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Lipoxin A₄ Redistributes Myosin IIA and Cdc42 in Macrophages: Implications for Phagocytosis of Apoptotic Leukocytes

Keira Reville,* John K. Crean,* Sharon Vivers,† Ian Dransfield,† and Catherine Godson²*

Lipoxins (LXs) are endogenously produced anti-inflammatory agents that modulate leukocyte trafficking and stimulate nonphlogistic macrophage phagocytosis of apoptotic neutrophils, thereby promoting the resolution of inflammation. Previous data suggest a role for altered protein phosphorylation and cytoskeletal rearrangement in LX-stimulated phagocytosis but the exact mechanisms remain unclear. In this study we examine the effects of LXA₄ on the protein phosphorylation pattern of THP-1 cells differentiated into a macrophage-like phenotype. THP-1 cells stimulated with LXA₄ (1 nM) exhibit dephosphorylation of a 220-kDa protein. Using mass spectrometry, this protein was identified as MYH9, a nonmuscle myosin H chain II isoform A, which is involved in cytoskeleton rearrangement. THP-1 cells treated with LXA₄ adopt a polarized morphology with activated Cdc42 localized toward the leading edge and MYH9 localized at the cell posterior. Polarized distribution of Cdc42 is associated with Akt/PKB-mediated Cdc42 activation. Interestingly, the annexin-derived peptide Ac₂–₂₆ε recently described agonist for the LXA₄ receptor, also stimulates macrophage phagocytosis, MYH9 dephosphorylation, and MYH9 redistribution. In addition, we demonstrate that LXA₄ stimulates the phosphorylation of key polarity organization molecules: Akt, protein kinase Cζ and glycogen synthase kinase-3β. Inhibition of LXA₄-induced Akt and protein kinase Cζ activity with specific inhibitors prevented LXA₄-stimulated phagocytosis of both apoptotic polymorphonuclear neutrophils and lymphocytes, highlighting a potential use for LXA₄ in the treatment of autoimmune diseases. Furthermore, phosphorylation and subsequent inactivation of glycogen synthase kinase-3β resulted in an increase in phagocytosis similar to that of LXA₄. These data highlight an integrated mechanism whereby LXA₄ regulates phagocytosis through facilitative actin cytoskeleton rearrangement and cell polarization.

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3 Abbreviations used in this paper: LX, lipoxin; PMN, polymorphonuclear neutrophil; PKC, protein kinase C; GSK, glycogen synthase kinase; BDM, 2,3-butanedione monoxime; PVDF, polyvinylidene difluoride; NMMHC, nonmuscle myosin H chain.
chemoattractant FMLP exhibited intense phosphorylation of a 55-kDa protein that was blocked by ATLa (aspirin-triggered lipoxin) treatment (10–50 nM) (13). This 55-kDa protein was identified as leukocyte-specific protein 1, a downstream component of the p38 MAPK cascade in neutrophils.

Remodeling of the actin cytoskeleton is a prerequisite for all phagocytic processes (14). We have previously reported that LXA4 induces changes in ultrastructure and rearrangement of actin in monocytes and macrophages (15). These observations support the hypothesis that modulation of protein phosphorylation and of cytoskeletal reorganization play an important role in LXA4-mediated phagocytosis of apoptotic cells.

In this study we have investigated the effects of LXA4 on the macrophage phosphoproteome. We describe the identification of MYH9, a nonmuscle myosin H chain (NMMHC) class IIA protein involved in phagocytosis of apoptotic leukocytes. We have previously reported that LXA4-stimulated phagocytosis of apoptotic leukocytes is associated with decreased phosphorylation of MYH9, MYH9 redistribution, and cellular polarization coupled to Cdc42 activation and phosphorylation of the key signaling proteins Akt, protein kinase C (PKC)ζ, and glycogen synthase kinase (GSK)-3β.

Materials and Methods

Materials

All cell culture materials were from Invitrogen Life Technologies and other reagents were from Sigma-Aldrich unless otherwise stated. LXA4, (5S,6S,8R,15R)-15-(S)-tri-hydroxy-7,9,13-trans-11-cis-eicosatetraenoic acid) was purchased from BIOMOL. The annexin I mimetic peptide Ac2-20 (Ac-AMVSE FLKQAWFIEQEEYQTVK) was prepared by the Advance Biotechnology Centre (Charing Cross and Westminster Medical School, London, U.K.) by using solid-phase stepwise synthesis. Anti-phospho-serine 16B4 Ab was purchased from Calbiochem. Anti-phospho-Akt (Ser473) Ab, anti-Akt Ab, anti-phospho-GSK-3β (Ser9) Ab, and anti-phospho-PKCζ (Thr410) Ab were obtained from Cell Signaling Technology. Anti-PKCζ Ab was purchased from Upstate Biotechnology. Anti-Cdc42 Ab was obtained from Santa Cruz Biotechnology and anti-β-actin Ab was purchased from Sigma-Aldrich. The specific Akt inhibitor 1L-6-RWRKL-OH were obtained from Calbiochem. The GSK-3β pseudosubstrate inhibitor Myr-SIYRRGAR WRRLK-OH were obtained from Calbiochem. The GSK-3β inhibitor SN50 was purchased from Tocris Cookson and the myosin II inhibitor 2,3-butanedione monoxime (BDM) was from Sigma-Aldrich. An activity assay kit for Cdc42 and Super Signal West Pico and Dura Chemiluminescent substrates were purchased from Pierce.

Reagents were dissolved in DMSO or ethanol and further diluted in medium (final concentration, <0.1%). Equivalent concentrations of DMSO or ethanol were used as vehicle controls. The effect of pharmacological treatments on cell viability was monitored by flow cytometry using propidium iodide.

Cell isolation and culture

Human PMN were isolated from peripheral venous blood drawn from healthy volunteers, after informed written consent in accordance with institutional ethical guidelines (Mater Misericordiae University Hospital, Dublin, Ireland). PMN were separated by density gradient centrifugation on Ficoll-Pague (Amersham Biosciences) and dextran sedimentation (16). Following dextran sedimentation and hypotonic lysis of RBCs, PMN were incubated at 2 × 10^6 cells/ml with 10 μM CMFDA (5-chloromethylfluorescein diacetate; Molecular Probes) at 37°C for 15 min. Spontaneous apoptosis of CMFDA-labeled PMN was achieved by culturing 4 × 10^6 PMN/ml of RPMI 1640 medium supplemented with 10% autologous serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C for 24 h.

The human leukemic T cell lymphoblast cell line (Jurkat) was obtained from the European Cell Collection of Cell Cultures (ECACC). Jurkat cells were maintained in suspension in RPMI 1640 medium supplemented with 10% FCS, 2 mM glutamine, and 1% penicillin/streptomycin. Apoptosis was induced in CMFDA-labeled Jurkat T cells (at a concentration of 4 × 10^6 cells/ml) by exposure to UV irradiation at 254 nm for 2 min. Irradiated cells were then cultured overnight in RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

The human myelomonocytic cell line THP-1 (ECACC) was maintained in suspension in RPMI 1640 supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS. THP-1 cells were differentiated into a macrophage-like phenotype by treatment with 10 nM PMA for 48 h at 37°C (15).

Immunoprecipitation and Western blotting

Differentiated THP-1 cells (2 × 10^6 cells/ml), were serum-starved for 24 h and exposed to various agents as indicated. Lysates were harvested in RIPA lysis buffer (20 mM Tris-HCl (pH 7.4), 50 mM NaCl, 5 mM ethylene diaminetetraacetic acid, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 5 mM NaF, 1 mM PMSF, 1 mM Na3VO4, 1 mM leupeptin, 0.3 mM aprotinin). The lysates were clarified by centrifugation at 14,000 rpm for 20 min and samples were normalized for total protein. For Western blot analysis, 60 μg of THP-1 protein extract was loaded onto each lane and separated by SDS-PAGE. Proteins were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Millipore), blocked with 5% (w/v) nonfat dried milk in TBST for 1 h at room temperature and probed overnight at 4°C with Abs raised against β-actin (1/20,000), myosin

FIGURE 1. LXA4 induces dephosphorylation of a 220-kDa protein. A, Differentiated THP-1 cells were treated with vehicle or LXA4 (1 nM) before lysis in RIPA buffer. Phosphoserine proteins were immunoprecipitated (IP) from 1 mg of whole cell protein lysate using mAb 16B4. Immunocomplexes were denatured in sample buffer, separated by 18% SDS-PAGE, transferred to PVDF membranes, and probed for phosphoserine (WB). Densitometry results are depicted graphically and represent the mean ± SEM (n = 3). Values given are expressed as fold induction relative to vehicle treated cells (+4 ± 0.005 relative to vehicle). B, Differentiated THP-1 cells were treated with vehicle or LXA4 (1 nM) before lysis in RIPA buffer. Phosphoserine proteins were immunoprecipitated from 1 mg of whole cell protein lysate using mAb 16B4. Immunocomplexes were denatured in sample buffer, separated by SDS-PAGE, and stained with colloidal Coomassie blue for subsequent analysis by mass spectrometry. The bands excised for MALDI-TOF mass spectrometry analysis are marked with an arrow. The location of molecular weight (MW) standards are as indicated.
FIGURE 2. Identification of p220/MYH9 by mass spectrometry. A. Peptides were analyzed by peptide mass fingerprinting and matched to sequences in human MYH9. The sequences of peptides are shown along with the total protein coverage of MYH9 (boldface). B. MALDI-TOF mass spectrometry of peptides obtained by in-gel trypsin digestion of phosphorylated protein is shown as spectrum of MYH9 220-kDa protein.
IA (1/1000), phosphoserine (1/500), total Akt (1/1000), phospho-Akt (1/500), phospho-GSK-3β (1/1000), total PKCζ (1/1000), and phospho-PKCζ (1/1000). Membranes were incubated with HRP-conjugated secondary Abs (1/2000) for 1 h at room temperature and proteins were visualized by chemiluminescence.

For experiments as shown in Fig. 1, THP-1 cells were seeded at a density of 3 x 10^6 cells/ml onto 150-mm plates (six plates per condition), differentiated with 10 nM PMA for 48 h, serum-deprived, and stimulated with LXA4 (1 nM, 15 min) or vehicle control (0.1% ethanol, 15 min). Phosphoserine proteins were immunopurified from 4.7 mg of precleared lysates with 17 μg of mouse anti-phosphoserine 16B4 Ab and rocked overnight at 4°C. The 150 μl of protein G-agarose beads were added to the protein-Ab mixture and samples were rocked for a further 2 h at 4°C. Precipitated immunocomplexes were washed three times in fresh lysis buffer, boiled in sample buffer (250 mM Tris-HCl (pH 6.8), 10% (w/v) SDS, 0.5% (w/v) bromophenol blue, 25% (v/v) glycerol, and 6% (v/v) 2-ME), and resolved by SDS-PAGE. The gels were either stained with colloidal Cooomassie blue (17) or transferred to PVDF membranes, and immunoblotted for phosphoserine.

For coimmunoprecipitation studies, THP-1 cells were differentiated, serum-starved, and stimulated with LXA4 (1 nM, 15 min) or vehicle control (0.1% ethanol, 15 min). Phosphoserine proteins were immunopurified from 1 mg of precleared lysate with 4 μg of mouse anti-phosphoserine 16B4 Ab and rocked overnight at 4°C. A total of 10 μl of protein G-agarose beads was added to the protein-Ab mixture and samples were rocked for a further 2 h at 4°C. Precipitated immunocomplexes were washed three times in fresh lysis buffer and boiled in sample buffer. Samples were subsequently resolved by SDS-PAGE, transferred onto PVDF membrane, and probed for myosin IIA or phosphoserine.

In-gel digestion

Gel bands were cut from colloidal Cooomassie blue-stained one-dimensional SDS-PAGE gels, destained, in-gel digested with trypsin, and the peptides extracted using previously described methods (18).

Protein identification by peptide mass fingerprinting

Before peptide mass fingerprinting, the volumes of peptide containing solutions were adjusted to 5 μl by addition of 0.1% trifluoroacetic acid in 50% AcN. One microliter of each sample was deposited on a 1 x 15 mm MALDI target plate and dried in a vacuum container. Equal volumes of matrix (10 mg/ml α-cyano-4-hydroxycinnamic acid in 50% AcN, 0.1% trifluoroacetic acid) were added to the previously loaded digest. Samples were dried using a vacuum container. Mass spectrometry measurements were conducted with a MALDI/TOF mass spectrometer Voyager super STR (Applied Biosystems equipped with a 337-nm nitrogen laser). The external calibration was performed on a tryptic digest of lysozyme C. The analyses were performed in the reflectron mode with an accelerating voltage of 20 kV, a delayed extraction parameter of 100–140 ns, and a low mass gate of 850 Da. Laser power was set slightly above threshold (10–15% higher than the threshold) for molecular ion production. Spectra were obtained by summation of 300 consecutive laser shots. Masses of the peaks were extracted from the spectra and used for protein identification using the MASCOT search engine (www.matrixscience.com) against SWISS-PROT and TrEMBL.

Cdc42 activity assay

Cdc42 activity was determined using an EZ-Detect Cdc42 activation kit. Briefly, THP-1 cells (2 x 10^6 cells/ml) were differentiated and stimulated as indicated. After treatment, cells were rinsed once with ice-cold PBS and scraped into 500 μl of lysis buffer. Samples were then vortexed and centrifuged at 16,000 x g for 15 min at 4°C and the supernatant transferred to a new tube. Active Cdc42 was then affinity purified with GST-Pak1-PBD (Pak1-p21-binding domain), separated by SDS-PAGE and measured by Western blotting using anti-Cdc42 Ab and compared with total Cdc42.

Immunochemistry

Differentiated THP-1 cells were grown at a concentration of 0.5 x 10^6 cells/ml on four-well Lab Tek permannox chamber slides (catalogue no. 177437; Nunc) and stimulated as indicated. Cells were washed with PBS and then fixed with 3.8% (w/v) paraformaldehyde in PBS for 20 min at room temperature. After rinsing in PBS, the cells were incubated with permeabilization buffer (0.1% (v/v) Triton X-100 in PBS) for 15 min at room temperature. Following incubation, fixed cells were washed twice with PBS. Cells were incubated in blocking buffer (3% (w/v) BSA in PBS) for 1 h at room temperature with gentle agitation. Slides were then incubated with primary Ab (1/100 dilution of a polyclonal rabbit anti-Cdc42 Ab or monoclonal mouse anti-myosin IIA Ab) in 1% (w/v) BSA/PBS overnight at 4°C. Slides were rinsed and incubated with secondary Ab (1/250 dilution of an anti-rabbit or anti-mouse FITC-conjugated Oregon green Ab) for 45 min at room temperature in dark conditions. Nuclei were counterstained with DAPI (4',6'-diamidino-2-phenylindole; 1 μg/ml, 30 s). Stained cells were visualized with a Leitz DM40 microscope and images captured using the Axiosem system and Axiovision 3.0.6 software (Carl Zeiss).

Phagocytosis of apoptotic leukocytes

THP-1 phagocytosis of apoptotic leukocytes was assessed using a flow cytometric assay as previously described (19). THP-1 cells (1 x 10^6 cells/ml) were differentiated to a macrophage-like phenotype by treatment with PMA (10 nM, 48 h) in 24-well plates (Costar). Differentiated THP-1 cells were exposed to experimental stimuli as indicated and incubated with CMFDA-labeled apoptotic cells (2 x 10^4 cells/ml) for 2 h at 37°C. After incubation of THP-1 cells with apoptotic cells, the medium was removed and wells were then incubated with 0.5% trypsin/0.2% EDTA for 5 min at 37°C to detach all THP-1 cells and remove noninternalized apoptotic cells. After 10 min incubation on ice, the proportion of THP-1 cells that exhibited increased fluorescence (corresponding to phagocytosis of fluorescent-labeled apoptotic cells) was determined by flow cytometry using an EPICS XL-MCL flow cytometer (Beckman Coulter) System II software for

![FIGURE 3](http://www.jimmunol.org/) Confirmation that p220 is MYH9 by Western blotting (WB) and immunoprecipitation (IP) experiments. A. Differentiated THP-1 cells treated with vehicle or LXA4 (1 nM). B. Freshly isolated PMN treated with vehicle or LXA4. C. Differentiated THP-1 cells treated with vehicle or Ac2–26 (100 nM) lysed in RIPA buffer. Phosphoserine proteins were immunoprecipitated from 1 mg of whole cell protein lysate using mAb 16B4. Immunocomplexes were denatured in sample buffer, separated by SDS-PAGE, transferred to PVDF membranes, and probed for MYH9 using a mAb against NMMHC IIA. Total cellular MYH9 was determined by immunoblotting for MYH9. Expression of β-actin was examined as a loading control.
EPICS XL-MCL v.3.0. Apoptotic target and THP-1 populations were identified by their distinct laser scatter properties. A minimum of 20,000 events within the THP-1 gate was acquired. The number of FL1-H-positive events in the THP-1 gate was divided by the total number of THP-1 cells to obtain the percentage of THP-1 cells that had internalized apoptotic cells.

For inhibitor studies, differentiated THP-1 cells were washed with RPMI 1640 before treatment with a specific Akt inhibitor, IL-6-hydroxymethyl-chiro-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate (10 μM, 30 min) (20), the GSK-3β inhibitor, SB-415286 (30 μM, 30 min) (21), a PKCδ pseudosubstrate inhibitor (10 μM, 60 min) (22), the myosin II inhibitor, BDM (10 μM, 30 min) (23), or appropriate vehicles before stimulation with LXA₄ (1 nM, 15 min, 37°C) or vehicle control (0.1% ethanol, 15 min, 37°C). The treated cells were washed with RPMI 1640 before incubation with CMFDA-labeled apoptotic cells as described earlier.

**FIGURE 4.** Effect of LXA₄ on MYH9 localization and function in phagocytosis of apoptotic leukocytes. Differentiated THP-1 cells were exposed to vehicle or LXA₄ (1 nM) (A) or vehicle or Ac₂-26 (100 nM) (B). Cells were fixed with paraformaldehyde and stained with a mAb against NMMHC IIA. Nuclei were counterstained with DAPI. Arrows indicate MYH9 localization. C. Differentiated THP-1 cells were treated with BDM (10 μM, 30 min) at 37°C before stimulation with vehicle or LXA₄ (1 nM). THP-1 cells were then incubated with apoptotic PMN or apoptotic Jurkat T cells for 2 h and phagocytosis was assayed by flow cytometry. Data represent mean ± SEM (n = 3) and are expressed as fold induction over basal (*, p < 0.005 vs vehicle; #, p < 0.005 vs LXA₄; and ##, p < 0.001 vs LXA₄).
Results

LXA₄ alters the protein phosphorylation pattern of THP-1 cells

To investigate the effect of LXA₄ on the macrophage phosphoproteome, differentiated THP-1 cells were stimulated with vehicle (0.1% (v/v) ethanol) or LXA₄ (1 nM) for 15 min at 37°C and serine-phosphorylated proteins were prefractionated by immunoprecipitation. This fraction was subsequently separated by SDS-PAGE on a 10% (v/v) gel. This method allowed us to obtain high concentrations of relatively low abundant proteins, membrane proteins, and proteins greater than 100 kDa (24). Following electrophoresis, gels were either transferred to PVDF membranes and immunoblotted with an anti-phosphoserine Ab to detect changes in protein phosphorylation (Fig. 1A) or stained with colloidal Coomassie blue for subsequent analysis by mass spectrometry (Fig. 1B). Both the colloidal Coomassie blue-stained gel and the phosphoserine immunoblot demonstrate a significant dephosphorylation of a protein ~220 kDa (p220) in size following stimulation with LXA₄.

Identification of p220 as MYH9

Colloidal Coomassie blue-stained polyacrylamide gel slices corresponding to the p220 serine-phosphorylated protein were excised (Fig. 1B; marked by black arrows) and subjected to in-gel digestion by trypsin. After digestion, a small portion of the supernatant was removed and analyzed by high-accuracy peptide mass fingerprinting using MALDI. The peptide masses obtained by MALDI analysis were used to search protein databases. Forty-eight tryptic peptides were identified by peptide mapping that matched sequences in human myosin, heavy polypeptide 9, nonmuscle type A, NMMHC IIA (MYH9), which amounted to a total protein coverage of 29% (Fig. 2A). A MALDI mass spectrum for the tryptic digest of p220 is presented in Fig. 2B. Both Western blotting and immunoprecipitation experiments were undertaken to confirm that p220 was MYH9 (Fig. 3). To determine whether treatment with LXA₄ dephosphorylates serine residues within MYH9, we prefractionated the phosphoserine proteins by immunoprecipitation before immunoblotting for MYH9 with a specific Ab. As shown in Fig. 3A, MYH9 undergoes significant serine dephosphorylation in response to stimulation with LXA₄ (1 nM). Total cellular levels of MYH9 remain unchanged after treatment with LXA₄ (Fig. 3A). In contrast, treatment of freshly isolated PMN with LXA₄ did not promote serine dephosphorylation of MYH9 (Fig. 3B). A peptide mimic of annexin 1, Ac2–26 also promotes significant serine dephosphorylation of MYH9 (Fig. 3C).

LXA₄ treatment induces cellular polarization and MYH9 rearrangement in differentiated THP-1 cells

Myosin IIA activity has been implicated as having a role in establishing cell polarity (25). Given this observation, we investigated whether treatment with LXA₄ and subsequent dephosphorylation of MYH9 altered myosin distribution within THP-1 cells. Differentiated THP-1 cells were exposed to LXA₄ (1 nM) or vehicle for 15 min at 37°C and stained for MYH9. Control THP-1 cells demonstrated a spherical shape and homogenous MYH9 staining (Fig. 4A). Stimulation with LXA₄ induced a shape change with cells adopting a polarized structure and MYH9 appearing to localize at the cell posterior (Fig. 4A). Microscopic analysis revealed that 30% of LXA₄-treated cells demonstrated changes in cell shape and MYH9 distribution. Stimulation of differentiated THP-1 cells with Ac2–26 induced a shape change and MYH9 distribution similar to that of LXA₄ (Fig. 4B). Microscopic investigation showed that 22% of Ac2–26-treated cells adopted a polarized shape with altered MYH9 distribution.

Myosin assembly is required for LXA₄-stimulated phagocytosis of apoptotic leukocytes

Myosin IIA activity has previously been demonstrated to play a role in particle internalization during both FcγR and complement receptor type 3-mediated phagocytosis (26). To determine whether myosin filament assembly is involved in LXA₄-induced phagocytosis of apoptotic cells, we made use of the chemical inhibitor BDM, which blocks the ATPase activity of myosin II (27). Differentiated THP-1 cells were treated with 10 μM BDM for 30 min before stimulation with vehicle or 1 nM LXA₄ for 15 min. THP-1 cells were then incubated with apoptotic CMFDA-labeled PMN or lymphocytes for 2 h at 37°C. As previously reported (9), LXA₄ stimulated an increase in phagocytosis of apoptotic PMN (Fig. 4C). Pretreatment of differentiated THP-1 cells with BDM reduced LXA₄-stimulated phagocytosis of apoptotic PMN (Fig. 4C). Similarly, LXA₄ stimulated an increase in phagocytosis of apoptotic lymphocytes and pretreatment of differentiated THP-1 cells with BDM reduced LXA₄-stimulated phagocytosis of apoptotic lymphocytes (Fig. 4C). Pretreatment of differentiated THP-1 cells with BDM did not affect cell viability (data not shown).

LXA₄ induces Akt-mediated activation of Cdc42, accompanied by localization of Cdc42 expression at the leading edge of THP-1 cells

Having previously observed that LXA₄ stimulates RhoA- and Rac-dependent cytoskeleton reorganization (15 and given data from other groups implicating Rho GTPases as important modulators of actin cytoskeleton function during phagocytosis (28, 29), we subsequently investigated the involvement of Cdc42 in LXA₄-induced actin rearrangement and polarization. Cdc42 has previously been

FIGURE 5. Effect of LXA₄ on Cdc42 localization and activation. A. Differentiated THP-1 cells were exposed to vehicle or LXA₄ (1 nM). Cells were fixed with paraformaldehyde and stained with a polyclonal Ab against Cdc42. Nuclei were counterstained with DAPI. Arrows indicate Cdc42 localization. B. Differentiated THP-1 cells were treated with a specific Akt inhibitor (10 μM, 30 min) at 37°C before stimulation with vehicle or LXA₄ (1 nM). Activity of the GTP-bound Cdc42 in THP-1 lysates was assessed by a pulldown assay using glutathione-Sepharose beads coupled to a GST-Pak1-PBD fusion protein. Bound and total Cdc42 proteins were detected by Western blotting with a polyclonal Cdc42 Ab.
FIGURE 6. Involvement of Akt, PKCζ, and GSK-3β in LXA₄-stimulated phagocytosis of apoptotic leukocytes. A. Differentiated THP-1 cells were treated with vehicle or LXA₄ (1 nM) for the indicated time period at 37°C before lysis in RIPA buffer. Phosphorylation of Akt was monitored by Western blotting with a polyclonal phospho-Akt Ab, and expression of total Akt was also examined by Western blotting with a polyclonal total Akt Ab. B, Differentiated THP-1 cells were treated with vehicle or the indicated concentration of LXA₄ for 15 min at 37°C. Phosphorylation of Akt was monitored by Western blotting with a polyclonal phospho-Akt Ab and expression of total Akt was also examined by Western blotting with a polyclonal total Akt Ab. C, Differentiated THP-1 cells were treated with a specific Akt inhibitor (AI, 10 μM for 30 min) at 37°C before stimulation with vehicle or LXA₄ (1 nM). THP-1 cells were then incubated with apoptotic PMN or apoptotic Jurkat T cells for 2 h and phagocytosis was assayed by flow cytometry. Data represent mean ± SEM (n = 3) and are expressed as fold induction over basal relative to vehicle-treated cells (*, p < 0.0005 vs vehicle; #, p < 0.005 vs LXA₄; **, p < 0.001 vs vehicle; ##, p < 0.001 vs LXA₄). D, Differentiated THP-1 cells were treated with vehicle or LXA₄ (1 nM) for (Figure legend continues)
shown to be active toward the front of polarized cells (30). We consequently examined Cdc42 distribution within LXA4-treated THP-1 cells. Differentiated THP-1 cells were exposed to LXA4 (1 nM) or vehicle for 15 min and stained for Cdc42. Control THP-1 cells exhibited uniform Cdc42 staining throughout the cytoplasm (Fig. 5A). Stimulation with LXA4 induced a polarized phenotype with Cdc42 accumulating at the leading edge of the cells (Fig. 5A). Microscopic analysis revealed that 27% of LXA4-treated cells demonstrated changes in cell shape and Cdc42 distribution.

To probe the role of Cdc42 in LXA4-induced actin cytoskeleton rearrangement and polarization, cells were treated with LXA4 and lysates assayed for active Cdc42 using a GST-Pak-PBD pulldown assay. LXA4 induced an increase in levels of active Cdc42 (Fig. 5B). The PI3K pathway, which is required for cell polarization, activates Rac and Cdc42 GTPases at the leading edge of migrating cells (31). To determine whether LXA4-stimulated activation of Cdc42 is mediated by Akt, we exposed differentiated THP-1 cells to a specific Akt inhibitor (10 nM) or vehicle for 15 min and stained for Cdc42. Control THP-1 cells (31). To determine whether LXA4-stimulated activation of Cdc42 is mediated by Akt, we exposed differentiated THP-1 cells to a specific Akt inhibitor (10 nM, 15 min). Pretreatment of differentiated THP-1 cells with the specific Akt inhibitor blocked LXA4-stimulated activation of Cdc42 (Fig. 5B).

This observation led us to further investigate the role of Akt in LXA4 signaling. Treatment of differentiated THP-1 cells with LXA4 (1 nM, 15 min) stimulated the rapid phosphorylation (within 2 min) of Akt (Fig. 6A). Furthermore, LXA4-triggered Akt phosphorylation is concentration-dependent (EC50 ~1 × 10−9 M; range 1 × 10−12 to 1 × 10−7 M) (Fig. 6B). To investigate the involvement of Akt phosphorylation in LXA4-stimulated phagocytosis, THP-1 cells were preincubated with a specific Akt inhibitor 30 min before treatment with LXA4 and incubation with either apoptotic CMFDA-labeled PMN or lymphocytes for 2 h at 37°C. LXA4-induced phagocytosis of both apoptotic PMN and apoptotic lymphocytes (Fig. 6C) was blocked by pretreatment with the inhibitor. Pretreatment of differentiated THP-1 cells with the Akt inhibitor did not affect cell viability (data not shown).

**LXA4 induces the phosphorylation of PKCζ and GSK-3β, regulating cell polarization and phagocytosis of apoptotic leukocytes**

To investigate the possibility that LXA4 may stimulate phagocytosis in part through cell polarization, we examined the phosphorylation status of components of the cell polarization regulatory pathway, namely PKCζ and GSK-3β. Addition of LXA4 to differentiated THP-1 cells caused the rapid and transient phosphorylation of PKCζ (Fig. 6D). Furthermore, LXA4-triggered PKCζ phosphorylation is concentration-dependent (EC50 ~1 × 10−9 M; range 1 × 10−12 to 1 × 10−7 M) (Fig. 6E). We then investigated whether the PKCζ pathway is involved in LXA4-stimulated phagocytosis of apoptotic leukocytes. THP-1 cells were preincubated with the specific myristoylated PKCζ pseudosubstrate peptide inhibitor (10 μM) for 1 h before treatment with LXA4 for 15 min and incubation with either apoptotic CMFDA-labeled PMN or lymphocytes for 2 h at 37°C. LXA4-induced phagocytosis of apoptotic PMN and apoptotic lymphocytes (Fig. 6F) was blocked by the addition of the inhibitor. Pretreatment of differentiated THP-1 cells with the pseudosubstrate inhibitor did not affect cell viability (data not shown).

Given the observations that LXA4 treatment induces THP-1 cell polarization and the rapid phosphorylation of Akt, we subsequently investigated another component of the polarization complex and known Akt substrate GSK-3β. Phosphorylated (activated) Akt phosphorylates (inactivates) GSK-3β. In this inactive state, phosphorylated GSK-3β promotes polarization (32). We demonstrate that GSK-3β is rapidly phosphorylated and consequently inactivated in response to treatment with LXA4 (Fig. 6G). The effect of phosphorylated GSK-3β on phagocytosis was investigated using the GSK-3β inhibitor SB-415286. SB-415286 phosphorylates, and therefore inactivates GSK-3β, resulting in an increase in phagocytosis of apoptotic PMN and apoptotic lymphocytes (Fig. 6H) mimicking that of LXA4. Pretreatment of differentiated THP-1 cells with SB-415286 did not affect cell viability (data not shown).

**Discussion**

In this study, we used a single-step affinity-based purification (phosphoserine immunoprecipitation) followed by one-dimensional electrophoresis and mass spectrometry to identify a protein involved in LXA4 signaling. In this study, we report that LXA4 (1 nM, 15 min) promotes dephosphorylation of MYH9 and alters phosphorylation/activation of components of a signaling cascade mediating cell polarization and phagocytosis. Therefore, MYH9 dephosphorylation may serve as a regulatory mechanism. The concentration of LXA4 that was effective against MYH9 was consistent with that shown to stimulate macrophage phagocytosis of apoptotic PMN (9, 10). Thus, we have identified a site of action for LXA4 in macrophages that may explain some of the physiological effects of this mediator.

MYH9 is a class IIA NMMHC (1961 amino acid polypeptide) with a molecular mass of 220 kDa. In humans, there are three known class II NMMHC (IIA, IIB, and IIC), encoded by distinct genes (MYH9, MYH10, and MYH14) (33). Most cell tissues contain both NMMHC IIA and NMMHC IIB (34), although some cell types are selectively enriched in either NMMHC IIA (platelets, lymphocytes, neutrophil granulocytes, and brush border cells) (35) or NMMHC IIB (neuronal tissue) (36).

Class II myosins have been proposed to have a crucial role in providing the motor activity for diverse functions such as cytokinesis, phagocytosis, and muscle contraction and polarity. Class II myosins exist as a hexameric complex composed of two myosin H chain subunits and two pairs of L chain subunits. The H chain
subunit contains the ATPase activity, providing energy that is the driving force for the contractile processes previously mentioned. To participate in contractility, nonmuscle myosin II must successfully assemble into functional bipolar filaments. First, individual H chains must dimerize by folding into an α-helical coiled-coil structure. The second assembly step involves lateral associations of H chain dimers to form functional bipolar filaments. In principle, defects in either step could result in the inability to form functional nonmuscle myosin IIA filaments (37).

Lower eukaryotes such as *Dictyostelium* and *Acanthamoeba* possess a single myosin II gene, and thus are suitable model organisms for genetic studies on the general cellular functions of myosin II (38, 39). *Dictyostelium* nonmuscle myosin II has been studied in detail and has a molecular structure very similar to that of mammalian myosin II. In lower eukaryotes, myosin II filament assembly, contraction, and disassembly are highly regulated by phosphorylation and are important in the establishment of cell polarity, the retraction of the posterior cell body and the regulation of pseudopod extension (40–42). Although the mechanisms of regulation of mammalian MYH9 phosphorylation are poorly understood, it has recently been proposed that Mts1, a member of the S100 family of Ca$^{2+}$-binding proteins, is involved in regulating filament assembly by binding to residues 1909–1924 of nonmuscle myosin IIA (43). It is possible that serine phosphorylation of MYH9 may regulate filament assembly by modulating the formation of the Mts1-MYH9 complex.

Treatment of THP-1 cells with LXA$_4$ resulted in MYH9 localization at the cell posterior (Fig. 4A). Such data are consistent with the localization of myosin II at the rear and sides of neutrophils (44). Similarly, MYH9 is enriched at the uropod in T cells, particularly at the head-tail junction (45).

Phagocytosis is a highly regulated process, which requires remodeling of the macrophage cytoskeleton for successful engulfment of the apoptotic target (14). The signals generated by the phagocyte-apoptotic cell interaction target the cytoskeleton (46), inducing changes that lead to engulfment, followed by a process of internalization (14). To our knowledge, this study is the first demonstration of a specific role for MYH9 in the regulation of phagocytosis of apoptotic cells by LX$_4$. Previously, a proteomics approach was adopted to identify the proteins associated with latex bead phagosomes (47). A number of actin binding proteins were identified, including NMMHC IIA. However, the phosphorylation status of MYH9 was not investigated in this study (47). These findings correlate with our hypothesis that MYH9 is critically involved in phagocytosis of apoptotic cells.

Myosins have previously been implicated in FcγR- and complement receptor-mediated phagocytosis, localizing on phagosomes (26, 48). Complement receptor-mediated phagocytosis specifically uses MYH9 to assist in the assembly of actin cups around particles (26). Our data demonstrate that, through specific inhibition of class II myosin, macrophages require MYH9 motor activity to successfully engage in LX$_4$-stimulated phagocytosis of apoptotic cells (Fig. 4B). We propose that LX$_4$-mediated dephosphorylation and localization of MYH9 induces a polarized phenotype, consequently priming the macrophage to rapidly respond to and ultimately phagocytose apoptotic cells.

It is noteworthy that pretreatment with a peptide mimetic of annexin 1, Ac$_2$-26 peptide induces severe dephosphorylation of MYH9 and redistribution of MYH9 within polarized cells. Recent data have demonstrated pretreatment with Ac$_2$-26 peptide promotes the nonphlogistic phagocytosis of apoptotic PMN acting through the pleiotropic agonist of the LX$_4$ receptor (11).

Cell polarity is important for phagocytosis. Establishment and maintenance of cell polarity requires the activation of separate molecular processes at the cell anterior and posterior (49). Primed mammalian and *Dictyostelium* cells have strongly biased axial polarity, with F-actin assembled primarily in the leading edge and myosin II in the rear cell body (50). The Rho GTPases, Rho, Rac, and Cdc42 have been shown to regulate actin assembly and rearrangement in response to external stimuli, hence providing the driving force for polarization, cell migration, and phagocytosis of particles (51). A role for the Rho GTPase Cdc42 in controlling cell polarization in diverse cell types including fibroblasts, macrophages, astrocytes, and mesangial cells is well established (52–54). Cdc42 has also been shown to regulate actin reorganization during phagocytosis by promoting pseudopod extension and phagosome closure (29, 55). Treatment of THP-1 cells with LX$_4$ resulted in Cdc42 localization at the cell anterior (Fig. 5A) and increased levels of active Cdc42 (Fig. 5B). These observations are consistent with previous studies demonstrating the localization of Cdc42 to the leading edge of polarized motile cells (53) and that Cdc42 localization to the leading edge is usually associated with activation (30).

There is growing evidence that a key step in mediating polarity is the activation of PI3K and the downstream effector Akt (56). Our results indicate that LX$_4$-induced activation of Cdc42 is mediated by the serine/threonine kinase Akt, which is a downstream target of PI3K (Fig. 5B). Activation of the PI3K pathway is also important in macrophage phagocytosis of apoptotic cells (29). We have previously observed that pretreatment of THP-1 cells with the PI3K inhibitor LY294002 inhibited LX$_4$-stimulated phagocytosis of apoptotic PMN (10). However, in LX$_4$-treated renal mesangial cells, inhibition of platelet-derived growth factor receptor phosphorylation is coupled to an attenuation of downstream PI3K activity and subsequent Akt phosphorylation. These differing observations may reflect the heterogeneity of PI3K isoform expression in cells of myeloid and nonmyeloid lineage (57) or the multiplicity of receptors. FPRL2, a receptor for peptide agonists, is expressed on monocytes/macrophages but not on PMN (58). LX and its analogs act via the agonists of the LX$_4$ receptor to inhibit PMN chemotaxis. In contrast, LXs have been reported to activate monocyte chemotaxis, and this effect is shared by agonists of the LX$_4$ receptor peptide ligands. LXs have been demonstrated to induce changes in the ultrastructure and F-actin reorganization in human monocytes and macrophages, but not in PMN (15). This contrasting data obtained from peptide and LX ligands in monocytes and macrophages, but not in PMN (15). This contrasting data obtained from peptide and LX ligands in monocytes and macrophages, but not in PMN (15). This contrasting data obtained from peptide and LX ligands in monocytes and macrophages, but not in PMN (15). This contrasting data obtained from peptide and LX ligands in monocytes and macrophages, but not in PMN (15). This contrasting data obtained from peptide and LX ligands in monocytes and macrophages, but not in PMN (15). This contrasting data obtained from peptide and LX ligands in monocytes and macrophages, but not in PMN (15). This contrasting data obtained from peptide and LX ligands in monocytes and macrophages, but not in PMN (15). This contrasting data obtained from peptide and LX ligands in monocytes and macrophages, but not in PMN (15). This contrasting data obtained from peptide and LX ligands in monocytes and macrophages, but not in PMN (15). This contrasting data obtained from peptide and LX ligands in monocytes and macrophages, but not in PMN (15). This contrasting data obtained from peptide and LX ligands in monocytes and macrophages, but not in PMN (15). This contrasting data obtained from peptide and LX ligands in monocytes and macrophages, but not in PMN (15). This contrasting data obtained from peptide and LX ligands in monocytes and macrophages, but not in PMN (15). This contrasting data obtained from peptide and LX ligands in monocytes and macrophages, but not in PMN (15). This contrasting data obtained from peptide and LX ligands in monocytes and macrophages, but not in PMN (15). This contrasting data obtained from peptide and LX ligands in monocytes and macrophages, but not in PMN (15). This contrasting data obtained from peptide and LX ligands in monocytes and macrophages, but not in PMN (15). This contrasting data obtained from peptide and LX ligands in monocytes and mac
this study we show that treatment of THP-1 cells with LXA₄ results in phosphorylation of PKCζ (Fig. 6C). This activated PKCζ appears to be involved in driving LXA₄-induced phagocytosis of apoptotic cells because exposure of THP-1 cells to the myristoylated peptide inhibitor Myr-SIYRRGARRWRKL-OH before apoptotic cells because exposure of THP-1 cells to the myristoylated peptide inhibitor Myr-SIYRRGARRWRKL-OH before LXA₄ stimulation was found to abrogate LXA₄-induced phagocytosis (Fig. 6D). We propose that PKCζ plays a role in regulating LXA₄-stimulated cytoskeletal rearrangements given previous observations that the Cdc42 target protein, PKCζ, is essential for defining cell polarity in migrating astrocytes (53) and mesangial cells (54).

Intriguingly, inhibitors directed against MYH9, Akt, and PKCζ failed to completely block phagocytosis of both apoptotic PMN and apoptotic Jurkat T cells, thus suggesting that activation of a pathway involving these signaling components is required for LXA₄-stimulated phagocytosis of apoptotic leukocytes. Previous data have also indicated that LXA₄-mediated augmentation of phagocytosis is both PI3K- and PKC-dependent (10). It is of interest to note that inhibitors directed against MYH9, Akt, and PKCζ did not affect phagocytosis of apoptotic cells by macrophages untreated with LXA₄. These data may suggest that these signaling proteins are not involved in normal clearance of apoptotic cells. However, in our experiments the level of phagocytosis observed basally is low (5%) so that it may not be possible to observe a further decrease in basal phagocytosis in response to inhibitors. Lack of detectable effect does not preclude a possible inhibition. We propose that LXA₄ specifically dephosphorylates MYH9 and activates Akt and PKCζ, causing the phagocyte to adopt a polarized phenotype, engaging the phagocyte for a rapid response to the presence of apoptotic cells and their subsequent engulfment.

Active Cdc42 further promotes polarization by recruiting and activating a cytoplasmic mPar6-PKCζ complex, which in turn interacts with and regulates GSK-3β activity (32). GSK-3β has also been implicated in the regulation of polarity governing connective tissue growth factor-mediated mesangial cell migration (54). Activated Akt specifically phosphorylates GSK-3β (an Akt substrate) on Ser⁹ thus inhibiting its catalytic activity (61). Following treatment with LXA₄, GSK-3β undergoes rapid phosphorylation at Ser⁹, and subsequent inactivation (Fig. 6E). Treatment with the GSK-3β inhibitor SB-415286 induces phosphorylation/inactivation of GSK-3β, resulting in an increase in phagocytosis mimicking that of LXA₄, thus highlighting the importance of GSK-3β in the establishment of LXA₄-induced polarity and phagocytosis (Fig. 6F).

Importantly, we demonstrate for the first time the ability of LXA₄ to stimulate macrophage phagocytosis of apoptotic lymphocytes. This observation is of significant interest in light of recent evidence that increased lymphocyte apoptosis and defects in macrophage removal of apoptotic cells have been proposed to contribute to the development of the autoimmune disease, systemic lupus erythematosus (62–64). Consequently, LXA₄-induced modulation of macrophage phagocytosis may be considered an attractive target for therapeutic intervention. A recent investigation into host defense against *Mycobacterium tuberculosis* further highlighted the involvement of LXA₄ in maintaining a balance between inflammation and resolution (65). Administration of the stable lipoxin analog ATLA2 to *M. tuberculosis*-infected 5-lipoxigenase knockout mice restored both pulmonary mycobacterial loads to levels comparable to those observed in infected wild-type animals, thereby negatively regulating protective Th1 responses.

In conclusion, this study has yielded the intriguing finding that MYH9 may be a regulatory site for LXA₄-induced effects on the macrophage cytoskeleton. THP-1 cells treated with LXA₄ adopt a polarized morphology with MYH9 and Cdc42 demonstrating a spatial distribution within the cell. In addition, our observations that LXA₄ stimulates the phosphorylation of key signaling molecules (Akt, PKCζ, and GSK-3β) concurrent with increased levels of activated Cdc42 demonstrates that THP-1 cell polarization is a key, early event in LXA₄-induced phagocytosis (Fig. 7). Collectively, these data provide an insight into the complex signal-transduction network controlling clearance of apoptotic cells and highlight the significant anti-inflammatory potential of LX in promoting the resolution of inflammation.

Disclosures

The authors have no financial conflict of interest.

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