Activation of Extracellular Signal-Related Protein Kinases 1 and 2 of the Mitogen-Activated Protein Kinase Family by Lipopolysaccharide Requires Plasma in Neutrophils from Adults and Newborns

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Neutrophils exposed to low concentrations of gram-negative lipopolysaccharide (LPS) become primed and have an increased oxidative response to a second stimulus (e.g., formyl-methionyl-leucyl-phenylalanine [fMLP]). In studies aimed at understanding newborn sepsis, we have shown that neutrophils of newborns are not primed in response to LPS. To further understand the processes involved in LPS-mediated priming of neutrophils, we explored the role of extracellular signal-related protein kinases (ERK 1 and 2) of the mitogen-activated protein kinase family. We found that LPS activated ERK 1 and 2 in cells of both adults and newborns and that activation was plasma dependent (maximal at ≥5%) through LPS-binding protein. Although fibronectin in plasma is required for LPS-mediated priming of neutrophils of adults assessed by fMLP-triggered oxidative burst, it was not required for LPS-mediated activation of ERK 1 and 2. LPS-mediated activation was dose and time dependent; maximal activation occurred with approximately 5 ng of LPS per ml and at 10 to 40 min. We used the inhibitor PD 98059 to study the role of ERK 1 and 2 in the LPS-primed fMLP-triggered oxidative burst. While Western blotting showed that 100 μM PD 98059 completely inhibited LPS-mediated ERK activation, oxidative response to fMLP by a chemiluminescence assay revealed that the same concentration inhibited the LPS-primed oxidative burst by only 40%. We conclude that in neutrophils, LPS-mediated activation of ERK 1 and 2 requires plasma and that this activation is not dependent on fibronectin. In addition, we found that the ERK pathway is not responsible for the lack of LPS priming in neutrophils of newborns but may be required for 40% of the LPS-primed fMLP-triggered oxidative burst in cells of adults.

Septicemia and shock leading to multiple organ failure remains one of the major causes of death of adults and newborn infants (21, 22). Many symptoms of septic shock, including vasodilation, myocardial dysfunction, and disseminated intravascular coagulation, are elicited by lipopolysaccharide (LPS), a membrane glycolipid from the cell wall of gram-negative bacteria. Septicemia is characterized by low levels (ng/ml) of LPS in the bloodstream (9, 29).

LPS interacts with specific cellular recognition proteins to modify cellular function (15). For example, neutrophils or polymorphonuclear leukocytes of adults exposed to LPS have increased response to bacterial oligopeptides such as formyl-methionyl-leucyl-phenylalanine (fMLP). This process is known as priming (26). It has been shown that neutrophils from newborn infants are not primed in response to LPS, in contrast to neutrophils from adults under similar in vitro conditions (2, 23, 24).

The immediate postreceptor events of LPS priming are largely unknown; however, protein tyrosine kinases have been implicated in the signaling pathways, and inhibitors of protein tyrosine kinases (e.g., genistein) block neutrophil priming (12, 13, 25). In primed neutrophils, proteins with molecular sizes in the range of approximately 40 to 46 kDa become phosphorylated on tyrosine residues (11–14, 20, 28). Thus, mitogen-activated protein kinases (MAPKs) with similar molecular sizes may be involved in LPS signaling (10, 20). This led us to question whether differences in these pathways are responsible for the biological difference between neutrophils of adults and newborns in response to LPS.

The term MAPK broadly refers to a family of serine/threonine kinases that are activated by multiple extracellular factors, respond to stress, and control cellular growth and differentiation (6, 18). The three major MAPK pathways identified in mammalian cells are p42/44 or extracellular signal-regulated kinases 1 and 2 (ERK 1 and 2), p38 MAPK, and c-Jun N-terminal kinase or stress-activated protein kinase (18). Although Nolan et al. reported that ERK 1 and 2 are activated in response to LPS, this trend was only evident with high concentrations of LPS (1 g/ml) or relatively long treatment periods (19). Others have found that in neutrophils, ERK 1 and 2 are not activated by low concentration of LPS (≤500 ng/ml) (8, 16, 17).

Here we report that in neutrophils, ERK 1 and 2 MAPK are activated by low concentrations of LPS (approximately 5 ng/ml) in a plasma- and time-dependent manner. Although plasma was required, fibronectin, a protein that plays a role in LPS priming of respiratory burst (3), was not the plasma factor responsible for LPS-mediated activation of ERK. In addition, there was no significant difference in the LPS-mediated ERK 1 and 2 activation between cells of adults and newborns. Furthermore, while we found that the ERK pathway-specific inhibitor PD 98059 completely prevented ERK activation at 100 M in cells of adults, at the same concentration it only partially
inhibited the LPS-primed fMLP-triggered oxidative burst in these cells. This work indicates that LPS activation of ERK 1 and 2 in neutrophils requires extracellular factors normally found in plasma and that the ERK pathway is not solely responsible for the LPS-primed fMLP-triggered oxidative burst.

MATERIALS AND METHODS

Reagents. Heparin was purchased from Organon Teknika (Toronto, Ontario, Canada). Dextran (6% w/v) was bought from Abbott Laboratories Inc. (Toronto, Ontario, Canada). Ficoll-Plaque Plus was acquired from Amersham Pharmacia Biotech, Inc. (Quebec, Quebec, Canada). Hank’s buffered salt solution (HBSS), 7.5% sodium bicarbonate, and 1M HEPEs buffer solution were purchased from Gibco Laboratories (Burlington, Ontario, Canada). PD 98059 was obtained from Calbiochem (through Cedar Lane Laboratories, Hornby, Ontario, Canada). Radioimmunoprecipitation assay (RIPA) lysis buffer consisted of 25 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.25% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 1 mM EDTA (pH 7.5). Prior to lysis, the following protease and phosphatase inhibitors were added to the RIPA: 1.57 mg of benzamidine per ml, 5 mM sodium fluoride, 200 mM sodium orthovanadate, 5 μg of pepstatin A per ml, 2 mM diisopropyl fluorophosphate, 5 μg of leupeptin per ml, 10 μM phenylarsine oxide, 1 μM phenethylmercuribenzoate fluoride, and 1 mM dithiothreitol; all were from Sigma Chemical (Oakville, Ontario, Canada). Purified recombinant human lipopolysaccharide binding protein (LBP) and mouse monoclonal antibody to human LBP (subclass immunoglobulin G1 [IgG1]) were obtained from Cedar Lane Laboratories. Lucigenin, fMLP, and human plasma fibronectin were also from Sigma Chemical. Escherichia coli LPS (serotype 0111:B4), obtained from List Biological Laboratories Inc. (Campbell, Calif.), was dissolved in HBSS at a concentration of 10 μg/ml and stored in aliquots at −70°C. Before use, LPS was thawed, diluted in HBSS to 1 μg/ml, and sonicated twice (2-s bursts). H-medium consisted of 145 mM NaCl, 5 mM KCl, 1 mM MgCl2, 0.8 mM CaCl2, 5 mM glucose, and 10 mM HEPEs (pH 7.4). Protein A-Sepharose 4B beads were obtained from Sigma Chemical, myelin basic protein was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, Calif.), and γ-32P-ATP was from Amersham Pharmacia Biotech Inc. All whole-cell solutions were prepared with human infusion-grade water (Abbott Laboratories Ltd.). Tris-buffered saline with 70°C. Before use, SDS-PAGE and Western blotting. SDS-PAGE was performed with 7.5% sodium bicarbonate, and 1M HEPES buffer solution were purchased from Ont. The column was prepared by washing beads with 70% ethanol followed by 3 washings with phosphate-buffered saline (PBS). Under sterile conditions, tubes were immediately placed on ice, and cold HBSS (without Ca2+ and Mg2+) was added to assay tubes, and oxidative burst was measured for 4 min.

RESULTS

LPS-mediated ERK 1 and 2 activation in neutrophils from adults and newborns. LPS is known to activate ERK in many cell types (27, 30) but results using neutrophils are controversial, with some studies indicating that ERK is not activated by LPS (8, 16, 17) and others showing activation occurs only at high concentrations (19). Using Western blotting to detect levels of ERK 1 and 2 phosphorylation, we examined the LPS-mediated activation of ERK 1 and 2 in neutrophils from both adults and newborns. We first stimulated neutrophils with 5 ng of LPS per ml for 20 min in the presence of 5% plasma, conditions that effectively prime neutrophils without activation (2). Under these conditions LPS induced dramatic activation of ERK 1 and 2 (Fig. 1). We also noted that levels of LPS-induced ERK 1 and 2 activation were similar between neutrophils from adults and newborns (Fig. 1A, upper blot). Stripping membranes and reprobing them with an antibody for nonphosphorylated ERK 2 showed that lanes had similar loading of this protein (Fig. 1A, lower blot). Data from several different individuals showed that there was no difference in the relative amount of ERK 1 and 2 activation (Fig. 1B). There was also no difference between cells from adults and newborns in the ratio of activated ERK 1 to ERK 2 (data not shown). In addition, assays of adult samples for ERK activity verified that ERK phosphorylation was indicative of ERK activity (Fig. 1C).

Preparation of fibronectin-depleted plasma and fibronectin. Fibronectin-depleted human plasma was prepared according to the manufacturer’s instructions by using a gelatin-Sepharose 4B affinity column (Amersham Pharmacia Biotech, Inc.). The column was prepared by washing beads with 70% ethanol followed by three washings with phosphate-buffered saline (PBS). Under sterile conditions, plasma from adults was passed through the column three times and was stored in aliquots at −70°C. The column was washed with PBS followed by a wash with 0.9% sodium chloride solution, and fibronectin was eluted from the column using 0.05 M sodium acetate and 1.0 M NaBr, pH 5.0. Fibronectin was dialyzed with PBS overnight and stored in aliquots at −70°C. Fibronectin depletion of plasma and fibronectin purification was confirmed by SDS-PAGE and Coomassie blue staining.
terms of ERK activation no difference was found between plasma from adults and newborns (Fig. 2).

**LPS-mediated activation of ERK 1 and 2 is time dependent.** Next, we examined the time course for LPS-mediated activation of ERK 1 and 2. As shown in Fig. 3, ERK 1 and 2 activation in neutrophils increased after a lag of 5 min and was maximal after 20 min of incubation with LPS (Fig. 3A and B). When cells were incubated with LPS for longer than 20 min ERK activation rapidly decreased, though it was still higher than that in unstimulated cells (Fig. 3). No significant difference was observed in the time course for LPS-mediated activation of ERK 1 and 2 between cells of adults and newborns (Fig. 3).

**LPS-mediated activation of ERK 1 and 2 is dose dependent.** LPS has previously been shown to activate ERK 1 and 2 in human neutrophils only when used at concentrations higher than 500 ng/ml (19), but as demonstrated above, in the presence of plasma low concentrations of LPS can induce ERK 1 and 2 activation in neutrophils from adults and newborns. Therefore, we sought to measure the LPS dose response under these conditions. We found that neutrophils from different individuals responded variably to LPS stimulation when used at doses lower than 2.5 ng/ml (data not shown). Maximal ERK 1 and 2 activation generally occurred in cells of both adults and newborns when the LPS dose was approximately 5 ng/ml (Fig. 4). LPS-mediated ERK 1 and 2 activation was similar for neutrophils from adults and newborns at 5 and 50 ng/ml (Fig. 4). Increasing the LPS concentration from more than 50 ng/ml to up to 1,000 ng/ml did not cause further enhancement of ERK 1 and 2 activation (data not shown).

**PD 98059 completely prevents LPS-mediated ERK 1 and 2 activation but only partially inhibits LPS priming of neutrophil respiratory burst.** To determine whether the ERK pathway was responsible for LPS-mediated neutrophil priming, we examined the effect of PD 98059, a MEK-specific inhibitor, on ERK activation and on fMLP-triggered oxidative burst in the presence or absence of LPS priming. We found that ERK activation was markedly inhibited (approximately 70%) at 10 M PD 98059 and completely inhibited at 100 M (Fig. 5A and B). However, in chemiluminescence assays for oxidative burst activity in neutrophils from adults primed with LPS then triggered with fMLP, only partial inhibition (approximately 40%) was seen at 100 M PD 98059 (Fig. 5B). These results indicate that pathways other than the ERK MAPK pathway are involved in the mechanism of LPS-mediated neutrophil priming.

**Fibronectin is not the essential plasma factor in LPS-mediated activation of ERK.** We have reported that fibronectin is required for LPS priming of the fMLP-triggered neutrophil
We have shown that ERK 1 and 2 are activated in neutrophils exposed to low concentrations of LPS (<5 ng/ml) and that this activation is plasma dependent (Fig. 1). These results differ from earlier reports that the ERK 1 and 2 pathway is not activated by low concentrations of LPS (<500 ng/ml) in neutrophils (8, 16, 17). This discrepancy is likely due to the fact that we reconstituted neutrophils with 5% plasma prior to incubation with LPS. In contrast, earlier studies used only 0.1% bovine serum albumin (8, 16) or 0.1% heat-inactivated platelet-poor plasma (17). Our experiments indicate that plasma concentrations of less than 1% do not result in detectable activation of ERK 1 and 2 (Fig. 2). Interestingly, LPS activation of p38 occurs in the presence or absence of plasma, suggesting that LBP is not required (16, 17). In comparison to earlier reports, our findings indicate that LPS-mediated ERK activation requires either a higher concentration of plasma containing LBP or other ERK-specific plasma factors than does p38 for activation. Differences in experimental conditions (e.g., the presence of specific protease or phosphatase inhibitors in the lysis buffer) may also explain why we were able to detect ERK 1 and 2 activation by LPS.

Previously it was shown that neutrophils from newborns, in contrast to those from adults, do not exhibit an LPS-primed increase in fMLP-triggered oxidative burst (2, 24). DeLeo et al. have shown that incubating neutrophils with 100 ng of LPS per ml results in the translocation of cytosolic p47<sub>phox</sub> (a component of the NADPH oxidase) to the plasma membrane of the cell (7). Furthermore, Benna et al. have found that ERKs are involved in phosphorylation and translocation of p47<sub>phox</sub> to the plasma membrane (1). This evidence led us to ask if neutrophils from newborns fail to prime in response to LPS due to deficiencies in ERK activation. Thorough comparisons of the conditions for ERK 1 and 2 activation in cells of adults and newborns indicates that there is no significant difference in LPS-mediated activation of ERK 1 and 2 between these cells.
We conclude that the inability of newborn neutrophils to prime in response to LPS is not due to deficiencies in the ERK MAPK pathway.

Although there was no detectable difference in ERK activation between cells from adults and newborns, we wondered if phosphorylated proteins downstream of ERK may be involved in the LPS-primed oxidative burst in neutrophils. We found that at a concentration of 100 μM PD 98059 completely blocked LPS-mediated activation of ERK 1 and 2 in neutrophils but inhibited the LPS-primed fMLP-triggered oxidative burst by only 40% in these cells (Fig. 5). This partial inhibition of the LPS-primed fMLP-triggered oxidative burst by PD 98059 and the kinase assay results (Fig. 1) support a conclusion that ERK activation plays a minor role in LPS priming in these cells. Approximately 60% of the priming activity remained when PD 98059 was used at concentrations shown to completely inhibit ERK. Thus, although ERK 1 and 2 are involved in the LPS-primed fMLP-triggered increase in oxidative burst, other pathways also must contribute to the majority of this response, further supporting the conclusion that deficiencies in the LPS priming of neutrophils from newborns are likely due to other processes and not the ERK pathway.

Previously it was shown that plasma with the adult isoform of fibronectin is involved in LPS priming of neutrophils but plasma with the newborn isoform is not (3). As preliminary studies indicated that a plasma protein or proteins of ≥100 kDa were involved in LPS-stimulated activation of ERK 1 and 2 (data not shown), we were interested in whether fibronectin was required for plasma-dependent LPS-mediated activation of ERK 1 and 2. Use of fibronectin-depleted plasma did not significantly affect ERK activation in adult cells. Furthermore, using plasma fractionated by protein size, we found that LPS-mediated ERK 1 and 2 activation is maximal in fractions with molecular sizes of 50 to 70 kDa that do not contain fibronectin (data not shown). In contrast to the studies with fibronectin, we found LBP to be an essential factor involved in LPS-stimulated activation of ERK 1 and 2 (Fig. 7). These observations are consistent with the above findings that plasma-dependent LPS-mediated ERK activation does not differ between neutrophils.
from adults and newborns. We conclude that fibronectin is not one of the plasma factors necessary for LPS-mediated activation of ERKs. Our findings demonstrate that ERK 1 and 2 activation occurs in neutrophils and contributes to some of the LPS-primed oxidative burst increase in these cells. We have shown that plasma is necessary for LPS-mediated ERK activation and that fibronectin is not involved in plasma-dependent activation of ERK. Furthermore, we have shown that there is no difference in LPS-mediated ERK 1 and 2 activation between cells from adults and newborns. This eliminates one of the major LPS-activated intracellular signaling pathways as being the source for the biological difference between neutrophils from adults and newborns in response to LPS. Future research is needed to identify the plasma factor or factors required for ERK 1 and 2 activation and other signaling pathways to explain the biological differences between neutrophils from adults and newborns.

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REFERENCES