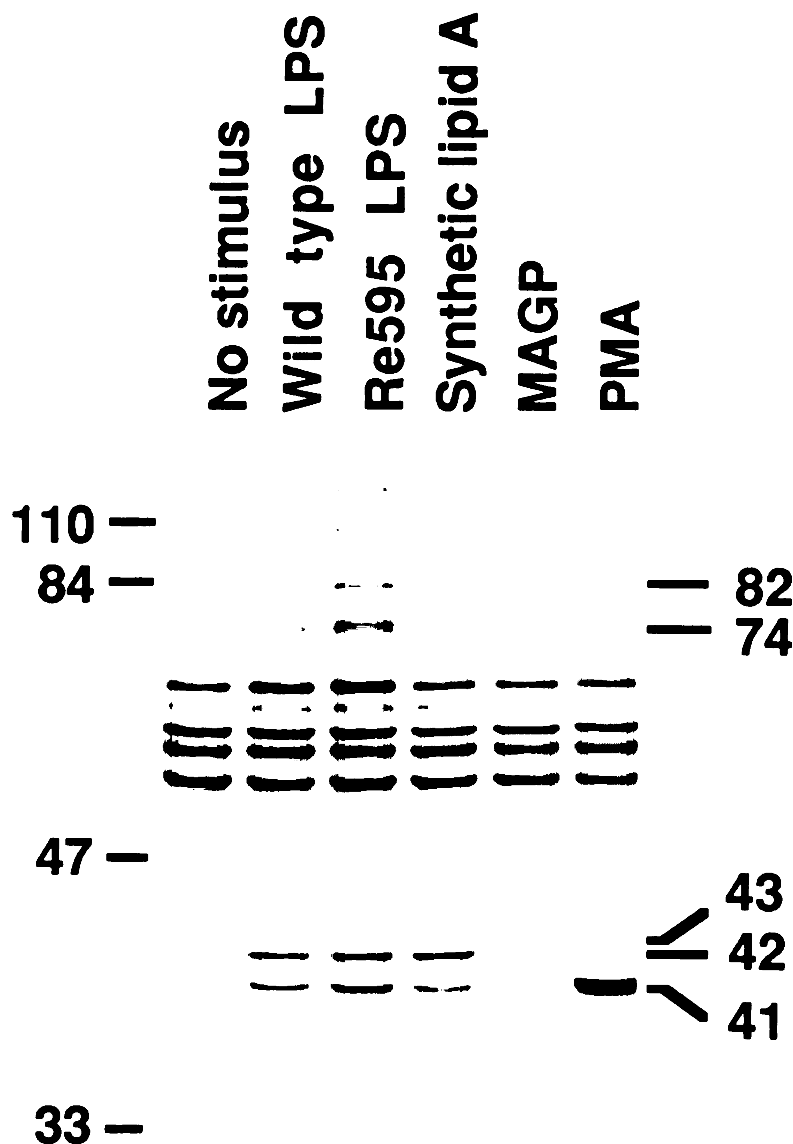


Figure 2 LPS-induced protein tyrosine phosphorylation in human macrophages. Macrophages were stimulated for 15 min with 100 nM PMA or with 1 μg/ml of the following: wild type *S. minnesota* LPS, rough type *S. minnesota* Re595 LPS, *E. coli* type synthetic lipid A or N^2-monoacylglucosamine 1-phosphate (MAGP). Following stimulation, Triton X-100 cell lysates were prepared and the detergent-soluble proteins were analyzed by anti-phosphotyrosine immunoblotting. Similar results were seen in duplicate experiments.

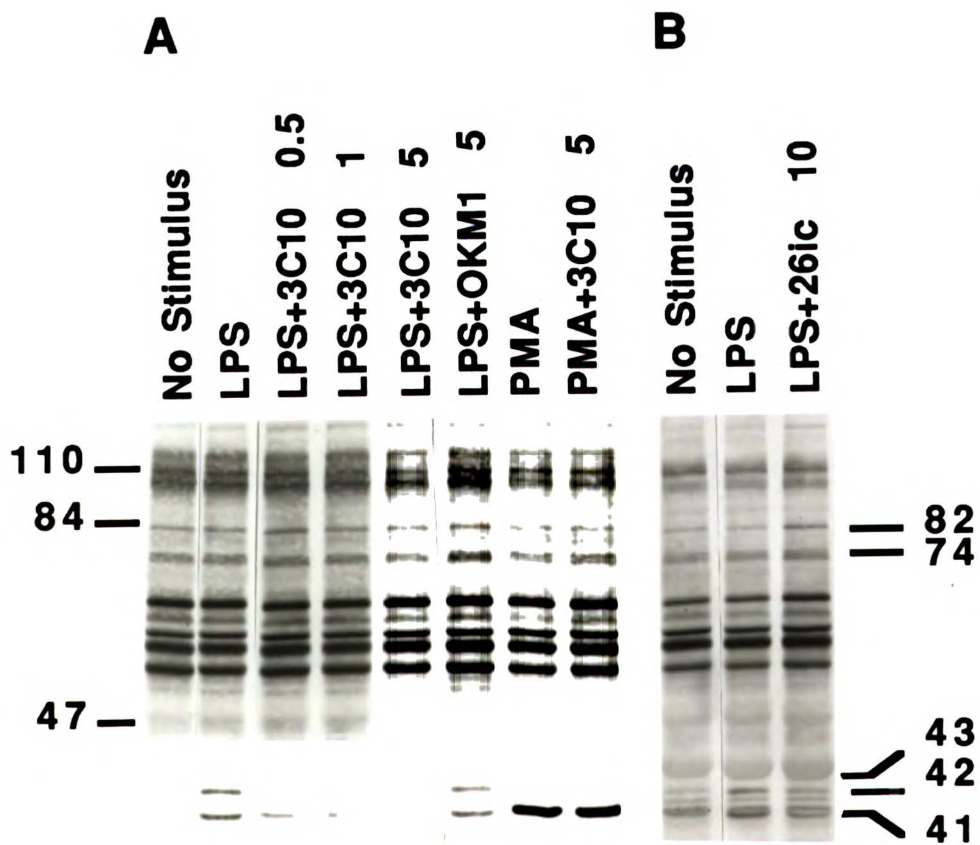
macrophages, although the pattern of tyrosine phosphoproteins induced by PMA was distinct from that observed following LPS treatment. Thus, LPS and PMA probably stimulate protein tyrosine phosphorylation by different mechanisms in human macrophages, as was the case in murine macrophages (16, 17; chapters 1 and 3). Together, these results indicate that the induced tyrosine phosphorylation response is very similar in human and murine macrophages. Finally, LPS also induced tyrosine phosphorylation in the THP-1 human monocytic cell line (data not shown). Interestingly, the induction of only a single tyrosine phosphoprotein of 40-45 kD was detected after LPS treatment in these cells. Nonetheless, induced protein tyrosine phosphorylation appears to be a common intracellular event that follows LPS exposure in human monocytes and macrophages.

Anti-CD14 antibodies inhibit LPS-induced protein tyrosine phsophorylation

Recent evidence suggests that the macrophage membrane protein, CD14, acts as a receptor for LPS (6). Antibodies to CD14 prevent LPS-LBP complexes from binding to macrophages and inhibit the LPS-stimulated release of TNF-α. Thus, LPS-LBP binding to CD14 appears to lead to some of the functional responses by macrophages. However, the intracellular signaling events triggered by CD14 activation are not known. Since induced protein tyrosine phosphorylation appears to be part of the LPS-activated signal transduction mechanism in macrophages, we tested whether CD14 mediates this response. In these experiments, human macrophages were pretreated with the 3C10 anti-CD14 monoclonal antibody, which prevents LPS-LBP complexes from binding to CD14 (6), and then the cells were stimulated with 1 ng/ml LPS. Following stimulation, the tyrosine phosphoproteins were analyzed by anti-phosphotyrosine immunoblotting. We found that anti-CD14 antibodies inhibited LPS-induced tyrosine

phosphorylation, with near complete inhibition achieved with an antibody concentration of 5 µg/ml (Fig. 3a). This antibody concentration has been reported to fully block LPS-LBP binding to CD14 (6). Additionally, two other anti-CD14 monoclonal antibodies, 26ic (Fig. 3b) and 63D3 (data not shown), produced similar inhibition of the response. In contrast, an isotype-matched control antibody, OKM1 (anti-CD11b), did not inhibit the LPS-stimulated response. Thus, the anti-CD14 antibody-mediated inhibition appeared to be a consequence of the antibodies binding to CD14, as opposed to them binding to Fc receptors. Also, 3C10 anti-CD14 antibodies did not inhibit PMA-stimulated protein tyrosine phosphorylation. Since PMA activates protein kinase C and by-passes membrane receptors, the PMA-induced response serves as a control for the specificity of the antibody-mediated inhibition. Thus, antibody blockade of CD14 specifically inhibited LPS-induced protein tyrosine phosphorylation. Additional evidence supporting the involvement of CD14 in the induced phosphorylation response was obtained with macrophages that had been treated to remove membrane-bound CD14. Since CD14 is attached to the membrane by a GPI-linkage, this protein can be cleaved from cells with a phosphatidylinositol-specific phospholipase C (PI-PLC). Human macrophages were incubated with PI-PLC, and then the LPS-stimulated tyrosine phosphorylation response was examined in these cells by anti-phosphotyrosine immunoblotting. PI-PLC treatment of the macrophages reduced surface CD14 by 45% (as determined by FACS, median fluorescence, data not shown) and caused a small but detectable decrease in the level of induction of protein tyrosine phosphorylation stimulated by LPS (data not shown). This effect was specific for the LPS-induced response as PMA- and CSF-1 -stimulated tyrosine phosphorylation responses were not

Figure 3 Anti-CD14 antibodies inhibited LPS-stimulated protein tyrosine phosphorylation. Human macrophages were incubated for 15 min with (a) either medium, 0.5, 1 or 5 µg/ml of the 3C10 anti-CD14 monoclonal antibody (IgG_{2b}), or 5 µg/ml of the OKM1 anti-CD11b monoclonal antibody (IgG_{2b}); or (b) 10 µg/ml of the 26ic anti-CD14 monoclonal antibody. The cells were then stimulated for 15 min with 1 ng/ml *S.minnesota* Re595 LPS or with 10 nM PMA. Following stimulation, Triton X-100 soluble cell lysates were prepared and were analyzed by anti-phosphotyrosine immunoblotting. Similar results to those shown in panel (a) and in panel (b) were obtained in three separate experiments and in duplicate experiments, respectively. In the other experiment with the 26ic monoclonal, this antibody was as effective as the 3C10 antibody in inhibiting LPS-stimulated protein tyrosine phosphorylation. Note that, in panel (b), two additional tyrosine phosphoproteins (41-42 -kD) were detected. The level of tyrosine phosphorylation of these proteins was not modulated by either LPS or anti-CD14 antibody treatments.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inhibited by PI-PLC treatment (data not shown). Thus, physically removing CD14 from the macrophage membrane or blocking CD14 with antibodies had similar inhibitory consequences on LPS-stimulated protein tyrosine phosphorylation. These results suggest that the binding of LPS-LBP to CD14 leads to increased intracellular protein tyrosine phosphorylation.

Inhibition of LPS-induced tyrosine phosphorylation by anti-CD14 antibodies is dependent on the LPS concentration

While LPS-LBP binding to CD14 appears to mediate some biological responses by macrophages, this pathway is not the only mechanism by which these cells respond to LPS. Several groups have shown that concentrations of LPS greater than 10 ng/ml can stimulate monocytes and macrophages to secrete TNF-α and IL-1 in the absence of CD14 or LBP (6, 28-30). Thus, macrophages seem to possess a second mechanism for responding to high concentrations of LPS that is independent of CD14. To examine the CD14-dependence of the LPS-induced tyrosine phosphorylation response at higher LPS concentrations, we pretreated macrophages with 3C10 anti-CD14 antibodies to block the CD14 -dependent pathway and then exposed the cells to increasing doses of LPS. As was observed in figure 3, the induction of protein tyrosine phosphorylation stimulated by doses of LPS of 1 ng/ml or less was completely inhibited by the anti-CD14 antibodies (Fig. 4). However, the extent of the inhibition diminished as the LPS concentration was raised to 10 ng/ml and very little inhibition was detected in cells treated with 1 μg/ml LPS. Thus, the protein tyrosine phosphorylation stimulated by high LPS concentrations appeared to occur independently of CD14. Interestingly, induction of tyrosine phosphorylation of the 74-kD species remained nearly completely inhibited by the anti-CD14 antibodies, even following stimulation with 1-10 μg/ml concentrations of LPS. This result implies that

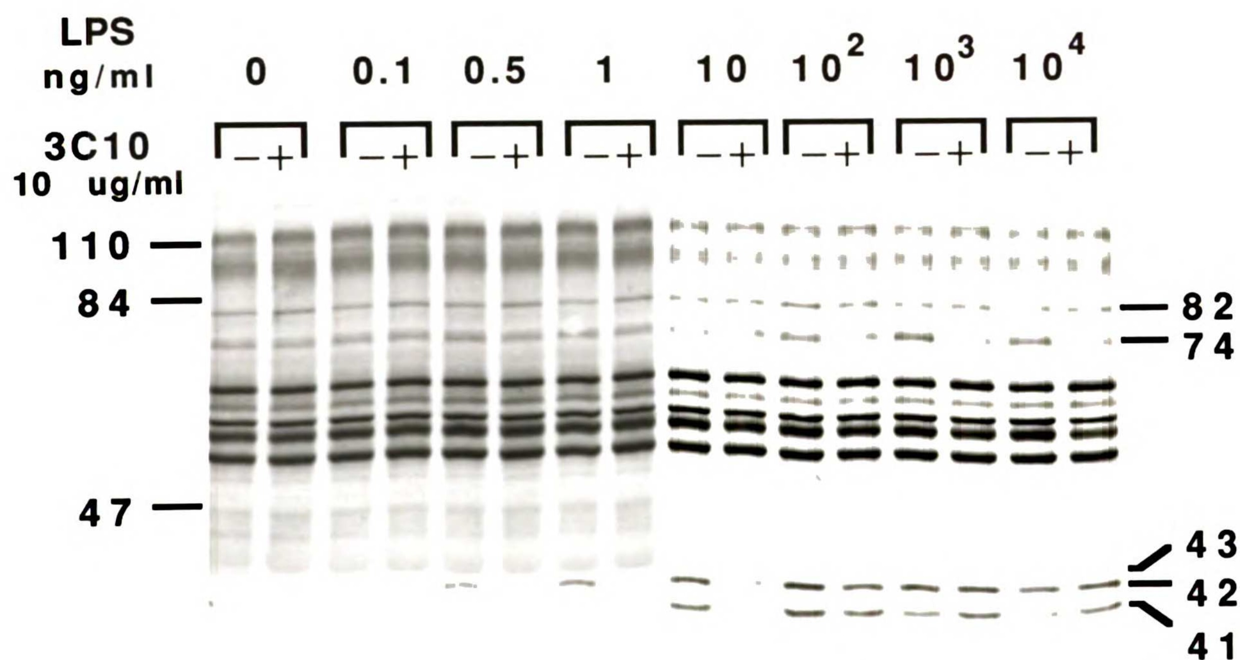

Figure 4 High LPS concentrations overcame anti-CD14 inhibition of induced protein tyrosine phosphorylation. Macrophages were incubated for 15 min with 10 µg/ml of the 3C10 anti-CD14 monoclonal antibody and then the cells were stimulated for 15 min with the indicated concentration of *S. minnesota* Re595 LPS. Following stimulation, cell lysates were prepared and were analyzed by anti-phosphotyrosine immunoblotting. The data shown is representative of results obtained in three separate experiments.

modulation of the 74-kD protein by LPS is strictly dependent on CD14, whereas induction of the other tyrosine phosphoproteins occurs by a CD14-dependent mechanism at low LPS concentrations and by a CD14-independent mechanism at high LPS concentrations.

Discussion

The experiments reported here demonstrate that LPS increases protein tyrosine phosphorylation in human macrophages. This response was rapidly stimulated by biologically active forms of LPS and lipid A, at concentrations of these bacterial components which trigger anti-bacterial responses by macrophages. These characteristics of the tyrosine phosphorylation response are very similar to those previously observed in murine macrophages. Thus, the induction of protein tyrosine phosphorylation is an evolutionarily conserved, intracellular response of LPS-stimulated macrophages. This conservation is consistent with this event playing an important role in LPS receptor signal transduction.

Previous work suggests that CD14 is an important membrane recognition structure for LPS on macrophages. We found that reducing the number of functional CD14 molecules on macrophages blunted the induction of protein tyrosine phosphorylation by LPS. Physically removing some of the membrane-attached CD14 with PI-PLC resulted in a diminished tyrosine phosphorylation response and blocking CD14 with antibodies had a more complete inhibitory effect. Thus, CD14 appears to be necessary for the induction of protein tyrosine phosphorylation at low LPS concentrations. These results suggest that the binding of LPS-LBP to CD14 triggers increased protein tyrosine phosphorylation in macrophages.

LPS-induced tyrosine phosphorylation, however, does not appear to occur solely through a CD14-mediated pathway. Under conditions where CD14 was blocked by anti-CD14 antibodies, doses of LPS greater than 10 ng/ml were able to stimulate much of the tyrosine phosphorylation response. This result did not appear to be a consequence of the high LPS

doses competing with the antibodies for binding to CD14. Even at high LPS concentrations, CD14 remained blocked by the antibodies, since one of the tyrosine phosphoproteins modulated by LPS could not be induced in the presence of the anti-CD14 antibodies. Thus, protein tyrosine phosphorylation can be stimulated by high concentrations of LPS through a CD14-independent pathway. Together, these results demonstrate that induced protein tyrosine phosphorylation is biochemically tied to the CD14-dependent and CD14-independent LPS recognition mechanisms that are believed to mediate functional responses by macrophages.

The intracellular signaling reactions that are triggered by the binding of LPS to macrophages and that lead to anti-bacterial responses by these cells are only beginning to be understood. One intracellular response to LPS that appears to have a role in signal transduction is the induction of protein tyrosine phosphorylation. This response is stimulated within minutes of LPS exposure, which is before anti-bacterial responses such as the secretion of TNF-α and eicosanoid mediators are detectable. Moreover, the induced tyrosine phosphorylation response does not require transcription or translation (16; chapter 1), in contrast to nearly all of the other responses of LPS-stimulated macrophages. These results are consistent with the induction of protein tyrosine phosphorylation being mechanistically upstream of most macrophage responses to LPS. In addition, the tyrosine phosphorylation response is initiated by the same extracellular LPS recognition events that are known to stimulate biological responses by macrophages. For example, LPS-LBP binding to CD14, which appears to mediate at least some anti-bacterial responses by macrophages, induces protein tyrosine phosphorylation. Similarly, LPS interaction with macrophages involving an as yet undefined, but CD14-independent,

recognition mechanism stimulates protein tyrosine phosphorylation as well as other functional responses. Thus, induced protein tyrosine phosphorylation could be an intermediate response that mechanistically connects CD14-dependent and CD14-independent recognition of LPS to the downstream anti-bacterial responses. Alternatively, induced protein tyrosine phosphorylation and the expression of anti-bacterial function could be completely independent responses initiated by the same LPS recognition events. The observation that blocking the induction of protein tyrosine phosphorylation with a tyrosine kinase inhibitor is accompanied by the inhibition of eicosanoid mediator release (16; chapter 1) suggests that at least some anti-bacterial reponses occur as a consequence of the tyrosine phosphorylation reaction. Thus, induced protein tyrosine phosphorylation appears to transduce both CD14-dependent and CD14-independent recognition of LPS into functional responses by macrophages.

The mechanisms by which the CD14-dependent and -independent pathways initiate intracellular protein tyrosine phosphorylation remain unknown. There are, however, some clues as to how CD14 might mediate signaling. CD14 is GPI-linked to the membrane and, therefore, lacks an intracellular domain which could directly transduce signals to the cell interior. However, GPI-linked proteins may associate with other transmembrane molecules that provide the signal transduction function. In fact, many GPI-linked proteins have increased mobility in the membrane which may facilitate establishing protein-protein interactions (31). Three observations support the notion that CD14 associates with other proteins resulting in the initiation of signaling. First, CD14 reportedly co-precipitates with tyrosine kinase activity (32), implying that CD14 associates directly or indirectly with a tyrosine kinase. This kinase potentially could mediate the

protein tyrosine phosphorylation response induced by LPS. Second, a monoclonal antibody to CD14 (26ic) that does not block the binding of LPS-LBP, nonetheless, inhibited LPS-induced tyrosine phosphorylation. One possible explanation for this result is that the antibody ablated signal transduction by sterically preventing CD14 from associating with critical signaling molecules. However, the 26ic antibody did not inhibit the stimulated secretion of TNF-α from LPS-treated whole human blood (6). While the reasons for these discrepant results are not clear, it is possible that the 26ic antibody prevented CD14-dependent signaling during the short time course experiments reported here, but was unable to fully prevent signaling during the several hour LPS incubation used to stimulate TNF-α secretion . A final piece of evidence that suggests that CD14 associates with other molecules has been reported by Lund-Johansen et al. (33). They have shown that cross-linking CD14 with an anti-CD14 monoclonal antibody followed by a secondary antibody triggers a spike in intracellular calcium and hydrogen peroxide production in human monocytes. In this experiment, the antibody-mediated cross-linking may mimic the effect of LPS-LBP binding and promote CD14 interaction with other proteins leading to intracellular effects. While these observations suggest a signaling mechanism involving the interaction of CD14 with other proteins, further experiments will be necessary to settle this issue.

Macrophages must recognize bacterial molecules such as LPS to make effective anti-bacterial responses, and CD14 appears to be a key molecule in this process. While macrophages can be activated by high doses of LPS in the absence of CD14, responses to sub-ng/ml concentrations of LPS depend on CD14. Thus, CD14 effectively increases the sensitivity of macrophages to LPS and enables these cells to make early responses to bacterial infection. On the

other hand, excessive stimulation of macrophages by LPS can contribute to life-threatening septic shock (34). One mechanism which may help macrophages to avoid this problem is to reduce the surface expression of CD14. Bazil and Strominger have shown that CD14 is proteolytically shed from macrophages as a consequence of treatment with LPS for long periods *in vitro* (35). This observation, also reported by Wright (36), suggests that the decreased expression of CD14 on the cell surface may be important for desensitizing macrophages to prolonged LPS exposure. In addition, other cells may influence CD14 expression on macrophages. Several groups have reported that IFN-γ and IL-4 down-modulate the expression of CD14 on monocytes and macrophages *in vitro* (37-41). Thus, other cells may reduce macrophage responsiveness to LPS by releasing these cytokines. Interestingly, IFN-γ generally enhances macrophage responsiveness to LPS, at least for some biological responses (3, 42). Thus, it is somewhat of a surprise that this cytokine may also have antagonistic effects on macrophage responses. Nonetheless, this type of regulation may allow cells that have been activated by chemical signals from LPS-stimulated macrophages to exert a degree of feedback control on the macrophages. Thus, cytokine-mediated down-modulation of CD14 may be a mechanism for deactivating macrophages during the late stages of an immune response to a bacterial infection. While a lot remains to be learned about the role of CD14 in macrophage responses to LPS during bacterial infections, these observations suggest that CD14-dependent recognition of LPS may be important for rapid and appropriate immune responses by macrophages.

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Chapter Three:

Bacterial Lipopolysaccharide Induces Tyrosine Phosphorylation and Activation of MAP Kinases in Macrophages

Summary

Bacterial lipopolysaccharide (LPS) is a potent activator of anti-bacterial responses by macrophages. Following LPS stimulation, the tyrosine phosphorylation of several proteins is rapidly increased in macrophages and this event appears to mediate some responses to LPS. We now report that two of these tyrosine phosphoproteins of 41 kDa and 44 kDa are isoforms of mitogen-activated protein (MAP) kinase. Each of these proteins was reactive with anti-MAP kinase antibodies and co-migrated with MAP kinase activity in fractions eluted from a MonoQ anion-exchange column. Following LPS stimulation, column fractions containing the tyrosine phosphorylated forms of p41 and p44 exhibited increased MAP kinase activity. Inhibition of LPS-induced tyrosine phosphorylation of these proteins was accompanied by inhibition of MAP kinase activity. Additionally, induction of p41/p44 tyrosine phosphorylation and MAP kinase activity by LPS appeared to be independent of activation of protein kinase C, even though phorbol esters also induced these responses. These results demonstrate that LPS induces the tyrosine phosphorylation and activation of at least two MAP kinase isozymes. Since MAP kinases appear to modulate cellular processes in response to extracellular signals, these kinases may be important targets for LPS action in macrophages.

Introduction

The major outer membrane component of Gram negative bacteria, lipopolysaccharide (LPS), is a potent activator of macrophage responses involved in the host defense against infection (1, 2). LPS-activated macrophages secrete several immunoregulators that promote anti-bacterial responses by other cells such as interleukin-1, tumor necrosis factor, and arachidonic acid metabolites (3, 4). In addition, the combination of LPS and interferon-γ induce macrophages to differentiate to a highly bactericidal state. However, excessive LPS stimulation of macrophages and other cells occurring during severe bacterial infections can also lead to extensive tissue damage and septic shock (5). Thus, a better understanding of the mechanisms by which macrophages are activated by LPS could provide important insight into the regulation of the host response to bacterial infection.

We have investigated the early biochemical events that are triggered in macrophages by LPS. Previously, we reported that LPS increases protein tyrosine phosphorylation in murine macrophages and that this early signaling event appears to mediate some downstream macrophage responses to LPS (6; chapter 1). To extend these findings, we have attempted to identify the molecular components involved in the induced phosphorylation response. One such component is the macrophage cell surface protein, CD14. This protein has been shown to bind complexes consisting of LPS and serum LPS-binding protein and has been implicated in the cellular response to LPS (7). Inhibition of LPS binding to CD14 with anti-CD14 antibodies also inhibited LPS-induced tyrosine phosphorylation in human macrophages (chapter 2). This observation suggests that CD14 plays a role in mediating

this signaling event. Also of interest are the identities of the proteins that undergo increased tyrosine phosphorylation following LPS stimulation. Among the most prominent tyrosine phosphorylated bands in LPS-stimulated macrophages are a series of 40-45 -kDa proteins (6; chapter 1). These molecular masses are similar to those of a family of serine/threonine protein kinases known as mitogen-activated protein (MAP) kinases (8-10). MAP kinases appear to participate in the signal transduction pathways activated by a variety of extracellular ligands. These kinases have been shown in several cell types to be rapidly phosphorylated on tyrosine residues following cellular activation and this modification contributes to the increased enzymatic activity of these proteins. The *in vitro* phosphorylation and activation of the 90 kDa ribosomal S6 protein kinase (11, 12) and the c-*jun* transcription factor (13) by MAP kinases suggest that they may regulate fundamental cellular processes.

Given the growing evidence that MAP kinases are important signal transduction components, we tested whether any of the tyrosine phosphorylated bands observed in LPS-stimulated macrophages correspond to MAP kinases and whether these enzymes become activated following LPS treatment. In this report, we show that MAP kinase activity is increased following LPS treatment and this response appears to occur as the result of tyrosine phosphorylation of at least two different MAP kinase isozymes. Thus, MAP kinases are the first identified substrates for LPS-induced tyrosine phosphorylation.

Methods

Materials - Preparations of LPS were purchased from List Biological Laboratories (Campbell, CA) and Diphosphoryl lipid A was purchased from Ribi Immunochemical Research (Hamilton, MT). Synthetic lipid A (diphosphoryl, *Escherichia coli* type), [γ-^{32}P]ATP and the PY-20 anti-phosphotyrosine monoclonal antibody were from ICN Biomedicals. The 4G10 anti-phosphotyrosine monoclonal antibody was a gift from D. Morrison (Frederick Cancer Institute). Goat anti-rabbit IgG and goat anti-mouse IgG conjugated to alkaline phosphatase were procured from Bio-Rad. Sheep anti-mouse IgG conjugated to horseradish peroxidase and the ECL detection kit for immunoblotting were purchased from Amersham. Herbimycin A was obtained from N.R. Lomax, Drug Synthesis and Chemical Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). Compound 3 was a gift from Dr. Michael Venuti, Department of Bio-Organic Chemistry, Genentech (San Francisco, CA). Complete and Incomplete Freund's Adjuvant were from GIBCO. The BCA protein assay kit was purchased from Pierce Chemical (Rockford, IL). Other reagents were purchased from Sigma.

Cell culture, stimulation and lysis -The murine macrophage cell line, RAW 264.7 (American Type Culture Collection, Rockville, MD), was cultured in Dulbecco's modified Eagle medium containing 10% heat-inactivated fetal bovine serum and 2mM glutamine at 37 ^{o}C in a 5% CO_2/air mixture. For each experimental sample, 2 x10^7 cells were seeded into a 150 mm dish in 20 ml of medium. The cells were then cultured for about 18 h to allow the cell number to approximately double. Cells were stimulated by the addition of the indicated activator for 15 min. In some experiments, prior to the

addition of stimulators, cells were pretreated with 10 μg/ml herbimycin A for 4 h or 10 μM Compound 3 for 20 min. Following stimulation, cells were washed *in situ* with ice-cold phosphate-buffered saline containing 1 mM Na_3VO_4, then lysed in 2 ml of 20 mM MOPS, pH 7.2, 5 mM [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA), 1% (wt/vol) NP-40, 1 mM dithiothreitol, 75 mM β-glycerol phosphate, 1 mM Na_3VO_4 and 1 mM phenylmethylsulfonylfluoride for 20 min at 4 ^{o}C. The detergent-insoluble material was pelleted by centifugation (10,000 xg, 15 min, 4 ^{o}C) and the soluble supernatant fraction was removed and stored at -80 ^{o}C.

For immunoprecipitation of MAP kinase, cultures were seeded at 4 $\times 10^6$ cells in a 100 mm dish in 10 ml medium. The cells were then cultured for about 18 h to allow the cell number to approximately double. Following stimulation, the cells were lysed in 0.3 ml boiling 0.5% SDS, 10 mM Tris, pH 7.3, 1 mM dithiothreitol. Lysates were boiled an additional 5 min and the insoluble material was removed by centrifugation (10,000 xg, 15 min, 25 ^{o}C).

The protein concentration of the macrophage lysates was determined using the BCA assay.

Isolation and assay of MAP kinases - Macrophage lysate protein (~1 mg) was loaded onto a MonoQ anion-exchange column (1 ml bed volume) equilibrated in column buffer (12.5 mM MOPS, pH 7.2, 0.5 mM EGTA, 2 mM dithiothreitol, 12.5 mM β-glycerol phosphate, 7.5 mM $MgCl_2$). The column was eluted with a 20 ml linear gradient of 0-0.8 M NaCl using a Pharmacia fast protein liquid chromatography system, and 250 ml fractions were collected. MAP kinase activity of MonoQ fractions was assessed by assaying the myelin basic protein (MBP) phosphorylating activity as described previously (14).

Production of MAP kinase antibodies. Antibodies were raised to synthetic peptides from the rat extracellular signal-regulated kinase 1 (ERK 1) sequence: subdomain III (*erk1*-III, PFEHQTYCQRTLREIQILLGFRHENVIGIRDILRAP-GGC), from the C-terminus (*erk1*-CT, CGG-PFTFDMELDDLPKERLKELIFQETARFQPGAPEAP), and from the ATP-binding site of the 44-kDa MAP kinase encoded by the sea star *mpk* gene ($p44^{mpk}$) (mpk-I, GLAYIGEGAYGMVC). New Zealand White rabbits were immunized subcutaneously at four sites with ~500 µg of KLH-coupled peptide emulsified in complete Freund's adjuvant (one ml final volume). Rabbits were subsequently boosted every four weeks intramuscularly at two sites with 500 µg of KLH-coupled peptide emulsified in incomplete Freund's adjuvant. Ear bleeding was performed two weeks after each boost. The blood was permitted to clot at 37°C for 30 min and then incubated at 4°C overnight to allow the clot to contract. The antisera were collected and stored at -20°C. Antipeptide antibodies were affinity purified on the appropriate peptide-agarose column by eluting with 0.1 M glycine, pH 2.5 and subsequent neutralization with a saturated Tris solution. Antibody titres were estimated by standard ELISA techniques. Rabbit polyclonal antibodies raised against the purified sea star $p44^{mpk}$ protein were prepared as described previously (15) and affinity purified on a $p44^{mpk}$-agarose column. The mouse monoclonal anti-MAP kinase antibody used for the immunoprecipitation experiment was purchased from Zymed (San Francisco, CA).

Electrophoresis and immunoblotting - Protein samples were prepared for electrophoresis by mixing with a concentrated sample buffer to obtain a final concentration of 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 100 mM dithiothreitol, 10% glycerol and 0.01% bromophenol blue. Samples were then separated on

12% SDS-polyacrylamide gels using the buffer system described by Laemmli (16). Following electrophoresis, the separating gel was soaked in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) for 5 min and then the proteins were transferred to nitrocellulose for 4 h at 0.5 A. Subsequently, the nitrocellulose membrane was blocked with Tris buffered saline (TBS) containing 3% gelatin or 2% bovine serum albumin for 2 h at room temperature. The membrane was washed twice with TBS containing 0.05% Tween 20 (TBST) for 5 min before overnight incubation with rabbit polyclonal anti-MAP kinase antibodies or the mouse monoclonal PY-20 anti-phosphotyrosine antibody (in 1 % gelatin-TBST; 1:1000 dilution) or the mouse monoclonal 4G10 anti-phosphotyrosine antibody (1:3 diluted culture supernatent in TBST). The next day, the membrane was washed twice with TBST before incubation with the second antibody (goat anti-rabbit IgG or goat anti-mouse IgG coupled to alkaline phosphatase in 1% gelatin-TBST; 1:3000 dilution) for 2 h at room temperature. The membrane was rinsed with 2 washes of TBST, followed by 1 wash with TBS prior to color development with 0.5 mg/ml 5-bromo-4-chloro-3-indolyl phosphate and 0.25 mg/ml nitro blue tetrazolium in 0.1 M $NaHCO_3$, 10 mM $MgCl_2$, pH 9.8. The color development was continued for 5 min to 4 h to give the desired darkness, and the reaction was stopped by rinsing the membrane in a large volume of water.

Immunoprecipitation of MAP kinase - Cell lysates in 0.5% SDS, 10 mM Tris pH 7.3, 1 mM dithiothreitol were diluted 1:5 with 12.5 mM Tris pH 7.3, 187.5 mM NaCl, 1.25% deoxycholate, 1.25% Triton X-100, 0.625% NP-40, 1.25 mM EDTA, 1.25 mM EGTA, 0.25 mM Na_3VO_4, 0.25 mM phenylmethylsulfonylfluoride. Diluted lysates (1.5 ml) were precleared with 3 µg of affinity purified rabbit anti-mouse IgG and 40 µl of packed Protein A-

Sepharose beads for 30 min. The lysates were then incubated with a combination of 5 mg of a mouse monclonal anti-MAP kinase antibody and 5 µl of *erk1*-CT anti-MAP kinase polyclonal antibodies for 2 h followed by the addition of 3 µg of affinity purified rabbit anti-mouse IgG for 1 h. Immune complexes were precipitated by transferring the lysates to tubes containing 40 µl of packed Protein A-Sepharose beads and incubating for 1 h. All incubations were performed at 4°C. The beads were washed once with 1 ml of wash buffer (10 mM Tris, pH 7.3, 2 M NaCl, 0.1% SDS, 1% deoxycholate, 1% Triton X-100, 0.5% NP-40, 1 mM EDTA, 1 mM EGTA, 0.2 mM Na_3VO_4, 0.2 mM phenylmethylsulfonylfluoride) and then 3 times with 1 ml of wash buffer containing 150 mM NaCl. The beads were resuspended in 40 µl of 2X concentrated SDS sample buffer and boiled 10 min. The supernatent fraction was resolved on a SDS-polyacrylamide gel and then transferred to a nitrocellulose membrane. The tyrosine phosphoproteins were visualized by immunoblotting with the 4G10 anti-phosphotyrosine monoclonal antibody followed by sheep anti-mouse IgG-horseradish peroxidase antibodies (1:15,000 dilution in TBST, 1 h, 25°C) and an enhanced chemiluminescence detection system used as directed by the manufacturer (Amersham). The membrane was stripped (2% SDS, 62.5 mM Tris pH 6.7, 100 mM 2-mercaptoethanol, 50°C, 30 min) and reprobed with a mouse monoclonal anti-MAP kinase antibody (1:5,000 dilution in TBST, overnight, 4°C), followed by sheep anti-mouse IgG-horseradish peroxidase antibodies (1:15,000 dilution in TBST, 1 h, 25°C). The immunoreactive proteins were then visualized on film by chemiluminescence.

Results

LPS- and PMA-induced tyrosine phosphorylated p41 and p44 correspond to MAP kinase isozymes

LPS rapidly increases the tyrosine phosphorylation of several proteins in the macrophage cell line, RAW 264.7, including four bands of 41 kDa, 42 kDa, 43.5 kDa and 44 kDa as assessed by anti-phosphotyrosine immunoblotting [(6); chapter 1 and Fig. 1A, F]. In addition, treatment of these cells with phorbol 12-myristate 13-acetate (PMA) induces tyrosine phosphorylation of the 41-kDa and 44-kDa-proteins. To test whether any of these LPS- and PMA-induced bands correspond to MAP kinase isozymes, parallel immunoblots were probed with a panel of antibodies specific for MAP kinases (Fig 1B-E). These antibodies were raised against the purified sea star MAP kinase, p44*mpk*, or against peptides derived from p44*mpk* or the rat ERK 1 gene product. These antibodies have been shown to detect a 41-42 -kDa isoform of MAP kinase as well as a 43-44 -kDa isoform in 3T3 fibroblasts (11) and in other cell types[1]. In RAW 264.7 cells, these antibodies detected two isoforms of MAP kinase which co-migrated with the 41-kDa and the 44-kDa tyrosine phosphoproteins induced by LPS and PMA. In contrast, the anti-MAP kinase antibodies did not react with bands corresponding to the 42-kDa or the 43.5-kDa tyrosine phosphoproteins induced by LPS but not PMA. Thus, RAW 264.7 cells have at least two isoforms of MAP kinase and each isoform co-migrates on SDS polyacrylamide gels with a protein that becomes tyrosine phosphorylated following treatment with LPS or PMA. Interestingly, the 41-kDa immunoreactive MAP kinase from LPS- and PMA-stimulated cells

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[1] unpublished results, S.L. Pelech.

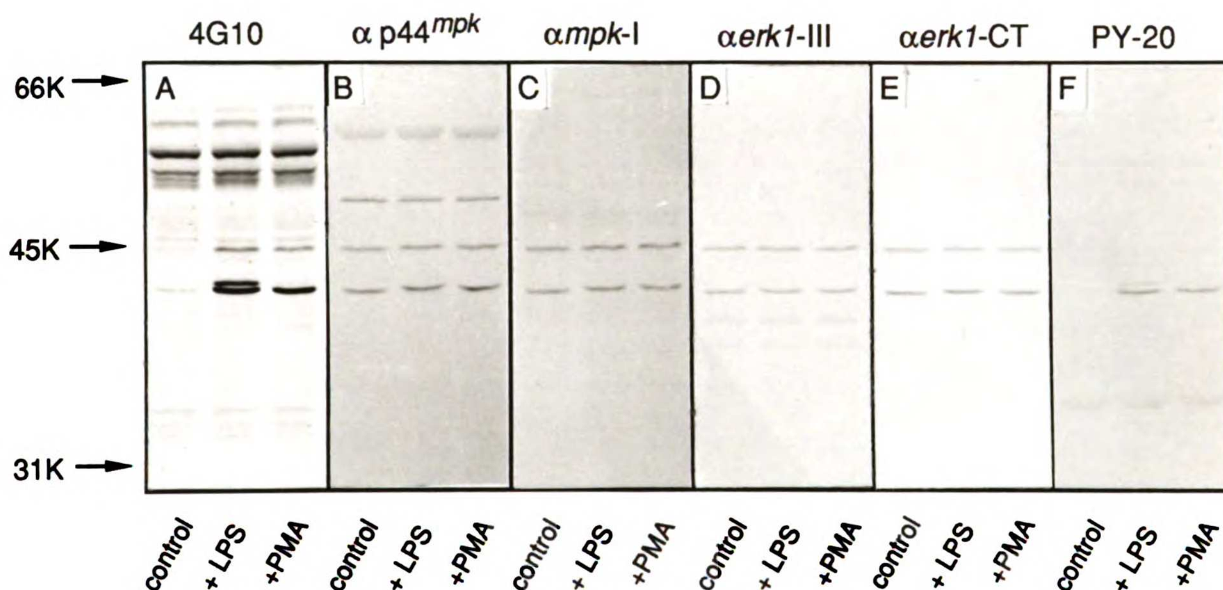


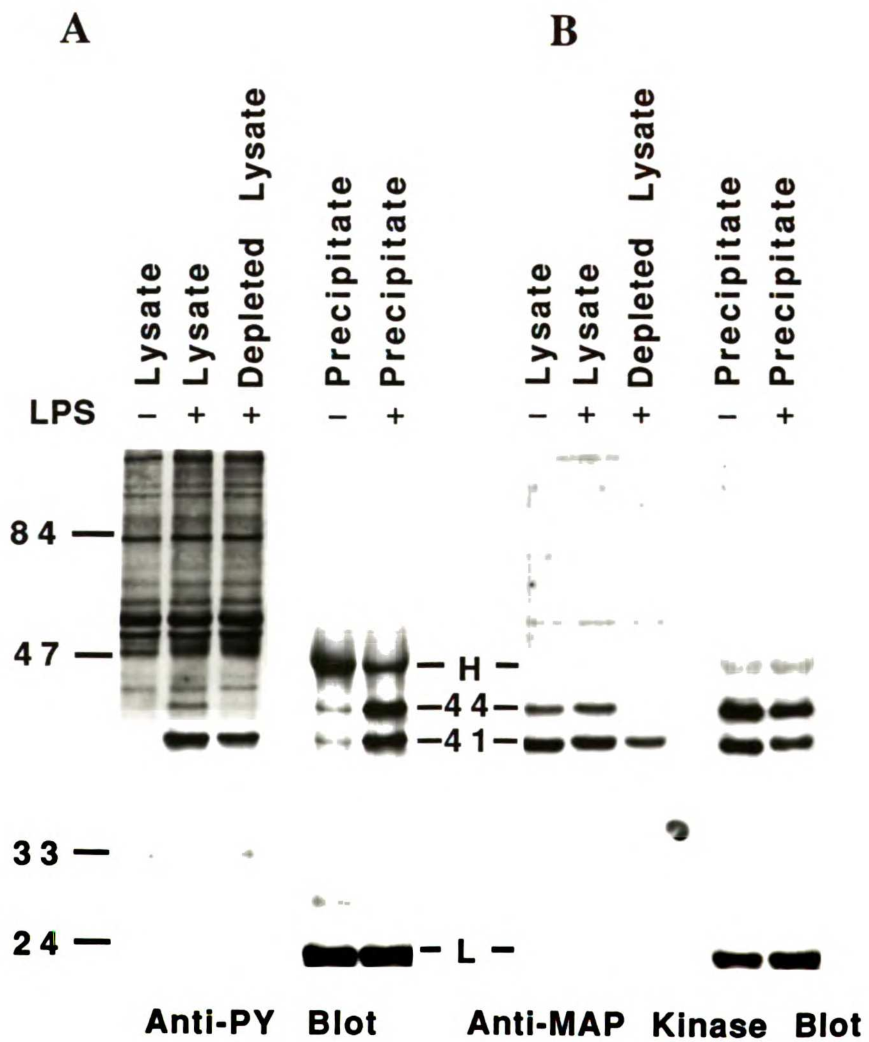
Figure 1 Anti-phosphotyrosine and anti-MAP kinase immunoblot analysis of proteins from RAW 264.7 cells. Cells were stimulated with 1 μg/ml LPS or 100 nM PMA for 15 min and then cell lysates were prepared. The soluble proteins were separated by SDS polyacrylamide gel electrophoresis, transferred to nitrocellulose and immunoblotted either with 4G10 or PY-20 anti-phosphotyrosine antibodies or with anti-MAP kinase antibodies as indicated. Panels shown were from one of two slab gels run in parallel and were aligned by the molecular weight markers whose positions are indicated on the left (M_r).

migrated slightly more slowly on the SDS-polyacrylamide gel than the corresponding protein from unstimulated cells. A similar mobility shift has been reported for a 42 kDa MAP kinase isozyme in *Xenopus* oocytes following progesterone treatment (17) and in platelet-derived growth factor-stimulated fibroblasts (18). It has been suggested that this mobility shift may be a consequence of increased phosphorylation.

To further assess the relationship between the 41-kDa and 44-kDa proteins detected by anti-phosphotyrosine antibodies and by anti-MAP kinase antibodies, RAW 264.7 cell lysates were immunoprecipitated with a mixture of monoclonal and polyclonal anti-MAP kinase antibodies and the resulting precipitated and unprecipitated fractions were separated on a SDS-polyacrylamide gel and immunoblotted with anti-phosphotyrosine antibodies. Following anti-MAP kinase immunoprecipitation, the 44-kDa tyrosine phosphoprotein was completely depleted and the 41-kDa phosphoprotein was partially depleted from the cell lysate (Fig. 2A). This result indicates that both of these proteins were recognized by the anti-MAP kinase antibodies. Depletion of these two proteins was specific as other tyrosine phosphoproteins were not affected. Moreover, when these blots were stripped and reprobed with anti-MAP kinase antibodies, the 41-kDa and 44-kDa MAP kinase isoforms were found to be depleted to the same extent as the corresponding tyrosine phosphoproteins (Fig 2B). These results suggest that the 41-kDa and 44-kDa tyrosine phosphoproteins induced by LPS are isoforms of MAP kinases.

The anti-MAP kinase immunoprecipitated fractions were also analyzed by immunoblotting with anti-phosphotyrosine antibodies (Fig. 2A). We found that the 41-kDa and 44-kDa MAP kinase isoforms were phosphorylated on tyrosine to a greater extent after stimulation with LPS.

Figure 2 Anti-MAP kinase immunoprecipitation of tyrosine phosphoproteins from LPS-stimulated RAW 264.7 cells. SDS-containing detergent lysates prepared from unstimulated and LPS-stimulated (1 µg/ml for 15 min) cells were immunoprecipitated with a combination of an anti-MAP kinase monoclonal antibody and erk1-CT anti-MAP kinase polyclonal antibodies. The immunodepleted lysates and precipitated fractions were immunoblotted with the 4G10 anti-phosphotyrosine monoclonal antibody and the tyrosine phosphoproteins were visualized by enhanced chemiluminescence (A). The blot was stripped and reprobed with an anti-MAP kinase monoclonal antibody and the immunoreactive proteins were detected by enhanced chemiluminescence (B). Note that, the 42-kD, LPS-induced tyrosine phosphoprotein was not detected on the anti-phosphotyrosine immunoblot. The phosphotyrosine epitope of this protein appears to have been destroyed by the denaturing conditions used for immunoprecipitation. Also, the samples were resolved on a 2 mm thick SDS-polyacrylamide gel which resulted in greater band separation than in Figure 1. The position of heavy chain (H) and light chain (L) of the immunoprecipitating antibodies and the position (M_r) of the LPS-induced tyrosine phosphoproteins are indicated between the blots. The position of M_r standards is indicated at the left. Similar results were obtained in three experiments.


A
B
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Lysate
Lysate
Depleted Lysate
Precipitate
Precipitate
84
47
33
24
H
44
41
L
Anti-PY Blot
Anti-MAP Kinase Blot

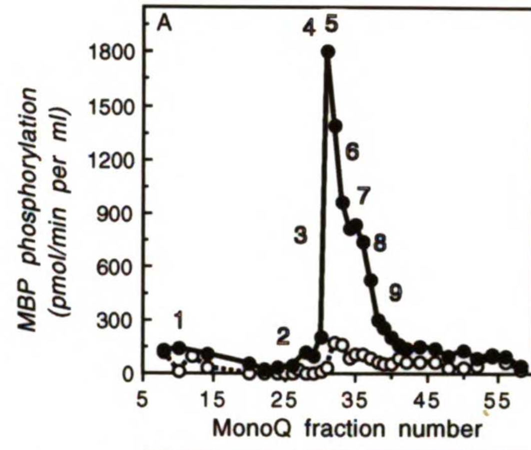
Since similar amounts of these MAP kinases were immunoprecipitated from the unstimulated and the LPS-stimulated cell lysates (Fig. 2B), these results directly demonstrate that LPS treatment increases the tyrosine phosphorylation of the 41-kDa and 44-kDa MAP kinases.

LPS activates MAP kinase isoforms

Since LPS increases the tyrosine phosphorylation of two MAP kinase isozymes and as tyrosine phosphorylation is thought to be a critical event activating these enzymes, we tested whether LPS alters MAP kinase activity. RAW 264.7 cell lysates were fractionated by MonoQ anion exchange chromatography and the resulting column fractions were analyzed for MAP kinase activity and immunoblotted with anti-phosphotyrosine antibodies or anti-MAP kinase antibodies. LPS treatment resulted in a large increase in MAP kinase activity as assessed by the phosphorylation of myelin basic protein (MBP), a standard substrate of MAP kinase (Fig. 3A). The increased activity was partially resolved into two peaks by Mono Q chromatography with a main peak of activity in column fractions 30-35 and a smaller, second peak in fractions 35-38. In some other experiments the resolution into 2 peaks was more evident (see below).

LPS treatment also resulted in the increased tyrosine phosphorylation of the 41-kDa and 44-kDa proteins that correspond to isoforms of MAP kinase (Fig. 3B, C). Induced phosphorylation of these isozymes was accompanied by changes in the migration of these proteins on the immunoblots (Fig. 3 B-G) . As was observed in Figure 1, most of the 41-kDa MAP kinase isoform was shifted to a higher apparent Mr as indicated by the arrow in Figure 3, panels B-G. In addition, the 44-kDa MAP kinase isoform from LPS-stimulated cells eluted from the MonoQ column in later fractions indicating that this protein was bound more tightly to the column. Both of

Figure 3 Anion exchange chromatography of MAP kinases from LPS-activated RAW 264.7 cells. NP-40 soluble proteins from untreated cells (O) and cells exposed to 1 μg/ml LPS for 15 min (●) were fractionated by MonoQ anion exchange chromatography as described in the Experimental Procedures. The column fractions were analyzed for MAP kinase activity using MBP as a substrate (A). Adjacent column fractions were combined as indicated by the outlined numbers in panel A and immunoblotted with the PY-20 anti-phosphotyrosine monoclonal antibody (B, C), anti-p44^{mpk} antibodies (D , E) or anti-erk1-CT peptide antibodies (F, G). Samples shown in panels B, D and F were from unstimulated RAW 264.7 cells and panels C, E and G were from LPS-stimulated cells. The top and bottoms of each box corresponds to the electrophoretic migrations of the prestained marker proteins ovalbumin (50K) and carbonic anhydrase (33K), respectively. The position of the shifted form of the 41-kDa MAP kinase is indicated with an arrow. Note that, this form of the 41-kDa MAP kinase was detected better by anti-p44^{mpk} antibodies than the lower Mr form of this protein. Similar results with this antibody have been reported previously (17). Two separate experiments yielded similar results.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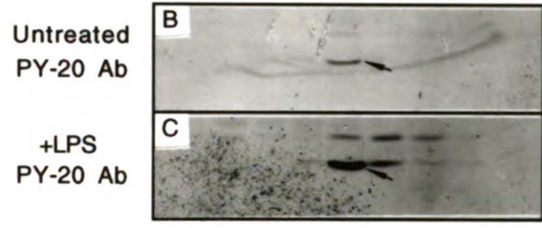
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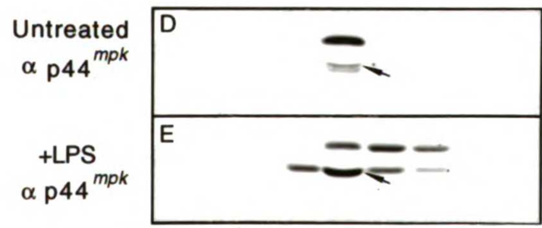
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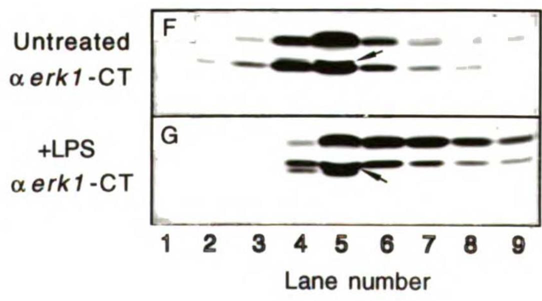
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these phenomena are thought to be indicative of increased phosphorylation of these proteins (17-20).

Comparison of the immunoblotting data and the MAP kinase activity profile revealed that column fractions with elevated activity contained both the tyrosine phosphorylated, 41-kDa and 44-kDa isoforms of MAP kinase. Thus, the contribution of each isozyme to a particular activity peak could not be determined. In any case, LPS increased MAP kinase activity in RAW 264.7 cells and this effect correlated with the induced tyrosine phosphorylation of at least two MAP kinase isoforms.

Similar increases in MAP kinase activity were observed following stimulation of RAW 264.7 cells with wild type (smooth) LPS or a rough form of LPS that lacks most of the carbohydrate portion of the molecule. In addition, purified and synthetic preparations of the lipid A subdomain of LPS elevated MAP kinase activity (Fig. 4). Thus, different biologically active forms of LPS activate MAP kinases and this response appears to be lipid A-dependent, as is the case for almost all the effects of LPS on macrophages (4).

Herbimycin A inhibits LPS-induced activation of MAP kinases

The enzymatic activity of MAP kinase isozymes is regulated in part by phosphorylation on tyrosine residues (21). Since LPS induces the tyrosine phosphorylation of the 41-kDa and 44-kDa MAP kinase isoforms, LPS may modulate MAP kinase activity by inducing p41/p44 MAP kinase tyrosine phosphorylation. To test this possibility, we examined the effect of preventing LPS-induced tyrosine phosphorylation of p41/p44 on the MAP kinase activity. Previously, we reported that herbimycin A, a protein tyrosine kinase inhibitor (22-24), completely blocks tyrosine phosphorylation

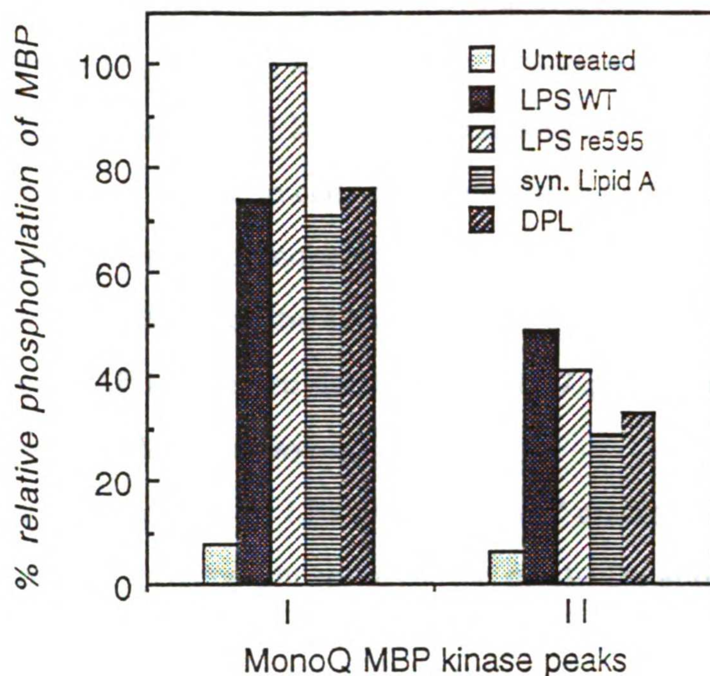


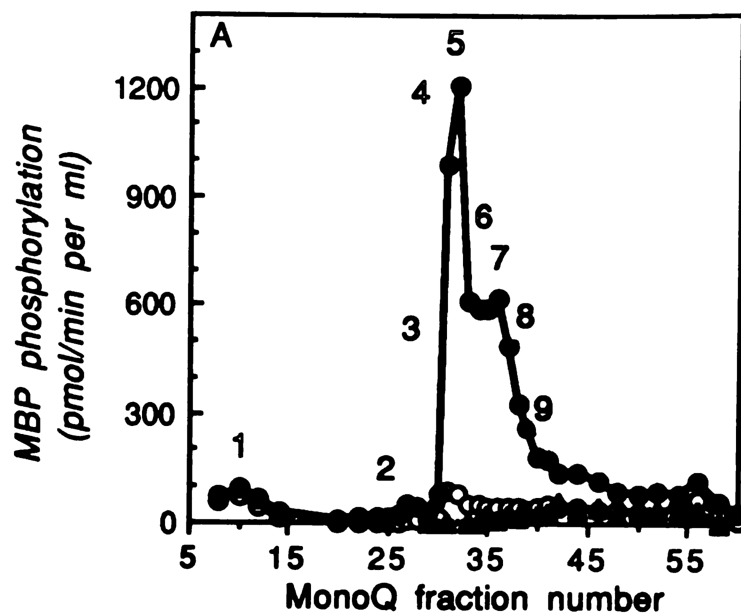
Figure 4 Capacity of different LPS forms to activate MAP kinases from RAW 264.7 macrophage cells. Detergent-soluble proteins from untreated cells or from cells treated for 15 min with 1 µg/ml of wild type *S. minnesota* LPS (WT), rough type LPS from *S. minnesota* Re 595, synthetic *E. coli* type lipid A or purified bacterial lipid A from *S. minnesota* (DPL) were subjected to MonoQ chromatography as described in the Experimental Procedures. The column fractions were assayed for MAP kinase activity using MBP as a substrate. Values are expressed relative to the maximal MBP phosphorylating activity in the first peak from MonoQ (fraction numbers 31-33). The relative MBP phosphorylating activity in the second MonoQ peak is also shown (fraction numbers 35-39). Similar results were obtained in two independent experiments.

of all the proteins modulated by LPS including the 41-kDa and 44-kDa proteins (6). Herbimycin A pretreatment also inhibited LPS-induced MAP kinase activity (Fig.5A). In addition, the LPS-induced mobility shift of the 41-kDa immunoreactive MAP kinase isoform and the delayed elution of the 44-kDa isoform were absent in cells pretreated with herbimycin A (Fig. 5C, D). Together, these results demonstrate a strong correlation between the tyrosine phosphorylation status of the 41-kDa and 44-kDa MAP kinase isozymes and the level of MAP kinase activity in RAW 264.7 macrophages.

PMA Stimulates MAP kinase Activity

Similar to the effects of LPS on RAW 264.7 macrophages, PMA treatment also induces the tyrosine phosphorylation of 41-kDa and 44-kDa species that correspond to MAP kinase isoforms (Fig. 1). Therefore, we tested whether MAP kinase activity is elevated in PMA-stimulated RAW 264.7 cells. PMA stimulation strongly increased the amount of MAP kinase activity with two peaks of activity partially resolved by MonoQ chromatography (Fig. 6A). A large peak of activity eluted in fractions 28-33 and a second, smaller peak that resolved as a shoulder of the first peak eluted in fractions 34-40. Thus, like LPS, PMA appeared to activate at least two isoforms of MAP kinase. Interestingly, following PMA treatment two 44-kDa MAP kinase isoforms with different MonoQ elution patterns were detected on anti-phosphotyrosine and anti-MAP kinase immunoblots (Fig. 6B-D). One of these proteins eluted from the column in the same fractions as p44 MAP kinase from unstimulated cells and appeared in lane 4 on the blots. The other protein was more strongly bound to the column and was detected in lanes 7 and 8. A similar phenomenon has been observed in nerve growth factor-stimulated PC12 cells (20) and in insulin-treated rat fibroblasts (19). It is unclear whether this observation results from the detection of two

Figure 5. Effect of herbimycin A on LPS activation of MAP kinases. MonoQ chromatography of detergent-soluble protein from untreated (O), LPS-treated (●), and LPS + herbimycin-treated (▲) cells was performed and the column fractions were assayed for phosphotransferase activity toward MBP (A). Combined, adjacent column fractions from LPS + herbimycin-treated macrophages designated by the outlined numbers in panel A, were immunoblotted with the PY-20 anti-phosphotyrosine antibody (B), anti-p44*mpk* antibodies (C) or anti-erk1-CT peptide antibodies (D). The unstimulated and LPS-stimulated controls are not shown, but were similar to those shown in Figure 3. The position of the shifted form of the 41-kDa MAP kinase is indicated with an arrow. The results shown are representative of data obtained in several experiments.


A
MBP phosphorylation (pmol/min per ml)
1200
900
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300
0
1
2
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4
5
6
7
8
9
5
15
25
35
45
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MonoQ fraction number

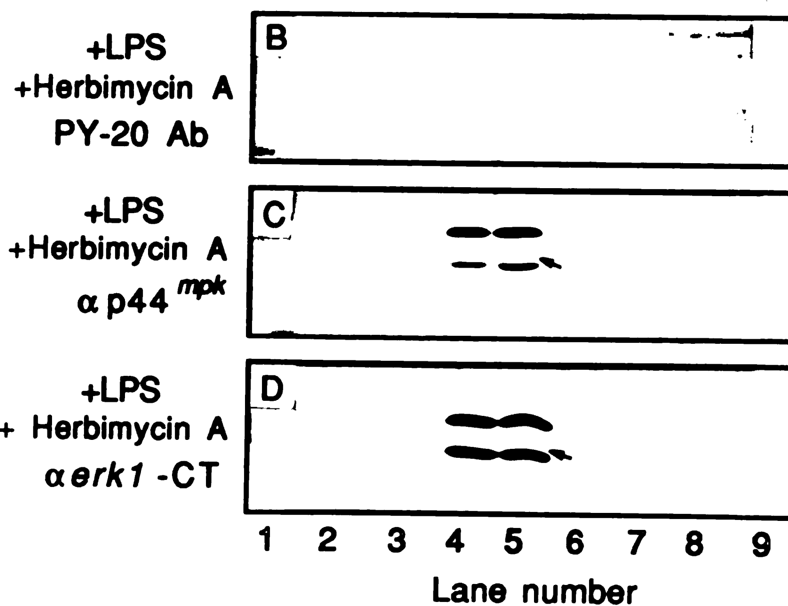
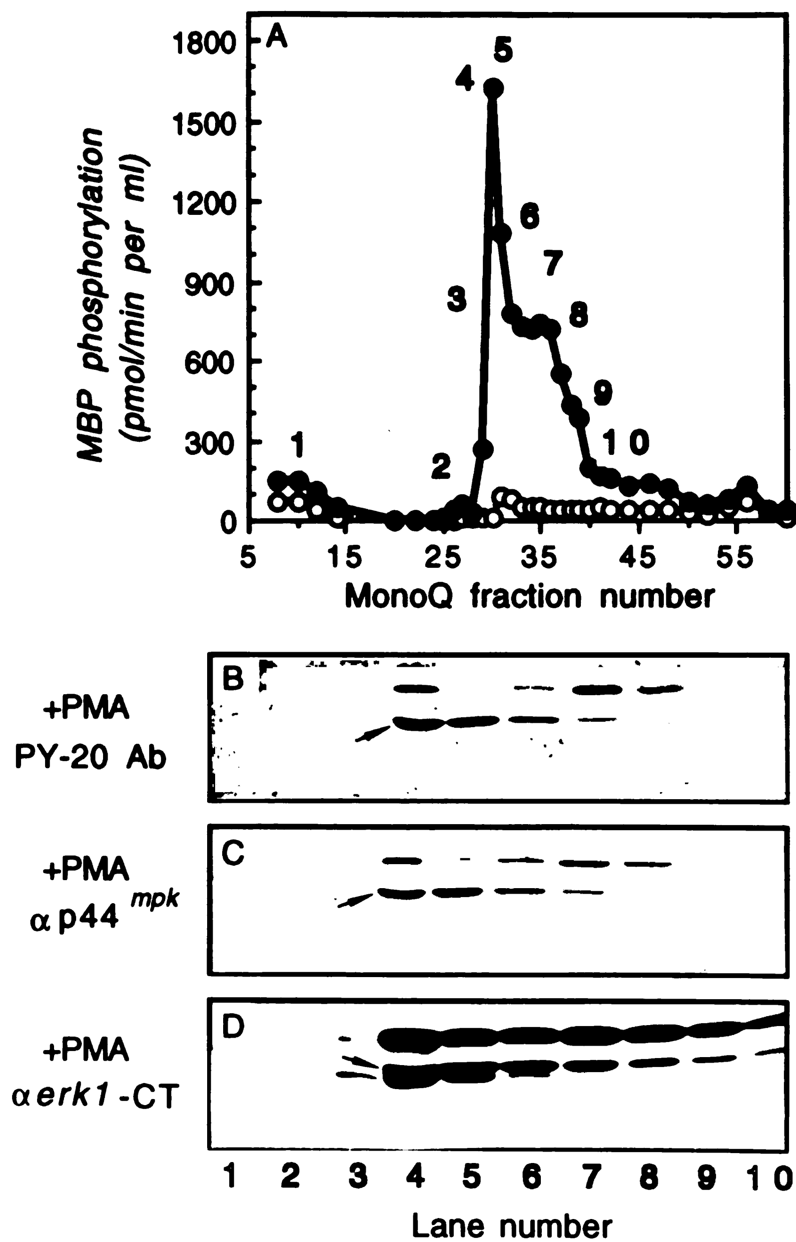

+LPS
+Herbimycin A
PY-20 Ab
B
+LPS
+Herbimycin A
α p44 mpk
C
+LPS
+ Herbimycin A
α erk1 -CT
D
1 2 3 4 5 6 7 8 9
Lane number

Figure 6. Activation of MAP kinases by PMA stimulation of RAW 264.7 macrophages. MonoQ chromatography of detergent-soluble protein from untreated cells (O) and cells treated with 100 nM PMA for 15 min (●) was performed and the column fractions were assayed for phosphotransferase activity with MBP (A). Pooled adjacent column fractions from PMA-treated cells designated by outlined numbers in panel A, were immunoblotted with the PY-20 anti-phosphotyrosine antibody (B), and two different MAP kinase antibodies, anti-p44^{mpk} antibodies (C) or anti-erk1-CT peptide antibodies (D). The unstimulated control is shown in Figure 3. The position of the shifted form of the 41-kDa MAP kinase is indicated with an arrow. Similar results were obtained in two independent experiments.


A
1800
1500
1200
900
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300
0
MBP phosphorylation
(pmol/min per ml)
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2
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4
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7
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9
1 0
5
15
25
35
45
55
MonoQ fraction number
B
+PMA
PY-20 Ab
C
+PMA
α p44 mpk
D
+PMA
α erk1 -CT
1 2 3 4 5 6 7 8 9 1 0
Lane number

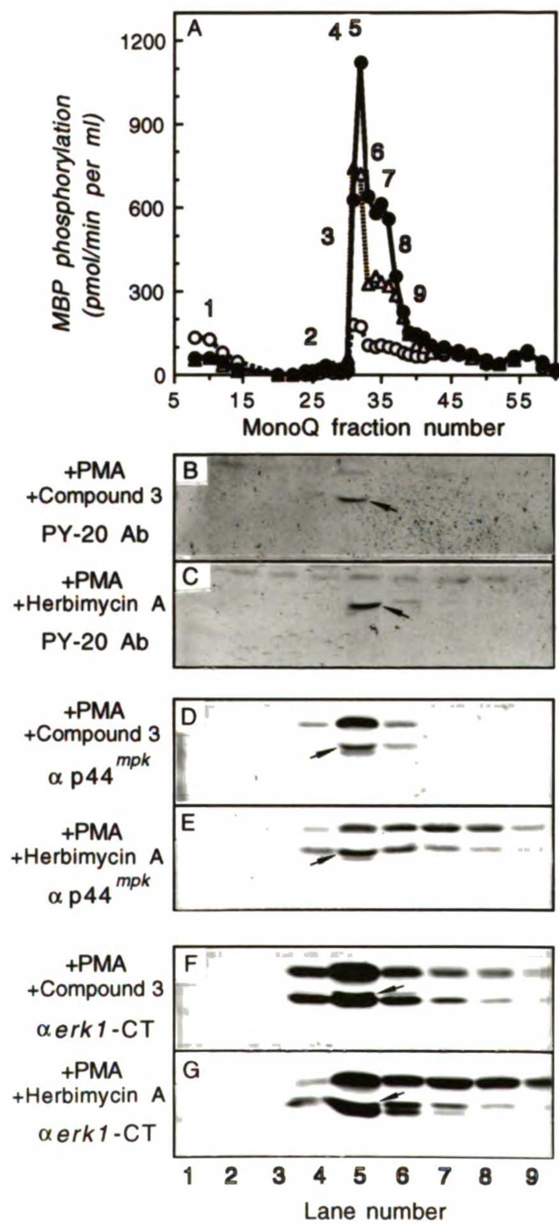
different MAP kinase isozymes of similar Mr or two different forms of the same isozyme. As mentioned earlier, LPS also induced some increased retention of p44 to the Mono Q column. However, LPS stimulation did not result in the appearance of two distinct 44-kDa MAP kinase species. Thus, the effects induced by LPS and PMA on the 44-kDa isoform(s) of MAP kinase were not identical in RAW 264.7 cells.

Protein Kinase Inhibitors Differentially Affect LPS- and PMA-stimulated MAP Kinase Activation

To further investigate the mechanism by which LPS and PMA activate MAP kinase isozymes, we examined the effect of herbimycin A on the response triggered by PMA. Herbimycin A treatment, which completely blocked LPS-induced tyrosine phosphorylation and activation of MAP kinase, only weakly inhibited PMA induction of these responses (Fig. 7A, C, E, G). Since, the targets of herbimycin A action are thought to be protein tyrosine kinases, the activation of MAP kinase by LPS appears to involve a herbimycin-sensitive tyrosine kinase whereas the PMA-induced response does not.

The results with a protein kinase C inhibitor provide further evidence that LPS- and PMA-induced activation of MAP kinase are mechanistically different. We found that pretreatment with the staurosporine analog, Compound 3 (25), inhibited PMA-induced tyrosine phosphorylation of p41 and p44 and also inhibited activation of MAP kinase (Fig. 7). This result was expected since phorbol esters are thought to exert their effects on cells by activating protein kinase C. In contrast, these LPS-induced responses were insensitive to Compound 3. (Fig. 8). Thus, the mechanism by which LPS activates MAP kinases in RAW 264.7 cells does not appear to be dependent on protein kinase C.

Figure 7 Effect of herbimycin A and Compound 3 on PMA activation of MAP kinases. MonoQ chromatography of RAW 264.7 cell protein from cells exposed to 100 nM PMA for 15 min in the absence (●) and presence of herbimycin A (Δ) or Compound 3 (O) was performed and the MAP kinase activity of column fractions using MBP as a substrate is shown in panel A. The combined column fractions from PMA + herbimycin A-treated cells (C, E, G) and PMA + Compound 3-treated cells (B, D, F) designated by outlined numbers in panel A, were immunoblotted with the PY-20 anti-phosphotyrosine antibody (B, C) or anti-MAP kinase antibodies, anti-p44^{mpk} antibodies (D, E), or anti-erk1-CT peptide antibodies (F, G). The unstimulated and PMA-stimulated controls are shown in Figure 3 and Figure 6, respectively. The position of the shifted form of the 41-kDa MAP kinase is indicated with an arrow. Similar results were obtained in duplicate experiments.



A
MBP phosphorylation (pmol/min per ml)
1200
900
600
300
0
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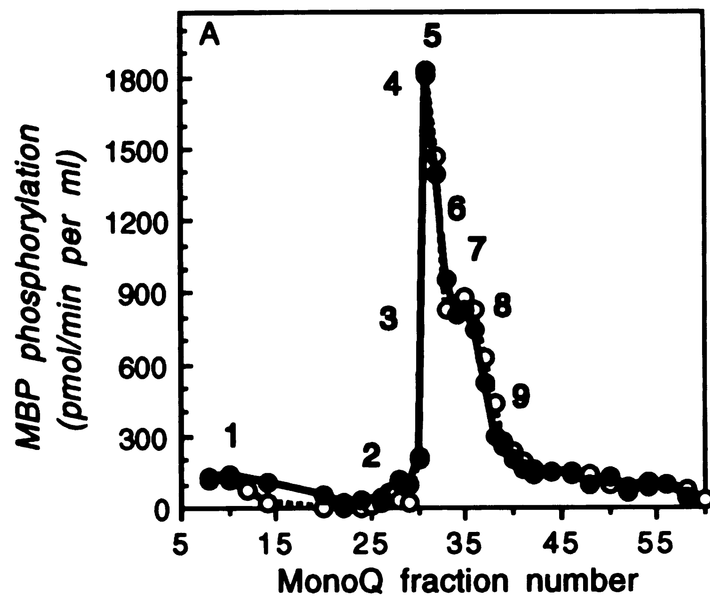
15
25
35
45
55
MonoQ fraction number
B
+PMA
+Compound 3
PY-20 Ab
C
+PMA
+Herbimycin A
PY-20 Ab
D
+PMA
+Compound 3
α p44 mpk
E
+PMA
+Herbimycin A
α p44 mpk
F
+PMA
+Compound 3
α erk1-CT
G
+PMA
+Herbimycin A
α erk1-CT
1 2 3 4 5 6 7 8 9
Lane number

Figure 8 Effect of a protein kinase C inhibitor on LPS activation of MAP kinases. MonoQ chromatography of RAW 264.7 cell protein from LPS-treated cells (●), and LPS + compound 3-treated (O) cells was performed and the column fractions were assayed for MAP kinase activity using MBP as a substrate (A). The pooled adjacent fractions from LPS + Compound 3-treated cells designated by outlined numbers in panel A, were immunoblotted with the PY-20 anti-phosphotyrosine antibody (B), anti-p44^{mpk} antibodies (C) or anti-erk1-CT peptide antibodies (D). The unstimulated and LPS-stimulated controls are shown in Figure 3. The position of the shifted form of the 41-kDa MAP kinase is indicated with an arrow. Similar results were obtained in two independent experiments.


A
MBP phosphorylation (pmol/min per ml)
MonoQ fraction number
1800
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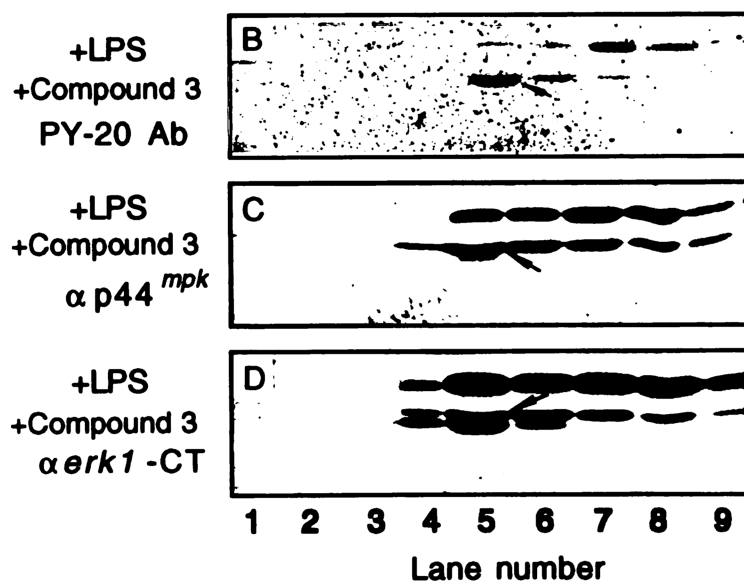
+LPS
+Compound 3
PY-20 Ab
B
+LPS
+Compound 3
α p44 mpk
C
+LPS
+Compound 3
α erk1 -CT
D
1 2 3 4 5 6 7 8 9
Lane number

Discussion

LPS stimulation of macrophages results in the increased tyrosine phosphorylation of several proteins. Here, we report that two of the induced tyrosine phosphoproteins, of 41 kDa and 44 kDa, correspond to isoforms of MAP kinase. Each of these proteins was immunoreactive with anti-MAP kinase antibodies and could be partially depleted from cell lysates by immunoprecipitation with these antibodies. In addition, both pp41 and pp44 co-eluted with MAP kinase activity following Mono Q chromatography. Together, these results show that the 41-kDa and 44-kDa proteins whose tyrosine phosphorylation is induced by LPS are isozymes of MAP kinase.

To date, at least four highly-related MAP kinases have been described in a variety of species by biochemical, immunological and molecular cloning data (8-10). One isoform has a mass of 42 kDa and is tyrosine phosphorylated in response to mitogenic stimulation in a wide variety of cells. This MAP kinase isoform has been designated $p42^{mapk}$ and corresponds to the ERK 2 gene product (20, 26). A slightly larger MAP kinase isoform (43-44 kDa) exhibits induced tyrosine phosphorylation often in parallel with increased phosphorylation of the 42-kDa isoform and is thought to correspond to ERK 1 (10). The existence of additional MAP kinase isoforms has been inferred from the molecular cloning of a third sequence-related cDNA (ERK 3, predicted Mr 63 kDa)(20) and from the immunoblotting of a 45-kDa polypeptide (ERK 4) with anti-ERK 1 antibodies[2] (20). The 41-kDa MAP kinase isoform from RAW 264.7 macrophages behaves most similarly to $p42^{mapk}$/ERK 2. In addition to the similar Mr, both MAP kinases exhibit

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[2] unpublished results, S.L. Pelech.

slightly decreased mobility on SDS polyacrylamide gels, following cellular activation. Moreover, anti-p44^{mpk} antibodies recognized the 41-kDa isoform more efficiently after LPS or PMA stimulation of RAW 264.7 cells. This property was previously observed with p42 MAP kinase from *Xenopus* oocytes (17). The 44-kDa MAP kinase isoform from RAW 264.7 cells behaves like p44 MAP kinase from PC 12 cells (20) and rat fibroblasts (19). For each of these 44-kDa isoforms, cellular stimulation, leads to stronger binding of the 44-kDa protein to a MonoQ column. Although positive identification will require additional experiments, it seems likely that the two isoforms of MAP kinase activated by LPS in macrophages correspond to the two major MAP kinase isoforms seen in other cell types.

LPS-induced tyrosine phosphorylation of the two MAP kinase isoforms in RAW 264.7 macrophages was accompanied by increased MAP kinase activity. This activity could be partially resolved into two peaks by MonoQ chromatography suggesting that at least two MAP kinase isoforms were activated by LPS. Each of the column fractions with elevated MAP kinase activity contained tyrosine phosphorylated forms of the 41-kDa and 44-kDa MAP kinase isozymes. Since tyrosine phosphorylation of MAP kinases is necessary for the activation of these proteins, both the 41-kDa and 44-kDa isoforms are likely to contribute to the observed MAP kinase activity. The loss of MAP kinase activity which accompanied inhibition of induced tyrosine phosphorylation of these proteins is consistent with this interpretation. Moreover, the anti-phosphotyrosine blots of the active column fractions did not reveal the presence of any other tyrosine phosphoproteins. This observation makes it unlikely that additional MAP kinase isozymes which were not detected by our MAP kinase antibodies were responsible for the observed MAP kinase activity. Therefore, LPS appears to

modulate MAP kinase activity in RAW 264.7 cells by inducing the tyrosine phosphorylation of the 41-kDa and 44-kDa MAP kinase isoforms. Since phosphorylation on threonine residues has been shown to be necessary for the full activation of MAP kinase (22, 27), LPS may additionally modulate MAP kinase activity by inducing increased threonine phosphorylation of these proteins. Alternatively, MAP kinases in RAW 264.7 cells may be phosphorylated on regulatory threonine residues prior to LPS stimulation.

The mechanism by which LPS treatment increases the tyrosine phosphorylation of MAP kinases is not clear. Recent evidence from several groups has indicated that MAP kinases can autophosphorylate on tyrosine residues as well as threonine residues (14, 28-30). Thus, LPS-induced tyrosine phosphorylation of MAP kinase isoforms may occur by a LPS-stimulated autophosphorylation mechanism, independent of other protein tyrosine kinases. Alternatively, increased MAP kinase tyrosine phosphorylation could be a consequence of LPS-activated protein tyrosine kinases.

The ability of herbimycin A to inhibit both LPS-induced tyrosine phosphorylation and activation of the MAP kinase proteins suggests that an activated protein tyrosine kinase is necessary for these LPS responses. While the mechanism of action of herbimycin A is not completely understood, this inhibitor appears to inactivate protein tyrosine kinases by irreversibly binding to thiol groups in the affected kinases (22, 23). In addition, herbimycin A binding to these kinases promotes their degradation (22, 31). Since the amount of MAP kinases detected by immunoblotting did not change following herbimycin A treatment, these kinases may not be targets of this inhibitor. Moreover, PMA-induced MAP kinase tyrosine phosphorylation and activation was only weakly affected by herbimycin A

treatment. This result demonstrates that herbimycin A does not directly inhibit MAP kinase. Thus, it seems most likely that herbimycin A inhibits an upstream protein tyrosine kinase that is necessary for the LPS-induced tyrosine phosphorylation of MAP kinases.

The effects of herbimycin A on LPS- and PMA-induced MAP kinase activity also suggest that MAP kinases may mediate some of the anti-bacterial responses of macrophages. First, herbimycin A, which prevented the LPS-stimulated increase in MAP kinase activity, also inhibits the release of arachidonic acid metabolites from LPS-treated RAW 264.7 macrophages (6; chapter 1). Arachidonic acid metabolites are potent inflammatory mediators and their secretion by macrophages is characteristic of the activated state. In contrast to the results with LPS, herbimycin A did not strongly inhibit PMA-stimulated MAP kinase activation or the release of arachidonic acid metabolites [Fig. 7. and (6; chapter 1)]. Thus, the induction of MAP kinase activity by LPS and PMA appears to be correlated with the appearance of at least some downstream macrophage responses. A further indication of the relationship between MAP kinase activity and macrophage activation was provided by the results obtained with the protein kinase C inhibitor, Compound 3, in PMA-stimulated cells. Compound 3, which blocked PMA-induced MAP kinase activation also inhibited the release of arachidonic acid metabolites (data not shown). However, the results with RAW 264.7 macrophages pretreated with Compound 3 and then stimulated with LPS do not fit this pattern. Compound 3 treatment, which did not inhibit LPS-induction of MAP kinase activity, did inhibit LPS-stimulation of arachidonic acid metabolite release (data not shown). Thus, in this case, an activated macrophage response was not triggered despite the induction of MAP kinase activity by LPS. This result, however, is still consistent with the hypothesis

that MAP kinases participate in LPS-stimulated signal tranduction. For example, one obvious explanation is that protein kinase C, or another protein kinase that is inhibited by Compound 3, participates in the LPS signaling pathway downstream of MAP kinase activation. Alternatively, the release of arachidonic acid metabolites induced by LPS in RAW 264.7 macrophages may require the generation of two independent intracellular signals-one provided by MAP kinases and the other by protein kinase C. Clearly the experiments presented here do not prove that MAP kinases mediate macrophage responses to LPS, but the results with the protein kinase inhibitors are consistent with this hypothesis.

The precise function of MAP kinases in macrophages and in other cells is, however, not yet known. Several proteins have been found to be efficient *in vitro* substrates of MAP kinases and these may be indicative of MAP kinase function *in vivo*. For example, phosphorylation of microtubule-associated protein 2 by MAP kinases (32, 33) may alter the cytoskeleton and could provide a molecular mechanism for the morphological changes induced by LPS in macrophages. Similarly, MAP kinases can phosphorylate and activate both the S6 ribosomal protein kinase (11, 12) and the c-*jun* product (13), proteins that are involved in the regulation of translation and transcription, respectively. If MAP kinases phosphorylate these targets *in vivo,* these kinases may contribute to the altered expression of LPS-modulated proteins. Since many of the responses triggered by LPS in macrophages depend on transcription and translation, LPS activation of MAP kinases could be a critical part of the mechanism by which LPS induces responses in macrophages. Despite the incomplete understanding of the role of MAP kinases in cells, our results suggest that these kinases could be very important targets of LPS action in macrophages

and lend support to the hypothesis that induced protein tyrosine phosphorylation is part of the signal transduction pathway that mediates macrophage responses to LPS.

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Conclusions

This investigation examined the potential role of protein tyrosine phosphorylation in the signal transduction pathway activated by LPS in macrophages. The first question addressed in these studies was simply whether protein tyrosine phosphorylation is induced by LPS in these cells. Several proteins were found to be rapidly phosphorylated on tyrosine in human and murine macrophages and macrophage cell lines (Chapters 1 and 2). In addition, increased protein tyrosine phosphorylation was detected in a human monocytic cell line and, in a preliminary experiment, in human peripheral blood monocytes, a result recently corroborated by Beaty and co-workers (1). Moreover, the doses and types of LPS that induced tyrosine phosphorylation were similar to those that initiate biological responses in macrophages. Thus, induced tyrosine phosphorylation is an evolutionarily conserved, early response to LPS that correlates with the expression of subsequent anti-bacterial responses by macrophages.

Another issue addressed in these studies was whether the induction of protein tyrosine phosphorylation was biochemically coupled to CD14, a molecule recently implicated as an LPS receptor. I found that LPS-induced tyrosine phosphorylation was exclusively mediated by CD14 at low LPS concentrations (Chapter 2). However, stimulation of the response by LPS doses ≥ 10 ng/ml was largely independent of CD14. A similar LPS dose-dependent shift from CD14-dependent to CD14-independent recognition of LPS has previously been observed for the secretion of tumor necrosis factor (2). Thus, once again, a correlation was seen between the induction of protein tyrosine phosphorylation and the expression of anti-bacterial responses. Moreover, these results demonstrated that the induction of

tyrosine phosphorylation is biochemically linked to the CD14-dependent and CD14-independent recognition mechanisms for LPS that are believed to initiate functional responses by macrophages.

To further explore the relationship between the induction of tyrosine phosphorylation and the development of anti-bacterial responses , the effects of blocking the phosphorylation response on the expression of subsequent cellular responses were examined. Preventing the induction of tyrosine phosphorylation with the tyrosine kinase inhibitor, herbimycin A, was accompanied by the inhibition of the release of arachidonic acid metabolites (Chapter 1). In addition, another group has reported that the LPS-stimulated secretion of tumor necrosis factor is inhibited in this type of blocking experiment (1). Together, these results suggest that at least some of the cellular responses elicited by LPS are mechanistically downstream of the tyrosine phosphorylation response. Thus, the induction of protein tyrosine phosphorylation appears to be an intermediate step in the signaling pathway leading to later anti-bacterial responses in macrophages.

The last part of this investigation focused on the identity of the proteins that are substrates for induced tyrosine phosphorylation and the effect that this modification has on their function. I found that two of the prominent tyrosine phosphoproteins induced by LPS corresponded to isoforms of MAP kinase and that tyrosine phosphorylation of these kinases increased their enzymatic activity (Chapter 3). Since MAP kinases are implicated in the regulation of translation, transcription and cytoskeletal dynamics, cellular processes known to be altered by LPS, these kinases could be key targets of LPS action in macrophages.

In summary, the induction of intracellular tyrosine phosphorylation by LPS has many of the hallmarks of a signaling reaction in macrophages.

First, the membrane recognition structures for LPS that are believed to initiate the cellular effects of LPS are coupled to the tyrosine phosphorylation response. Second, protein tyrosine phosphorylation is induced prior to nearly all of the other known cellular responses to LPS. Third, at least some of the anti-bacterial responses stimulated by LPS are dependent on increased tyrosine phosphorylation. Fourth, tyrosine phosphorylation alters the activity of proteins that are likely to be involved in producing the anti-bacterial responses. Taken together, these data strongly argue that the induction of protein tyrosine phosphorylation is part of the signal transduction pathway activated by LPS in macrophages.

The induction of protein tyrosine phosphorylation, however, does not appear to be a universal event in cells responding to LPS. For example, this response is conspicuously absent in LPS-treated B cells (3). The reasons why LPS does not induce tyrosine phosphorylation in these cells are not clear. However, in contrast to macrophages and neutrophils, B cells do not express CD14 (4). Therefore, B cells appear to have another receptor for LPS and this molecule apparently does not couple to the tyrosine phosphorylation pathway. In this respect, the cellular recognition of LPS may resemble that of many hormones and neurotransmitters, which have two or more distinct receptors, each coupled to a different signaling pathway. Nevertheless, the lack of a tyrosine phosphorylation response in LPS-stimulated B cells does not in any way diminish the significance of this response in macrophages.

These studies also provided information concerning the relationship between the induction of protein tyrosine phosphorylation and the activation of protein kinase C (PKC) in LPS-stimulated signal transduction. While many cellular responses to LPS appear to be mediated by PKC (5-11), inactivating this enzyme with the PKC inhibitor, Compound 3, did not

prevent the induction of tyrosine phosphorylation by LPS. In contrast, this inhibitor effectively blocked the cellular responses elicited by the PKC activator, PMA. Thus, the activation of PKC does not occur upstream of the tyrosine phosphorylation response. However, both PKC activation and induced tyrosine phosphorylation are necessary for the LPS-stimulated secretion of tumor necrosis factor (1) and release of arachidonic acid metabolites (Chapter 1, data not shown). These observations suggest that the two signaling reactions function together in some fashion to produce downstream responses. Thus, either PKC is activated as a consequence of tyrosine phosphorylation or the two events are triggered separately.

The induction of protein tyrosine phosphorylation appears to be a widespread intracellular signaling event, in the sense that this response is stimulated in a variety of cells by a myriad of extracellular ligands. Based on available evidence, the general features of the tyrosine phosphorylation response, in these different cells, appear to be very similar. While it is too soon to know for certain whether LPS-induced tyrosine phsophorylation in macrophages shares these characteristics, this appears to be the case. For example, one manifestation of the apparently conserved nature of the tyrosine phosphorylation response in different cells is the phosphorylation of a common set of proteins such as MAP kinases, c-raf, ras-GTPase-activating protein (GAP), phospholipase C-γ and phosphatidylinositol 3-kinase (12,13). As demonstrated by these studies, LPS induces the tyrosine phosphorylation of MAP kinases (Chapter 3) and, in preliminary experiments not shown, GAP was phosphorylated on tyrosyl residues. Thus, it seems likely that other aspects of LPS-induced tyrosine phosphorylation will be similar to the tyrosine phosphorylation responses elicited in other cells. Therefore, the tyrosine phosphorylation response induced by LPS in

macrophages may prove to be a useful model for understanding the role of this response in all cells.

Several questions in the signal transduction field may be answerable through further investigation of LPS action in macrophages. For example, determining how CD14 is coupled to the induction of tyrosine phosphorylation should provide new insight into the mechanism by which GPI-linked proteins transduce signals. Second, determining the role of MAP kinases and the other phosphorylated proteins in macrophages may tell us how these molecules function in all cells. Third, learning how the tyrosine phosphorylation response in macrophages is coupled to expression of LPS-modulated genes would provide insight into one of the major questions in the signaling field. Finally, the protein tyrosine kinase/phosphatase regulated by LPS may be encoded by an oncogene (e.g., a src-family tyrosine kinase). Since the cellular function of these proteins is largely unknown, identification of the LPS-activated tyrosine kinase or phosphatase might have important ramifications for understanding the connection between signal transduction and the control of cell growth. Thus, further investigation of LPS-induced tyrosine phosphorylation in macrophages could produce results that have relevance to general signal transduction mechanisms.

Aside from providing insights into basic biological mechanisms, further study of LPS signaling in macrophages is imperative for medical reasons. The LPS-macrophage interaction plays a critical role in the host response to bacterial infection. Generally, macrophage responses to LPS facilitate resolution of infections. However, prolonged stimulation of macrophages by LPS can contribute, instead, to life-threatening septic shock (14). Thus, a better understanding of the biochemical events that mediate

macrophage responses to LPS may lead to the development of more effective therapeutic intervention for septic shock.

While the results presented here are certainly consistent with induced protein tyrosine phosphorylation being part of the LPS-activated signal transduction pathway in macrophages, several aspects of LPS signaling require further investigation to definitively establish its role. For example, the molecules that mediate CD14-independent recognition of LPS need to be identified. Since these molecules may also cooperate with CD14 to trigger CD14-dependent signaling, identification of these molecules could be crucial to the understanding of LPS signal transduction. A related issue that needs to be examined is the molecular coupling between the LPS recognition structures and the induction of intracellular protein tyrosine phosphorylation. With regard to this coupling, it is interesting that the tyrosine phosphorylation response induced by LPS in macrophages is considerably slower (minutes versus seconds) than the tyrosine phosphorylation responses elicited by extracellular stimulators in other cell types (15), including stimuli that activate receptors that do not contain intrinsic tyrosine kinase domains. These observations suggest that the initiation of protein tyrosine phosphorylation by LPS may not be a proximal event following receptor activation; instead, additional intracellular reactions may intervene between the LPS receptor and the protein tyrosine kinase/phosphatase regulated by LPS. A third issue warranting additional investigation concerns how the protein substrates for LPS-induced tyrosine phosphorylation such as the MAP kinases are involved in producing the the cellular effects of LPS. Clearly, new information in each of these areas is needed to ultimately determine what role tyrosine phosphorylation plays in LPS signaling.

Fortunately, it is likely that much of this new information can be generated by standard biochemical approaches and/or genetic strategies. For example, to identify the molecules that intervene between CD14 and the tyrosine kinase/phosphatase regulated by LPS, it may be possible to co-precipitate CD14 and the associated signaling molecules (16). An alternative biochemical strategy to find these signaling molecules would be to identify the cellular fractions that contain the components that phosphorylate MAP kinase. With this approach, it may be possible to isolate the critical molecules from the appropriate cell fraction and then to iteratively work back toward the membrane, identifying the upstream signaling elements. Thus, these two biochemical strategies approach the problem from opposite directions, but are, nonetheless, complemetary. A biochemical strategy may also be useful for identifying the molecules that are phosphorylated and regulated by MAP kinases. Since c-jun, c-myc, c-raf and S6 kinase have been shown to be *in vitro* substrates of MAP kinase (references in Chapter 3) and as these molecules could clearly play important roles in cellular responses to LPS, it would be reasonable to determine whether any of these proteins is phosphorylated in LPS-stimulated macrophages. If one or more of these proteins is phosphorylated, then the phosphorylated residues can be compared with the amino acids phosphorylated by MAP kinase *in vitro.* Identical phosphopeptide maps would imply that MAP kinases phosphorylate these molecules *in vivo.* Genetic approaches involving overexpression and /or functional knockout (e.g. dominant negative mutant) of potential signaling molecules is also likely to yield valuable information about the LPS signaling pathway. For example, the role of MAP kinases in LPS signaling might be investigated by expressing forms of MAP kinase with dominant negative mutations in macrophages. Cells expressing

these mutant MAP kinases would then be examined for potential LPS signaling deficits. Thus, combining biochemical and genetic strategies may be very useful for obtaining a better understanding of the role protein tyrosine phosphorylation in LPS-signaling.

The mechanisms by which cells recognize and respond to LPS have proven to be an enigma. Despite many years of investigation, very little was known about the receptors for LPS or the intracellular signaling reactions activated by this bacterial molecule. The recent efforts of Wright, Ulevitch, Morrison and others (17-20) have, however, brought new insight regarding LPS receptors. Similarly, the results presented here, illustrating the role of protein tyrosine phosphorylation in LPS-activated signaling, represent an important advance in the LPS field. Combined, the work in these two areas has begun to unravel the mystery surrounding the mechanisms of cellular responses to LPS, and it provides a foundation for more directed investigation of LPS signal tranduction in the future.

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