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# Lipoxins Induce Actin Reorganization in Monocytes and Macrophages But Not in Neutrophils

## *Differential Involvement of Rho GTPases*

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**Lipoxins (LXs) are endogenously produced eicosanoids that inhibit neutrophil trafficking and stimulate nonphlogistic phagocytosis of apoptotic neutrophils by monocyte-derived macrophages. In this study we assessed the effect of LXs on cell ultrastructure and actin reorganization in human leukocytes and investigated the signaling events that subserve LX bioactivity in this context. $LXA_4$ ($10^{-9}$ mol/L), the stable synthetic analogues 15-(*R/S*)-methyl-$LXA_4$ and 16-phenoxy-$LXA_4$ ($10^{-11}$ mol/L), but not the LX precursor 15-(*S*)-HETE, induced marked changes in ultrastructure and rearrangement of actin in monocytes and macrophages. In contrast, $LXA_4$ did not modify actin distribution in neutrophils under basal conditions and after stimulation with leukotriene $B_4$. Blockade of Rho kinases by the inhibitor Y-27632 prevented $LXA_4$-triggered actin reorganization in macrophages. To investigate the role of the specific small GTPases in LX-induced actin rearrangement we used THP-1 cells differentiated to a macrophage-like phenotype. THP-1 cells stimulated with LXs, but not with 15-(*S*)-HETE, showed an increase in membrane-associated RhoA and Rac by immunoblotting. Additionally, a twofold increase in Rho activity was seen in response to $LXA_4$. LX-induced actin rearrangement and RhoA activation were inhibited by the cell permeable cAMP analogue 8-Br-cAMP, whereas Rp-cAMP, an inhibitor of protein kinase A, mimicked the effect of $LXA_4$. These data demonstrate that LXs stimulate RhoA- and Rac-dependent cytoskeleton reorganization, contributing to the potential role of LXs in the resolution of inflammation. *(Am J Pathol 2002, 160:2275–2283)***

Lipoxins (lipoxygenase interaction products, LXs) are trihydroxytetraene-containing eicosanoids generated via biosynthetic pathways catalyzed initially by the sequential actions of two lipoxygenases (LO), either 5-LO and 15-LO or 5-LO and 12- LO, on arachidonic acid.[1,2] In a cytokine-primed milieu, aspirin acetylation of cyclooxygenase type 2 switches the catalytic activity of the enzyme from a prostaglandin endoperoxide synthase to an *R*-LO that initiates the biosynthesis of the 15-epimer LXs (aspirin-triggered LXs, ATL) that share many of the bioactions of the nonepimeric forms.[3] The rapid metabolic degradation of the native LXs has prompted the development of stable analogues that retain the biological activity of the native compounds.[4]

Because of their effects on modulation of leukocyte trafficking, LXs have been proposed to act as braking signals in inflammation.[5] With respect to polymorphonuclear neutrophils (PMNs), LXs do not influence cell motility or adhesiveness under basal conditions, but are potent inhibitors of chemotaxis, adhesion to endothelium, and transmigration across endothelial cell and epithelial cell monolayers induced by leukotriene $B_4$ ($LTB_4$) and other chemoattractants *in vitro*.[6–9]

In contrast to their inhibitory effects on PMN function, LXs are potent activators of monocytes, stimulating their chemotaxis and adherence without causing degranulation or release of reactive species.[10,11] We recently reported that LXs also rapidly stimulate the nonphlogistic phagocytosis of apoptotic PMNs by monocyte-derived macrophages (M$\phi$), both *in vitro* and *in vivo* (Mitchell et al, manuscript submitted).[12] Such phagocytic clearance without the provocation of an inflammation response plays an essential role in the resolution of inflammation.[13] In addition to the clearance of apoptotic cells and their potential noxious contents, such phagocytosis is associ-

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ated with the production of quiescing intermediates such as transforming growth factor-$\beta$ that actively suppress cytokine release.[14] Thus LXs are not only braking signals for PMN-mediated inflammation[5] but also actively promote the resolution of inflammatory processes on monocytes and M$\phi$.

Multiple cell surface molecules have been implicated in the recognition of apoptotic cells by phagocytic M$\phi$.[13,15,16] We have demonstrated that LX-stimulated phagocytosis may modulate several of these including CD36 and CD51/61 integrins.[12] Remodeling of the actin cytoskeleton is a prerequisite for all phagocytic processes.[17] Such cytoskeletal rearrangement may reflect a response to the phagocytic target.

In this article we have examined the effects of LXs on actin cytoskeleton rearrangement in monocyte/M$\phi$ and PMNs. We report that the native $LXA_4$ and the stable analogues 15-(*R/S*)-methyl-$LXA_4$ and 16-phenoxy-$LXA_4$ induce changes in the actin cytoskeleton in monocytes and in M$\phi$, but not in PMNs and that these changes correlate to differential activation of the small GTPases RhoA and Rac.

## Materials and Methods

### Materials

5*S*,6*R*,15*S* - trihydroxy - 7,9,13 - *trans* - 11 - *cis* - eicosatetraenoic acid ($LXA_4$), $LTB_4$, and (5*Z*,8*Z*,11*Z*,13*E*,15(*S*))-15-hydroxyeicosatetraenoic acid (15-(*S*)-HETE) were purchased from Cascade Biochem Ltd. (Berkshire, UK). Synthetic analogues 15(*R/S*)-methyl-$LXA_4$ (5*S*,6*R*,15*R/S*-trihydroxy-15-methyl-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid methyl ester) and 16-phenoxy-$LXA_4$ (15*S*−16-phenoxy-17,18,19,20-tetranor-$LXA_4$ methyl ester) were prepared by total organic synthesis as previously described.[4] The anti-RhoA, anti-Rac2, and anti-Cdc42 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The compounds Rp-cAMP and LY294002 were purchased from Calbiochem, San Diego, CA; 8-bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP) was obtained from Sigma (St. Louis, MO). The cDNA for the GTPase-binding domain (RBD) from Rhotekin was a gift from Dr. Joan Heller Brown (Dept. of Pharmacology, University of California, San Diego, CA). Reagents were dissolved in dimethyl sulfoxide or ethanol and further diluted in medium (final concentration, 0.1%) or phosphate-buffered saline (PBS) as appropriate. Equivalent concentrations of dimethyl sulfoxide or ethanol, used as vehicle in controls, had no effects in our experiments.

### Isolation of Peripheral Blood Monocytes and Cell Culture

Human PMNs and monocytes were isolated from peripheral venous blood drawn from healthy volunteers, after informed written consent in accordance with institutional ethical guidelines (Mater Misericordiae Hospital, Dublin, Ireland). PMNs and mononuclear cells were separated by centrifugation on Ficoll-Paque (Pharmacia, Uppsala, Sweden). PMNs, after dextran sedimentation (Dextran T500, Pharmacia) and hypotonic lysis of red cells, were suspended in PBS (Bio-Whittaker, Walkersville, MD) at $2 \times 10^6$ cells/ml. Monocytes were isolated from the mononuclear cell fraction. To eliminate platelet contamination, mononuclear cells were washed twice with PBS containing 5 mmol/L of ethylenediaminetetraacetic acid. Cells were then resuspended at $2 \times 10^6$ cells/ml in RPMI 1640 supplemented with 10% autologous serum, 2 mmol/L glutamine, 100 IU/ml penicillin, and 100 $\mu$g/ml streptomycin (Life Technologies, Inc., Grand Island, NY). Cells were placed on 18-mm glass coverslips for fluorescence studies or in Lab-Tek chamber slides (Nunc, Naperville, IL) for electron microscopy studies. Monocytes were allowed to attach for 2 hours at 37°C in a 5% $CO_2$ humidified atmosphere and nonadherent cells were removed by two washes with RPMI 1640. M$\phi$ were obtained by culturing monocytes for 7 days in RPMI 1640 supplemented with 10% autologous serum, 2 mmol/L glutamine, 100 IU/ml penicillin, and 100 $\mu$g/ml streptomycin.

The human myelomonocytic cell line THP-1 (European Collection of Cell Cultures, Salisbury, UK) was maintained in suspension in RPMI 1640 supplemented with 2 mmol/L glutamine, 100 IU/ml penicillin, 100 $\mu$g/ml streptomycin, and 10% fetal calf serum (Life Technologies, Inc.). THP-1 cells at $2 \times 10^6$/ml were differentiated to a M$\phi$-like phenotype by treatment with phorbol 12-myristate, 13-acetate (PMA) (16 nmol/L, Sigma) for 48 hours in 12- or 6-well plates (Costar, Cambridge, MA).

### Electron Microscopy

Monocytes or M$\phi$ were treated with vehicle or $LXA_4$ ($10^{-9}$ mol/L) for 15 minutes at 37°C and washed twice with PBS. PMNs were treated with vehicle or $LXA_4$ ($10^{-9}$ mol/L) for 15 minutes at 37°C followed by further treatment with vehicle or $LTB_4$ ($10^{-7}$ mol/L) for 30 seconds at 37°C. All samples were fixed in 2.5% glutaraldehyde in PBS, pH 7.4, postfixed in 1% osmium tetroxide and embedded in Epon using standard methods. The plastic of the Lab-Tek chamber was removed from the polymerized Epon to expose the basal region of the cells and sections to a depth of 5 $\mu$ were discarded. Thus podosomal regions of cells were excluded before 50-nm ultrathin sections were taken for analysis. Sections were stained with uranyl acetate and lead citrate and examined in a JEOL 2000EX microscope (Tokyo, Japan) using an acceleration potential of 80 kV and objective aperture of 20 $\mu$.

### Actin Staining

Adherent monocytes or M$\phi$ on glass coverslips were incubated with the appropriate stimuli ($LXA_4$, $10^{-9}$ mol/L; 15-(*R/S*)-methyl-$LXA_4$, $10^{-11}$ mol/L; 16-phenoxy-$LXA_4$, $10^{-11}$ mol/L; 15-(*S*)-HETE, $10^{-9}$ mol/L) for 15 minutes at 37°C. 8-Br-cAMP (2 mmol/L), Rp-cAMP (100 $\mu$mol/L), and LY294002 (10 $\mu$mol/L) were co-incubated with vehicle or $LXA_4$ ($10^{-9}$ mol/L) for 15 minutes at 37°C. M$\phi$ were pretreated with Y-27632 (10 $\mu$mol/L) for 30 minutes at 37°C before exposure to $LXA_4$ (15 minutes at 37°C). At

the end of the incubations, cells were rinsed with PBS and fixed in 3.8% paraformaldehyde-PBS for 20 minutes at room temperature.

PMNs were incubated with $LXA_4$ ($10^{-9}$ mol/L) or vehicle for 15 minutes at 37°C before the addition of $LTB_4$ ($10^{-7}$ mol/L) or the appropriate vehicle for 10, 30, 60, or 300 seconds at 37°C. Cells were fixed in 3.8% paraformaldehyde-PBS for 20 minutes at room temperature and spread on poly-L-lysine-coated glass slides.

Cells were permeabilized with 0.1% Triton X-100 in PBS for 5 minutes and stained with Oregon Green-phalloidin (0.33 μmol/L; Molecular Probes, Eugene, OR) for 30 minutes at room temperature. Coverslips were mounted on microscope glass slides with Probing Antifade medium (Molecular Probes). Cells were viewed on a Leica fluorescent microscope and photographed with Ektachrome 400 film. Images of the cells were recorded at focal planes that excluded cell/substrate interfaces.

### *Western Blotting*

Differentiated THP-1 cells were incubated with the different LXs or with 15-(*S*)-HETE for 15 minutes at 37°C. The reaction was stopped by washing twice with ice-cold PBS and cells were scraped with ice-cold lysis buffer (20 mmol/L HEPES, 2 mmol/L ethylenediaminetetraacetic acid, 1 mmol/L $MgCl_2$) containing 50 μl/ml of a protease inhibitor cocktail [2 mmol/L 4-(2-aminoethyl) benzenesulfonyl fluoride, 1 mmol/L ethylenediaminetetraacetic acid, 130 μmol/L bestatin, 1.4 μmol/L *trans*-epoxysuccinyl-L-leucyl-amido(4-guanidino)butane (E64), 1 μmol/L leupeptin, 0.3 μmol/L aprotinin; Sigma]. Cells were incubated for 20 minutes at 4°C, homogenized and centrifuged at 22,000 × *g* for 60 minutes at 4°C. The supernatants were retained as cytosolic fractions and the membrane pellets were resuspended in lysis buffer and Laemmli sample buffer. Samples from the same number of cells ($1 \times 10^6$ cells) were resolved by electrophoresis on a 12% sodium dodecyl sulfate polyacrylamide gel and transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were incubated with blocking buffer [Tris-buffered saline (20 mmol/L Tris, 137 mmol/L NaCl) containing 0.1% Tween-20 (TBS-T) and 5% milk] before probing with either 1 μg/ml of monoclonal anti-RhoA antibody, rabbit anti-Rac2, or anti-Cdc42 antibodies in TBS-T. After incubation with a horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody as appropriate (1/2000 in TBS-T; New England Biolabs, Beverly, MA), bound antibody was visualized with an enhanced chemiluminescence detection system (Santa Cruz Biotechnology) and blots were quantified by densitometry.

PMNs stimulated with the chemoattractant fMLP (1 μmol/L for 30 seconds) were used as positive controls for Cdc42 Western blotting.

### *RhoA Activity Assay*

For expression and purification of GST-RBD (Rho binding domain of Rhotekin),[18] bacteria expressing GST-RBD were cultured to the log phase and protein expression induced on incubation with 0.5 mmol/L of isopropyl-β-D-thiogalactopyranoside for 2 hours. Proteins were purified on glutathione-coupled Sepharose 4B beads (Pharmacia). After six washes in washing buffer (50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 5 mmol/L $MgCl_2$, 0.5% Triton X-100, 1 mmol/L dithiothreitol, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 0.1 mmol/L phenylmethyl sulfonyl fluoride), the beads were resuspended in washing buffer supplemented with 10% glycerol, aliquoted, and stored at −80°C. The purity and the concentration of the preparation were determined by Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gels by comparison to bovine serum albumin standards.

Differentiated THP-1 cells ($4 \times 10^6$ cells), after exposure to $LXA_4$ ($10^{-9}$ mol/L) or vehicle for 15 minutes at 37°C, were washed with ice-cold PBS and lysed in cold lysis buffer (50 mmol/L Tris, pH 7.2, 0.5% Triton X-100, 150 mmol/L NaCl, 5 mmol/L $MgCl_2$) containing 1 mmol/L of phenylmethyl sulfonyl fluoride and 10 μg/ml each of leupeptin and aprotinin. Cell lysates were centrifuged at 13,000 × *g* for 10 minutes at 4°C. Twenty μg of bacterially expressed GST-RBD fusion protein bound to glutathione-coupled Sepharose 4B beads were incubated with equal volumes of lysates for 45 minutes at 4°C. The beads and protein bound to the fusion protein were washed four times with ice-cold lysis buffer containing 0.1 mmol/L of phenylmethyl sulfonyl fluoride and 10 μg/ml each of leupeptin and aprotinin. The bead pellets were resuspended in Laemmli sample buffer. Bound and total Rho proteins were detected by Western blotting with an antibody against RhoA as described above.

## *Results*

### *LXs Induce Ultrastructural Changes and Actin Reorganization in Human Monocytes and Mϕ, But Not in PMNs*

To evaluate the effect of LXs on monocytes and Mϕ, cells were exposed to $LXA_4$ ($10^{-9}$ mol/L) or vehicle for 15 minutes at 37°C and F-actin staining and cellular ultrastructure were investigated. In control monocytes, the staining of F-actin was homogenous with an even distribution of actin at the level of the surface of the cell (Figure 1A). Stimulation with $LXA_4$ induced a shape change with the formation of lamellipodia and focal intensities across the cytoplasm (Figure 1B). These morphological changes were confirmed by electron microscopy. Control monocytes had smooth cellular surfaces with few short pseudopodia (Figure 1C). $LXA_4$-stimulated monocytes showed a profusion of elongated pseudopodia and cell organelles peripherally distributed (Figure 1D).

Adherent monocytes were cultured in medium for 7 days to obtain mature Mϕ. The latter were more heterogeneous in shape compared to monocytes, however, ~70% of the control Mϕ showed a spherical shape with a diffuse distribution of actin and discrete extensions (Figure 1E). After $LXA_4$ treatment, the cells appeared more polarized and presented extensive filopodia and lamelli-

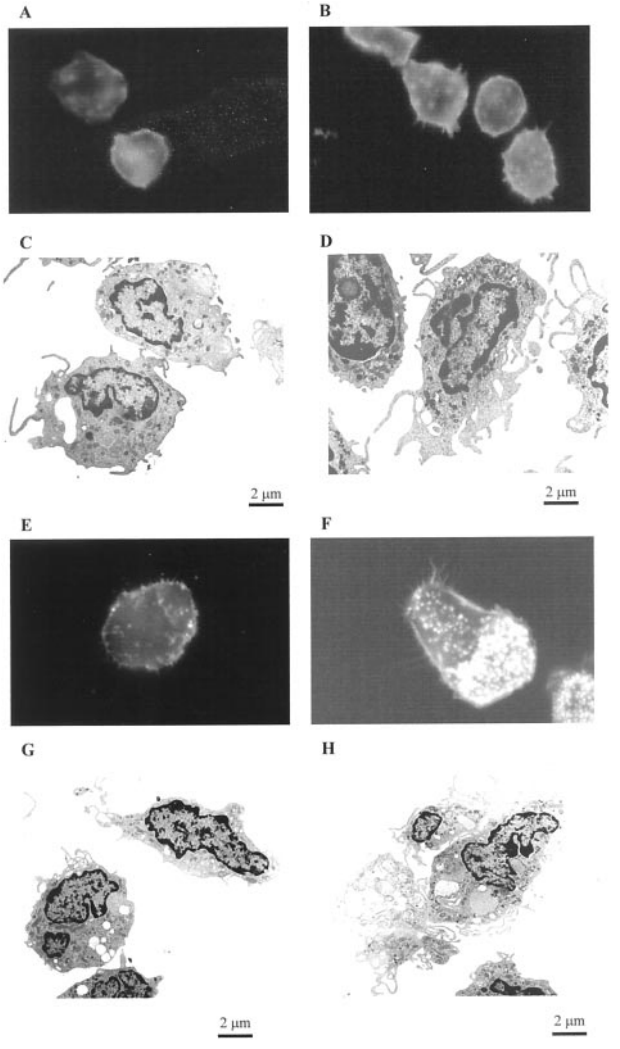


**Figure 1.** Effects of $LXA_4$ on ultrastructure and actin reorganization in human monocytes and monocyte-derived macrophages. Adherent monocytes (**A–D**) or monocyte-derived macrophages (**E–H**) were treated with vehicle (**A**, **C**, **E**, **G**) or with $LXA_4$ ($10^{-9}$ mol/L) (**B, D, F, H**) for 15 minutes at 37°C. Cells were fixed with glutaraldehyde and osmium tetroxide for electron microscopy and with paraformaldehyde for fluorescence microscopy. Localization of actin was determined using Oregon Green phalloidin and visualized by fluorescence microscopy using a ×100 oil objective. The electron microscopy images were all taken at the same magnification. These images are representative of several fields derived from two independent experiments for electron microscopy and of five for fluorescence microscopy.

podia, with punctate F-actin staining on the apical surface, representative of filopodia (Figure 1F). Electron microscopy of control M$\phi$ showed smooth-surfaced cells almost bereft of pseudopodia (Figure 1G), whereas $LXA_4$-stimulated M$\phi$ exhibited innumerable filamentous membrane protrusions of filopodia and a broad membrane-structure characteristic of lamellipodia (Figure 1H).

LXs are rapidly inactivated by monocytes, the major route of degradation being the formation of the inactive metabolites 15-oxo-$LXA_4$, 13,14-dihydro-15-oxo-$LXA_4$, and 13,14-dihydro-$LXA_4$.[19] 15-(*R/S*)-methyl-$LXA_4$ and 16-phenoxy-$LXA_4$ are synthetic stable $LXA_4$ analogues that resist to the metabolic inactivation and retain many of the anti-inflammatory activities of the native compound with respect to PMNs and M$\phi$ including stimulation of M$\phi$

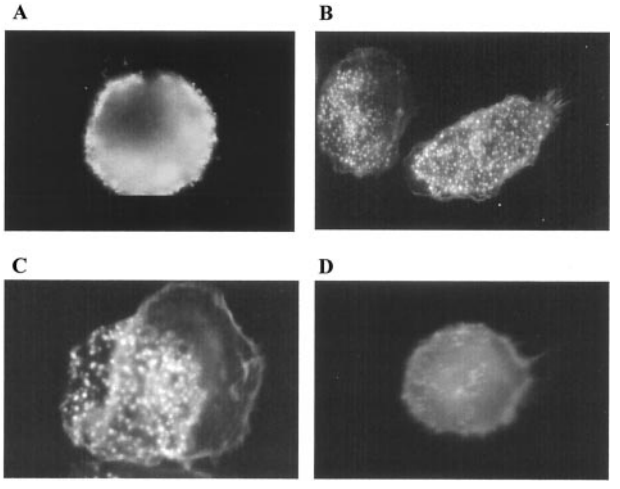


**Figure 2.** Effects of stable analogues of $LXA_4$ and 15-(S)-HETE on actin reorganization in human monocyte-derived macrophages. M$\phi$ were exposed to vehicle (**A**), 15-(*R/S*)-methyl-$LXA_4$ ($10^{-11}$ mol/L) (**B**), 16-phenoxy-$LXA_4$ ($10^{-11}$ mol/L) (**C**), or 15-(*S*)-HETE ($10^{-9}$ mol/L) (**D**) for 15 minutes at 37°C. Cells were fixed with paraformaldehyde and localization of actin was determined using Oregon Green phalloidin and visualized by fluorescence microscopy using a ×100 oil objective. These images are representative of several fields derived from one experiment of five independent experiments.

phagocytosis of apoptotic PMNs.[4,12] In the present study, both analogues ($10^{-11}$ mol/L, 15 minutes at 37°C) stimulated actin reorganization in monocytes (data not shown) and M$\phi$ (Figure 2, B and C). In contrast, when M$\phi$ were exposed to the $LXA_4$ precursor, 15-(*S*)-HETE (Figure 2D) the actin distribution was similar to that seen in unstimulated control cells (Figure 2A).

Given the evidence that LXs stimulate the functions of monocytes and M$\phi$, but inhibit many of the bioactivities of PMNs, it was of interest to investigate the effects of LXs on PMN ultrastructure and actin rearrangement. PMNs were exposed to $LXA_4$ ($10^{-9}$ mol/L) or appropriate vehicle (0.1% ethanol) for 15 minutes before the addition of $LTB_4$ ($10^{-7}$ mol/L) for different times (10, 30, 60, 300 seconds). Control PMNs were round with actin filaments homogeneously distributed through the cytoplasm and a minor enrichment at the cell periphery (Figure 3A) and electron microscopy revealed cells with few pseudopodia (Figure 3B). After 30 seconds of exposure to $LTB_4$, PMNs showed accumulation of F-actin on the margin of the cells (Figure 3C) and a large increase in the number of pseudopodia (Figure 3D). $LXA_4$ did not affect actin rearrangement in PMNs (Figure 3E), an observation confirmed by electron microscopy that showed PMNs greatly resembling control cells (Figure 3F). In addition, $LXA_4$ did not inhibit the actin reorganization (Figure 3G) and the ultrastructural changes (Figure 3H) induced by $LTB_4$. Similar results were observed for all of the incubation times (data not shown).

## *Cell Signaling Pathways Involved in $LXA_4$-Induced Actin Reorganization*

We have previously demonstrated that LX-stimulated phagocytosis was modulated by cAMP.[12] In the present study, the involvement of cAMP in $LXA_4$-triggered actin

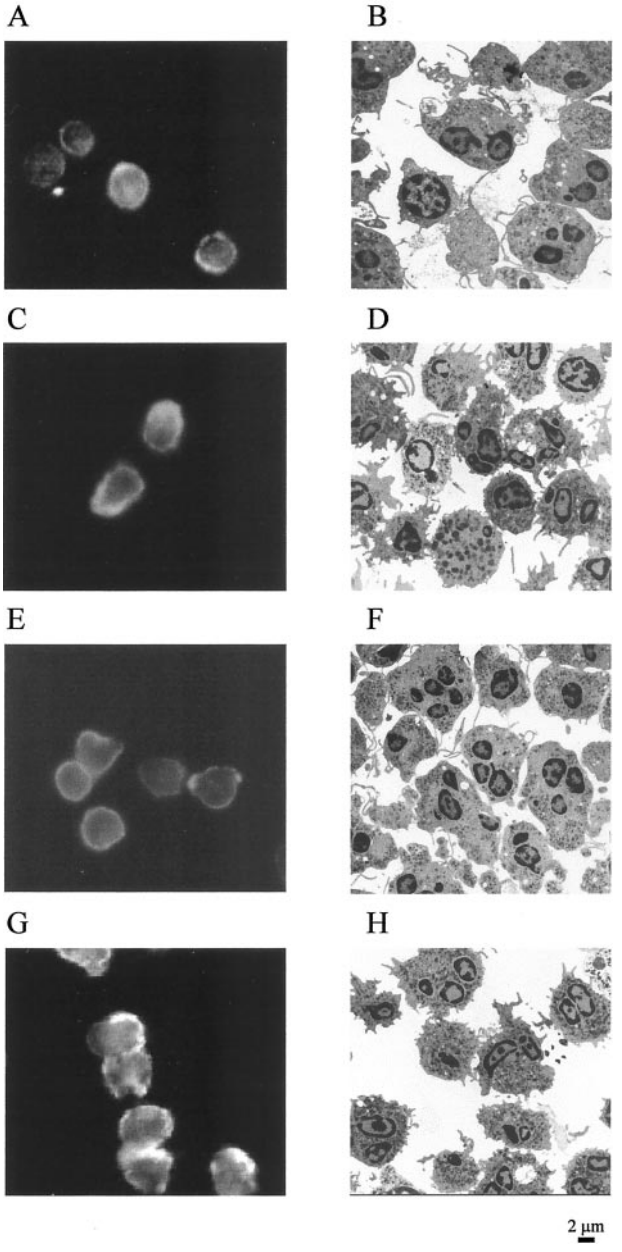


**Figure 3.** Effects of $LXA_4$ on actin reorganization in human PMNs. Human PMNs ($2 \times 10^6$ cells/ml) were treated with vehicle (**A** and **B**) or $LXA_4$ ($10^{-9}$ mol/L) (**E** and **F**) for 15 minutes at 37°C before the addition of $LTB_4$ ($10^{-7}$ mol/L) for 30 seconds at 37°C [vehicle + $LTB_4$ (**C** and **D**); $LXA_4$ + $LTB_4$ (**G** and **H**)]. At the end of the incubation time, cells were fixed with 3.8% paraformaldehyde-PBS for 20 minutes at room temperature and spread on poly-L-lysine-coated glass slides for fluorescence studies or with glutaraldehyde for electron microscopy. Localization of actin was determined using Oregon Green phalloidin and visualized by fluorescence microscopy using a ×100 oil objective. The electron microscopy images were all taken at the same magnification. These images are representative of several fields derived from two independent experiments for electron microscopy and of three for fluorescence microscopy.

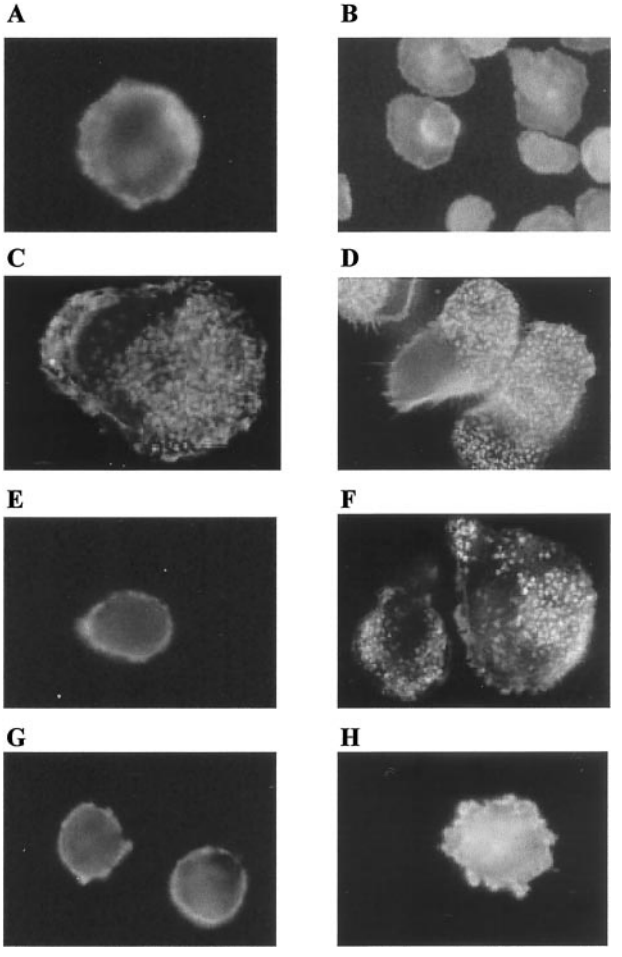


**Figure 4.** Roles of cAMP, PI 3-kinase, and Rho kinase in $LXA_4$-induced rearrangement of the actin cytoskeleton in human monocyte-derived macrophages. Mϕ were treated with 8-Br-cAMP (2 mmol/L), Rp-cAMP (100 μmol/L), or LY294002 (10 μmol/L) simultaneously with vehicle or $LXA_4$ ($10^{-9}$ mol/L) for 15 minutes at 37°C. Mϕ were incubated with the inhibitor Y-27632 (10 μmol/L) for 30 minutes before exposure to $LXA_4$ ($10^{-9}$ mol/L) for 15 minutes. Cells were fixed with paraformaldehyde and localization of actin was determined using Oregon Green phalloidin and visualized by fluorescence microscopy using a ×100 oil objective. These images are representative of several fields derived from three independent experiments. **A:** vehicle + 8-Br-cAMP; **B:** $LXA_4$ + 8-Br-cAMP; **C:** vehicle + Rp-cAMP; **D:** $LXA_4$ + Rp-cAMP; **E:** vehicle + LY294002; **F:** $LXA_4$ + LY294002; **G:** Y-27632 + vehicle; **H:** Y-27632 + $LXA_4$.

reorganization in Mϕ was studied using both a stable analogue of cAMP, 8-Br-cAMP to mimic elevation of intracellular levels of cAMP and Rp-cAMP, a cAMP-dependent protein kinase (PKA) inhibitor. Incubation of Mϕ for 15 minutes with 8-Br-cAMP (2 mmol/L) did not result in significant changes in F-actin distribution (Figure 4A). Co-incubation of 8-Br-cAMP and $LXA_4$ resulted in an inhibition of $LXA_4$-induced actin reorganization, leading to round cells with incomplete development of cytoskeletal extensions (Figure 4B). In contrast, Rp-cAMP (100 μmol/L, 15 minutes) mimicked the effect of $LXA_4$ on actin cytoskeleton, increasing the accumulation of F-actin throughout the cytoplasm (Figure 4C). Co-incubation of Mϕ with Rp-cAMP and $LXA_4$ induced similar effects on F-actin distribution as $LXA_4$ alone (Figure 4D compared to Figure 1F). These data parallel our observations that LX-stimulated phagocytosis is inhibited by 8-Br-cAMP and mimicked by Rp-cAMP.[12]

PI 3-kinases have been implicated in many cellular responses such as macrophage chemotaxis[20] and phagocytosis.[21,22] To evaluate the effects PI 3-kinases have on actin cytoskeleton, Mϕ were co-incubated for 15 minutes with the inhibitor LY294002 (10 μmol/L) alone or in combination with $LXA_4$ ($10^{-9}$ mol/L). Inhibition of PI-3

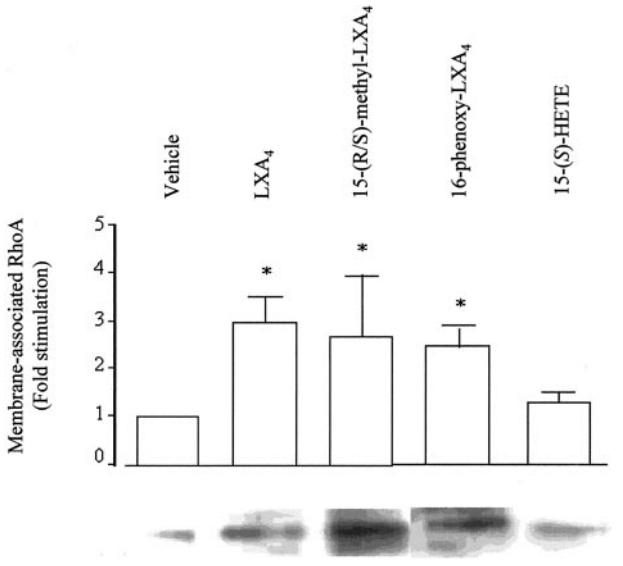


**Figure 5.** LX-induced translocation of RhoA. THP-1 cells were differentiated into a M$\phi$-like phenotype by treatment with PMA (16 nmol/L) for 48 hours. Cells were exposed to vehicle, $LXA_4$ ($10^{-9}$ mol/L), 15-(*R/S*)-methyl-$LXA_4$ ($10^{-11}$ mol/L), 16-phenoxy-$LXA_4$ ($10^{-11}$ mol/L), or 15-(*S*)-HETE for 15 minutes at 37°C. Cells were lysed and a crude preparation of membranes was isolated. Samples from the same number of cells ($1 \times 10^6$ cells) were resolved by electrophoresis on a 12% sodium dodecyl sulfate-polyacrylamide gel and Western blot analysis was performed with an antibody against RhoA. Blots were quantified by densitometry. Values are expressed as fold stimulation relative to vehicle (unity) and are the means ± SEM of five independent experiments. A representative blot is shown. *, $P < 0.05$ *versus* vehicle.

kinase did not affect actin reorganization in control and $LXA_4$-stimulated M$\phi$ (Figure 4, E and F).

## *$LXA_4$-Induced F-Actin Reorganization Is Blocked by Y-23632 and Involves RhoA and Rac Activation*

Rho GTPases seem to be important modulators of actin cytoskeleton function during many phagocytic responses.[17,22–26] Rho kinases are effectors of Rho and they can be specifically inhibited by the compound Y-27632.[27,28] In the present study we evaluated the effect of Y-27632 on $LXA_4$-induction of actin rearrangement. Whereas Y-27632 itself (10 $\mu$mol/L) did not influence F-actin distribution (Figure 4G), previous exposure of M$\phi$ to this inhibitor almost completely inhibited the $LXA_4$-induced changes in the actin reorganization (Figure 4H), implicating the involvement of small GTPases in the $LXA_4$-elicited effect on actin cytoskeleton.

To examine the ability of $LXA_4$ to activate small GTPases of the Rho family, we measured changes in membrane-associated RhoA, Rac, and Cdc42 in THP-1 cells differentiated to a M$\phi$-like phenotype by treatment with PMA. In THP-1 cells, $LXA_4$ induces ultrastructural changes and actin redistribution comparable to those observed in M$\phi$ (data not shown). When cells were stimulated with $LXA_4$ ($10^{-9}$ mol/L) or the two analogues ($10^{-11}$ mol/L) for 15 minutes, a threefold increase in membrane-associated RhoA, an index of Rho activation,[27] was observed, whereas 15-(*S*)-HETE was without effect (Figure 5). Consistent with the observation of others

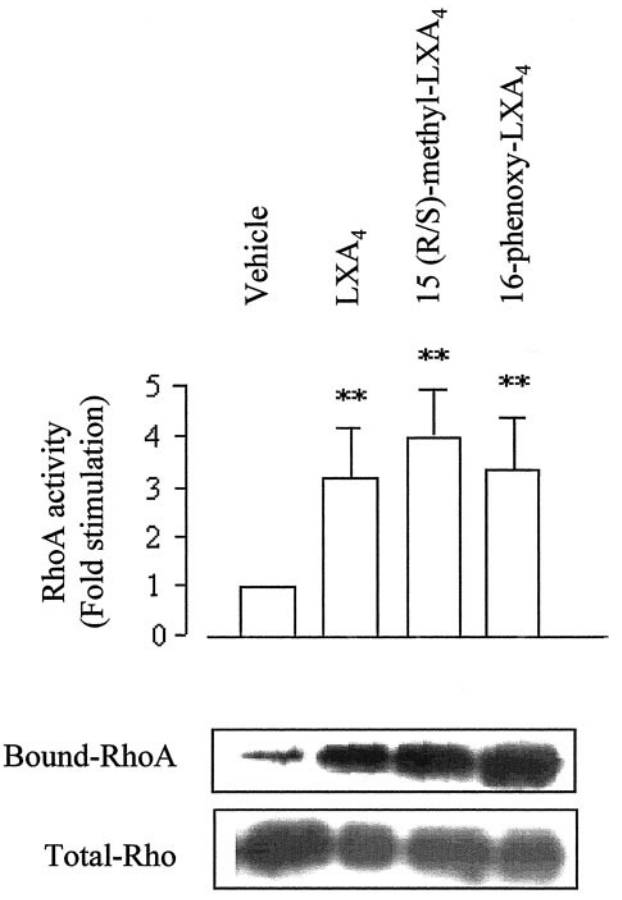


**Figure 6.** LXs activate RhoA in THP-1 cells. THP-1 cells were differentiated into a M$\phi$-like phenotype by treatment with PMA (16 nmol/L) for 48 hours. Cells were exposed to vehicle, $LXA_4$ ($10^{-9}$ mol/L), 15-(*R/S*)-methyl-$LXA_4$ ($10^{-11}$ mol/L), 16-phenoxy-$LXA_4$ ($10^{-11}$ mol/L), or 15-(*S*)-HETE for 15 minutes at 37°C. Activity of the GTP-bound Rho in THP-1 lysates was assessed by a pull-down assay using glutathione-Sepharose 4B beads coupled with bacterially expressed GST-RBD fusion protein. Bound and total Rho proteins were detected by Western blotting with an antibody against RhoA. RhoA activity is indicated by the amount of RBD-bound Rho normalized to the amount of Rho in whole-cell lysates. Values represent Rho activity relative to vehicle. Results are the means ± SEM of three independent experiments and a representative blot is shown. **, $P < 0.01$ *versus* vehicle.

on Rho activation, a decrease in cytosolic RhoA could not be detected in our experimental conditions (data not shown) because of the fact that a large fraction of the total Rho protein still remained cytosolic.[29] Using a pull-down assay with Rho-binding domain of Rhotekin we found that $LXA_4$ and the stable analogues caused a significant increase in Rho activity in THP-1 lysates (Figure 6). There is not any statistically significant difference in the effect of native $LXA_4$ and the stable analogues on *in vitro* Rho activation assay. However, the difference in the active concentrations between $LXA_4$ and the analogues can be related to the rapid inactivation of $LXA_4$[19] and the enhanced stability of the analogues, bypassing the initial rapid inactivation, can lead to more sustained actions in *in vivo* models of inflammation.

As discussed above, cAMP/PKA pathways are involved in $LXA_4$-stimulated rearrangement of the actin cytoskeleton. Inhibition of RhoA translocation to the membrane was observed in differentiated THP-1 cells treated with 8-Br-cAMP and $LXA_4$ (Figure 7). When THP-1 cells

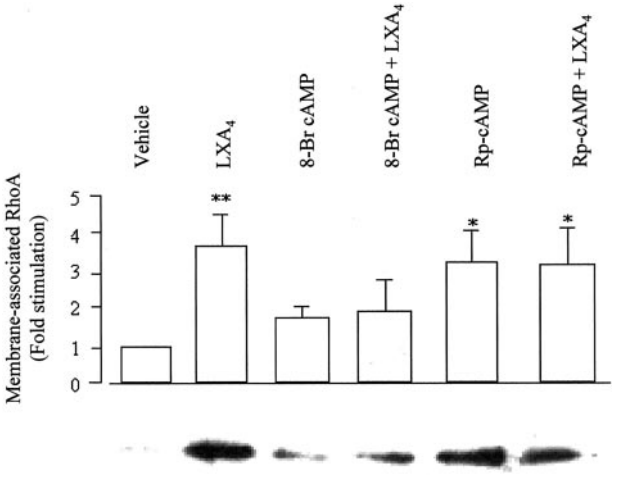


**Figure 7.** Role of cAMP in $LXA_4$-induced Rho translocation. THP-1 cells were differentiated into a M$\phi$-like phenotype by treatment with PMA (16 nmol/L) for 48 hours. Cells were treated with 8-Br-cAMP (2 mmol/L) or Rp-cAMP (100 $\mu$mol/L), simultaneously with vehicle or $LXA_4$ ($10^{-9}$ mol/L) for 15 minutes at 37°C. Cells were lysed and a crude preparation of membranes was isolated. Samples from the same number of cells ($1 \times 10^6$ cells) were resolved by electrophoresis on a 12% sodium dodecyl sulfate-polyacrylamide gel and Western blot analysis was performed with an antibody against RhoA. Blots were quantified by densitometry. Values are expressed as fold stimulation relative to vehicle (unity) and are the means ± SEM of three independent experiments. A representative blot is shown. *, $P < 0.05$; **, $P < 0.01$ *versus* vehicle.

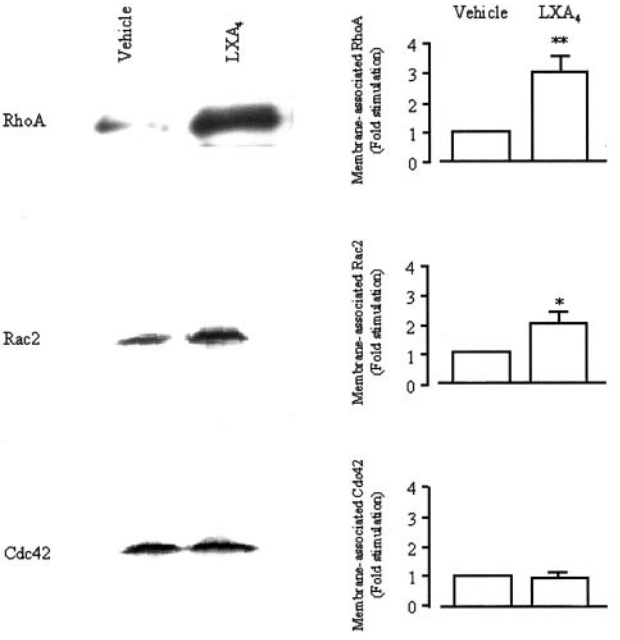


**Figure 8.** LX-induced translocation of Rho small GTPases. THP-1 cells were differentiated into a M$\phi$-like phenotype by treatment with PMA (16 nmol/L) for 48 hours and then exposed to vehicle or $LXA_4$ ($10^{-9}$ mol/L) for 15 minutes at 37°C. Cells were lysed and a crude preparation of membranes was isolated. Samples from the same number of cells ($1 \times 10^6$ cells) were resolved by electrophoresis on a 12% sodium dodecyl sulfate-polyacrylamide gel and Western blot analysis was performed with antibodies against RhoA, Rac2, or Cdc42. Blots were quantified by densitometry. Values are expressed as fold stimulation relative to vehicle (unity) and are the means ± SEM of three independent experiments and in each case a representative blot is shown. *, $P < 0.05$; **, $P < 0.01$ *versus* vehicle.

were exposed to Rp-cAMP alone or in combination with $LXA_4$, a threefold increase in membrane-associated RhoA was detected, an increase comparable to that observed with $LXA_4$ alone (Figure 7).

THP-1 cells exposed to $LXA_4$ ($10^{-9}$ mol/L) showed a twofold increase in the membrane-associated Rac, whereas the levels of membrane-associated Cdc42 were unaffected by $LXA_4$ (Figure 8).

## *Discussion*

In this study we present evidence that LXs induce remarkable changes in the ultrastructure and F-actin reorganization in human monocytes and M$\phi$, but not in PMNs. LXs are endogenous mediators that play an important role in the resolution of inflammation through distinct effects on specific cell types.[1,2] The anti-inflammatory activities of LXs on PMN trafficking have been demonstrated *in vitro* and *in vivo*.[1–9,30–32] LXs inhibit PMN chemotaxis,[8] adhesion, and transmigration across endothelia and epithelia in response to pro-inflammatory stimuli such as $LTB_4$ and fMLP.[7,9] The data presented here, indicating that LXs are without effect on $LTB_4$-induced changes in the actin cytoskeleton on PMNs, suggest that the locus of LX inhibition of $LTB_4$-stimulated PMN activation is not at the level of actin cytoskeletal rearrangement and migration and provides further circumstantial evidence for LX modulating PMN trafficking predominantly through inhibition of $LTB_4$-stimulated $\beta_2$ integrin-mediated adhesion.[9,33,34]

In contrast to the inhibitory effects of LXs on PMN function, LXs are potent stimuli for monocytes. $LXA_4$ is rapidly metabolized by monocytes to the inactive metabolites 15-oxo-$LXA_4$, 13–14-dihydro-15-oxo-$LXA_4$, and 13,14-dihydro-$LXA_4$.[11,19] $LXA_4$ activates Rainbow trout macrophages, increasing the intracellular calcium concentrations.[35] The effect of $LXA_4$ on the calcium levels correlates with the chemotactic mechanisms, but not with phagocytosis of yeast particles in this system.[35] $LXA_4$ and $LXB_4$ stimulate human monocyte chemotaxis and adhesion, without degranulation or release of reactive oxygen species,[10,11] suggesting that the actions of LXs may be related to the recruitment of monocytes to sites of injury. LXs were shown to accelerate the resolution of allergic pleural edema[36] and to stimulate nonphlogistic phagocytosis of apoptotic PMN by M$\phi$ *in vitro*[12] and *in vivo* in a mouse thioglycollate-elicited peritonitis model (Mitchell et al, manuscript submitted). These data provide compelling evidence for a role for LXs in promoting the resolution of inflammation. The locus of the effect of LXs on promoting phagocytosis of apoptotic PMNs is unresolved. Multiple cell surface molecules on the phagocyte have been implicated in the recognition and binding of apoptotic cells[13,15,16] and functional inhibition of several of these is associated with modulation of LX-stimulated phagocytosis.[12] The work presented here has investigated whether $LXA_4$ and its stable analogues might modulate rearrangement of the actin cytoskeleton in monocytes and M$\phi$. Our data demonstrate that LXs induce significant changes in the reorganization of actin in human monocytes and M$\phi$ resulting in the promotion of cytoplasmic extensions and in the formation of pseudo-

podia. Given that the process of phagocytosis is highly dependent on the localized polymerization of actin filaments that facilitate the formation of filopodia that surround the cells or the microorganisms to be engulfed,[17] our data are of a particular significance. It is reasonable to propose that enhanced phagocytosis of apoptotic PMNs is an inevitable consequence of interaction with such primed M$\phi$.

The small Rho GTPases (Rho, Rac, and Cdc42) regulate the assembly and reorganization of the actin cytoskeleton in response to external stimuli, providing the driving force for cells to migrate, to adhere, and to phagocytose particles.[37] Rho GTPases cycle between an inactive GDP-bound form and an active GTP-bound form and once activated, they interact with downstream effectors,[38,39] regulating different signal transduction pathways linking various membrane receptors to the assembly of actin.[40] In fibroblasts Rac is involved in the formation of lamellipodia causing actin polymerization at the plasma membrane, Cdc42 mediates the formation of filopodia, and Rho mediates the formation of stress fibers and focal adhesions.[41,42] However the function of the Rho family in shape control, actin reorganization, and integrin activity might be different in cells that do not possess stress fibers such as leukocytes.[37,43] Differential involvement of specific Rho family members has been demonstrated in phagocytosis in response to stimulation of immunoglobulin (Fc$\gamma$) or complement receptors. Rac and Cdc42 have been shown to regulate actin reorganization during Fc$\gamma$ receptor-mediated phagocytosis by promoting pseudopod extension and phagosome closure,[22–26] whereas complement-mediated phagocytosis, a process not associated with the release of pro-inflammatory molecules, requires the activation of RhoA.[24] Phagocytosis of apoptotic cells shares some of the characteristics of both mechanisms. Similar to the Fc$\gamma$ receptor-mediated phagocytosis, internalization of the apoptotic cells is because of extension of the phagocyte membrane around the cell to be engulfed[44] and require Rac and Cdc42, whereas inhibition of Rho enhanced phagocytosis.[22] However uptake of apoptotic cells does not evoke release of inflammatory mediators,[14] a scenario comparable to complement-mediated phagocytosis. We have investigated whether LX-stimulated actin reorganization is associated with Rho small GTPases. The effect of $LXA_4$ on actin cytoskeleton was impaired by Y-27632, an inhibitor of Rho kinase, a downstream kinase of Rho, suggesting a role for these signaling molecules in LX-triggered cytoskeletal reorganization. In addition, we have measured the activation of the small GTPases in THP-1 cells treated with $LXA_4$ by evaluating the membrane-associated levels of RhoA, Rac, and Cdc42. To measure Rho activation, we have also used a more sensitive assay by measuring the ability of RhoA to associate with the Rho-binding domain of Rhotekin exploiting the fact that Rho effectors intersect only with GTP-bound Rho.[18] Our data demonstrate that $LXA_4$ activates RhoA and Rac, but not Cdc42 in THP-1 cells, a M$\phi$-like phenotype. It has been proposed that inhibition of Rho enhances phagocytosis, but reduces monocyte transmigration and M$\phi$ chemotaxis.[22,37] The effect of $LXA_4$ on Rho activation suggests that RhoA might be differentially involved in the stimulatory activity of LXs on monocyte/M$\phi$ such as chemotaxis and phagocytosis. The increase of the levels of active RhoA is of interest in the context of the nonphlogistic phagocytosis induced by $LXA_4$, a characteristic shared with the complement-receptor mediated phagocytosis. On the other hand, the increase in Rac levels may be responsible for the protrusion of the plasma membrane around the apoptotic cell.[17,22]

cAMP-dependent mechanisms have been found to produce changes in the rearrangement of the actin cytoskeleton in different cell types through RhoA.[45–47] Elevated intracellular cAMP levels cause activation of PKA, which phosphorylates many target proteins including RhoA. It has been suggested that such phosphorylation down-regulates the binding of Rho to Rho kinases.[45,46] We have previously demonstrated that the effect of LXs on phagocytosis involves cAMP/PKA pathways.[12] A role for cAMP/PKA pathways on the effects of $LXA_4$ on actin cytoskeleton are suggested by the observation that $LXA_4$-induced actin reorganization is inhibited by the cell permeable cAMP analogue, 8-Br-cAMP, and is mimicked by an inhibitor of PKA.

Interestingly, we find that LX-stimulated actin cytoskeletal rearrangement is insensitive to the PI 3-kinase inhibitor LY294002, whereas LX-stimulated phagocytosis is PI 3-kinase-dependent (Michell et al, manuscript submitted). This may reflect the independence of PI 3-kinase in the regulation of the initial actin polymerization, but its critical role is in more distal events in the phagocytic process involving membrane fusion.[22]

In conclusion, we demonstrate that LXs and LX-stable analogues induce reorganization of the actin cytoskeleton in monocytes and M$\phi$ but not in PMNs and that this effect is associated with differential activation of small Rho GTPases. This observation is of particular interest in view of the fact that LXs stimulate nonphlogistic phagocytosis of apoptotic PMN and that a role for LX in wound healing has been suggested on the basis of activation of monocyte function and chemotaxis.[11] Given the role of Rho small GTPases in cell motility it may be proposed that LX stimulation of RhoA and Rac activity modulates chemotactic and phagocytic responses. In particular, data presented here suggest that LX pretreatment of M$\phi$ might prime them for chemotaxis and phagocytosis by promoting RhoA- and Rac-dependent cytoskeleton reorganization and pseudopodia formation, contributing to the potential role of LXs in the resolution of inflammation.

## *Acknowledgments*

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