Endotoxin Induces Rapid Protein Tyrosine Phosphorylation in 70Z/3 Cells Expressing CD14*

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CD14, a glycosylphosphatidylinositol-anchored glycoprotein of leukocytes, binds endotoxin (lipopolysaccharide (LPS)) with high affinity. After the murine pre-B cell line 70Z/3 is transfected with DNA encoding human CD14 (hCD14), the resultant stably transfected cell line, 70Z/3-hCD14, responds to 1000-fold lower LPS concentrations than the parental CD14-negative line. We have used 70Z/3-hCD14 cells, RAW264.7 cells, and elicited murine peritoneal exudate macrophages (PEM) to study LPS-induced protein tyrosine phosphorylation. LPS induces the rapid tyrosine phosphorylation of a 38-kDa protein (p38) in 70Z/3-hCD14 cells, PEM, and RAW264.7 cells and of two isoforms of mitogen-activated protein kinases (MAPK) in only RAW264.7 cells and PEM. p38 can be distinguished from the MAPK isoforms based on differences in mobilities on SDS-polyacrylamide gel electrophoresis and the lack of reactivity of p38 with anti-MAPK antibody even after dephosphorylation with potato acid phosphatase. Synthetic lipid A induces p38 phosphorylation in 70Z/3-hCD14 cells, whereas phorbol 12-myristate 13-acetate and interferon-γ fail to induce tyrosine phosphorylation of p38. Pretreatment of 70Z/3-hCD14 cells with anti-hCD14 monoclonal antibody or the tyrosine kinase inhibitor herbimycin A inhibits LPS-induced tyrosine phosphorylation of p38. These results suggest that increased protein tyrosine phosphorylation occurs rapidly after LPS binds to CD14 and is likely to be an important event in mediating LPS-induced cell activation.

Endotoxin (lipopolysaccharide (LPS)) is a complex glycolipid found in the outer membrane of Gram-negative bacteria (1). Picomolar to nanomolar concentrations of LPS induce multiple biological responses in man and animals by stimulating cells of monocytic lineage (MO) to release cytokines (2, 3). Recent studies from our laboratory have shown that interaction of LPS with CD14 can be directly demonstrated (6, 9) and that the concentration of LPS required to stimulate the cells is up to 1000-fold lower than that required to stimulate the parental CD14-negative cell line (6). Thus, the 70Z/3-hCD14 cells resemble MO and provide a novel model system to study the effects of LPS binding to CD14 on intracellular changes involved in cell activation.

Ligand-induced protein tyrosine phosphorylation is a very rapid event that mediates subsequent intracellular changes for many different receptors (10). Recently, Weinstein et al. (11,12) reported that LPS treatment of RAW264.7 cells, a murine MO-like cell line, rapidly increases tyrosine phosphorylation of multiple proteins including the 41- and 44-kDa isoforms of mitogen-activated protein kinase (MAPK), that these protein tyrosine phosphorylations occur rapidly after addition of LPS to cells, and that pretreatment of RAW264.7 cells with the protein-tyrosine kinase inhibitor herbimycin A blocks MAPK phosphorylation. However, these studies did not address the role of CD14 in protein-tyrosine kinase activation.

Herein, we show that stimulation of 70Z/3-hCD14 cells and two different sources of MO with LPS, but not other agonists, rapidly induces protein tyrosine phosphorylation of a 38-kDa protein (p38). Phosphorylated p38 is distinct from the 41- and 44-kDa MAPK isoforms expressed in 70Z/3 and MO. Inhibition of LPS-induced tyrosine phosphorylation of p38 occurs when cells are pretreated with the protein-tyrosine kinase inhibitor herbimycin A or with anti-CD14 monoclonal antibody. These data provide evidence for LPS-specific protein tyrosine phosphorylation that is linked to cell stimulation via a CD14-dependent pathway.

MATERIALS AND METHODS

Reagents—B6595 LPS was isolated from yopihylus Salmonella minnesota B6595 bacteria as described (13). LPS from Escherichia coli 0111:B4 was purchased from List Biological Laboratories (Campbell, CA), and synthetic lipid A was purchased from ICN Biomedicals, Inc. (Costa Mesa, CA). Recombinant mouse interferon-γ (IFN-γ) was a gift of R. D. Schreiber (Washington University, St. Louis, MO). FB2, an anti-phosphotyrosine monoclonal antibody (mAb), was purified from

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The abbreviations used are: LPS, lipopolysaccharide; MO, cells of monocytic lineage, macrophage; LBP, LPS-binding protein; hCD14, human CD14; MAPK, mitogen-activated protein kinase; IFN-γ, interferon-γ, mAb, monoclonal antibody; ECL, enhanced chemiluminescence; CHO, Chinese hamster ovary; RSV, Rous sarcoma virus; PEM, peritoneal exudate macrophages; FCS, fetal calf serum; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; TBS, Tris-buffered saline; FMA, phorbol 12-myristate 13-acetate.

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FB2 hybridoma (ATCC CRL1891) culture supernatant by chromatography on a protein G column. 4G10 anti-phosphotyrosine monoclonal antibody was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Rabbit anti-extracellular signal-regulated kinase (MAPK) polyclonal antibody was from Santa Cruz Biotech (Santa Cruz, CA). Goat anti-mouse IgG conjugated to horseradish peroxidase was purchased from Cappel (Durham, North Carolina). Enhanced chemiluminescence (ECL) Western blotting detection reagents and Hybond-ECL nitrocellulose membranes were purchased from Amersham Corp. and used as described by the manufacturer. MY4, an anti CD14 monoclonal antibody to hCD14, was purchased from Coulter Diagnostics (Hialeah, FL), and hCD14, an antigenic CD14 monoclonal antibody, was a gift from Dr. A. Arfors (Pharmacia LKB Biotechnology Inc.). An oligonucleotide specifying the NF-κB consensus sequence was purchased from Promega Biotec. The membrane was then rinsed with three changes of TBS (containing 0.05% Tween 20 and 1% BSA), was applied to the membrane. After 60 min at room temperature, the membrane was removed by heating at 100 °C for 20 min, 4 °C). The proteins detected by FB2 differed in CHO-RSV and 70Z3-RSV cells, but addition of 1 ng/ml LPS to these cell lines did not alter the pattern of the constitutively tyrosine-phosphorylated proteins in both CHO and 70Z3 cells. The pattern of the constitutively tyrosine-phosphorylated proteins in both CHO and 70Z3 cells was radiolabeled with [32P]ATP and incubated at room temperature with nuclear extracts in 10 mM Tris, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1 μg/ml poly(dI-dC), pH 7.5, for 20 min before analyzing samples on a 4% non-denaturing polyacrylamide gel as described (18).

De phosphorylation of p38 and MAPK—Detergent extracts were prepared as described above from either 70Z3-hCD14 or RAW264.7 cells stimulated for 15 min with 1 ng/ml Re595 LPS and 0.1 μg phorbol 12-myristate 13-acetate (PMA). The samples were electrophoresed by SDS-PAGE as described (12) and electrotransferred to polyvinylidene difluoride membranes (Bio-Rad) using transfer buffer lacking methanol (19). The resultant blots were stained with anti-phosphotyrosine mAb FB2 as described above, and after visualizing immunoreactive proteins using LAS, the region of the gel containing banding according to p38 or MAPK2 was cut out. Elution of protein from the filter was accomplished by placing the filter in a small tube containing 0.5 ml of elution buffer/cm² of polyvinylidene difluoride membrane (elution buffer = 50 mM Tris-HCl, 2% SDS, 1% Triton X-100, and 100 μg/ml BSA, pH 9.0) for 30 min with shaking at room temperature. The resultant solution was centrifuged for 10 min, and the eluted proteins were concentrated by precipitation with 4 volumes of acetone at −80 °C for 18 h. Precipitated proteins were recovered by centrifugation and dissolved in 10 mM HEPES, 0.1% Triton X-100, pH 6.0. A portion of the solubilized proteins were treated with potato acid phosphatase (600 units/ml; Lot 207215, Sigma) for 15 min at 30 °C; a ratio of 1 unit of potato acid phosphatase to 200 μl of original cell lysate was utilized. The dephosphorylation reaction was terminated by the addition of an equal volume of SDS-PAGE sample buffer (16). After heating at 100 °C for 5 min, the resultant samples were subjected to SDS-PAGE as described (16). After SDS-PAGE, the samples were electrotransferred to nitrocellulose and stained with either anti-phosphotyrosine or anti-MAPK antibody as described above.

RESULTS

Characteristics of LPS-induced Protein Tyrosine Phosphorylation in CD14-Transfected Cells—Transfected CHO-K1 or 70Z3 cells expressing human CD14 (CHO-hCD14 or 70Z3-hCD14 cells, respectively) or the same cells transfected with empty vector (CHO-RSV or 70Z3-RSV cells, respectively) (6) were treated with 1 ng/ml Re595 LPS for 15 min, and detergent lysates were prepared from LPS-treated or -untreated cells. An aliquot of the cell-free lysate was subjected to SDS-PAGE, transfer to nitrocellulose membranes, and identification of tyrosine-phosphorylated proteins by staining with anti-phosphotyrosine mAb FB2 as described under “Materials and Methods.” FB2 staining (Fig. 1a) revealed a complex pattern of phosphotyrosine-containing proteins in both CHO and 70Z3 cells. The pattern of the constitutively tyrosine-phosphorylated proteins detected by FB2 differed in CHO-RSV and 70Z3-RSV cells, but addition of 1 ng/ml LPS to these cell lines did not alter the cell-specific patterns. Expression of CD14 did not change the patterns of constitutively phosphorylated proteins in either cell type. However, LPS treatment of 70Z3-hCD14 cells resulted in tyrosine phosphorylation of a protein with an apparent molecular mass of 38 kDa, and we refer to this protein as p38 in the remainder of this report. In contrast, LPS addition (1 ng/ml) to CHO-hCD14 cells did not stimulate detectable changes in the patterns of protein tyrosine phosphorylations observed in unstimulated cells.

These and all subsequent results, with the exception of data shown in Fig. 1b, were obtained from experiments in which the cells were stimulated in medium containing 10% FCS. As noted below in Fig. 1b, LPS also stimulated p38 tyrosine phosphorylation in serum-free medium supplemented with purified LBP. We have previously reported that LPS-induced stimulation of 70Z3-hCD14 cells is identical in serum-free medium supplemented with BSA or containing 10% heat-inactivated FCS (6).

Although the intensity of p38 staining on Western blots was somewhat variable from experiment to experiment, increased tyrosine phosphorylation of p38 was always noted when 70Z/
Materials and Methods. CHO-K1 and protein tyrosine phosphorylation; thus, for simplicity, in all obtained from LPS-treated 70W3-hCD14 cells in more than 12 Methods. actinomycin D anti-phosphotyrosine mAb staining of immunoblots as described under "Materials and Methods." Where noted (+), cells were pretreated with anti-hCD14 mAb MY4 for 30 min prior to LPS stimulation.

or other anti-CD14 mAbs also inhibit LPS-induced NF-κB activation.²

We next determined complete time course and LPS dose-response studies of p38 phosphorylation in the absence and presence of anti-hCD14 mAb MY4. When 1 ng/ml Re595 LPS was added to 70Z3-hCD14 cells, the maximum increase in tyrosine phosphorylation of p38 was detected 15 min after LPS addition (Fig. 2a). Although the onset of p38 phosphorylation is rapid, we typically detected tyrosine-phosphorylated p38 up to 120–180 min after LPS addition; studies beyond this time period have not been performed. Pretreatment of the cells with MY4 prevented LPS-induced tyrosine phosphorylation of p38.

70Z3-hCD14 cells maintained in the absence or presence of MY4 were also exposed to varying amounts of Re595 LPS (0.01–1000 ng/ml) for 15 min, and the extent of p38 tyrosine phosphorylation was evaluated. Maximum p38 phosphorylation was noted with 1 ng/ml Re595 LPS; the presence of MY4 completely inhibited p38 phosphorylation induced by 1 or 10 ng/ml LPS and nearly completely blocked p38 phosphorylation observed with 100 or 1000 ng/ml LPS (Fig. 2b). For all subsequent experiments, unless otherwise noted, we treated 70Z3-hCD14 for 15 min with 1 ng/ml Re595 LPS.

Agnost Specificity of p38 Tyrosine Phosphorylation—The previous experiments were performed with LPS isolated from the R-form mutant of S. minnesota, Re595. We next sought to determine whether LPS isolated from S-form bacteria or synthetic lipid A also induce tyrosine phosphorylation of p38 (Fig. 3). Because 70Z3 cells are also stimulated by IFN-γ and PMA (20, 21), we evaluated the effect of a single concentration of these substances on tyrosine phosphorylation of p38. The two LPS isolates (1 ng/ml) and synthetic lipid (10 ng/ml) all induced p38 phosphorylation in 70Z3-hCD14 cells, but not in 70Z3-RSV cells. Treatment of either 70Z3-RSV or 70Z3-hCD14 cells with IFN-γ did not result in tyrosine phosphorylation of p38. PMA also failed to induce p38 phosphorylation, but did induce tyrosine phosphorylation of a moiety clearly distinct from p38 with an apparent molecular mass of 41 kDa. In contrast to the results observed with LPS in which increased protein tyrosine phosphorylation only occurred in 70Z3-hCD14, the effects of PMA on protein tyrosine phosphorylation were identical in 70Z/3-RSV and 70Z3-hCD14 cells.

LPS-induced Tyrosine Phosphorylation in Macrophages—Several recent reports describe LPS-induced tyrosine phosphorylation in macrophages and in a macrophage-like cell line (11, 12, 22). These studies showed that the 41- and 44-kDa isofoms of MAPK (MAPK2 and MAPK1, respectively) are

Fig. 2. Time and concentration dependence of LPS-induced p38 tyrosine phosphorylation: effect of anti-CD14 monoclonal antibody. 70Z3-hCD14 cells were incubated with 1 ng/ml Re595 LPS for the indicated times (+) or with the indicated concentrations of Re595 LPS for 15 min (×). The time of the cell-free detergent lysates were analyzed for protein tyrosine phosphorylation as described under "Materials and Methods." Where noted (+), cells were pretreated with anti-hCD14 mAb MY4 for 30 min prior to LPS stimulation.

Fig. 1. LPS-induced protein tyrosine phosphorylation in CHO-K1 and 70Z3 cells transfected with hCD14 or empty vector (pRe/RSV). Protein tyrosine phosphorylation in the Triton X-100-soluble lysates of control or LPS-treated cells was determined using anti-phosphotyrosine mAb staining of immunoblots as described under "Materials and Methods." A, cells were incubated with (+) or without (-) Re595 LPS (1 ng/ml) for 15 min; protein molecular mass standards (in kilodaltons) are noted on the left. B, 70Z3-hCD14 cells were pretreated with anti-hCD14 mAb MY4 (10 ng/ml), anti-hCD18 mAb IB4 (10 ng/ml), actinomycin D (10 μg/ml), or cycloheximide (10 μg/ml) for 30 min in serum-free medium containing 500 ng/ml rabbit LBP. These cells were incubated with (+) or without (-) 1 ng/ml Re595 LPS or with medium alone for 15 min, and the cell-free detergent lysates were analyzed for protein tyrosine phosphorylation as described under "Materials and Methods."
prominent targets among the proteins displaying increased tyrosine phosphorylation after LPS addition. Thus, we next compared the effects of LPS (1 ng/ml Re595 LPS) and PMA (0.1 μM) on protein tyrosine phosphorylation in five groups of cells: 70Z/3-RSV and 70Z/3-hCD14 cells, RAW264.7 cells, and PEM obtained from C3HeB/FeJ and C3H/HeJ mice (Fig. 4a) and specifically examined the effects of these agonists on tyrosine phosphorylation of MAPK and p38. As expected, this concentration of LPS induced p38 phosphorylation in 70Z/3-hCD14 cells, but not in 70Z/3-RSV cells, whereas PMA also induced phosphorylation of a 41-kDa protein in both cell lines. Addition of LPS to RAW264.7 cells or PEM obtained from C3HeB/FeJ mice induced tyrosine phosphorylation of p38 and two additional proteins with apparent molecular masses of 41 and 44 kDa, respectively. LPS failed to induce tyrosine phosphorylation of any of these proteins in PEM obtained from C3H/HeJ mice. In PEM from C3H/HeJ mice, PMA induced tyrosine phosphorylation of a protein with an apparent size near that of p38, but careful analysis showed that this protein band does not correspond to p38 (data not shown). Addition of PMA to all five cell types resulted in tyrosine phosphorylation of a 41-kDa protein. We also observed that PMA induced tyrosine phosphorylation of a 44-kDa moiety in RAW264.7 cells. The effects of LPS and PMA on protein tyrosine phosphorylation in these five groups of cells are summarized in Table I.

To confirm previous reports that LPS induces tyrosine phosphorylation of MAPK1 and MAPK2 in MO cells (12, 22) and to ascertain if p38 is related to MAPK, we stripped the anti-phosphotyrosine mAb from the Western blot shown in Fig. 4a and reprobed the filter with an anti-MAPK antibody that recognizes murine MAPK1 and MAPK2 (Fig. 4b). Each of the five cell types contains MAPK1 and MAPK2, and the bands detected on the Western blot with anti-MAPK correspond to the 44- and/or 41-kDa protein phosphorylated by LPS or PMA (Fig. 4a and Table I). In contrast, no anti-MAPK reactivity in the region corresponding to p38 was noted. Thus, p38 appears to be distinct from these MAPK isozymes based on the lack of reactivity with anti-MAPK antibody and its mobility on SDS-PAGE. Interestingly, in RAW264.7 cells, it appeared that LPS- or PMA-induced protein tyrosine phosphorylation reduced the reactivity of MAPK2 with anti-MAPK antibody.

Dephosphorylation of p38 and MAPK2: Effect on Reactivity with Anti-MAPK Antibody—We next performed experiments to rule out the possibility that p38 is a hypophosphorylated form of MAPK2 that has anomalous behavior on SDS-PAGE and reacts poorly with anti-MAPK antibody. To do this, we treated 70Z/3-hCD14 cells with 1 ng/ml Re595 LPS and 0.1 μM PMA and recovered the tyrosine-phosphorylated bands that correspond to p38 or MAPK2 from polyvinylidene difluoride membranes as described under “Materials and Methods.” After treatment with potato acid phosphatase, the phosphorylated and dephosphorylated samples were subjected to SDS-PAGE, electrotransfer to nitrocellulose, and immunoblotting with anti-phosphotyrosine mAb FB2 or anti-MAPK antibody using the procedures described under “Materials and Methods.” Potato acid phosphatase has broad specificity of action in dephosphorylating various phosphoproteins (23–26). After treatment of either MAPK2 or p38 with potato acid phosphatase, these proteins failed to stain with FB2 (Fig. 5a). Dephosphorylation of p38 with potato acid phosphatase did not result in staining with anti-MAPK antibody, whereas potato acid phosphatase-treated MAPK2 was still recognized by anti-MAPK antibody (Fig. 5b). The same results were obtained with the protein bands corresponding to p38 and MAPK2 obtained from LPS-treated RAW264.7 cells (data not shown).

Inhibition of Protein Tyrosine Phosphorylation Blocks LPS-induced NF-κB Activation—In previous studies with 70Z/3-hCD14 cells, we showed that as little as 0.1–1.0 ng/ml LPS causes marked activation of NF-κB (6). Studies to be described elsewhere show that maximum NF-κB activation occurs 15 min after LPS addition and that pretreatment of cells with anti-CD14 mAb inhibits LPS-induced NF-κB activation. Here, we determined the effect of a protein-tyrosine kinase inhibitor and a protein kinase C inhibitor on LPS-induced protein tyrosine phosphorylation and NF-κB activation. 70Z/3-hCD14 cells were pretreated for 4 h with the protein-tyrosine kinase inhibitor herbimycin A or with the protein kinase C inhibitor GF 109203X (27) and then stimulated for 15 min with either Re595 LPS or PMA. Cell-free detergent lysates were prepared for analysis of protein tyrosine phosphorylation (Fig. 6a), and nuclear extracts were prepared to measure NF-κB activity (Fig. 6b). Herbimycin A inhibited LPS-induced tyrosine phosphorylation of p38 in a dose-dependent manner, with strong inhibition observed at 1–10 μg/ml; LPS-induced NF-κB activation was also inhibited in a similar manner. The effects of herbimycin A were not due to cellular toxicity as determined by trypan blue exclusion. In contrast, 10 μM GF 109203X had no effect on LPS-induced p38 tyrosine phosphorylation and NF-κB activation. However, GF 109203X completely blocked PMA-induced tyrosine phosphorylation of MAPK2, whereas herbimycin A failed to inhibit this. NF-κB activation was not induced by PMA in the 15-min time period used in this experiment. In additional studies (data not shown), other protein-tyrosine kinase inhibitors (genistein (10 μM), erbstatin analog (5 μM), and lav-
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Tyrosine phosphorylations induced by LPS or PMA

| Cell      | LPS | PMA | p38 | MAPK1 (44 kDa) | MAPK2 (41 kDa) |
|-----------|-----|-----|-----|---------------|---------------|
| 70Z/3-RSV | +   | -   | -   | -             | -             |
| 70Z/3-hCD14| + | - | - | - | - |
| RAW264.7  | +   | -   | -   | -             | -             |
| PEM (FeJ) | -   | +   | -   | -             | -             |
| PEM (HeJ) | -   | +   | -   | -             | -             |
| CHO-RSV   | +   | NT  | -   | -             | -             |
| CHO-hCD14 | +   | NT  | -   | -             | -             |

* Not tested.

**Fig. 5.** Dephosphorylation of p38 and MAPK2 with acid phosphatase: effect on reactivity with anti-MAPK antibody. The tyrosine-phosphorylated forms of p38 and MAPK2 were obtained from LPS (1 ng/ml)- and PMA (0.1 μM)-stimulated 70Z/3-hCD14 cells, respectively, and treated with potato acid phosphatase (Ptase) as described under "Materials and Methods." Tyrosine-phosphorylated and resultant dephosphorylated p38 and MAPK2 were subjected to SDS-PAGE and electrotransfer to nitrocellulose, and the Western blots were probed with anti-phosphotyrosine mAb FB2 (a) or anti-MAPK antibody (b) as described under "Materials and Methods."

**Fig. 6.** Effects of herbimycin A and GF 109203X on LPS- or PMA-induced tyrosine phosphorylation and NF-κB activation. 70Z/3-hCD14 cells were pretreated for 4 h with the indicated concentrations of herbimycin A or GF 109203X; the cells were stimulated with Re585 LPS (1 ng/ml) or PMA (0.1 μM) for 15 min, and the cell-free detergent lysates were analyzed for protein tyrosine phosphorylation as described under "Materials and Methods." (a). NF-κB activity in nuclear lysates was determined by electrophoretic mobility shift assay as described under "Materials and Methods." (b). Addition of the inhibitors alone had no effect (data not shown).

endustin A (1.7 μM) and the protein kinase A inhibitor H89 (30 μM) failed to block p38 tyrosine phosphorylation and NF-κB activation.

**DISCUSSION**

Herein, we show that LPS induces tyrosine phosphorylation of a 38-kDa protein (termed p38) in 70Z/3-hCD14 cells. Phosphorylation of p38 is detected within 15 min after LPS addition and is blocked by pretreatment of cells with an anti-CD14 mAb or with a protein-tyrosine kinase inhibitor (herbimycin A). Because p38 has an electrophoretic mobility that differs from MAPK1 and MAPK2 and because neither phosphorylated nor dephosphorylated p38 reacts with anti-MAPK antibodies, we conclude that p38 is distinct from the 41- and 44-kDa isoforms of MAPK. Although 70Z/3 cells contain both MAPK isoforms, LPS does not induce tyrosine phosphorylation of these proteins. We also observed LPS-induced tyrosine phosphorylation of a protein in the MO-like cell line RAW264.7 and in PEM obtained from C3HeB/FeJ mice with an electrophoretic mobility comparable to p38.

p38 phosphorylation is induced by R- and S-forms of LPS as well as by synthetic lipid A. In 70Z/3-hCD14 cells, the phosphorylation of p38 appears to be LPS-specific since other agonists for these cells such as IFN-γ and PMA fail to induce tyrosine phosphorylation of p38. Pretreatment of 70Z/3-hCD14 cells with anti-hCD14 mAb MY4 nearly completely inhibited p38 phosphorylation when the cells were challenged with 0.01–1000 ng/ml LPS. Therefore, binding of LPS to CD14 initiates transmembrane signaling, resulting in tyrosine phosphorylation of p38. Although the mechanism whereby CD14 induces transmembrane signaling leading to protein tyrosine phosphorylation is not understood, several models have been proposed, which include the participation of additional membrane proteins that function together with CD14 to compose a high-affinity heteromeric membrane receptor for LPS (8). These additional proteins may also act as a low affinity LPS receptor and mediate LPS-induced cell activation in the absence of CD14 expression.

We showed that addition of LPS to RAW264.7 cells or to PEM from C3HeB/FeJ mice also resulted in tyrosine phosphorylation of a 38-kDa protein that we tentatively conclude is related to that observed in 70Z/3-hCD14 cells. PMA did not induce p38 phosphorylation in these cell types. Thus, p38 tyrosine phosphorylation appears to be a specific target of LPS action in three different LPS-sensitive cell types. Further evidence suggesting that p38 tyrosine phosphorylation is an LPS-specific event derives from the observation that LPS failed to induce p38 phosphorylation in PEM obtained from LPS-hyporesponsive C3HeB/FeJ mice.

Data supporting the contention that protein tyrosine phosphorylation is involved in LPS signaling in MO have been provided by Weinstein et al. (11, 12). These investigators described LPS-induced protein tyrosine phosphorylation of several proteins in the murine macrophage-like cell line RAW264.7 and have identified the 41 (MAPK2)- and 44 (MAPK1)-kDa isoforms of MAPK as specific targets of LPS action. Inclusion of herbimycin A prevented tyrosine phosphorylation of these proteins, and since a protein kinase C inhibitor (compound 3) did not block LPS-induced tyrosine phosphorylations, Weinstein et al. concluded that the LPS effects are mediated by a herbimycin A-sensitive protein-tyrosine kinase and are not dependent on protein kinase C. The recent reports of Manthey et al. (22) and Dong et al. (28) also confirmed and extended these observations. Tyrosine phosphorylation of MAPK isoforms was noted with elicited PEM from LPS-responsive C3H mice, but not with PEM from the LPS-hyporesponsive mouse strain, C3H/HeJ. Results described herein with PEM from C3HeB/FeJ (LPS-responsive) and C3H/HeJ (LPS-hyporesponsive) mice and with RAW264.7 cells demonstrating LPS- or PMA-induced tyrosine phosphorylation of proteins that correspond to MAPK1 and MAPK2 isoforms are generally in agreement with the findings of these other investigators (11, 12, 22, 28). Inspection of the published Western blots in the studies of Weinstein et al. (11, 12) and Manthey et al. (22) reveals the presence of additional
tyrosine-phosphorylated proteins present in lysates of LPS-stimulated cells that might correspond to p38. It is difficult, however, to unequivocally assign a specific band corresponding to p38 in these previous studies. Nevertheless, because we have duplicated all of the published findings from these other investigators using comparable cell preparations, it is likely that tyrosine phosphorylation of a protein corresponding to p38 also occurred.

Western blot analysis established that 70Z/3 cells contain the two MAPK isoforms and that PMA induces the tyrosine stimulation of 70Z/3 cells. Although PMA can mimic some effects of LPS, PMA failed to induce p38 phosphorylation. Moreover, the effects of PMA on MAPK phosphorylation are blocked by the herbimycin A.

The western analysis showed that anti-CD14 antibody blocks LPS-induced tyrosine phosphorylation and, in studies to be described elsewhere, that anti-CD14 antibody blocks LPS-induced NF-kB activation. Herbimycin A treatment also blocks LPS-induced tyrosine phosphorylation of p38 and LPS-induced NF-kB activation. Determining the exact relationship among p38 phosphorylation, NF-kB activation, and LPS-induced cell activation will require additional studies. However, our findings suggest that after LPS binds to CD14, transmembrane signaling occurs, which leads to activation of a protein-tyrosine kinase or, alternatively, reduced activity of a protein-tyrosine phosphatase. Additional studies are needed to distinguish between these possibilities.

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