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Involvement of the Rho-Kinase/Myosin Light Chain Kinase Pathway on Human Monocyte Chemotaxis Induced by ATL-1, an Aspirin-Triggered Lipoxin A4 Synthetic Analog

Rafael L. Simões and Iolanda M. Fierro

Lipoxins (LXs) are arachidonic acid metabolites able to induce monocyte chemotaxis in vitro and in vivo. Nonetheless, the signaling pathways mediating this process are yet unclear. In this study, we have investigated the mechanisms associated with human monocyte activation in response to 15-epi-16-(para-fluoro)-phenoxy-LXA4 (ATL-1), a stable 15-epi-LXA4 analog. Our results demonstrate that ATL-1-induced monocyte chemotaxis (10–300 nM) is inhibited by pertussis toxin, suggesting an effect via the G-protein-linked LXA4 receptor. Monocytes stimulated with the analog presented an increased ERK-2 phosphorylation, which was reduced by PD98059, a selective inhibitor of the MEK 1/2 pathway. After exposure of the cells to ATL-1, myosin L chain kinase (MLCK) phosphorylation was evident and this effect was inhibited by PD98059 or Y-27632, a specific inhibitor of Rho kinase. In addition, Y-27632 abolished ERK-2 activation, suggesting that the MAPK pathway is downstream of Rho/Rho kinase in MLCK activation induced by ATL-1. The specific MLCK inhibitor ML-7, as well as Y-27632, abrogated monocyte chemotaxis stimulated by the analog, confirming the central role of the Rho kinase/MLCK pathway on ATL-1 action. Together, these results indicate that ATL-1 acts as a potent monocyte chemoattractant via Rho kinase and MLCK. The present study clarifies some of the mechanisms involved in the activation of monocytes by LXs and opens new avenues for investigation of these checkpoint controllers of inflammation. The Journal of Immunology, 2005, 175: 1843–1850.

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3 Abbreviations used in this paper: LX, lipoxin; ALT, aspirin-triggered LX; ATL-1, 15-epi-16-(para-fluoro)-phenoxy-LXA4; PMN, polymorphonuclear cell; MLCK, myosin L chain; MLCK, MLCK kinase; PVP, polyvinyl pyrrolidone; PTX, pertussis toxin.
involved in chemotaxis induced by MCP-1 and E-selectin but not by lysophosphatidylcholine (17, 18).

The Rho protein family is part of the Ras superfamily of small GTPases, playing a major role in regulating actin cytoskeleton and cell adhesion (19, 20). Rho-kinase (Rho-associated protein kinase—p160ROCK), one of the target proteins of Rho, is implicated in many downstream processes of Rho, such as stress fiber and focal adhesion formation (21, 22) and transendothelial migration of monocytes (23). Although large numbers of studies have demonstrated a role for Rho family proteins on cell motility, little is known about how Rho family members influence cell migration.

In the present study, we have investigated possible mechanisms associated with monocyte activation in response to 15-epi-16-(para-fluoro)phenoxy-LXA₄ (ATL-1), a stable 15-epi-LXA₄ analog, as well as the signaling pathways involved in this process. We have shown that ATL-1 was able to induce monocyte chemotaxis in a Rho-dependent manner. The analog-induced myosin L chain kinase (MLCK) phosphorylation, which was inhibited by PD98059 and Y-27632, specific inhibitors of the MAPK and Rho/Rho kinase pathways, respectively. ATL-1 also promoted actin cytoskeleton reorganization, a key event in cell migration. These results indicate that the LX analog is a potent monocyte chemoattractant, acting via ALXR and using the Rho/MAPK/MLCK pathway to exert its biological actions.

**Materials and Methods**

**Reagents**

Ficoll-Hypaque and Percoll were purchased from Amersham Biosciences. PD98059, ML-7, and Y-27632 were obtained from Calbiochem. Abs and protein A/G agarose were purchased from Santa Cruz Biotechnology and streptavidin from Caltag Laboratories. All other reagents and chemicals were purchased from Sigma-Aldrich. ATL-1, the stable 15-epi-LXA₄ analog, was a generous gift from Brigham and Women’s Hospital (Harvard Medical School, Boston, MA).

**Isolation of human monocytes**

Isolated PBMC were obtained from EDTA (0.5%)-treated venous blood of healthy volunteers by Ficoll-Hypaque density gradient centrifugation. The PBMC monolayer was collected, washed twice, and resuspended in RPMI 1640. Monocytes were obtained from this suspension by Percoll density gradient centrifugation as described (24). Isolated monocytes (98% purity), estimated to be ≈96% viable by trypan blue exclusion, were resuspended in RPMI 1640 medium, in the absence of serum, and kept in an ice bath before chemotaxis or signaling experiments.

**Monocyte chemotaxis**

Monocyte chemotaxis was assayed in a 48-well Boyden chamber (Neuroprobe Microchemotaxis System) using a 5-μm polyvinyl pyrrolidone (PVP)-free polycarbonate filter as previously described (25). For chemotaxis assays, the chemotactic stimuli, fMLP (100 nM) and ATL-1 (1–1000 nM) were added to the bottom wells of the chamber. Cells suspended in RPMI 1640 medium (2 × 10⁵/ml) were added to the top wells of the Boyden chamber and allowed to migrate for 1.5 h at 37°C in a 5% CO₂ atmosphere. In some experiments, monocytes were preincubated with genistein (100 μM) or PD98059 (10 μM) for 5 min; pertussis toxin (PTX) (1 μg/ml), ML-7 (300 nM), or Y-27632 (10 μM) for 15 min at 37°C before the chemotactic assay. After the incubation time, the filter was removed from the chamber, fixed and stained with a Diff-Quick stain kit (Baxter Travenol Laboratories). Cells that migrated through the membrane were counted under light microscopy (×100 objective) on at least five random fields. The results, expressed as the number of monocytes per field, were representative of three independent experiments performed in triplicate for each test group. Monocyte migration toward RPMI 1640 medium plus 0.05% ethanol (random chemotaxis) was used as a negative control.

**Cytchemistry**

To evaluate the effect of ATL-1 on the distribution of F-actin, monocytes suspended in RPMI 1640 were incubated in the presence of ATL-1 (1–1000 nM) at 37°C. After 5 min, aliquots with 100 μM of monocyte suspension (10⁶ cells/ml) were plated onto cytoreps and centrifuged at 500 rpm for 5 min. The role of Rho kinase was assessed by pretreatment of the cells with Y-27632 (10 μM) for 15 min before ATL-1 addition (100 nM). Cells were fixed with 4% paraformaldehyde and 4% sucrose in PBS for 20 min at room temperature. After fixation, cells were permeabilized for 5 min in PBS containing 0.2% Triton X-100, washed with PBS, and incubated with rhodamine-conjugated phalloidin (1:1000), which binds specifically to F-actin, for 1 h at room temperature. Cytoreps were mounted on a slide using a solution of N-propylgallate (20 mM) and glycerol (80%) in PBS before examination under an epifluorescence microscope (Olympus Model BX40F4).

**Preparation of cell extracts**

Monocytes (3 × 10⁶/ml) were incubated with medium plus 0.05% ethanol (vehicle) or ATL-1 (100 nM) for different periods of time (0.5–30 min) at 37°C, followed by immediate freezing in an ice bath to stop the reaction and then centrifuged at 10,000 rpm for 5 min at 4°C. To obtain whole cell extracts, monocytes were resuspended in a proper lysis buffer (50 mM MES, pH 6.4, 1 mM MgCl₂, 10 mM EDTA, 1% Triton X-100, 1 μg/ml DNase, 0.5 μg/ml RNase and the following protease inhibitors: 1 mM PMSF, 1 mM benzamidine, 1 μM soybean trypsin inhibitor). Proteins present in the whole cell extract were obtained by acidic precipitation and dissolved in a 1% (v/v) SDS solution. The total protein content in the cell extracts was determined by the Bradford method (26).

**Immunoprecipitation**

Monocytes (5 × 10⁵/ml) were incubated with medium plus 0.05% ethanol (vehicle), fMLP (100 nM), or ATL-1 (100 nM) for 5 min at 37°C. In the experiments to evaluate ERK-2 or MLCK phosphorylation, monocytes were preincubated with PD98059 (10 μM) for 5 min; Y-27632 (10 μM) for 30 min, or PTX (1 μg/ml) for 15 min at 37°C before incubation with ATL-1 (100 nM) for 15 min. After incubation, cells were lysed in a lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1.5 mM MgCl₂, 1.5 mM EDTA, Triton X-100 (1% v/v), glycerol (10% v/v), aprotinin (10 μg/ml), leupeptin (10 μg/ml), pepstatin (2 μg/ml), and 1 mM PMSF. Lysates (2 μg/ml) were incubated overnight at 4°C with specific Abs (1:200). Then, protein A/G agarose (20 μg/ml protein) was added and samples were incubated at 4°C under rotation for 2 h. The content of total and phosphorylated protein was analyzed by immunoblotting.

**Western blots**

Cell lysates were denatured in Laemmli’s sample buffer (50 mM Tris-HCl, pH 6.8, 1% SDS, 5% 2-ME, 10% glycerol, 0.001% bromphenol blue) and heated in a boiling water bath for 3 min. Samples (50 μg of total protein from cell extracts) were resolved by SDS-PAGE and proteins were transferred to nitrocellulose membranes. Membranes were blocked with Tween TBS (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.1% Tween 20) containing 2% BSA and probed with the specific primary Abs: polyclonal anti-ERK-2, polyclonal anti-MLCK, biotin-conjugated monoclonal anti-phosphoserine (1:1000), biotin-conjugated monoclonal anti-phosphotyrosine (1:200). After extensive washing in Tween TBS, nitrocellulose sheets were incubated with anti-goat or anti-rabbit IgG Ab biotin-conjugated (1:1000) for 1 h and then incubated with streptavidin-conjugated HRP (1:1000). Immunoreactive proteins were visualized by 3,3'-diaminobenzidine staining and the bands were quantified by densitometry using Scion Image Software.

**Statistical analysis**

Statistical significance was assessed by ANOVA followed by Bonferroni’s t test, and p < 0.05 was taken as statistically significant.

**Results**

ATL-1 induces human monocyte chemotaxis via a G-protein-coupled receptor

Maddox and Serhan (2) demonstrated that native LXA₄ and B₄ are able to stimulate monocyte chemotaxis. In this study, we show for the first time that ATL-1, a 15-epi-LXA₄ stable analog, stimulates human monocyte chemotaxis. A representative assay is shown in Fig. 1A. ATL-1 induced chemotaxis in a dose-dependent manner giving a maximum effect at 100 nM. At this concentration, the effect was similar to that obtained with 100 nM of fMLP, a well-appreciated chemoattractant for monocytes, that was used here as a reference agonist for the purpose of direct comparison (27).
Involvement of the MAPK pathway on ATL-1-induced monocyte chemotaxis

We next investigated the signaling pathways involved in the ATL-1-stimulated monocyte chemotaxis. ATL-1 (100 nM) induced protein tyrosine phosphorylation in monocytes in a time-dependent manner, showing a significant effect already at 1 min, peaking at 10 min, and declining thereafter. Proteins phosphorylated at tyrosine residues corresponding to molecular sizes of 14, 42, and 50 kDa were detected by Western blot analysis using an anti-phosphotyrosine mouse mAb (Fig. 2A). The involvement of tyrosine kinases was confirmed using genistein (100 μM), a general inhibitor of protein tyrosine kinases, which inhibited ~65% of ATL-1-induced monocyte chemotaxis (Fig. 2B).

Because the predominant band (42 kDa) observed with the anti-phosphotyrosine Ab corresponded to the molecular size of the ERK-2, a member of the MAPK family, we next evaluated the role of this kinase on ATL-1-induced monocyte chemotaxis. We performed a study on ERK-2 activation and a chemotaxis assay using PD98059, a specific inhibitor of MEK 1/2. Using an immunoprecipitation assay, cells treated with ATL-1 (100 nM) showed a significantly higher rate of phosphorylated ERK-2 when compared with vehicle-treated cells (Fig. 3A). The phosphorylation levels from ATL-1-stimulated monocytes were not different from those obtained with fMLP (100 nM), used as a positive control. Correlating with chemotaxis results, prior exposure of the cells to PTX (1 μg/ml) for 15 min reduced ERK-2 activation (Fig. 3A), suggesting that ATL-1 actions must be ALXR-dependent. Although it has been reported that incubation of monocytes for 1–2 h with PTX completely abolishes chemokine-induced ERK activation, in our hands, long periods of incubation affected cell viability and consequent responses (28).

To assess the role of ERK activation on monocyte chemotaxis, cells were pretreated for 5 min with PD98059 (10 μM) before stimulation with the analog. ATL-1-induced chemotaxis was impaired in ~60% (Fig. 4), as compared with the ATL-1-stimulated monocytes in the absence of the inhibitor.
ATL-1 induces MLCK phosphorylation in an ERK-2-dependent manner

ERK-2 activation by ATL-1 and its involvement on ATL-1-induced monocyte chemotaxis points to a cytoplasmatic action of this kinase, which prompted us to examine the activation profile of MLCK. The phosphorylation of MLCK, a Ca$^{2+}$/calmodulin-dependent enzyme, is a critical step for cell migration, promoting increased myosin filament formation and leading to its association with actin filaments. In addition, MLCK contains multiple MAPK phosphorylation sites, which could be directly phosphorylated by ERK-2 (29). After exposure of the cells to ATL-1 (100 nM), MLCK phosphorylation was evident when compared with the negative control (Fig. 5). To test the hypothesis that MLCK activation might be downstream of ERK-2 phosphorylation, we studied the MLCK phosphorylation profile using an ERK activation inhibitor.

Pretreatment of the monocytes with PD98059 (10 μM) before ATL-1 (100 nM) stimulation partially reduced MLCK activation. The enhanced phosphorylation of MLCK could not be explained by increased protein expression because immunoblotting experiments revealed that lysates contained the same levels of MLCK.

ATL-1 induces MLCK phosphorylation in a Rho-dependent manner

Several studies have demonstrated that the phosphorylation state of myosin L chain (MLC) can be regulated by the small GTP-binding protein, Rho (19, 20). Rho kinase, one of the target proteins of Rho, is implicated in many downstream processes of Rho. To examine the role of Rho kinase in the ATL-1-induced MLCK activation, we used Y-27632, a specific inhibitor of this kinase. In response to ATL-1 (100 nM) stimulation, a rapid increase in the levels of phosphorylated MLCK was observed after 15 min when

FIGURE 3. ATL-1 induced ERK-2 activation via G-protein-coupled receptor. A, Monocytes were incubated with medium plus 0.05% ethanol (vehicle) and with ATL-1 (100 nM) or fMLP (100 nM) for 5 min at 37°C. The content of phosphorylated ERK-2 was determined by immunoprecipitation using an anti-ERK-2 Ab and immunoblotted with an anti-phosphotyrosine Ab. B, The cellular content of phosphorylated ERK-2 was evaluated in monocytes pretreated with pertussis toxin (1 μg/ml) or DMSO for 15 min followed by stimulation with ATL-1 (100 nM) for 5 min at 37°C. Cell lysates were immunoprecipitated with an anti-ERK-2 Ab and immunoblotted with an anti-phosphotyrosine Ab. Blots were analyzed by densitometry, and the content of phosphorylated ERK-2 was expressed in arbitrary units. The data show mean ± SEM of three similar experiments. *p < 0.05 in comparison with vehicle. **p < 0.05 in comparison with ATL-1 alone.

FIGURE 5. ATL-1 induced MLCK activation in an ERK-2-dependent manner. The cellular content of phosphorylated MLCK was evaluated in monocytes pretreated with PD98059 (10 μM) or DMSO by immunoprecipitation with medium plus 0.05% ethanol (vehicle) or ATL-1 (100 nM) for 15 min at 37°C. Cell lysates were immunoprecipitated with an anti-MLCK Ab and immunoblotted with an anti-phosphoserine Ab. Blots were analyzed by densitometry, and the content of phosphorylated MLCK was expressed in arbitrary units. Results are representative of three similar experiments. *p < 0.05 in comparison with cells nonstimulated with ATL-1. **p < 0.05 in comparison with ATL-1 alone.
ATL-1-induced MLCK phosphorylation is dependent on Rho activation. The cellular content of phosphorylated MLCK was evaluated in monocytes pretreated with Y-27632 (10 μM) followed by stimulation with medium plus 0.05% ethanol (vehicle) or ATL-1 (100 nM) for 15 min at 37°C. Cell lysates were immunoprecipitated with an anti-MLCK Ab and immunoblotted with an anti-phosphoserine Ab. Blots were analyzed by densitometry and the content of phosphorylated MLCK was expressed in arbitrary units. Results are representative of three similar experiments. *, p < 0.05 in comparison with vehicle (Fig. 6). Treatment of the cells with Y-27632 (10 μM) completely abolished ATL-1-induced MLCK phosphorylation. The inhibitor alone had no effect on the MLCK phosphorylation state.

ATL-1-induced ERK-2 phosphorylation is dependent on Rho kinase activation

To investigate whether Rho kinase and ERK-2 were acting concurrently or in a sequential way on MLCK activation, we assessed the phosphorylation state of ERK-2 following treatment of the monocytes with the Rho kinase inhibitor for 15 min. In response to ATL-1 stimulation, as already seen, cells presented an increase in phosphorylated ERK-2, which was abrogated by the treatment with Y-27632 (10 μM) (Fig. 7), indicating that Rho/Rho kinase and ERK-2 could be sequentially activating MLCK.

Using a pharmacological approach, we confirmed a central role for Rho kinase/MLCK on monocyte chemotaxis toward ATL-1. As shown in Fig. 8, treatment of the cells with ML-7 (300 nM), a specific MLCK inhibitor, or with the Rho-kinase inhibitor Y-27632 (10 μM), ablated ATL-1-induced cell migration.

ATL-1 induces actin polymerization in human monocytes

Phosphorylation of MLC by MLCK is a critical regulatory step in myosin function because it promotes myosin ATPase activity and consequent cytoskeleton contraction necessary for cell movement (30, 31). To examine whether ATL-1 was able to induce a rearrangement of the actin cytoskeleton, we measured the intracellular contents of F-actin. ATL-1 induced a marked increase on fluorescence intensity, an indicator of actin polymerization, in a dose-dependent manner, peaking at 100 nM (Fig. 9A). The actin mobilization induced by the analog was completely inhibited by the pretreatment of the cells with Y-27632 (10 μM) (Fig. 9B).

Discussion

Monocyte migration into inflammatory sites is a complex process mediated by a cascade of cellular interactions in which the generation of chemotactic gradients is thought to play a critical role. LX have been shown to stimulate in vitro monocyte chemotaxis but the signaling pathways involved in the regulation of this process are still poorly defined (2, 3). In this study, we have demonstrated that ATL-1 induces human monocyte chemotaxis in a dose-dependent manner, an effect blocked by pretreatment of the cells with PTX, suggesting that the analog effect involves the activation of ALXR, the G-protein linked LXA4 receptor. LXA4 binding to its receptor activates both phospholipase A2 and phospholipase D, responses that are inhibited by treatment of the cells with PTX (3).

Leukocyte migration is highly dependent on the magnitude of Ca2+ mobilization and, in monocytes, LXA4 induces increases in intracellular Ca2+ >50% of that induced by equimolar fMLP, a well-known chemoattractant (32). Both LX and fMLP exert their chemotactic activity by binding to specific receptors, which subsequently activate a number of intracellular signal transduction pathways. To date, the interrelationship between these assorted pathways in mediating the leukocyte chemotactic response...
remains unclear. In the present study, we have shown that ATL-1 induces tyrosine phosphorylation of a number of monocyte proteins in a time-dependent manner. Marked increases in tyrosine phosphorylation were detected as early as 1 min after stimulation with the analog and peaked at 10 min. It is known that the modulatory actions of LX and ATL in transendothelial neutrophil migration can be attenuated by prior exposure of the cells to tyrosine kinase inhibitors (33). Along these lines, we found that ATL-1-stimulated chemotaxis is partially reversed by genistein, a tyrosine kinase inhibitor.

MAPK is a key signaling point at which a number of pathways converge and is involved in a number of physiological phenomena, including apoptosis, cell cycle and gene expression in response to chemokines and adhesion molecules (34–37). Moreover, MAPK is implicated on monocyte chemotaxis and the associated actin cytoskeleton rearrangement (38). Our results show that ATL-1 induces phosphorylation of ERK-2, a member of the MAPK family and this effect is partially inhibited by prior exposure of the cells to PTX, indicating an action via ALXR. This observation is consistent with previous results, which showed that LXA4 induces the activation of ERK-2 and p-38 MAPK in renal mesangial cells (39). Furthermore, distinct peptide ligands and serum amyloid A were also shown to increase ERK-2 phosphorylation through the activation of ALXR/N-formyl-peptide receptor-like-1 (40, 41). Of interest, it has been shown that annexin-1, a peptide that can bind ALXR/N-formyl-peptide receptor-like-1 specifically regulates signaling components of the ERK pathway, resulting in the modulation of biochemical functions in RAW macrophages (42, 43).

MAPK is a key signaling point at which a number of pathways converge and is involved in a number of physiological phenomena, including apoptosis, cell cycle and gene expression in response to chemokines and adhesion molecules (34–37). Moreover, MAPK is implicated on monocyte chemotaxis and the associated actin cytoskeleton rearrangement (38). Our results show that ATL-1 induces phosphorylation of ERK-2, a member of the MAPK family and this effect is partially inhibited by prior exposure of the cells to PTX, indicating an action via ALXR. This observation is consistent with previous results, which showed that LXA4 induces the activation of ERK-2 and p-38 MAPK in renal mesangial cells (39). Furthermore, distinct peptide ligands and serum amyloid A were also shown to increase ERK-2 phosphorylation through the activation of ALXR/N-formyl-peptide receptor-like-1 (40, 41). Of interest, it has been shown that annexin-1, a peptide that can bind ALXR/N-formyl-peptide receptor-like-1 specifically regulates signaling components of the ERK pathway, resulting in the modulation of biochemical functions in RAW macrophages (42, 43).

In a recent work, Kumar et al. (17) presented evidence that ERK-2 activation is necessary to monocyte chemotaxis, and we next confirmed the role of this kinase on ATL-1-induced monocyte chemotaxis using PD98059, a selective and noncompetitive inhibitor of MEK 1/2. This result corroborates previous data demonstrating that MCP-1-induced chemotaxis of human monocytes involves the ERK/MAPK (44, 45). However, these findings differ from other reports where ERK-2 is not involved on monocyte chemotaxis (18, 46).

Activation of the ERK-2 pathway by different chemotactic agents suggest that the kinase action could lead to direct activation of the intracellular motility machinery independent of de novo gene transcription. The phosphorylation of MLC by MLCK is a critical regulatory step in myosin function because it promotes myosin ATPase activity and polymerization of actin cables. This results in a fully functional actin-myosin motor unit involved in generating contractile force necessary for cell motility (30, 31). In our study, ATL-1 increases MLCK phosphorylation in an ERK-2-dependent fashion, suggesting that MLCK could be a downstream step of the MAPK pathway in monocytes. We, then,
propose that ERK-2 could directly phosphorylate MLCK, increasing its ability to phosphorylate MLC, which promotes cytoskeleton contraction necessary for cell movement. In accordance with our data, Klemke et al. (47) have also reported that ERK phosphorylates and thereby activates MLCK, promoting cellular migration. Also, MLCK functions downstream of Ras/ERK have been shown to promote migration of urokinase-type plasminogen activator-stimulated cells (48).

The Rho family of small GTPases, Rho, Rac, and Cdc42, plays a major role in regulating cytoskeleton dynamics and cell motility in response to external stimuli (49, 50). In this work, we found that a Rho kinase inhibitor abrogated both MLCK phosphorylation and chemotaxis stimulated by the LX analog, indicating that the Rho/Rho kinase pathway is implicated on ATL-1-induced monocyte chemotaxis via MLCK. Interestingly, while MLCK phosphorylation was completely abrogated by the treatment with Y-27632, an ERK-2 inhibitor only partially inhibited the kinase activation, implying that MLCK phosphorylation via the MAPK pathway could be a secondary route. The total inhibition of ERK-2 activation by the Rho-kinase inhibitor suggested that the MAPK pathway is downstream of Rho/Rho kinase in MLCK activation induced by ATL-1. These results point to a central role of Rho/Rho kinase on ATL-1-induced monocyte chemotaxis.

Activation of Rho is associated with phosphorylation of MLC, which might result from activation of MLCK downstream of Rho kinase or from inhibition of a MLC phosphatase (22, 51). Members of the Rho family serve as universal regulators of the actin cytoskeleton, linking extracellular signals to changes in cell shape and movement. In addition, Rho family members can activate MAPK signaling pathways, functioning as regulators of cytoskeleton remodeling and gene expression.

The driving force for the motile activities of leukocytes (chemotaxis and phagocytosis) is generated by dynamic alterations of the actin filament system (52). Thus, different chemotactic stimuli can induce extensive polymerization of actin filaments. Consequently, a functional actin cytoskeleton is necessary for monocytes to perform properly in the inflammatory process. In this study, we show that treatment of human monocytes with ATL-1 induces evident alterations in the actin network, with an increase on F-actin content. Our results corroborate a recent study demonstrating that LX4 analogs and analogs were able to induce Rho- and Rac-dependent actin filament reorganization in monocytes and macrophages but not in neutrophils (53). The involvement of the Rho/Rho kinase pathway on ATL-1-induced actin polymerization was confirmed by the use of a Rho kinase inhibitor.

LXs are a unique class of chemoattractants due to particular characteristics such as Ca\textsuperscript{2+} mobilization and stimulation of monocyte chemotaxis without impact on cell-mediated cytotoxicity and generation of reactive species. Cell migration results from significant changes in the reorganization of the actin cytoskeleton inducing the promotion of cytoplasmatic extensions and formation of pseudopodia. Moreover, the process of phagocytosis is highly dependent on the localized polymerization of actin filaments that facilitate the formation of filopodia surrounding the cells or microorganisms to be engulfed (54). It was recently reported that LX rapidly stimulate the nonphlogistic phagocytosis of apoptotic neutrophils by monocyte-derived macrophages (12). Such phagocytic clearance without the provocation of an inflammatory response corroborates the first results from Maddox et al. (2, 3) indicating that LX play an essential role in the resolution of inflammation.

In summary, we have demonstrated that ATL-1 is a potent monocyte chemotactic agent, acting via ALXR, and using the Rho kinase/MLCK pathway to exert its biological function (Fig. 10). LX and its analogs inhibiting several neutrophil functions and stimulating a self-limiting monocyte migration contribute to the resolution phase of the inflammatory response. Our data provide a better comprehension of the exact mechanisms involving the anti-inflammatory action of LX analogs and may lead to new anti-inflammatory therapies.

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Disclosures

The authors have no financial conflict of interest.

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