Differential Activation of Extracellular Signal–Regulated Kinase (ERK) 1, ERK2, p38, and c-Jun NH$_2$-Terminal Kinase Mitogen-Activated Protein Kinases by Bacterial Peptidoglycan

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Soluble staphylococcal peptidoglycan (sPGN) is an inducer of cytokine secretion and may activate macrophages through the CD14 lipopolysaccharide (LPS) receptor. To elucidate sPGN-activated signal transduction pathways, stimulation of mitogen-activated protein (MAP) kinases by sPGN was studied in mouse RAW264.7 macrophages. sPGN strongly activated extracellular signal–regulated kinase (ERK) 1 and ERK2, moderately activated c-Jun NH$_2$ terminal kinase (JNK), and weakly activated p38 MAP kinase, in contrast to LPS, which strongly activated all of these kinases, and phorbol 12,13-dibutyrate (PDB), which strongly activated ERK1 and ERK2 but did not activate p38 or JNK. sPGN- and LPS-induced activation of ERK1 and ERK2, unlike PDB-induced activation, was sensitive to inhibition by herbimycin A and insensitive to inhibition by increased intracellular cAMP. These results demonstrate differential activation of MAP kinases by sPGN, similar but not identical activation of signal transduction pathways by sPGN and LPS, and different mechanisms of MAP kinase activation by bacterial stimulants and phorbol esters.

Peptidoglycan (PGN) is the major constituent of the cell wall of gram-positive bacteria. PGN, similarly to lipopolysaccharide (LPS) from the cell walls of gram-negative bacteria, can reproduce most of the clinical manifestations of bacterial infections, including fever, acute-phase response, inflammation, septic shock, leukocytosis, sleepiness, malaise, abscess formation, and arthritis. Most of these effects are due to the release of cytokines and other mediators from macrophages and other cells [1–6].

Recent efforts to develop a new generation of drugs for the treatment of several diseases mediated by cytokines, including septic shock, have focused on interruption of signal transduction pathways in host cells [7]. However, very little is known about the signal transduction pathways activated by PGN. Therefore, to elucidate the PGN-activated signal transduction pathways, we focused on protein phosphorylation and dephosphorylation, which is one of the major mechanisms of regulating the activity of various signal transduction molecules and transcription factors [8]. Many signal transduction pathways lead to the activation of mitogen-activated protein (MAP) kinases, which in turn transmit the activation signal to the nucleus. There are three major families of MAP kinases: extracellular signal–regulated kinase (ERK), p38, and c-Jun NH$_2$-terminal kinase (JNK) [9]. ERK1 and ERK2 are typically activated by growth factors or protooncogene products, whereas p38 and JNK MAP kinases are typically activated by proinflammatory cytokines and stress [9, 10]. These MAP kinases are activated by dual phosphorylation on threonine and tyrosine in the Thr-Glu-Tyr (ERK1 and ERK2), Thr-Gly-Tyr (p38), or Thr-Pro-Tyr (JNK1 and JNK2) motifs [9].

We have shown that stimulation of macrophages by PGN involves increased tyrosine phosphorylation of ERK1 and ERK2, accompanied by increased activity of MAP kinases in cell lysates [4]. We compared these effects to the effects induced by LPS to determine if PGN and LPS activate the same signal transduction pathways because of the controversial reports that both LPS and PGN may [11–13] or may not [14–16] activate cells through the same CD14 cellular receptor. We also compared the effects of these bacterial stimulants with the effects of phorbol 12,13-dibutyrate (PDB), a phorbol ester that directly activates protein kinase C and bypasses the receptor-mediated cell activation mechanisms. Our results showed that all three stimulants (PGN, LPS, and PDB) induced strong tyrosine phosphorylation of ERK1 and ERK2 [4].

It was also recently shown that LPS, but not phorbol esters, induced tyrosine phosphorylation of p38 MAP kinase in mouse macrophages and CD14-transfected nonmacrophage cells [17, 18]. LPS also induced modest activation of p38 and JNK MAP kinases in transiently transfected cells overexpressing recombinant constructs of p38 or JNK plus Flag epitope [10]. However, it was not determined if other bacterial components, such as PGN, activated p38 and JNK MAP kinases and if LPS activated these kinases in untransfected macrophages.

Therefore, the first objective of this study was to determine which MAP kinases were tyrosine-phosphorylated and activated in response to bacterial PGN in mouse macrophages and to what extent and to compare PGN-induced effects with the...
effects of other MAP kinase activators (LPS, phorbol esters, stress). Since there are several signal transduction pathways with multiple regulation points that lead to the activation of MAP kinases [8–10], our second objective was to determine if activation of these MAP kinases by PGN, LPS, and phorbol esters was sensitive to the same pharmacologic modulators of various kinases and other signal transduction molecules. We have found differential activation and regulation of MAP kinases by PGN, LPS, and phorbol esters.

Materials and Methods

Materials and cells. Soluble (s) PGN was purified by affinity chromatography from culture supernatants of Staphylococcus aureus Rb grown in the presence of penicillin [6, 19]. It contained <12 pg of endotoxin/mg [4]. LPS from Salmonella minnesota Re 595 (ReLPS, a minimal naturally occurring endotoxic structure of LPS), PDB, and all other chemicals were from Sigma (St. Louis) unless otherwise indicated. [γ-32P]ATP was from DuPont (Boston). Murine macrophage RAW264.7 cell line was cultured in HL-I medium as before [4]. RAW264.7 cells were seeded at 0.35–0.4 x 10⁶/mL, 2 mL/well, in 24-well tissue culture plates (4 mL/well in 12-well plates for p38 immunoprecipitations) and cultured for 20–24 h, followed by activation with stimulants as indicated in Results, using optimal concentrations of the stimulants and times of stimulations, which were determined in previous [4] or preliminary experiments. The cells were washed, lysed with 1% NP-40, and centrifuged as before. [4]. The supernatants were precleared with normal rabbit IgG and S. aureus Cowan 1 cells [4] and immunoprecipitated for 4 h at 4°C with one of the following affinity-purified rabbit IgG antibodies (all from Santa Cruz Biotechnology, Santa Cruz, CA): anti-ERK1+2, specific to aa 305–327 of human ERK1 and reactive with 44-kDa ERK1 and 42-kDa ERK2 of mouse and other mammals; anti-p38, specific to aa 341–360 of mouse p38 (for ERK1+2 or p38 immunoprecipitations, the cellular proteins were denatured by boiling in 1% SDS, diluted five times with the lysis buffer, and precipitated with 0.5 μg of antibody/500 μL); anti-JNK1, specific to amino acids 368–384 of human JNK1 and reactive with mouse and human p46 JNK1 (0.25 μg/100 μL); or normal rabbit IgG as a control (which did not precipitate any of the above phosphoproteins). The immune complexes were adsorbed to S. aureus cells and washed four times with lysis buffer or kinase buffer [4].

Tyrosine phosphorylation of immunoprecipitated MAP kinases was detected by Western blotting. Proteins were released from the immune complexes by boiling with 2X SDS—sample buffer with 10% 2-mercaptoethanol and subjected to SDS-PAGE, blotting with 4G10 antiphosphotyrosine monoclonal antibody (MAb), and detected with enhanced chemiluminescence system as described [4]. The blots were then stripped, reprobed with either ERK1+2, anti-p38, or anti-JNK1 antibodies, and developed with goat anti-rabbit–horseradish peroxidase conjugate and the enhanced chemiluminescence system as described [4]. The antibodies were specific for the indicated MAP kinases and did not cross-react with other MAP kinases or other proteins, as determined by the manufacturer and confirmed by us in cross-precipitation—blotting experiments, in which MAP kinases immunoprecipitated with each antibody were checked for the lack of reactivity with all other antibodies by Western blotting.

ERK1, ERK2, p38, and JNK kinase assays. RAW264.7 cells were cultured and stimulated as indicated above, lysed at 4°C in 100 μL of MAP kinase lysis buffer (100 mM TRIS-HCl, pH 7.2, with 2 mM EDTA, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride (PMSF), 18 μg/ml aprotinin, 1% NP-40), and centrifuged at 6000 g. ERK1 and ERK2 were immunoprecipitated for 4 h at 4°C with agarose conjugates of affinity-purified rabbit antibodies specific to aa 352–367 of ERK1 or 345–358 of ERK2 (Santa Cruz Biotechnology) or normal rabbit IgG—agarose conjugate as a control (2.5 μg/100 μL of cell lysate). The agarose immune complexes were washed four times at 4°C and suspended in 30 μL of the following ERK kinase phosphorylation buffer: kinase lysis buffer with 20 mM MgCl₂, 3 mM MnCl₂, 3 mM 2-mercaptoethanol, 200 μM ATP, 0.1 mg/ml bovine serum albumin, 2.7 μCi of [γ-32P]ATP/tube, and 0.4 mg/ml synthetic ERK1/2 kinase substrate peptide (APRTPGGR), corresponding to aa 95–98 of bovine myelin basic protein (Upstate Biotechnology, Lake Placid, NY), which is readily phosphorylated by ERK1 and ERK2 kinases [20, 21]. The mixture was incubated for 20 min at 30°C, chilled in ice water, and centrifuged, and 25 μL of the supernatant was spotted on P81 ion exchange phosphocellulose paper disks (Whatman, Hillsboro, OR). The disks were washed nine times in 0.85% phosphoric acid and once in acetone, dried, and counted in a scintillation counter for 32P incorporated into the substrate peptide [20]. The results are expressed as counts per minute in groups with anti-ERK antibody minus counts in control groups with rabbit IgG.

p38 and JNK were immunoprecipitated for 4 h at 4°C with affinity-purified rabbit IgG antibodies (Santa Cruz Biotechnology) specific to aa 341–360 of mouse p38 (the cellular proteins were denatured by boiling in 1% SDS, diluted five times with the lysis buffer, and precipitated with 0.5 μg of antibody/500 μL), rabbit IgG antibodies specific to aa 368–384 of human JNK1 and reactive with mouse and human p46 JNK1 (0.25 μg/100 μL of a non-denatured lysate), or normal rabbit IgG as a control (which did not precipitate any of the above phosphoproteins). The immune complexes were adsorbed to S. aureus cells and washed with the lysis buffer and then with the kinase buffer at 4°C (p38 immunoprecipitates were allowed to renature for 20 min at 4°C and then incubated for 30 min at 30°C with 30 μL of the following kinase buffer: 33.3 mM HEPES, pH 7.2, with 0.1 mg/mL bovine serum albumin, 10 mM MgCl₂, 1.5 mM MnCl₂, 2 mM diithiothreitol, 50 μM ATP, 6 μCi of [γ-32P]ATP/tube, and 1 μg/tube affinity-purified recombinant 70-kDa fusion protein of full-length (1–505) human activating transcription factor-2 (ATF-2) tagged with polyhistidine (Santa Cruz Biotechnology), which is an excellent substrate for p38 and JNK kinase activity. Cells exposed to UV light [10] or 300 mM sorbitol [10] were used as positive controls. No incorporation of 32P into the 70-kDa band was observed in lysates from stimulated cells following precipitation with normal rabbit IgG. To confirm equal immunoprecipitation of p38 and JNK in all groups, p38
and JNK were released from S. aureus–bound immune complexes by being boiled with 2× sample buffer with 10% 2-mercaptoethanol and subjected to SDS-PAGE and blotting with anti-p38 or anti-JNK antibodies.

Effect of pharmacologic agents on ERK1 and ERK2 tyrosine phosphorylation and kinase activity. Optimal preincubation times and concentrations for all pharmacologic agents were established in preliminary experiments. RAW264.7 cells, grown in DMEM with 10% FCS, were preincubated with the following inhibitors or cAMP modulators as indicated in Results: herbimycin A, staurosporine, H89, 3-isobutyl-1-methylxanthine (IBMX), 4-(butoxy-4-methoxybenzyl)-2-imidazolidinone (RO20-1724; BIONOL Research Laboratories, Plymouth Meeting, PA), rolipram (Berlex Laboratories, Wayne, NJ), or forskolin. The stimulants (sPGN, ReLPS, or PDB) were then added for the indicated lengths of time, the cells were lysed, and tyrosine phosphorylation or kinase activity of ERK1 and ERK2 was determined as described above. The extent of tyrosine phosphorylation was quantified by scanning [19] the ERK1 and ERK2 bands.

Protein kinase A assay. RAW264.7 cells were cultured in DMEM with 10% FCS as described above and then preincubated with or without cAMP modulators (indicated in Results), incubated with PDB, ReLPS, sPGN, or no stimulant, and lysed in 50 mM TRIS-HCl, pH 7.5, with 5 mM EDTA, 1 mM PMSF, 18 μg/mL aprotinin, and 1% NP-40. The lysates were centrifuged at 6000 g, and the activity of protein kinase A in the supernatants was measured as the difference in incorporation of 32P (counts per minute) into the substrate peptide LRRASLG [22] in the absence and presence of a specific protein kinase A inhibitor (PKI 6-22 peptide) [23], using the protein kinase A assay system (Life Technologies GIBCO BRL, Gaithersburg, MD) as directed by the manufacturer, with the exception that cAMP was not added to the assay mixture in order to determine the amount of protein kinase A that was preactivated in the cells by various treatments.

Results

Differential activation of ERK1, ERK2, p38, and JNK MAP kinases by sPGN, ReLPS, and PDB. Our previous results indicated that sPGN, ReLPS, and PDB induce tyrosine phosphorylation of ERK1 and ERK2 MAP kinases and that they increase the overall activity of MAP kinases in unfraccionated cell lysates [4]. To determine the extent of ERK1 and ERK2 activation by these stimuli, immune complex kinase assays were done on ERK1 and ERK2 immunoprecipitated from stimulated and unstimulated cells. sPGN stimulated ERK1 and ERK2 kinase activity 11- and 27-fold, respectively, whereas ReLPS and PDB induced even higher stimulation (23- and 44-fold ReLPS and 24- and 48-fold PDB) (figure 1). These results are in agreement with strong tyrosine phosphorylation of ERK1 and ERK2 induced by all three stimuli [4]. Activation of ERK1 and ERK2 by sPGN, ReLPS, and PDB was serum-independent, since similar results were obtained in RAW264.7 cells cultured in serum-free medium (not shown).

We next wanted to determine the effects of sPGN on p38 and JNK kinases and to compare the effects of sPGN and ReLPS on these kinases in untransfected macrophages, because it has been shown that LPS induces tyrosine phosphorylation of p38 MAP kinase in macrophages [17, 18] and weakly increases the activity of p38 and JNK MAP kinases in transient transfectants overexpressing recombinant p38- or JNK-Flag constructs [10]. sPGN induced weak tyrosine phosphorylation (figure 2) and activation (figure 3) of p38 MAP kinase in RAW267.4 cells, compared with ReLPS, which was a very strong inducer of p38 tyrosine phosphorylation and activation, and with UV light, which was an intermediate inducer (figures 2, 3). PDB induced neither tyrosine phosphorylation nor activation of p38 MAP kinase (figures 2, 3). sPGN-induced tyrosine phosphorylation (figure 2) and activation (not shown) of p38 were not inhibited by polymyxin B, which indicates that the effect was not due to contamination with endotoxin. Similar results were obtained in six experiments, with cells grown in the presence (figures 2, 3) or absence (not shown) of serum (with the exception that in the absence of serum, UV light was as strong an inducer of p38 phosphorylation and activation as ReLPS).

sPGN was a moderately strong activator of JNK kinase, compared with strong activation of JNK kinase by ReLPS and osmotic shock (300 mM sorbitol) and no activation by PDB (figure 3). Similar results were obtained in five experiments with cells grown in the presence (figure 3) or absence (not shown) of serum. Stimulant-induced tyrosine phosphorylation of JNK could not be directly visualized on Western blots with antiphosphotyrosine MAb, because these MAb do not react with phosphorylated tyrosine residues in JNK, as shown by Derijard et al. [24] in human cells and confirmed by us in

Figure 1. Soluble peptidoglycan (sPGN), lipopolysaccharide from Salmonella minnesota Re 595 (ReLPS), and phorbol 12,13-dibutyrate (PDB) stimulate kinase activity of ERK1 and ERK2. RAW264.7 cells were incubated for 15 min with 10 μg/mL sPGN, 1 μg/mL ReLPS, or 100 nM PDB or in medium alone (control). Results are means of 4 experiments ± SEs.
mouse RAW264.7 macrophages (not shown). Stimulant-induced phosphorylation of JNK, however, was confirmed by an appearance on Western blots of a slower-mobility (shifted upward) JNK band in sPGN-, ReLPS-, and osmotic shock-stimulated but not in unstimulated and PDB-stimulated cells (figure 3).

Our current and previous [4] results, therefore, demonstrate that sPGN strongly activates ERK, moderately activates JNK, and weakly activates p38, in contrast to ReLPS, which strongly activates all three families of MAP kinases (ERK, p38, and JNK), and to PDB, which strongly activates ERK and does not activate p38 and JNK. This differential activation of MAP kinases by sPGN, ReLPS, and PDB was not due to the differences in the kinetics of activation of MAP kinases or concentrations of the stimulants, because optimal stimulation times and optimal concentrations of the stimulants were used in figures 1-3. These concentrations of sPGN and ReLPS were previously shown to induce similar amounts of tumor necrosis factor (TNF)-α mRNA and TNF-α bioactivity [4]. Similar differences in activation of MAP kinases by sPGN, ReLPS, and PDB were also apparent with suboptimal concentrations of the stimulants and suboptimal stimulation times (not shown). These results, therefore, indicate that the mechanisms of activation of MAP kinases by these stimulants are not identical.

Figure 2. Differential induction of tyrosine phosphorylation of p38 mitogen-activated protein kinase by soluble peptidoglycan (sPGN), lipopolysaccharide from Salmonella minnesota Re 595 (ReLPS), and phorbol 12,13-dibutyrate (PDB). RAW264.7 cells were incubated for 30 min in medium alone (Nil) or with 10 μg/mL sPGN, 1 μg/mL ReLPS, or 100 nM PDB or were irradiated with UV light, in the presence (+) or absence (−) of 25 μg/mL polymyxin B; lysed; and immunoprecipitated with rabbit anti-p38 antibody (in the far right lane, normal rabbit IgG instead of anti-p38 antibody was used). Immunoprecipitates were subjected to SDS-PAGE, blotted on Immobilon membrane, and reacted with antiphosphotyrosine 4G10 monoclonal antibody (upper). Blots were then stripped and reprobed with anti-p38 antibody (lower).

Figure 3. Differential activation of p38 and JNK mitogen-activated protein (MAP) kinases by soluble peptidoglycan (sPGN), lipopolysaccharide from Salmonella minnesota Re 595 (ReLPS), and phorbol 12,13-dibutyrate (PDB). RAW264.7 cells were incubated for 30 min in medium alone (Nil) or with 10 μg/mL sPGN, 1 μg/mL ReLPS, 100 nM PDB, or 300 mM sorbitol or were irradiated with UV light. Cells were lysed, and activities of p38 and JNK MAP kinases were determined in the immunoprecipitates by incorporation of 32P into activating transcription factor-2 (ATF-2) substrate, visualized by autoradiography (upper). In far right lanes, no ATF-2 substrate was added. Immunoprecipitation of p38 and JNK was confirmed by Western blots of immunoprecipitates with anti-p38 or anti-JNK antibodies (lower).
Effects of increased cAMP. We tested the effects of increased intracellular cAMP on sPGN-, ReLPS-, and PDB-induced tyrosine phosphorylation and activation of ERK1 and ERK2, because other investigators recently discovered that growth factor–induced activation of MAP kinases was inhibited by increased concentrations of intracellular cAMP [27–29].

PDB-induced tyrosine phosphorylation of ERK1 and ERK2 was inhibited by pretreatment of RAW264.7 cells with a combination of an adenylate cyclase activator, forskolin [30], and cAMP phosphodiesterase inhibitors, IBMX (a nonselective inhibitor) [31], or RO20-1724 or rolipram (selective inhibitors of type IV phosphodiesterase) [31] (figure 5). Each of these cAMP modulators alone was not effective in inhibiting PDB-induced tyrosine phosphorylation of ERK1 and ERK2 (figure 5). By contrast, sPGN- and ReLPS-induced phosphorylation of ERK1 and ERK2 was not significantly inhibited by combinations of forskolin and phosphodiesterase inhibitors or by any of these cAMP modulators individually (figure 5). Inhibition of PDB-induced tyrosine phosphorylation of ERK1 and ERK2 was not dependent on the duration of preincubation with a combination of forskolin and IBMX and was consistently inhibited after 15, 30, 60, and 120 min of preincubation (33% ± 2.7%, 23% ± 4%, 16% ± 3.5%, and 16% ± 3.5% of PDB without forskolin + IBMX, respectively). sPGN-induced tyrosine phosphorylation of ERK1 and ERK2 was not significantly affected by 15-, 30-, 60-, and 120-min preincubation periods with forskolin and IBMX (100% ± 8% to 120% ± 10% of sPGN without forskolin + IBMX), whereas ReLPS-induced ERK1 and ERK2 tyrosine phosphorylation was even significantly enhanced by 60-min pretreatment with forskolin and IBMX (121% ± 7%, 125% ± 7.5%, 175% ± 10%, and 120% ± 7% of ReLPS without forskolin + IBMX, respectively). sPGN-induced tyrosine phosphorylation of ERK1 and ERK2 was not dependent on the duration of preincubation with forskolin and IBMX (121% ± 7%, 125% ± 7.5%, 175% ± 10%, and 120% ± 7% of ReLPS without forskolin + IBMX, respectively, means of 3–5 experiments ± SE).

Combination of forskolin and IBMX or RO20-1724 also inhibited PDB-induced activation of ERK1 and ERK2, whereas it did not significantly inhibit sPGN- and ReLPS-induced activation of ERK1 and ERK2 (figure 6).

Because the inhibitory effect of increased cAMP on growth factor–induced activation of MAP kinases is mediated by protein kinase A, which blocks activation of Raf1 [32, 33], we then tested if in our system the inhibitory effects of cAMP modulators correlated with the activation of protein kinase A by these agents. The inhibition of ERK1 and ERK2 tyrosine phosphorylation and activation correlated with the ability of the mixture of forskolin and cAMP phosphodiesterase inhibitors to substantially increase the activity of intracellular protein kinase A (figure 7). Each drug individually did not inhibit tyrosine phosphorylation (figure 5) or activation (not shown) of ERK1 and ERK2 and was also much less effective in increasing the activity of protein kinase A (figure 7). Moreover, the increases in the activities of protein kinase A in unstimulated, PDB- or ReLPS-stimulated (figure 7), or sPGN-stimulated cells (not shown) were similar, indicating that the lack of inhibition of ReLPS- or sPGN-induced effects was not due to ReLPS- or sPGN-induced decrease in the activity of protein kinase A.
Figure 5. Forskolin plus cAMP-phosphodiesterase inhibitors inhibit phorbol 12,13-dibutyrate (PDB)-induced but not lipopolysaccharide from Salmonella minnesota Re 595 (ReLPS)- and soluble peptidoglycan (sPGN)-induced tyrosine phosphorylation of ERK1 and ERK2. RAW264.7 cells were preincubated for 2 h with or without indicated cAMP modulators: forskolin, 100 μM; 3-isobutyl-1-methylxanthine (IBMX), 500 μM; 4-(butoxy-4-methoxybenzyl)-2-imidazolidinone (RO20-1724), 50 μM; rolipram, 100 μM. Cells were then stimulated for 15 min with 10 μg/mL sPGN, 1 μg/mL ReLPS, or 60 nM PDB. Blots of ERK1 and ERK2 immunoprecipitates were developed with antiphosphotyrosine 4G10 monoclonal antibodies (upper), and blots were then stripped and reprobed with anti-ERK antibodies (lower). Phosphorylated 44-kDa (ERK1) and 42-kDa (ERK2) bands (arrows) are shifted upward and react more weakly with anti-ERK antibodies. Bar graph shows intensity of tyrosine-phosphorylated ERK1 and ERK2 bands quantified by scanning analysis. Results are means of 3–9 experiments ± SE and are expressed as % = (intensity of ERK1+ERK2 bands with cAMP modulators) × 100/(intensity of ERK1+ERK2 bands without cAMP modulators).

These results, therefore, indicate that the pathways that result in ERK1 and ERK2 phosphorylation and activation are different in PDB- and in sPGN- or ReLPS-stimulated cells and that the PDB-stimulated pathway includes a cAMP-sensitive step, whereas the sPGN- and ReLPS-stimulated pathways do not.

Discussion

Our results demonstrate similar, but not identical, activation of macrophage MAP kinases by sPGN and ReLPS, two bacterial cell wall products that have been postulated to activate
macrophages through interaction with CD14 [11, 13]. sPGN strongly activates ERK1 and ERK2, moderately activates JNK, and weakly activates p38, whereas ReLPS strongly activates all of these MAP kinases. These data are novel not only for sPGN but also for LPS, because other investigators [10] have studied activation of p38 and JNK by LPS only in transfectants overexpressing recombinant p38- or JNK-Flag constructs, and they found this LPS-induced activation to be rather weak [10], in contrast to very strong activation observed by us in untransfected RAW264.7 cells. PDB, a protein kinase C activator, strongly activated ERK1 and ERK2 but did not activate p38 or JNK, which is in agreement with previously shown strong phosphorylation and activation of ERK1 and ERK2 [4, 34, 35] but no or very weak phosphorylation [17, 18] and activation [10] of p38 and JNK.

Differential activation of p38 and JNK by ReLPS, sPGN, and PDB suggests that the mechanisms of cell activation by these stimuli are at least partially different. Signal transduction pathways that result in activation of p38 and JNK are different than the pathways that result in the activation of ERK1 and ERK2 [9, 10]. Therefore, it can be concluded that ReLPS generates signals that activate multiple signal transduction pathways that culminate in strong activation of all three families of MAP kinases (ERK, p38, and JNK), whereas sPGN generates signals that predominantly activate ERK and only partially activate p38 and JNK MAP kinases.

This difference in activation of MAP kinases by sPGN and LPS, however, does not exclude the possibility that both sPGN and LPS activate cells through interaction with CD14 [11–13]. Our most recent data indicate that CD14 does act as a functional...
sPGN receptor, because sPGN binds to CD14 on human monocytes (Weidemann B, Dziarski R, Kusumoto S, Rietschel ET, Flad HD, Ulmer AJ, unpublished data) and monocye activation by sPGN is inhibited by anti-CD14 MAbs and LPS partial structures [11], and because sPGN-unresponsive mouse 70Z/3 pre-B cells acquire responsiveness to sPGN after transfection with human CD14 or with the 1-151 N-terminal fragment of CD14 (Gupta D, Kirkland TN, Viriyakosol S, Dziarski R, unpublished data). This activation results in induction of nuclear factor-κB (NF-κB) and expression of surface IgM. However, either the binding of sPGN and LPS to CD14 or the activation of cells by sPGN-CD14 and LPS-CD14 complexes, or both, are not identical, because a series of CD14 deletion mutants transfected into 70Z/3 cells shows a different pattern of responsiveness to sPGN and LPS (Gupta D, Kirkland TN, Viriyakosol S, Dziarski R, unpublished data). These data, therefore, are consistent with the results presented here that show similar, but not identical, signals delivered by sPGN and LPS in macrophages.

ReLPS, sPGN, and PDB all strongly activate ERK1 and ERK2. Several signal transduction pathways can result in the activation of ERK MAP kinases [8, 9]. Therefore, to determine if ERK1 and ERK2 are activated by ReLPS, sPGN, and PDB through the same or different signal transduction pathways, we studied the effects of several inhibitors and second messenger modulators on ReLPS-, sPGN-, and PDB-induced activation of ERK1 and ERK2.

Herbimycin A, an inhibitor of receptor and Src-family tyrosine kinases [7], strongly inhibited sPGN- and ReLPS-induced and weakly inhibited PDB-induced ERK1 and ERK2 tyrosine phosphorylation. These results support the hypothesis that sPGN- and ReLPS-induced (but not PDB-induced) ERK activation involves Lyn [4, 36], a member of Src-family kinases. Low sensitivity of sPGN- and ReLPS-induced ERK1 and ERK2 tyrosine phosphorylation to staurosporine and H89 (inhibitors of protein kinase C and protein kinase A, respectively [25, 26]) indicates that activation of these MAP kinases by sPGN and ReLPS does not require activation of protein kinase C or protein kinase A (some inhibition by staurosporine is most likely due to its nonspecific effect on tyrosine kinases).

Our results indicate that the mechanism of ERK1 and ERK2 activation by bacterial stimulants (ReLPS and sPGN) is different from the mechanism of ERK1 and ERK2 activation by growth factors and phorbol esters, because, in contrast to growth factor- and phorbol-induced activation [27–29, 32, 33], sPGN- and LPS-induced activation was not inhibited by increased intracellular cAMP. Both growth factors and phorbol esters activate Raf1 (phorbols directly activate protein kinase C, which activates Raf1), which activates Mek1 and Mek2, which in turn activate ERK1 and ERK2 [8, 9, 37]. The inhibitory effect of cAMP on growth factor- and phorbol-induced ERK activation is mediated by protein kinase A, which is activated by an increased level of intracellular cAMP. Protein kinase A phosphorylates Raf1, which inhibits Raf1 activation [32, 33]. Because protein kinase A is equally well activated by cAMP-increasing agents in PDB-, ReLPS-, and sPGN-stimulated cells, the lack of inhibition of ReLPS- and sPGN-induced ERK activation by increased intracellular cAMP indicates that ERK activation by ReLPS and sPGN is not sensitive to protein kinase A and, therefore, may not require Raf1 activation. This possibility differs from the previously proposed pathway of LPS-induced MAP kinase activation (Raf1→Mek1/2→ERK1/2) [38–40] and requires further study.

Our recent data also demonstrate that both sPGN and LPS strongly induce NF-κB, in contrast to PDB, which does not induce NF-κB in RAW264.7 cells (Gupta D, Dziarski R, unpublished data). All of our data, therefore, are consistent with the hypothesis that complex bacterial stimulants trigger multiple signal transduction pathways that are all needed for maximal induction of cytokine production [4]. This hypothesis is supported by very strong induction of cytokine production [4, 11] by both PGN and LPS (which activate ERK1, ERK2, JNK, p38, and NF-κB) and only very weak induction of cytokine production [4] by PDB (which strongly activates ERK1 and ERK2 but does not activate JNK, p38, and NF-κB).

In summary, our results demonstrate strong activation of ERK1 and ERK2, moderate activation of JNK, and weak activation of p38 by sPGN. Our data also indicate differential activation of ERK, JNK, and p38 MAP kinases by LPS, PGN, and PDB and different mechanisms of ERK activation by bacterial stimulants (PGN and LPS) than by phorbol esters and growth factors.

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References