A Differential Role for the Mitogen-activated Protein Kinases in Lipopolysaccharide Signaling

THE MEK/ERK PATHWAY IS NOT ESSENTIAL FOR NITRIC OXIDE AND INTERLEUKIN 1β PRODUCTION*

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Endotoxin (lipopolysaccharide, LPS) is a component of the outer membrane of Gram-negative bacteria and promotes the activation of macrophages and microglial cells. Although these cells are highly LPS-responsive, they serve unique tissue-specific functions and exhibit different LPS sensitivities. Accordingly, it was of interest to evaluate whether these biological differences reside in variations within LPS signaling pathways between these two cell types. Because the mitogen-activated protein kinases ERK-1 and ERK-2 have been implicated in the control of many immune responses, we tested the concept that they are a key indicator for differences in cellular LPS sensitivity. We observed that murine RAW 264.7 macrophages and murine BV-2 microglial cells both respond to LPS by exhibiting increased iNOS degradation, enhanced NF-κB DNA binding activity, and elevated nitric oxide and interleukin-1β production. Although LPS potently stimulates ERK activation in RAW 264.7 macrophages, it does not activate ERK-1/2 in BV-2 microglia. Moreover, antagonism of the MEK/ERK pathway potentiates LPS-stimulated nitric oxide production, suggesting that LPS-stimulated ERK activation can exert inhibitory effects in macrophage-like cells. These data support the idea that ERK activation is not a required function of LPS-mediated signaling events and illustrate that alternative/additional pathways for LPS action exist in these cell types.

Lipopolysaccharide (endotoxin, LPS)† is a component of the outer membrane of Gram-negative bacteria and can promote the activation of macrophage and microglial cells, which are important sensors of infection by bacteria, fungi, and viruses, in both the periphery and the central nervous system (1–3). Microglia are resident macrophages with wide distribution in nervous tissues, and upon activation by endotoxin or various microorganisms, these cells and other mononuclear phagocytes produce several toxic mediators, such as nitric oxide (NO) and free radicals, in addition to numerous cytokines, including interleukin 1β (IL-1β) and tumor necrosis factor α (TNFα). The production of these mediators, although beneficial for killing bacteria and further activating the immune system, can also contribute to significant tissue damage, especially in the brain. For example, the generation of NO by microglial cells may promote brain damage during infection by enhancing vascular permeability and potentially compromising the blood brain barrier (4–6). Additionally, the activation of microglial cells may contribute to neurodegenerative disorders as a result of their production of β-amyloid protein (7–9), which is deposited in plaques thought to be important in the pathogenesis of Alzheimer’s disease. Moreover, microglial cells have also been implicated in neuronal injury such as stroke, meningitis, and multiple sclerosis. Thus, a further understanding of microglial cell activation and function should allow for the identification of potential targets for therapeutic intervention in these neurological disorders.

Multiple signaling pathways are known to be activated in macrophages upon LPS exposure (10). Among these pathways are the mitogen-activated protein kinases (MAPKs), which are a highly conserved family of protein serine/threonine kinases and include the extracellular signal-regulated kinases ERK-1 and ERK-2 (11, 12). Along with nuclear factor-κB (NF-κB) activation, ERK activation is often used as a hallmark of LPS-induced signal transduction in many cell types (13–19). It has been proposed that ERK (p42/p44 MAPK) activation is involved in LPS-induced cellular responses, such as the increased production of TNFα, inducible nitric-oxide synthase (iNOS) and NO, and interleukin-6 (IL-6) (20–22). Recent data suggest, however, that the ERKs are not involved in iNOS or NO production in macrophages nor in the activation of NF-κB DNA binding activity (23, 24), but rather other MAPKs, specifically p38, have been postulated to be important in the control of these end points. Therefore, given the powerful responses of macrophages and microglia to LPS, it is important to establish the relative contribution of the ERKs and p38 in the LPS sensitivity of these two cell types.

Because NF-κB activity regulates the expression of several interleukin, TNFα, tumor necrosis factor α, HEK 293, human embryonic kidney 293; RT, reverse transcription.
cytokine genes including TNFα, IL-6, and IL-1β (25–31), and because LPS modulates its activity, the status of NF-κB DNA binding activity is a key issue when considering the mechanisms of LPS signaling in murine macrophages and microglial cells. NF-κB is a homo- or heterodimeric transcription factor whose function is regulated by the binding of an inhibitory protein, IκB. Upon serine phosphorylation of the IκB protein, it is targeted for proteasome-mediated degradation, thus allowing the now transcriptionally active NF-κB protein complex to translocate into the nucleus where it can bind to various regulatory elements present in gene promoter regions (32). LPS is known to stimulate the degradation of one of the isoforms of IκB, IκBo, and accordingly, LPS promotes the activation of NF-κB DNA binding activity in numerous myelocytic cell types, including phagocytes (19). Additionally, it has been documented that NF-κB not only plays a role in the regulation of cytokine genes but also in the expression of other genes important for macrophage and microglial cell function. For example, binding sites for NF-κB are present in the promoter of the iNOS gene, and these have been shown to be involved in iNOS gene up-regulation (33, 34).

There are several known LPS-binding proteins present on macrophage membranes, including CD14, CD11b/18, and Toll-like receptors (35). Toll-like receptors are mammalian homologs of the Drosophila Toll receptor and are involved in initiating innate immune defense against bacteria and fungi (36). Because CD14 lacks a transmembrane domain and is unlikely to initiate signals on its own, it is noteworthy that the Toll-like receptor-4 (TLR-4) has emerged as a potential signaling mechanism. Although LPS is a potent activator of several cell types, such as macrophages and microglial cells, these cells often play unique tissue-specific roles and exhibit different sensitivities to LPS. These types of biological differences may be inherent in variations within LPS signaling pathways. Because ERK-1 and ERK-2 have been implicated in the control of a wide variety of macrophage mediators, the present studies were initiated to evaluate the hypothesis that variations in the activation pattern of the ERKs and NF-κB can serve as indicators for differences in LPS sensitivity between macrophage-like cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—LPS (Escherichia coli 0111:B4), 3’-O-(4-benzoylbenzoyl)-ATP (BzATP), asinasoycin, and the phorbol ester PMA were purchased from Sigma.

**Cell Culture**—Murine RAW 264.7 macrophage cells were grown to ~80% confluency and routinely passed in RPMI 1640 medium containing 5% cosmic calf serum (Mediatech, Herndon, VA) and 100 units/ml penicillin/streptomycin (Invitrogen). RAW 264.7 cells were cultured in 100-mm Falcon plates (Becton Dickinson, Franklin Lakes, NJ). CHO-K1 stable cell lines expressing vector (CHO-K1Neo) or human CD14 (CHO-K1/CD14) were generously provided to us by Dr. Douglas Golenbock (Boston University School of Medicine) (45) and were grown as described for RAW 264.7 cells. Prior to each experiment, cells were plated overnight in PBS to allow adherent cells to attach. RAW 264.7 cells (5 × 10^4 cells/well) were used in all experiments with LPS. Equal protein loading was confirmed by staining and re-probing blotts with anti-ERK antibodies. For IL-1β production, cells were treated for 24 h with 10 μM UO126 followed by stimulation with either 1 μM PMA (a potent activator of ERK-1 and -2) or 10 μM UO126 followed by stimulation with both 10 μM PMA and 1 μM UO126.

Human embryonic kidney 293 (HEK 293) cells were grown to 80% confluency and routinely passed in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (Mediatech) and 100 units/ml penicillin/streptomycin (Invitrogen) and were used as a negative control cell line for TLR-4 expression.  

**Immunoblotting for MAPKs, BzATP, and TLR-4**—Whole cell lysates were prepared by lysing CHO-K1, CHO-CD14, BV-2, and RAW 264.7 cells, plated as indicated above, in SDS-PAGE sample buffer without bromphenol blue (20 mM Tris, 2 mM EDTA, 1 mM NaVO₃, 2 mM diithietolre, 2% SDS, 20% glycerol). Protein content was determined using the Micro-BCA Protein Assay (Fierce). Equal amounts of protein were loaded per lane and separated by 10% SDS-PAGE as described by Laemmli (48). Proteins in the gels were transferred to Immobilon polyvinylidene difluoride (PVDF) membrane (Millipore Corp., Bedford, MA), and the membranes were subsequently blocked in 5% non-fat milk/TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20). Anti-ERK antibodies (Promega, Madison, WI) that recognize the dually tyrosine- and threonine-phosphorylated, and thus enzymatically active forms of ERK-1 and ERK-2, were used at a dilution of 1:5000. Anti-IL-1β antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were diluted 1:1000 in 5% milk/TBST and used to measure BzATP degradation following treatment with LPS. For detection of p38 and JNK activation, membranes were blocked in 1% IgG and protease-free BSA (Jackson ImmunoResearch, Bar Harbor, ME). Anti-active p38 antibody (Santa Cruz Biotechnology) and anti-active JNK antibodies were employed at a final concentration of 1:5000 in 0.1% BSA/TBST. Cell lysates (100 μg/ml of protein) were separated by 10% SDS-PAGE and transferred to PVDF membranes for immunoblotting using anti-TLR-4 antibodies (Santa Cruz Biotechnology) at a final dilution of 1:1000 in 5% milk/TBST. The immunoreactive bands were visualized using secondary antibodies conjugated to horseradish peroxidase (Santa Cruz Biotechnology) and Lumi-Glo chemiluminescence detection methods (Kirkgaard & Perry Laboratories, Gaithersburg, MD). To confirm equal protein loading, membranes were stripped at 70 °C for 30 min with a buffer consisting of 62.5 mM Tris-HCl, pH 6.7, 2% SDS, and 100 mM diithietolre. The immunoblots were re-blocked in 5% milk/TBST followed by incubation with antibodies that react with both active and inactive forms of ERK-1 and ERK-2 proteins (Santa Cruz Biotechnology). Bands were visualized using chemiluminescence. The data shown are representative of at least three separate experiments.

**Measurement of iNOS, NO, and IL-1β Production**—Murine BV-2 and RAW 264.7 cells were cultured in 24-well plates as described above. Cells were treated for 20 h with LPS at the concentrations indicated in the figure legends. For NO determinations, the medium was determined using the Griess reagent as described previously (49). For experiments investigating the involvement of MAPK signaling pathways, the MEK1/MEK2 inhibitor U0126 (10 μM, Promega) or the p38 MAPK antagonist SB 202190 (10 μM, Calbiochem) were used to pretreat RAW 264.7 or BV-2 cells prior to incubation with LPS. In some experiments, additional pulses of UO 126 were also given at several hour intervals following LPS stimulation, as indicated in the figure legends. To confirm the activity of these inhibitors, BV-2 cells were treated with 10 μM UO126 or 10 μM SB 20190 followed by stimulation with either 1 μM PMA (a potent activator of ERK-1 and -2) or 10 μM UO126 followed by stimulation with both 10 μM PMA and 3 μM SB 20190. Western blotting was performed on the membrane blots with antibodies that recognize both active and inactive forms of ERK-1 and ERK-2 proteins. Additionally, to ascertain the time point at which UO126 is capable of inhibiting ERK activation in culture, RAW 264.7 cells were treated for 2, 4, 6, or 18 h with 10 μM UO126 followed by stimulation with 100 nM PMA for 5 min, and ERK-1/ERK-2 and/or p38 activation was measured as described above. To assess NO levels, the breakdown product nitrite was measured in the medium as described above. The Student’s t test was used to evaluate the statistical differences between various treatment groups, with the level of significance being set at not more than p = 0.02. The levels of iNOS were measured by immunoblotting, as outlined above. Equal amounts of protein (~25 μg) were loaded and separated in each lane of a 10% SDS-polyacrylamide gel. The separated proteins were transferred to a PVDF membrane, and the membrane was blocked in 5% milk/TBST. Anti-iNOS antibodies (1:2000) (Transduction Laboratories, Lexington, KY) were used to detect iNOS protein expression. TLR-4 protein expression was detected with LPS. Equal protein loading was confirmed by staining and re-probing blotts with anti-ERK antibodies. For IL-1β level determinations, cells were treated for 3–18 h with either 10 or 1000 ng/ml LPS, and then Quantikine-M ELISA assays (R & D Systems, Minneapolis, MN) were performed according to the manufacturer’s protocol. This assay system measures both the processed and immature forms of IL-1β.
Cells were lysed in 1 ml of Tri- Reagent (Sigma), and total RNA was prepared from RAW 264.7 macrophages and BV-2 microglial cells were grown in 100-mm plates as detailed above. FACS analysis was performed in a Becton-Dickinson FACScan Flow Cytometer gating 10,000 live events.

**Reverse Transcription (RT)-PCR—** Murine RAW 264.7 macrophages and BV-2 microglia were grown in 100-mm Falcon plates as described previously (50). In these experiments, the cells were treated with 1 μg/ml LPS for 30, 60, or 90 min and lysed, and 10 μg of nuclear protein extract was incubated with 32P-labeled double-stranded oligonucleotide probe encoding two consensus NF-κB DNA-binding sites. The DNA-protein complexes were separated using non-denaturing PAGE techniques, and the gels were dried and apposed to film.

**CD14 Flow Cytometric Analysis—** Murine RAW 264.7 macrophages and BV-2 microglial cells were removed from 100-mm Falcon plates using a non-enzymatic cell dissociation solution (Sigma catalog number C-1544). Cells (5 × 10^6) were washed and resuspended in PBS containing 1% BSA. Rat anti-mouse CD14 (PharMingen International, San Diego, CA) or isotype control antibodies (Caltag Laboratories, Burlingame, CA) were added to the cells at a final concentration of 1 μg/tube and incubated for 1 h at 4 °C. After washing with PBS, 1% BSA, the cells were again incubated for 30 min at 4 °C with 1 μg/tube of anti-rat IgG phycoerythrin-labeled secondary antibodies (Caltag Laboratories, Burlingame, CA). Cells were washed and resuspended in PBS, 1% BSA. Propidium iodide (10 μg/tube) was added to differentiate live from dead cells. FACS analysis was performed in a Becton-Dickinson FACScan Flow Cytometer gating 10,000 live events.

**Electrophoretic Mobility Shift Assays—** Nuclear extracts were prepared from RAW 264.7 macrophages and BV-2 microglial cells, and prepared as described above. Cells were lysed in 1 ml of Tri-Reagent (Sigma), and total RNA was harvested as described by the manufacturer’s protocol, followed by DNase I treatment for 60 min and heat inactivation at 95 °C for 10 min. Five μg of total RNA were used as templates for the RT-PCR using random hexamers and Superscript Reverse Transcriptase II (Invitrogen). Reactions were performed according to the manufacturer’s protocol except that the reverse transcriptase enzyme was omitted in one sample for control purposes. Following reverse transcription, 1/10 of the total RT reaction was utilized for PCR of MD-2 mRNA using the following murine primers: 5’TCT GCA ACT CCT CGG ATG CAA TTA TTT CCT AC and 5’TGT ATT CAC GTC TCT TTT CAG AGC TCT GC. The expected size of the amplified fragment is 274 bp. As a control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH, expected amplicon size 982 bp) was also amplified using the following human primer sequences: 5’ TGA AGG TCG GAG TCA ACG GAT TTG GT and 5’ CAT GTG GGC CAT GAG GTC CAC CAC.

**RESULTS**

**Effect of LPS on iNOS, NO, and IL-1β Production in BV-2 Microglial and RAW 264.7 Macrophage Cells—** The murine macrophage cell line RAW 264.7 recapitulates the behavior of peripheral macrophage cells, whereas BV-2 cells retain the characteristics of activated microglial cells. These cells have similar antigen profiles, phagocytic capacities, and anti-inflammatory activities and respond to LPS in terms of NO production (51–53). Although the production of cytokines and mediators by macrophages and microglia are known to be critical for their function (53–55), it is unclear how these cells compare with regard to LPS responsiveness. Treatment of both RAW 264.7 macrophages and BV-2 microglial cells for 16–20 h with increasing concentrations of LPS (10–1000 ng/ml) resulted in increased iNOS protein levels (Fig. 2A) and subsequent NO production (Fig. 1B). However, as illustrated in Fig. 1B, on a per cell basis, BV-2 microglial cells respond more robustly to LPS than do RAW 264.7 macrophages in terms of NO production. This effect is most notable at the lower concentrations of LPS (10 and 100 ng/ml) where BV-2 cells produce more NO over the course of the assay than do RAW 264.7 cells.

Interestingly, a different pattern of LPS responsiveness is also observed with regard to the accumulation of the cytokine IL-1β by RAW 264.7 macrophages and BV-2 microglial cells (Fig. 2). In these studies, ELISAs were used to measure immunoreactive IL-1β forms of the cytokine. When RAW 264.7 and BV-2 cells are treated with LPS (10 and 1000 ng/ml) for 3–18 h, a large increase in immunoreactive IL-1β is observed in the tissue culture medium at 18 h (Fig. 2A). However, in addition to the differential LPS sensitivities of these two cell types, i.e. BV-2 microglia generate more immunodetectable IL-1β in the tissue culture medium on a per cell basis following treatment with 1000 ng/ml LPS for 18 h, we also detected a substantial difference in the kinetics of IL-1β appearance. In contrast to RAW 264.7 macrophages, IL-1β levels in the medium were detectable in the BV-2 microglial cell cultures within 3 h of treatment, while after 9 h of LPS (1000 ng/ml) exposure, the medium from both cell types contained approximately equal levels of IL-1β (Fig. 2A). Although the present ELISAs cannot discriminate between processed and immature forms of IL-1β, more IL-1β is detected in the medium of RAW 264.7 cells over time in response to 10 ng/ml LPS than is found with BV-2 microglial cells. In fact, the amount of immunoreactive IL-1β produced by BV-2 microglial cells in response to 10 ng/ml LPS appears unchanged over an 18-h period. Because this observation is different from what has been reported regarding IL-1β production by macrophages and macrophage cell lines (56–62), we evaluated the kinetics of IL-1β accumulation in the tissue culture medium in BV-2 microglial cells in response to 10 and 1000 ng/ml LPS (Fig. 2B). Again, as illustrated in Fig. 2, A and B, similar levels of detectable IL-1β were found in the medium from BV-2 cells following treatment with 10 ng/ml LPS for 6–18 h.

**Expression of CD14, TLR-4, and MD-2 on RAW 264.7 and BV-2 Cells—** Although LPS potently stimulates both RAW 264.7 macrophages and BV-2 microglial cells to produce cytokines and NO, BV-2 cells exhibit an altered pattern of LPS responsiveness, especially at lower LPS concentrations and...
The degradation of IκB proteins is involved in the activation and nuclear translocation of NF-κB protein complexes. IκB proteins maintain the transcription factor NF-κB in the cytosol until they are dissociated primarily because of their serine phosphorylation and subsequent ubiquitination targeting them for proteasome degradation (32). LPS is known to induce the degradation of IκBα in macrophages (63), thereby facilitating NF-κB DNA binding activity. The stimulation of NF-κB DNA binding activity by LPS is believed to be part of the mechanism employed by endotoxin to stimulate NO and IL-1β production in immune cells. The data in Fig. 4A indicate that LPS, at concentrations of 100 and 1000 ng/ml, induces the degradation of IκBα in BV-2 microglial cells within 15 min of treatment. Fig. 4B illustrates a comparable effect in RAW 264.7 macrophages, where 100 and 1000 ng/ml LPS stimulate the degradation of IκBα. Similarly, NF-κB DNA binding activity in the two cell types is apparent within 60 min of LPS treatment (Fig. 4C). Interestingly, RAW 264.7 macrophages display a higher level of basal NF-κB DNA binding activity than is observed in BV-2 cells.

The effect of LPS on the mitogen-activated protein kinases ERK-1, ERK-2, p38, JNK-1, and JNK-2—Because the levels of NO produced on a per cell basis by BV-2 cells treated with LPS were greater than those produced by RAW cells, and given that differences in CD14 expression were likely not the basis for this observation, we further investigated LPS-induced signaling pathways in BV-2 cells. Surprisingly, LPS did not detectably stimulate ERK-1 and ERK-2 in BV-2 cells (Fig. 5A) regardless of the concentration used, whereas a dose-dependent effect was observed in RAW 264.7 cells (Fig. 5B). To confirm that receptor-mediated activation of ERK-1 and ERK-2 was intact in BV-2 cells, BzATP (250 μM), an agonist of the purinergic receptor P2X5, was used as a positive control for ERK activation in both cell types and was found to greatly induce ERK-1 and ERK-2 activation. These blots were stripped and re-probed with an antibody that cross-reacts with ERK-1 and ERK-2 and confirmed that equal protein loading was obtained (data not shown). In summary, ERK activation was observed in both RAW 264.7 and BV-2 cells following stimulation with BzATP, indicating that BV-2 cells have the capacity to exhibit ERK activation in response to agents other than LPS.

To confirm further that LPS fails to stimulate ERK activation in BV-2 cells, an extended treatment with both LPS and the phorbol ester PMA was performed over a period ranging from 5 min to 4 h. As shown in Fig. 6A (upper panel), RAW 264.7 cells exhibited a strong ERK activation following stimulation with both 100 nM PMA and 1 μg/ml LPS, although the kinetics of activation by these stimuli are different. PMA maximally promotes ERK-1 and ERK-2 activation within 5 min of treatment, and active ERK levels remain elevated above control levels even after 4 h. In contrast, LPS does not measurably stimulate ERK activation until ~15 min following treatment, wherein it remains elevated above that observed in vehicle-treated cells until 60–120 min later, after which time active ERK levels return to basal levels. The lower panel shows the same blot stripped and re-probed with an antibody that cross-reacts with ERK-1 and, to a lesser degree, ERK-2 to confirm equal protein loading.

Interestingly, the kinetics of PMA-stimulated ERK-1 and ERK-2 activation in BV-2 cells is different from that observed in RAW 264.7 macrophages (Fig. 6B, upper panel). In BV-2 cells it is primarily ERK-2 activity that is stimulated upon PMA (100 nM) treatment, although some ERK-1 activation is observed. Active ERK-2 levels are increased upon PMA treatment of BV-2 cells within 5 min, and these remain elevated for

![Graph](http://www.jbc.org/)

**Fig. 2. Influence of LPS on IL-1β release by murine RAW 264.7 macrophages and BV-2 microglial cells.** A, total (processed and immature) immunodetectable IL-1β levels, as determined by ELISA, were measured in the culture medium of both RAW 264.7 and BV-2 cells treated with 10 or 1000 ng/ml LPS as indicated in the figure for 3–18 h. B, total IL-1β levels, as determined by ELISA, were measured in the culture medium of BV-2 cells treated with 10 or 1000 ng/ml LPS as indicated in the figure for 3–18 h. The data are expressed as the mean (femtograms of IL-1β per 5000 cells) ± S.E. of triplicate or quadruplicate samples. The results shown are representative of experiments performed in at least three separate trials. This ELISA detection method does not differentiate between the pro- (immature) and cleaved (mature) forms of IL-1β released into the culture medium.

Earlier time points. To determine whether the differential sensitivity of BV-2 cells to LPS is dependent upon the expression of the known LPS-binding protein CD14, we evaluated the expression levels of CD14 by flow cytometry. Both RAW 264.7 cells (Fig. 3A) and BV-2 cells (Fig. 3B) exhibited a comparable level of cell surface CD14 expression. Next, the expression of another LPS receptor, TLR-4, and its associated signaling partner MD-2 were evaluated using immunoblotting and RT-PCR analysis, respectively. As illustrated in Fig. 3C, TLR-4 protein expression does not appear to be measurably different between the two cell types. Because of the lack of availability of antibodies to evaluate MD-2 protein levels, RT-PCR was performed in both RAW 264.7 and BV-2 cells (Fig. 3D). Although this assay is not quantitative, both cell types exhibited similar levels of amplified MD-2 transcripts. These data suggest that the differential sensitivity to LPS by both RAW 264.7 and BV-2 cells is not due to substantial differences in the expression of the LPS-binding proteins CD14, TLR-4, or MD-2.
30 min. After this time they begin to decrease until after 4 h of treatment, whereupon active ERK levels return to approximately those observed in vehicle-treated cells. Again, as shown in Fig. 6B, LPS (1 μg/ml) does not detectably stimulate ERK activation within a 4-h period, indicating that LPS has different signaling effects in RAW 264.7 macrophages versus BV-2 microglial cells. The lower panel shows the same blot stripped and re-probed with an antibody that cross-reacts with ERK-1 and to a lesser degree, ERK-2, to confirm equal protein loading. Because LPS does not promote the activation of ERK-1 and ERK-2 in BV-2 cells, it was of interest to evaluate its effects on the activation of other MAPKs in BV-2 cells. As illustrated in Fig. 7, within 15 min of treatment, LPS can promote p38 (Fig. 7A) and JNK (Fig. 7B) activation in BV-2 cells. It is noteworthy that the activation of p38, but not the ERKs, has recently been implicated in LPS-stimulated NO production (24). This observation is in contrast to previous studies suggesting that both ERK and p38 activation are involved in NO and TNFα production (20, 22).

Recapitulation of the BV-2 Cell Phenotype in Transfected CHO-K1 Cells—To determine whether the lack of LPS-stimulated ERK activation is a phenotype specific to BV-2 microglial cells, we tested the ability of CHO-K1 cells to respond to LPS with regard to this end point. CHO-K1 cells are known to express the LPS receptor TLR-4 (64–66). This receptor has been reported to be essential for LPS-stimulated NF-κB DNA binding activity and many of the cell signaling events initiated upon LPS treatment, such as activation of ERK-1 and ERK-2 (41, 42, 44). CHO-K1/Neo and CHO-K1/CD14 cells were found to express TLR-4 protein and MD-2 mRNA (data not shown). As illustrated in Fig. 8A, LPS promotes IκBα degradation in RAW 264.7 cells and in CHO-K1 cells transfected with a CD14 expression vector (CHO-K1/CD14) but not in cells transfected with empty vector alone (CHO-K1/NEO). In contrast, ERK activation was not observed in either CD14-expressing CHO-K1 cells or in the parental vector-expressing cells at either concentration of LPS (1 or 10 μg/ml), but LPS-stimulated ERK activation was observed in RAW 264.7 cells (Fig. 8B). PMA, a phorbol ester, achieves ERK activation by receptor-independent mechanisms and was observed to stimulate the activation of ERK-1 and ERK-2 in all the three cell lines, whereas LPS was without effect in the CHO-K1/NEO- and CHO-K1/CD14-expressing cells (Fig. 8, B and C). Together, these data suggest that ERK activation is not a necessary part of LPS-mediated signal transduction because in both BV-2 cells and in CHO-K1 cells LPS can elicit the degradation of IκBα as...
Because LPS was observed to activate p38 MAPKs
in BV-2 cells, ERK activation does not lead to
the production of IL-1β and NO in the absence of ERK
activation. Additionally, these data suggest that LPS stimulation
of CD14/TLR-4 receptors in CHO-K1 cells does not lead to
activation. Additionally, these data suggest that LPS stimula-
tion of NO production is unaffected by the MEK inhibitor
UO126, whereas NO production is profoundly diminished in
the presence of the p38 inhibitor SB 202190. Interestingly, in
RAW 264.7 cells, addition of UO126 does not diminish the
production of NO, but rather it potentiates the effect of LPS
(p < 0.02) (Fig. 9, B and C), whereas inhibition of p38 by SB
202190 significantly reduced (p < 0.02) the amount of NO
produced by RAW 264.7 cells following LPS stimulation (Fig.
9B). As shown in Fig. 9C, subsequent treatment of RAW 264.7
cells with additional pulses of UO126 (4 and/or 8 h) after the
initial 15-min pretreatment (Fig. 9C, UO 15') does not alter the
ability of LPS to promote NO production (p < 0.01), further
supporting the idea that ERK activation is not required for LPS
induction of NO production. As controls for the ability of these
inhibitors to block ERK and p38 activation, BV-2 cells were
pretreated with the inhibitors for 15 min prior to stimulation
with either 1 μM PMA or 10 μg/ml anisomycin. The upper panel
of Fig. 9D demonstrates that UO126 blocks PMA-stimulated
ERK-1 and ERK-2 activation, and the lower panel shows block-
ade of p38 activation stimulated by anisomycin using
SB202190. Furthermore, to ascertain the length of time that
UO126 will inhibit ERK activation following treatment of RAW
264.7 macrophages in cell culture, Fig. 9E demonstrates that 2,
4, 6, and 18 h after addition of UO126 to cell culture PMA is
unable to elicit a detectable increase in ERK activation, sug-
gesting that the inhibitor is functional in cell culture even after
18 h of addition. These data also imply that any autocrine
factor that might be released by macrophages following LPS
stimulation in BV-2 cells after the 4-h period tested in Fig. 6
and 7 demonstrates that these results are not due to
uncontrolled degradation of p38 MAPKs. Therefore, the
SDS-PAGE confirms that LPS stimulation of RAW 264.7
cells, although it very potently promotes p38 activation.

Fig. 4. Effect of LPS on IκBα degradation and NF-κB DNA binding
activity in murine RAW 264.7 macrophages and BV-2 microglial
cells. A, murine BV-2. B, RAW 264.7 cells were treated
with 10, 100, or 1000 ng/ml LPS for 5, 10, or 15 min. Whole cell lysates
were prepared and proteins (~25 μg) were resolved by SDS-PAGE
for immunoblot analysis. Membranes were probed with an antibody to
IκBα and visualized using chemiluminescence to evaluate degradation
of IκBα. C, NF-κB DNA binding activity was evaluated using nuclear
extracts prepared from both BV-2 cells and RAW 264.7 cells treated
with 1 μg/ml LPS for 60 min. Nuclear protein extracts (10 μg) were
incubated with 32P-labeled double-stranded oligonucleotides encoding a
consensus NF-κB-binding site. The DNA-protein complexes were sepa-
rated by non-denaturing PAGE techniques, and the gels were dried and
apposed to film. The data shown are representative of experiments that
have been performed on at least three separate occasions.

Fig. 5. Dose response of LPS effects on ERK-1 and ERK-2 act-
ivation in murine RAW 264.7 macrophages and BV-2 microglial
cells. Murine BV-2 microglia (A) and murine RAW 264.7 macrophages
(B) were treated with 10, 100, or 1000 ng/ml LPS for 5, 10, or 15 min.
Cells were stimulated with either 1 μM PMA or 10 μg/ml anisomycin. The
upper panel of Fig. 5A demonstrates that BV-2 cells require higher
concentrations of LPS to elicit ERK-1 and ERK-2 activation than
RAW 264.7 cells. In contrast, RAW 264.7 cells, although it very potently
promotes p38 activation.
**DISCUSSION**

The data in the present study support the concept that ERK activation is not a necessary component of LPS-stimulated signaling events in the microglial cell line BV-2. Conversely, LPS-induced p38 activation does appear to be important both in this cell line and in RAW 264.7 macrophages because an inhibitor of p38 (SB 202190) greatly reduces LPS-stimulated NO production. Although BV-2 cells exhibit an LPS-induced production of critical cytokines and mediators, such as IL-1β and NO, as well as LPS-stimulated iNOS, the iNOS enzyme are involved in these effects. However, the present studies suggest that alternative pathways are linked to LPS-induced ERK activation because both TLR-4 and MD-2 appear to be expressed in BV-2 microglia and in CHO-K1 cells expressing CD14, it is possible that the expression of the MD-2 can contribute to the LPS-induced activation of the MAPKs in a heterologous system. Nonethe-
less, our data using both BV-2 microglial cells and CHO-K1

**Fig. 6.** Extended time course of ERK activation following LPS and phorbol ester treatment of murine RAW 264.7 macrophages and BV-2 microglial cells. **A,** murine RAW 264.7 macrophages were treated with 1 μg/ml LPS or 100 nM PMA for 5–240 min as indicated in the figure. Whole cell lysates were prepared, and proteins (~25 μg) were resolved by SDS-PAGE for immunoblot analysis. ERK-1 and ERK-2 activation was assessed using a phospho-specific anti-active MAPK antibody (upper panel). To confirm equal protein loading, the lower panel shows the same blot stripped and re-probed with an antibody that cross-reacts with ERK-1 and, to a lesser degree, ERK-2. B, murine BV-2 microglia were treated with 1 μg/ml LPS or 100 nM PMA for 5–240 min as indicated in the figure. Whole cell lysates were prepared, and proteins (~25 μg) were resolved by SDS-PAGE for immunoblot analysis. ERK-1 and ERK-2 activation was assessed using a phospho-specific anti-active MAPK antibody (upper panel). To confirm equal protein loading, the lower panel shows the same blot stripped and re-probed with an antibody that cross-reacts with ERK-1 and, to a lesser degree, ERK-2. The data shown are representative of experiments that have been performed on at least three separate occasions.

**Lack of ERK Involvement in Microglial Cell NO Production**

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**DISCUSSION**

The data in the present study support the concept that ERK activation is not a necessary component of LPS-stimulated signaling events in the microglial cell line BV-2. Conversely, LPS-induced p38 activation does appear to be important both in this cell line and in RAW 264.7 macrophages because an inhibitor of p38 (SB 202190) greatly reduces LPS-stimulated NO production. Although BV-2 cells exhibit an LPS-induced production of critical cytokines and mediators, such as IL-1β and NO, as well as LPS-stimulated iNOS, the iNOS enzyme are involved in these effects. However, the present studies suggest that alternative pathways are linked to LPS-induced ERK activation because both TLR-4 and MD-2 appear to be expressed in BV-2 microglia and in CHO-K1 cells expressing CD14, it is possible that the expression of either Toll-like receptors or their co-receptors, such as MD-2, may be involved in explaining these effects. However, the present studies suggest that alternative pathways are linked to LPS-induced ERK activation because both TLR-4 and MD-2 appear to be expressed in BV-2 and CHO-K1 cells, yet LPS is unable to stimulate ERK-1 and ERK-2 activation. In this regard, TLR-4 expression in HEK 293/CD14-expressing cells confers upon these cells the ability to induce iNOS, NF-κB activation (43). In these studies, Yang et al. (43) also showed that the MAPKs ERKs 1 and 2, p38, and JNKs 1 and 2 are absent in LPS-stimulated HEK 293/CD14 cells that express TLR-4. Upon expression of MD-2 in these cells, NF-κB activation was augmented and conferred to LPS the ability to stimulate all the MAPK members listed above. MD-2 is a protein that appears to be required for TLR-4 function. It is secreted from macrophages (40) and is believed to be important in mediating LPS-induced signaling events (67, 68). The results presented by Yang et al. (43) suggest that MD-2 can contribute to the LPS-induced activation of the MAPKs in a heterologous system. Nonetheless, our data using both BV-2 microglial cells and CHO-K1...
cells illustrate that although MD-2 may be involved in the LPS-induced activation of p38 and JNKs 1 and 2, another factor/protein is also likely to be involved in ERK activation following LPS treatment.

In this regard, one receptor system that may play an important role in LPS-stimulated ERK activation is the purinergic receptor P2X7. The P2X7 receptor is an ATP-gated cation channel, and previous work from our laboratory (46) has demonstrated that it plays a role in LPS-stimulated ERK activation.

**FIG. 7.** Time course and dose response of LPS on the activation of p38 and JNK MAPKs in murine BV-2 cells. A, murine BV-2 microglia were treated with 10, 100, or 1000 ng/ml LPS for 5, 10, or 15 min. Whole cell lysates were prepared, and proteins (~25 µg) were resolved by SDS-PAGE for immunoblot analysis. Activation of p38 was assessed using a phospho-specific anti-active p38 antibody that recognizes the dually tyrosine- and threonine-phosphorylated and enzymatically active form of p38. Equal protein loading was confirmed by re-probing these blots with an anti-ERK-1 antibody that recognizes total ERK-1 and ERK-2 protein (data not shown). B, murine BV-2 microglia were treated with 10, 100, or 1000 ng/ml LPS for 5, 10, or 15 min. Whole cell lysates were prepared, and proteins (~25 µg) were resolved by SDS-PAGE for immunoblot analysis. Activation of JNK-1 and JNK-2 was assessed using a phospho-specific anti-active JNK antibody that recognizes the dually tyrosine- and threonine-phosphorylated and enzymatically active forms of JNK-1 and JNK-2. Equal protein loading was confirmed by re-probing these blots with an anti-ERK-1 antibody that recognizes total ERK-1 and ERK-2 protein (data not shown).

**FIG. 8.** Effect of LPS treatment on ERK activation and IκBα degradation in CD14 expressing CHO-K1 cells. A, RAW 264.7, CHO-K1/Neo, and CHO-K1/CD14 cells were treated with 1 µg/ml LPS for 30 min. Whole cell lysates were prepared, and proteins (~25 µg) were resolved by SDS-PAGE for immunoblot analysis. Membranes were probed with an antibody to IκBα and visualized using chemiluminescence to evaluate degradation of IκBα. B, RAW 264.7, CHO-K1/Neo, and CHO-K1/CD14 cells were treated with 1 or 10 µg/ml LPS or 1 µM PMA for 15 min as indicated in the figure. Whole cell lysates were prepared, and proteins (~25 µg) were resolved by SDS-PAGE for immunoblot analysis. Activation of ERK-1 and ERK-2 proteins was assessed using a phospho-specific anti-active MAPK antibody. Equal protein loading was confirmed by re-probing these blots with an anti-ERK-1 antibody that recognizes total ERK-1 and ERK-2 protein (data not shown). C, graphical representation of optical densities obtained from the measurement of active ERK-1 and ERK-2 immunoreactive bands (n = 6 separate experiments) in RAW 264.7 macrophages and CHO-K1/neo and CHO-K1/CD14 cells treated with either vehicle or 1 µg/ml LPS for 15 min. The data were obtained using the program NIH Image and are expressed in arbitrary units, as the mean ± S.E.
Fig. 9. Effect of LPS treatment on nitric oxide production in BV-2 and RAW 264.7 macrophages in the presence of MEK and p38 inhibitors. A, BV-2 cells were treated with 100 or 1000 ng/ml LPS for 16 h in the presence or absence of SB 202190 or UO126 administered 15 min prior to stimulation with LPS. NO levels in the medium were determined using the Griess reagent as described under “Experimental Procedures.” The data are expressed as the mean ± S.E. of triplicate samples. B, RAW 264.7 cells were treated with 100 ng/ml LPS for 16 h in the presence or absence of SB 202190 or UO126 administered 15 min prior to stimulation with LPS. NO levels in the medium were determined using the Griess reagent as described under “Experimental Procedures.” The data are expressed as the mean ± S.E. of triplicate samples. *, p < 0.02 with respect to LPS stimulation alone. C, RAW 264.7 cells were treated with 100 ng/ml LPS for 16 h in the presence or absence of UO126 administered 15 min prior to stimulation with LPS and/or 4 or 8 h after LPS treatment, as indicated in the figure. NO levels in the medium were determined using the Griess reagent as described under “Experimental Procedures.” The data are expressed as the mean ± S.E. of triplicate samples. *, p < 0.01 with respect to LPS + UO126 treatments at all time points. D, BV-2 cells were pretreated with the MEK inhibitor UO126 (10 μM), the p38 inhibitor SB 202190 (10 μM), or Me2SO (DMSO) (vehicle) for 15 min prior to stimulation with either PMA (1 μM) or anisomycin (10 μg/ml) for 15 min. Whole cell lysates were prepared, and proteins (~25 μg) were resolved by SDS-PAGE for immunoblot analysis. Activation of p38 and ERK-1/ERK-2 proteins was assessed using phospho-specific anti-active antibodies. Equal protein loading was confirmed by re-probing these blots with an anti-ERK-1 antibody that recognizes total ERK-1 and ERK-2 protein (data not shown). The data are representative of three separate experiments.
strated that this nucleotide receptor is involved in certain LPS-stimulated end points in RAW 264.7 macrophages, such as NF-κB and ERK activation as well as iNOS up-regulation and NO production. Interestingly, the LPS-binding protein CD14 may act to potentiate P2X, receptor signaling with respect to NF-κB, p38, and JNK activation, but not ERK activation.2 Although the expression levels of the P2X7 receptor appear to be comparable in both cell lines (data not shown), it is conceivable that the expression of other proteins that may play an adaptive role between P2X, and CD14, or that are involved in transducing the signals from the P2X/CD14-containing protein complex are different or absent in BV-2 cells.

Another explanation for the negligible effect of LPS on ERK activation in BV-2 and CHO-K1/CD14-expressing cells may be that they lack a critical signaling component involved in regulating the ERK cascade. However, because PMA, LPS, and BzATP most likely utilize overlapping signaling mechanisms to stimulate the ERKs, it is probable that the signaling molecules required for ERK activation are present in BV-2 cells, given that PMA and BzATP retained the capacity to activate the ERKs. However, it is also possible that the activation of ERK-1 and ERK-2 is mediated in macrophage-like cells, such as RAW 264.7 cells, by an autocrine factor/receptor that is missing in BV-2 and CHO-K1/CD14-expressing cells. Due to the delayed onset of ERK activation in RAW 264.7 cells stimulated with LPS (10–15 min), compared with that of PMA or BzATP (<5 min), it is possible that a factor(s) is released upon LPS stimulation of macrophages that, in turn, results in ERK activation in RAW 264.7 cells. This factor may not be produced or secreted by BV-2 cells. Alternatively, both BV-2 and CHO-K1/CD14 cells may lack the ability to respond to this factor, perhaps because they do not express the receptor for this factor.

An additional consideration in the present investigations is the concept that a critical ratio of CD14 or MD2 to Toll-like receptors that may act to potentiate P2X7 receptor signaling with respect to cytokine and mediator production in BV-2 microglial cells in a manner that is at least as sensitive as that observed in the highly LPS-responsive RAW 264.7 macrophages (which is a cell type wherein LPS exposure promotes ERK-1 and ERK-2 activation). In fact, because both cell types express Toll-like receptors and MD-2, but have differential LPS signaling responses, it is conceivable that additional receptors/proteins are required for mediating/modulating LPS actions.

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9087

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A Differential Role for the Mitogen-activated Protein Kinases in Lipopolysaccharide Signaling: THE MEK/ERK PATHWAY IS NOT ESSENTIAL FOR NITRIC OXIDE AND INTERLEUKIN 1β PRODUCTION

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