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Role of Protein Tyrosine Kinase p53/56^{lyn} in Diminished Lipopolysaccharide Priming of Formylmethionylleucylphenylalanine-Induced Superoxide Production in Human Newborn Neutrophils

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Human newborns are more susceptible than adults to bacterial infection. With gram-negative bacteria, this may be due to a diminished response of newborn leukocytes to lipopolysaccharide (LPS). Since protein tyrosine kinase inhibition abolishes LPS priming in adult cells, we hypothesized that protein tyrosine kinases may have a critical role in LPS priming of polymorphonuclear neutrophils (PMNs) and that newborn PMNs may have altered protein tyrosine kinase activities. In the present study, we investigated the role of *src* family protein tyrosine kinases in the LPS response of newborn PMNs compared to adult cells. In a respiratory assay, the LPS-primed increase in formylmethionylleucylphenylalanine (fMLP)-triggered O_2^- release by adult PMNs was greatly decreased by PP1 [4-amino-5-(4-methyphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine], a *src* kinase inhibitor, to the level of untreated newborn PMNs, in which LPS failed to prime. LPS activated the *src*-like kinases p59^{hck} (HCK) and p58^{fgr} (FGR) in both adult and newborn PMNs but increased the activation of p53/56^{lyn} (LYN) only in adult cells. In newborn PMNs, LYN was highly phosphorylated independent of LPS. We evaluated subcellular fractions of PMNs and found that the phosphorylated form of LYN was mainly in the Triton-extractable, cytosolic fraction in adult PMNs, while in newborn cells it was located mainly in Triton-insoluble, granule- and membrane-associated fractions. In contrast, the phosphorylated mitogen-activated protein kinases ERK1/2 and p38 were mainly detected in the cytosol in both adult and newborn PMNs. These data indicate a role for LYN in the regulation of LPS priming. The trapping of phosphorylated LYN in the membrane-granule fraction in newborn PMNs may contribute to the deficiency of newborn cells in responding to LPS stimulation.

Polymorphonuclear neutrophils (PMNs) are the first line of host defense against bacterial infection. Upon stimulation by bacterial products, PMNs migrate extravascularly and accumulate at sites of infection, where they phagocytose and kill invading microorganisms. Importantly, these PMN functions can be modulated by cytokines (from the host) and toxins such as the lipopolysaccharide (LPS) from gram-negative bacteria. For example, following exposure to LPS in vitro, PMNs are primed for increased production of oxidative radicals, which are important in the effective killing of engulfed microorganisms (1, 46). A diminished response to LPS will affect the host's response to bacterial infection and may be one of the mechanisms accounting for the increased susceptibility of human newborns to gram-negative bacterial infection (20, 44). As we reported earlier, PMNs from newborns are primed less effectively in vitro with LPS than PMNs from adults (7, 38).

Over the past decade, the mechanism of LPS interaction with the phagocytic cell membrane has become more clearly understood. For adult PMNs, monocytes, and macrophages, CD14 is the principal cell membrane receptor for the LPS/LPS-binding protein complex (41). Indeed, the presence of CD14 and the LPS-binding protein greatly enhances cellular activation with LPS (23, 25, 37, 42, 47, 49, 50, 59). In addition to CD14, a family of transmembrane receptors with homology to Toll proteins of *Drosophila melanogaster* are known to trigger inflammatory reactions, including secretion of proinflammatory cytokines (30, 40). Toll-like receptor-4 (TLR-4) imparts ligand-specific recognition of LPS by mammalian cells (18, 26). Through CD14/TLR-4 interactions, LPS induces several intracellular responses, including activation of the mitogen-activated protein kinase family, particularly extracellular-signal-regulated kinases (ERKs) and p38 (48), which may eventually increase O_2^- production in response to additional stimuli such as formylmethionylleucylphenylalanine (fMLP) (5, 52). However, the intracellular processes involved in signal transduction following priming by LPS are less well understood.

It is well established that protein tyrosine kinases play a central role in PMN signaling (3). Not only is PMN activation accompanied by tyrosine phosphorylation of several proteins, including paxillin, mitogen-activated protein kinases, p58^{fgr} (FGR), and PYK2 (14, 15), but tyrosine kinase inhibitors also block PMN production of O_2^- as well as the effect of LPS priming (4, 13–15, 24, 39, 45). In PMNs, the activation of the *src* family kinases FGR, p53/56^{lyn} (LYN), and p59^{hck} (HCK) are associated with PMN stimulation (3, 4, 54), and their inhibition with genistein or PP1 [4-amino-5-(4-methyphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine] attenuates a variety of

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PMN functions (4, 13–15, 24, 39, 45, 57). Moreover, double mutation of FGR and HCK (*fgr*$^{-/-}$ *hck*$^{-/-}$) in mice greatly attenuates the production of O_2^- from PMNs stimulated with fMLP or tumor necrosis factor (28). These mutant mice show high resistance to the lethal effect of LPS, probably due to diminished PMN function (27). Other protein tyrosine kinases, such as p72^{syk} (SYK), have also been associated with PMN signaling (31, 32, 51, 56). Therefore, the *src* and *syk* family protein tyrosine kinases appear to be essential mediators that transmit intracellular signals involved in PMN activation.

The immune system develops continuously in utero and after birth. For example, leukocyte activation in response to LPS by preterm infants is more severely impaired than that of term infants and adults (16, 21). Because newborn cells appear to have several deficiencies in receptor-associated signaling, we hypothesized that the signaling systems in newborn PMNs may not be fully matured. In the present study, we investigated the possible role for the *src* family kinases FGR, HCK, and LYN in the diminished response of newborn PMNs to LPS priming. Through a comparative study on the activity and subcellular distribution of LYN between adult and newborn PMNs, we found that confinement of active LYN to the cytoskeletal fraction in newborn cells may be one of the underlying causes of their hyporesponsiveness to LPS.

MATERIALS AND METHODS

Reagents. *Escherichia coli* LPS (serotype O111:B4) was obtained from List Biological Labs (Campbell, Calif.). ECL Western blotting reagents, nitrocellulose membranes, reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and [γ-^{32}P]ATP were from Amersham Pharmacia Biotech (Baie d'Urfé, Canada). Therapeutic-grade sterile water (no preservative, nonpyrogenic) was purchased from Abbott Laboratories (Montreal, Canada). Flat-bottomed multiwell plates for cell culture were purchased from Costar (Cambridge, Mass.). Dithiothreitol, HEPES buffer, sodium bicarbonate solution, and Hanks' balanced salt solution (HBSS) were obtained from Gibco-BRL. PP1 [4-amino-5-(4-methyphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine] was from Biomol (Plymouth Meeting, Pa.). Rabbit anti-phosphorylated LYN (catalog number 2731), anti-phosphorylated *src* family kinases (catalog number 2101), and anti-phospho-p38 antibodies (catalog number 9211) and a horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G antibody were obtained from Cell Signaling Technology (Beverly, Mass.). Rabbit anti-LYN protein, anti-HCK protein, anti-phosphorylated HCK (catalog number sc-12958), anti-elastase, antilactoferrin, anti-p38 protein, and anti-ERK2 protein antibodies and mouse anti-phosphorylated ERK1/2, anti-CD66b, and anti-CD14 monoclonal antibodies were purchased from Santa Cruz Biotech (Santa Cruz, Calif.). Rabbit anti-FGR protein and anti-p47^{PHOX} antisera were kindly provided by G. Berton (University of Verona, Verona, Italy) (4). Horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G antiserum, E-Toxate kits, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.). Buffers and plasma were checked for endotoxin by a *Limulus* amebocyte lysate assay to ensure that LPS was not detectable (limit of detection, $<$12 pg/ml).

PMN preparation. Fresh peripheral venous blood from healthy adult volunteers and umbilical cord blood from normal full-term newborns delivered by caesarean section were collected aseptically and anticoagulated with heparin (10 U/ml). PMNs were isolated with a Ficoll gradient technique followed by hypotonic lysis of contaminated red blood cells as described previously (5), with purity of $>$95% and viability of $>$98% in approximately 2 h. After isolation, PMNs were washed and resuspended in HBSS$^{+/+}$ (with calcium and magnesium) supplemented with 1% autologous plasma before use. In some experiments, PMNs were treated with 1 μM PP1 or its solvent (dimethyl sulfoxide, 0.1%) alone for 10 min at room temperature.

Adult and newborn neutrophil superoxide production. PMNs were resuspended at a density of 10^6 cells/ml in HBSS$^{+/+}$ supplemented with 1% autologous plasma and 80 μM cytochrome *c*, with or without 1 μM PP1. Cells treated with 5 μg of superoxide dismutase were used as controls. The PMNs were primed or not with 5 ng of LPS/ml for 20 min at 37°C and then stimulated or not with 1 μM fMLP for another 15 min. The reaction was stopped by adding *N*-ethylmaleimide to a final concentration of 10 μM. The O_2^- produced by the cells was calculated from the superoxide dismutase-inhibitable changes in absorbance of the culture medium, which was measured at a wavelength of 550 nm with a spectrophotometer (Amersham Pharmacia Biotech, Quebec, Canada) as described earlier (52).

Cell lysate preparation. PMNs, at a density of 10^7 cells/ml in HBSS$^{+/+}$ plus 1% autologous plasma, were incubated in the presence or absence of 5 ng of LPS/ml for 20 min at 37°C and washed once with ice-cold phosphate-buffered saline containing 1 mM diisopropyl fluorophosphate. The cells were then pelleted by centrifugation at 10,000 $\times$ *g* for 10 s, and cellular proteins were prepared in different ways (stored at $-$80°C before use). The protein concentrations of the samples were assessed with the Bradford method (Bio-Rad Laboratories Ltd., Ontario, Canada) and equalized by adjusting with appropriate lysis buffer.

(i) Whole-cell lysates. PMN pellets (6×10^6 cells) were resuspended in 100 μl of phosphate-buffered saline plus inhibitors (10 μg of aprotinin per ml, 5 μg of pepstatin A per ml, 5 μg of leupeptin per ml, 1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 0.1 mM Na_3VO_4). The cells were then lysed by adding 100 μl of 2$\times$ SDS-PAGE sample buffer (62.5 mM Tris, pH 6.8, 2% SDS, 100 mM 2-mercaptoethanol, heated to 100°C) and boiled for 10 min.

(ii) Radioimmunoprecipitation assay lysates. PMN pellets were lysed with radioimmunoprecipitation assay (RIPA) buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 25 mM Tris-HCl, pH 7.5, 150 mM NaCl) plus the inhibitors listed above for 30 min on ice. The lysates were vortexed for 5 s and centrifuged at 12,000 $\times$ *g* at 4°C for 10 min to remove insoluble materials.

(iii) Triton-soluble and -insoluble fractions. As reported earlier (55), PMN pellets were lysed with a cytoskeletal stabilization buffer (CSK) consisting of 20 mM piperazine-*N,N′*-bis(2-ethanesulfonic acid) (PIPES, pH 6.8), 4 M glycerol, 1 mM $MgCl_2$, and 0.2% Triton X-100 plus the inhibitors listed above for 4 min on ice, followed by centrifugation at 2,000 $\times$ *g* for 4 min. The supernatants were designated the Triton-soluble fraction. The pellets were washed once with CSK and resuspended in CSK as the Triton-insoluble fraction.

(iv) Subcellular fractionation. PMN pellets (4×10^7 to 5×10^7 cells) were resuspended in 0.4 ml of ice-cold relaxation buffer (80 mM PIPES, pH 7.3, 100 mM KCl, 3 mM NaCl, 7.5 mM $MgCl_2$, freshly supplemented with the inhibitors mentioned above) and disrupted by sonication for two bursts of 10 s on ice with a 30-s interval at 60% maximum power with a Sonic Dismembrator (Fisher Scientific, Ontario, Canada). Nuclei and unbroken cells were removed by centrifugation at 200 $\times$ *g* for 6 min at 4°C, and the resultant supernatants were centrifuged over discontinuous Percoll gradients (bottom, 86%; top, 32.4% Percoll in relaxation buffer) at 43,000 $\times$ *g* for 2 min on a TLX Ultracentrifuge (Beckman Instrument Inc., Ontario, Canada). The sonicated mixture was then separated into four major layers: cytosol, membrane, β granules, and α granules (from top to bottom). The cytosolic fraction was further cleared by centrifugation at 100,000 $\times$ *g* for 10 min to remove any sediment. The membrane and granule fractions were collected, washed once with relaxation buffer, and harvested by centrifugation at 100,000 $\times$ *g* for 10 min; the pellets were then resuspended in relaxation buffer. All fractions were brought to a final concentration of 1% with NP-40.

Western blot. Aliquots of each sample prepared as described above were analyzed by Western blotting following SDS-PAGE and electrotransfer, with antibodies specific for phosphorylated LYN, ERK1/2, or p38 or for other proteins as indicated. The binding of the antibodies was detected with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse immunoglobulin G and ECL Western blotting reagents, followed by exposure to Kodak X-ray film. To confirm equal protein loading, the membranes were stripped and reblotted with antibodies specific for LYN, ERK2, and p38 proteins on β-actin, as described earlier (5).

In vitro kinase assay. LYN, HCK, or FGR was immunoprecipitated from PMN RIPA lysates (400 μl containing 100 μg of protein) with rabbit antibodies (2 μg/sample) specific for LYN, HCK, or FGR immobilized on protein A-Sepharose 4B beads. After intensive washing, kinase activity in the precipitates was assayed by autophosphorylation in the presence of [γ-^{32}P]ATP and detected by autoradiography following SDS-PAGE as described previously (57). The scanning images of developed films obtained from autoradiography and Western blotting were analyzed with the software Quantity One (Bio-Rad Laboratories Ltd., Ontario, Canada).

Statistical analysis. Data are presented as the mean $\pm$ standard deviation. For comparison between groups, an unpaired analysis of variance was used, and a *P* value of $<$0.05 was considered significant.

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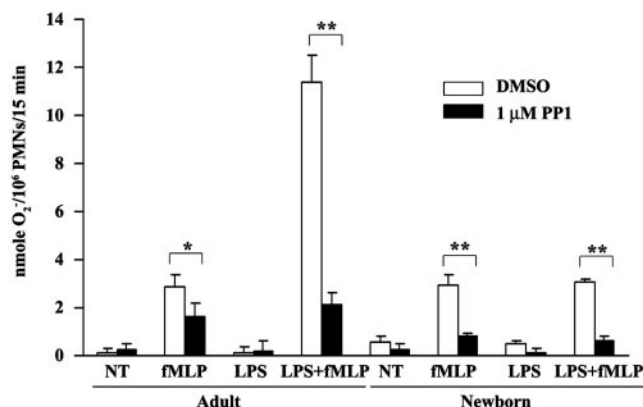

FIG. 1. LPS priming of fMLP-induced O_2^- release in adult PMNs is inhibited by PP1. PMNs from adults and newborns were resuspended at a density of 10^6 cells/ml in $HBSS^{+/+}$ supplemented with 1% autologous plasma and 80 μM cytochrome *c* and treated with 1 μM PP1 or solvent (dimethyl sulfoxide [DMSO], 0.1%) for 10 min at room temperature. The cells were then incubated or not with 5 ng of LPS/ml for 20 min at 37°C before treatment or not with 1 μM fMLP for 15 min. The O_2^- released was calculated from measurement of the superoxide dismutase-inhibitable changes in absorbance at 550 nm as described in the text and expressed as mean ± standard deviation ($n = 4$). *, $P < 0.05$; **, $P < 0.01$ control versus PP1. NT, not treated.

RESULTS

Production of O_2^- by adult and newborn PMNs is inhibited by PP1. Although human newborn neutrophils are able to secrete O_2^- in response to certain stimuli, unlike adult cells they cannot be primed by LPS to increase fMLP-induced O_2^- release. Since *src* family tyrosine kinases have an essential role in PMN function, we wondered if they contribute to the unresponsiveness of newborn cells to LPS. We examined the effects of PP1, a selective inhibitor of *src*-like kinases, on the respiratory burst in both adult and newborn cells. In agreement with previous observations (5, 57), fMLP by itself (1 μM) increased the respiratory burst to a similar extent in both adult and newborn PMNs (Fig. 1). LPS by itself (5 ng/ml) did not increase the release of O_2^- in either cell type. However, pretreatment with LPS dramatically increased fMLP-induced production of O_2^- in adult but not in newborn PMNs (Fig. 1). PP1 (1 μM) by itself did not affect PMN viability (as examined by trypan blue exclusion after 1 h of PP1 treatment; data not shown) or O_2^- release in control or LPS-stimulated PMNs from either adults or newborns. In adult PMNs, PP1 lowered fMLP-induced O_2^- production but had a much greater effect on LPS-primed fMLP-induced release, which was inhibited by approximately 80% ($P < 0.01$). Thus, adult PMNs treated with PP1 failed to respond to LPS priming. In this respect they are similar to newborn PMNs without PP1 treatment. Notably, the response of newborn PMNs to fMLP was more sensitive than that of adult cells to inhibition by PP1, which decreased the fMLP-induced production of O_2^- (with or without LPS) by approximately 75% ($P < 0.01$).

Activation of *src* family kinases in adult and newborn PMNs. The inhibition of PMN O_2^- production by PP1 suggests an important role for *src* family kinases in the LPS priming of PMNs. Next, we compared the response of *src* family kinases to LPS in adult and newborn PMNs. As shown in Fig. 2, two of the *src* family kinases (HCK and FGR) responded to LPS similarly in adult and newborn PMNs. As it has been observed previously that LYN is more sensitive to PP1 than HCK and FGR (57), the O_2^- data described above suggest a role for LYN in regulation of PMN priming and O_2^- secretion. We therefore compared the levels of LYN protein and its activation in adult and newborn PMNs with and without LPS stimulation.

Although the amount of LYN protein was similar in adult and newborn PMNs, as shown in Fig. 3, activated LYN was markedly lower in adult than newborn PMNs without LPS treatment. However, following LPS stimulation, adult PMNs showed increased activation of LYN compared to newborn cells, in which no obvious response was noted. The activation of LYN was assessed with an in vitro kinase assay, which monitors LYN activity directly (54), and with Western blotting with phosphorylation-specific antibodies, including catalog number 2731 (Cell Signaling Technology, anti-phosphorylated LYN), catalog number 2101 (Cell Signaling Technology, anti-phosphorylated *src* family kinases, which recognizes phosphorylated LYN and HCK), and catalog number SC-12958 (Santa Cruz Biotechnology, anti-phosphorylated HCK, which also recognizes phosphorylated LYN and Lck). The results obtained with these antibodies were comparable (data not shown). As shown in Fig. 3A (obtained with antibody 2731), Western blotting results correlated very well with those obtained by the in vitro kinase assay (Fig. 3B).

Differential Triton solubility of phosphorylated LYN in adult and newborn PMNs. It has been proposed that the distribution of LYN between Triton-soluble (equivalent to cytosolic and most membrane proteins) and Triton-insoluble (mainly cytoskeletal and some lipid raft-located proteins) fractions

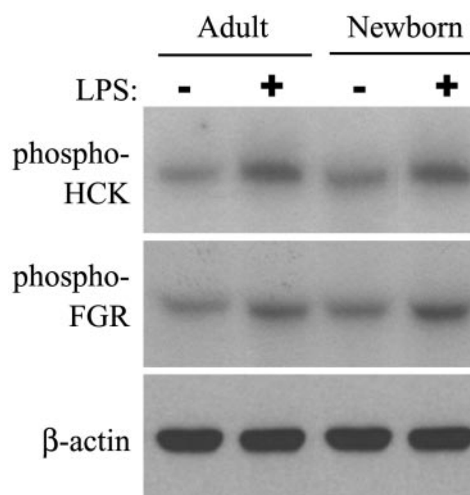

FIG. 2. Activation of HCK and FGR in adult and newborn PMNs. Adult and newborn PMNs were resuspended in $HBSS^{+/+}$ supplemented with 1% autologous plasma and incubated or not with LPS (5 ng/ml) for 20 min. HCK or FGR was immunoprecipitated from radioimmunoprecipitation assay lysates and assayed for kinase activity (autophosphorylation) with [γ-^{32}P]ATP, which was visualized by autoradiography following SDS-PAGE (upper two panels). Forty microliters (= 10 μg protein) of the supernatant of lysates after precipitation was analyzed by Western blotting for β-actin (lower panel). The results shown represent three independent experiments.

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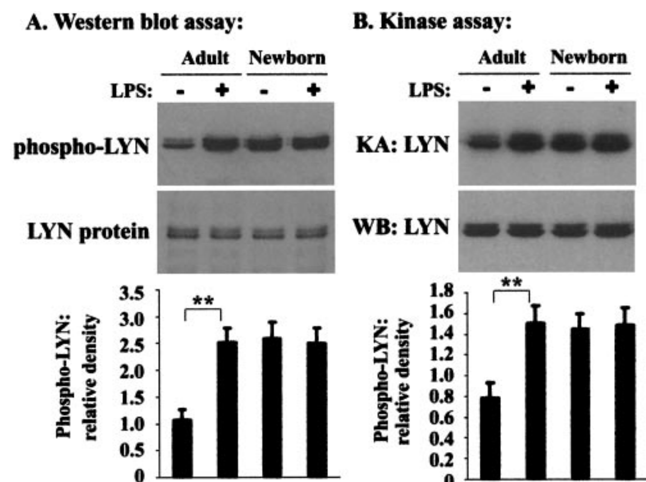

FIG. 3. Activation of LYN in adult and newborn PMNs. Adult and newborn PMNs were resuspended in HBSS$^{+/+}$ supplemented with 1% autologous plasma and incubated with or without LPS (5 ng/ml) for 20 min. A. Total cellular protein was prepared and analyzed by Western blotting for phosphorylated LYN with a phosphorylation-specific antibody (upper panel). The membrane was then stripped and reblotted for LYN protein (middle panel). B. LYN was immunoprecipitated from radioimmunoprecipitation assay lysates and assayed for kinase activity (KA) (autophosphorylation) with [γ-^{32}P]ATP, which was visualized by autoradiography following SDS-PAGE (upper panel). The radioimmunoprecipitation assay lysates from the same donors were also analyzed by Western blotting (WB) for LYN protein (middle panel). The bottom panels of A and B graph the relative densities of phospho-LYN (dividing the density of the phospho-LYN band by that of the corresponding LYN protein band) pooled from four independent experiments. **, $P < 0.01$.

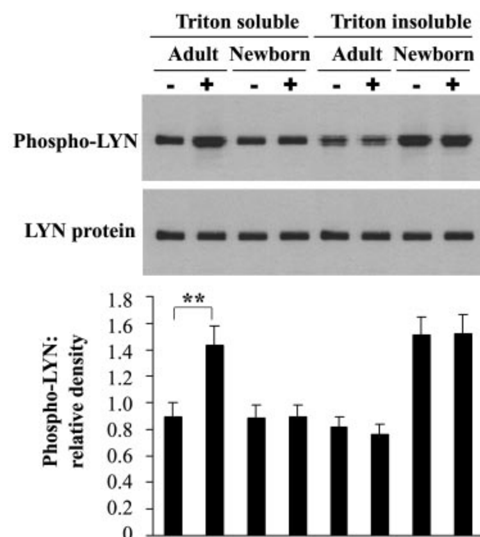

FIG. 4. Triton solubility of LYN in adult and newborn PMNs. Adult and newborn PMNs were resuspended in HBSS$^{+/+}$ supplemented with 1% autologous plasma and incubated with or without LPS (5 ng/ml) for 20 min. PMNs were fractionated into Triton-soluble and Triton-insoluble fractions with a Triton-containing buffer, CSK, as described in Materials and Methods. Both fractions (25 μg/sample) were analyzed by Western blotting for phosphorylated LYN (upper panel) and for total LYN protein (middle panel). The bottom panel graphs the relative densities of phospho-LYN (dividing the density of the phospho-LYN band by that of the corresponding LYN protein band) pooled from three independent experiments. **, $P < 0.01$.

may reflect its functional status (55). LYN protein was equally distributed between Triton-soluble and -insoluble fractions in both adult and newborn PMNs, independent of LPS treatment (Fig. 4). However, phosphorylated LYN was distributed differently in adult and newborn cells. In adult PMNs, phosphorylated LYN was found predominantly in the Triton-soluble fraction and increased significantly following LPS stimulation ($P < 0.05$). In newborn cells, phosphorylated LYN was found predominantly in the Triton-insoluble fraction, and no effect of LPS was observed (Fig. 4).

Subcellular distribution of LYN in adult and newborn PMNs. In order to examine the subcellular distribution of phosphorylated LYN and LYN protein, we prepared subcellular fractions from adult and newborn PMNs. Western blot analysis for elastase (a marker for the primary granule), lactoferrin (a marker for the secondary granule), CD66b (presented in secondary granule and membrane), CD14 (predominantly a membrane protein), and p47^{PHOX} (a cytosolic protein that translocates to the membrane upon activation) (6) demonstrated that α layer granules constituted mainly primary granules and β layer mainly secondary granules and that the membrane and cytosolic fractions were clearly distinct (Fig. 5). Western blotting with equal amounts of proteins loaded in each lane revealed that LYN protein was enriched in membrane fractions in both adult and newborn PMNs. Significant amounts of LYN were also noted in the cytosolic fraction and to a lesser extent in β granules in newborn cells (Fig. 6A, upper panel). In contrast, phosphorylated LYN was enriched primarily in the cytosol in both adult and newborn PMNs, independent of LPS treatment (Fig. 6B, upper panel).

In order to evaluate the actual subcellular distribution of total and phosphorylated LYN, i.e., on a per-cell basis, the above results were recalculated according to the proportion of total cellular proteins in each fraction (Fig. 6, lower panels). The total distribution of proteins in PMNs in α-granule (pri-

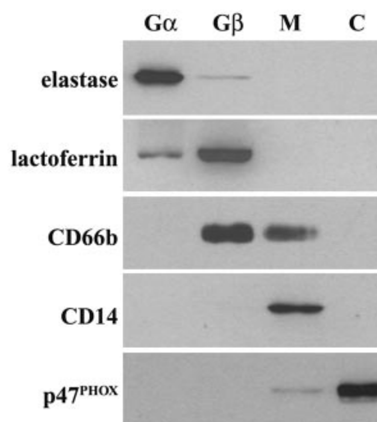

FIG. 5. Distribution of protein markers among different fractions prepared from adult PMNs. Adult PMNs (50×10^6 cells) were disrupted by sonication and then fractionated by centrifugation over discontinuous Percoll gradients as described in the text. Equal amounts of proteins (10 μg) from each fraction (Gα, α granules; Gβ, β granules; M, membrane; C, cytosol) were analyzed by Western blotting for elastase, lactoferrin, CD66b, CD14, and p47^{PHOX}. A representative result of three independent experiments is shown.

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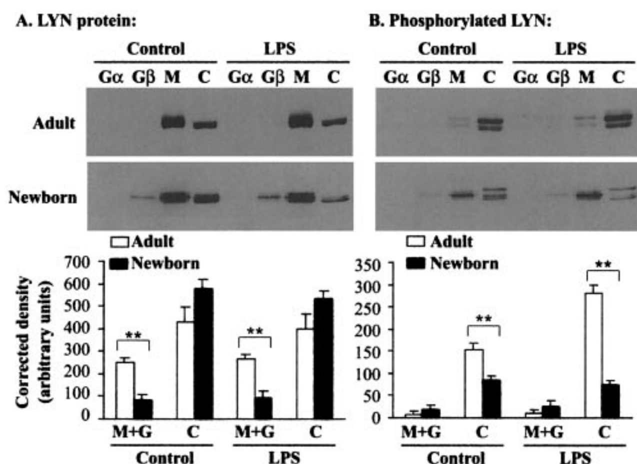

FIG. 6. Subcellular distribution of LYN in adult and newborn PMNs. Adult and newborn PMNs (50×10^6 cells for each) were resuspended in $HBSS^{+/+}$ supplemented with 1% autologous plasma and incubated with or without LPS (5 ng/ml) for 20 min. PMNs were disrupted by sonication and then fractionated by centrifugation over discontinuous Percoll gradients as described in the text. Upper panels: equal amounts of proteins (10 μg) from each fraction (Gα, α granules; Gβ, β granules; M, membrane; C, cytosol) were analyzed by Western blotting for LYN protein (A) and phosphorylated LYN (B) as described in the legend to Fig. 2. Lower panels: the corrected amounts of LYN protein and phosphorylated LYN in the cytosolic (C) and combined membrane and granule (M+G) fractions were calculated by multiplying the band densities (above) by the proportion of total protein in the corresponding fractions (see text). Results are expressed in arbitrary units (mean ± standard deviation for four independent experiments). **, $P < 0.01$.

mary), β-granule (secondary), membranous and cytosolic fractions were 17 ± 2%, 14 ± 1%, 16 ± 2%, and 53 ± 2%, respectively, for adult PMNs and 8 ± 1%, 10 ± 1%, 9 ± 1% and 73 ± 2%, respectively, for newborn PMNs. On this basis, the majority of LYN protein was cytosolic in both adult and newborn PMNs, although significantly more LYN protein was in the combined membrane-granule fractions of adult versus newborn cells (40% versus 15%; Fig. 6, lower panel). LPS treatment did not cause a translocation of LYN protein. As noted above, phosphorylated LYN was found predominantly in the cytosolic fraction in both cell types (Fig. 6, lower panel). In this case, a higher proportion of phosphorylated LYN was in the membrane-granule fractions of newborn compared to adult PMNs (25% versus 7% of total phosphorylated LYN). Consistent with Triton X-100 separation (Fig. 4), LPS induced LYN phosphorylation only in adult PMNs.

Subcellular distribution of mitogen-activated protein kinases in adult and newborn PMNs. Since the mitogen-activated protein kinases ERK1/2 and p38 may also have a role in regulation of PMN response to LPS priming (5, 52), the subcellular distribution of these kinases was examined for comparison. In adult PMNs, the subcellular distribution of ERK2 and p38 proteins was similar, mainly in the cytosol with small amounts associated with membranes (Fig. 7). In newborn PMNs, the subcellular location of these two mitogen-activated protein kinase proteins differed from that of adult cells, with somewhat greater amounts in granules and membranes. Following LPS stimulation, the phosphorylated forms of these mitogen-activated protein kinases were almost exclusively cytosolic in both adult and newborn PMNs. Phosphorylated p38 was higher in cytosolic and membrane fractions in adult than in newborn PMNs.

DISCUSSION

The increased susceptibility of human newborn infants to bacterial and viral infections is due to a variety of humoral or cellular factors. One possible factor for their susceptibility is that newborn PMNs function less effectively than adult cells during infection. This possibility is supported by in vitro experiments showing that newborn PMNs exhibit decreased LPS priming of the respiratory burst, i.e., radicals such as O_2^- in pretreatment with LPS (5, 7). We have previously shown that impaired LPS signaling responses, including decreased MyD88 expression and mitogen-activated protein kinase (p38) activation, may contribute to attenuated LPS priming in newborn PMNs (58). In the present study, we focused on the integrity of *src* family tyrosine kinase signaling in newborn and adult PMNs and demonstrated that LPS activation of LYN, one of the most common nonreceptor protein tyrosine kinases in hematopoietic cells (3), is significantly different between these cell types.

The pivotal role of *src* family tyrosine kinases in the regulation of PMN function has been demonstrated with gene disruption approaches (12, 27, 28) and selective kinase inhibitors such as PP1 (57). In our study, PP1 partially inhibited the respiratory burst triggered by fMLP in both adult and newborn PMNs. This is consistent with a requirement for *src* family protein tyrosine kinases for maximum response to fMLP (34). However, the LPS priming of O_2^- which occurs in adult but not in newborn PMNs was completely blocked by PP1. Adult and newborn cells exhibited no difference in expression of the *src*-like kinases (HCK, FGR, and LYN) known to be present in human PMNs. Of these, two (HCK and FGR) were activated

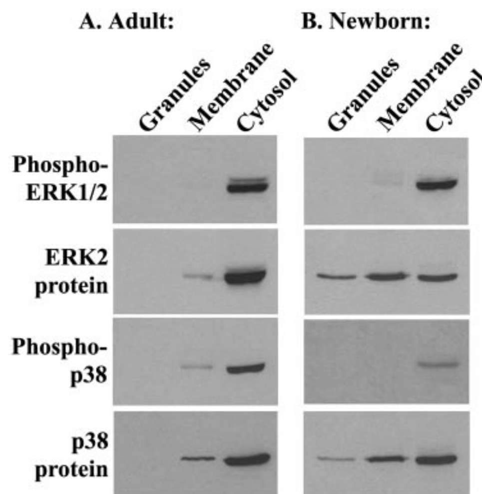

FIG. 7. Subcellular distribution of mitogen-activated protein kinases in adult and newborn PMNs. Subcellular fractions were prepared from LPS-stimulated PMNs of adults and newborns as described in the legend to Fig. 5. Proteins from each fraction (10 μg/sample) were analyzed by Western blotting for phosphorylated ERK1/2 or p38 and, as described in the legend to Fig. 2, the membranes were stripped and reblotted for ERK2 or p38 proteins. A representative result of three independent experiments is shown.

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by LPS to a similar extent in both adult and newborn PMNs. We therefore hypothesized that LYN, which is also more sensitive to PP1 inhibition (57), may be an important mediator of LPS priming and could contribute to the attenuated response of newborn PMNs. Indeed, LYN was activated in adult but not newborn PMNs stimulated with LPS, as assessed by both autophosphorylation and kinase activity. Nevertheless, LYN exhibited elevated basal activity and altered localization in newborn compared to adult cells, suggesting that the precise role of this kinase in PMN signaling (and the nature of its impairment in newborns) might not be straightforward.

CD14 and TLR-4 are major receptors for LPS on leukocytes (23, 25, 37, 42, 47, 49, 50, 59). After engagement of these receptors, a signaling complex forms on the cytoplasmic side of the cell membrane with recruitment of several key proteins, including MyD88 and IRAK. LPS binding with CD14 has been associated with activation of *src* family kinases in human PMNs (2, 52). Moreover, since CD14 signaling is altered in animals with inactivated genes for *src* family kinases (12, 27), these kinases appear to be required for the regulation of LPS responses. However, although much recent progress has been made in elucidating the downstream pathways leading to activation of NF-κB and mitogen-activated protein kinases, how this complex interacts with *src* family kinases or what specific role protein tyrosine kinases play in LPS signaling is less clearly understood. CD14 does not have a cytoplasmic domain and is believed to utilize TLR-4 to transmit its signals intracellularly. Although TLR-4 has been found associated with JNK, a mitogen-activated protein kinase (29), no direct interaction between TLR-4 and protein tyrosine kinases has been demonstrated by coimmunoprecipitation. Thus, integration of LPS and protein tyrosine kinase signaling may involve targeted subcellular localization of signaling complexes.

One of the major differences that we observed between adult and newborn PMNs is the apparent constitutive localization of phosphorylated LYN to a Triton X-100-insoluble fraction in the latter. Resistance to extraction with nonionic detergents such as Triton X-100 is traditionally a hallmark of both the cortical actin cytoskeleton and lipid rafts. *src* family protein tyrosine kinases (including LYN) are known to become associated with cytoskeletal elements upon activation by integrin engagement (53, 56). On the other hand, LYN is also a dually acylated cytoplasmic kinase which can be targeted to and activated in PMN lipid rafts, leading to enhanced superoxide generation (19). This would provide a basis for colocalization with the glycosylphosphatidylinositol-anchored CD14 receptor.

Interestingly, a recent proteomic analysis indicated that LYN is loosely associated with a cholesterol-rich but relatively dense subdomain of the PMN plasma membrane (33). This fraction exhibits features of both cytoskeleton and rafts and is enriched in actin and actin-binding proteins (e.g., fodrin, myosin, and actinin) as well as known raft components (flotillin and Gα_i). In the present study, phosphorylated LYN induced by LPS in adult cells was located exclusively in the Triton X-100-soluble fraction and was (along with phosphorylated ERK1/2 and p38) enriched in the cytosolic compartment of cells fractionated with Percoll gradients. Despite significant differences in LPS response and distribution of phosphorylated LYN between adult and newborn PMNs, we did not observe significant translocation of phosphorylated LYN or LYN protein between cellular fractions in either cell type upon LPS treatment.

The underlying cause of elevated basal phosphorylation of LYN in newborn PMNs remains to be established. Membrane-bound phosphorylated LYN can recruit other cytosolic proteins, including negative regulatory molecules such as the SH2 homologue containing phosphatase 1 (SHP-1) (8, 35, 43). SHP-1 can dephosphorylate protein tyrosine kinases and subsequently downregulate receptor signaling (36). Another regulatory protein that could influence LYN activity is CSK (C-terminal *src* kinase), which normally phosphorylates the C-terminal tyrosine residues of *src* family kinases and maintains protein tyrosine kinases in an inactive state (9, 17). Thus, increased phosphorylation of LYN in newborn PMNs might simply reflect phosphorylation of C-terminal tyrosine residue Tyr507 by CSK and thus a functionally inactive kinase. However, increased phosphorylation was correlated with increased kinase activity (Fig. 3). The interpretation is even more complicated in that activation of LYN can lead to autophosphorylation of both the activating site Tyr396 and the C-terminal Tyr507 residues, resulting in LYN hyperactivity and resistance to CSK downregulation (11). Although altered function of CSK or SHP-1 in newborn PMNs cannot be ruled out, it should be noted that the normal responses of HCK and FGR to LPS in these cells argues against a general defect in *src* family kinase regulation.

Our data are consistent with a graduated role for LYN in LPS priming and PMN function. According to this model, initial activation of LYN by LPS in adult PMNs would occur primarily in a cytosolic, Triton-soluble compartment and without major subcellular redistribution (at least, not apparent with our methodology). Based on PP1 sensitivity, this activation appears to be essential for LPS-primed, fMLP-induced O_2^- production and is perhaps involved in maintaining optimal cytoskeletal flexibility for coordination of LPS and integrin signaling events during the respiratory burst. In contrast, pre- or hyperactivation of LYN in newborn PMNs might lead to LPS-independent translocation and trapping of this kinase in a Triton X-100-insoluble membrane-granule compartment, where it may no longer be able to positively influence cytoskeletal dynamics essential for priming. Indeed, it has been observed that LPS priming of PMNs is enhanced by inhibition of action polymerization (10, 55) and that LYN moves from heavy to light (i.e., cytosolic) cellular fractions in human PMNs upon tumor necrosis factor alpha priming (22). Therefore, for PMN priming by LPS, it may be more important to maintain the mobility of certain signaling mediators in the cytoplasm rather than to integrate these molecules into a rigid cytoskeleton.

In summary, we observed that the *src* kinase inhibitor PP1 preferentially inhibited LPS-primed, fMLP-induced O_2^- production in adult PMNs. In these cells, the active form of LYN, either basal or activated by LPS, was mainly located in a cytosolic (Triton-soluble) fraction. In contrast, LYN in newborn PMNs was unresponsive to LPS stimulation, although it showed higher basal phosphorylation than in adult cells. More importantly, the phosphorylated form of LYN in newborn PMNs was largely confined to a Triton-insoluble fraction. Therefore, phosphorylated LYN in newborn PMNs appears to be trapped in cytoskeleton-like structures (including membrane and granules). Delineation of the mechanisms involved in LPS unre-

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sponsiveness in newborn PMNs may lead to a better understanding of the susceptibility of newborn infants to gram-negative bacterial infection and insight into the normal process in adults.

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