12/15-Lipoxygenase Translocation Enhances Site-specific Actin Polymerization in Macrophages Phagocytosing Apoptotic Cells*

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The enzyme 12/15-lipoxygenase (12/15-LO) introduces peroxyl groups in a position-specific manner into unsaturated fatty acids in certain cells, but the role of such enzymatic lipid peroxidation remains poorly defined. Here we report a novel function for 12/15-LO in mouse peritoneal macrophages. When macrophages were coincubated with apoptotic cells, the enzyme translocated from cytosol to the plasma membrane and was more extensively concentrated at sites where macrophages bound apoptotic cells, colocalizing with polymerized actin of emerging filopodia. Disruption of F-actin did not prevent the 12/15-LO translocation. In contrast, inhibition of the 12/15-LO activity, or utilization of genetically engineered macrophages in which the 12/15-LO gene has been disrupted, greatly reduced actin polymerization in phagocytosing macrophages. Lysates of 12/15-LO-deficient macrophages had significantly lower ability to promote in vitro actin polymerization than the lysates of wild type macrophages. These studies suggest that the 12/15-LO enzyme plays a major role in local control of actin polymerization in macrophages in response to interaction with apoptotic cells.

12/15-Lipoxygenase (LO) is a member of the LO family of enzymes that insert peroxyl groups into double bonds of free and phospholipid-bound polyunsaturated fatty acids. The exact role of these enzymes in biological processes has remained elusive, but increasingly evidence has accumulated that they play important roles in specific cellular functions. For example, 15-LO activity in reticulocytes at the stage of organelle degradation may contribute to membrane destabilization and contribute to pore formation in intracellular membranes (1, 2). Fatty acid products of 12/15-LO are powerful agonists for the nuclear receptor PPAR-γ, which helps regulate glucose metabolism and adipocyte and macrophage differentiation and function (3). A remarkable feature of 12/15-LO is that its expression is not constant during the cell life span but rather turns on at certain points during cell development. While circulating human monocytes do not express 15-LO, monocyte-derived macrophages exposed to interleukin-4 or interleukin-13 express 15-LO (4, 5). In addition, mouse macrophages residing for a long time in the peritoneum (resident macrophages) also highly express the mouse homologue, 12/15-LO, although the pathway leading to 12/15-LO expression may differ somewhat from that which occurs with human monocytes (6).

Macrophages of atherosclerotic lesions express high levels of 15-LO (7), and recent evidence utilizing apoE−/− mice in which the 12/15-LO gene was disrupted demonstrated its importance in the pathogenesis of atherosclerosis (8). Another characteristic of atherosclerotic tissue but not of normal vascular wall is the high abundance of apoptotic cells (9). This fact might reflect either an increased rate of formation of such cells, a decreased rate of clearance, for example by arterial macrophages, or both. In either case, phagocytosis and the degradation and metabolism of the ingested contents of dying cells are crucial for preventing the release of toxic cellular compounds and consequent inflammation. Inhibition of efficient phagocytosis would presumably lead to the accumulation of pro-inflammatory necrotic debris, plaque instability, and thrombogenesis.

During the complex process of phagocytosis, major changes in the cytoskeleton of the cell occur leading to the formation of filopodia surrounding an apoptotic cell or a microorganism to be engulfed. Changes in actin polymerization play a vital role in this process. Remarkably, 12-LO products are found in many tumor cells and have been suggested to have effects on actin polymerization (10) and cytoskeleton reorganization during cell transformation (10, 11). Therefore, it was tempting to propose that the activity of 12/15-LO in non-malignant cells, such as macrophages, was also related to a cytoskeleton function and to phagocytosis. Indeed, we now demonstrate that upon exposure to apoptotic cells, 12/15-LO translocates from the cytosol to sites of apoptotic cell binding and furthermore that actin polymerization itself is dependent on activity of 12/15-LO.

EXPERIMENTAL PROCEDURES

Cell Culture and Materials—Peritoneal macrophages were harvested from 8- to 10-week-old female mice, either Swiss Webster or C57BL/6 strains. The latter were strain-, age-, and sex-matched to 12/15-LO knockout mice. Resident or thioglycollate-elicited macrophages were plated in RPMI 1640 (BioWhittaker) supplemented with 10% fetal bovine serum (FBS) (Omega Scientific). Murine fibroblast cell lines overexpressing either human 15-LO (clone 12) or β-galactosidase (LacZ) were cultured in Dulbecco’s modified Eagle’s medium (BioWhittaker) with 10% FBS and 0.2 mg/ml G418 (Calbiochem) to maintain selection (12). Thymocytes were harvested from the thymuses of 4-week-old mice of the same strain as used for macrophage isolation and treated with 1 μM dexamethasone in 10% FBS/RPMI 1640 for 4 h to induce apoptosis (13). The appearance of condensed nuclei was a marker for apoptosis. We previously demonstrated that concentrations up to 20 μM of the specific lipooxygenase inhibitor PD 146176 (a gift from J. Cornicelli of...
Parke-Davis® were non-toxic for macrophages (14). Cytochalasin D was from Sigma, and Iatruclina A was from Molecular Probes. 13(S)-Hydroxyoctadecadienoic acid (13(S)-HODE), 15(S)-hydroxyeicosatetraenoic acid (15(S)-HETE), 12(S)-hydroxyeicosatetraenoic acid (12(S)-HETE), and linoleic acid were from Cayman Chemical.

Western Blot—Cells were lysed on the plate with 5% SDS in phosphate-buffered saline. Protein content was determined with a BCA kit (Pierce), and 15–100 μg of the cell lysate was run on a precast 4–12% gradient polyacrylamide gel (Novex) and then transferred to a nitrocellulose membrane (Millipore). The membrane was blocked with 5% non-fat milk and incubated with a protein A-purified polyclonal guinea pig anti-rabbit 15-LO antibody (7). This antibody cross-reacts not only with human 15-LO (7) but also with mouse 12/15-LO; it stained the band well for 12/15-LO. It did not yield any such band when lysates from 12/15-LO−/− mice were used. Guine pig preimmune IgG produced no specific staining.

Immunocytochemistry and Imaging—Macrophages plated overnight on coverslips were fixed with 3.7% paraformaldehyde for 10 min at 37 °C, permeabilized with 0.2% Triton X-100 for 5 min, blocked with 0.8 μg/ml Fc block (PharMingen) in 5% non-fat milk, 0.2% Triton X-100, stained for 30 min with the guinea pig anti-rabbit 15-LO antibody and for another 30 min with a rhodamine red-X-conjugated Fab′ fragment donkey anti-guinea pig Ig G (H + L) antibody (Jackson ImmunoResearch). Alternatively, a guinea pig anti-rabbit ApoA1 antibody was used as a negative control. Filamentous actin (F-actin) was stained by addition of 1.5 μM FITC-conjugated phallolidin (Sigma) to the solution of the secondary antibody. Cytosol was labeled by incubation of live cells with 0.5 μM, 5-chloromethylfluorescein diacetate (CellTracker green CMFDA from Molecular Probes) in serum-free medium for 30 min followed by a 30-min incubation in the regular culture medium. This green staining remained in fixed cells. Cell nuclei were stained blue with 1 μg/ml Hoechst 33258 (Sigma) for 15 min. The coverslips were mounted on microscopic glass slides with ProLong antifade medium (Molecular Probes). Images were captured by deconvolution microscopy (15) using a DeltaVision deconvolution microscopy system operated by SoftWorx software (Applied Precision). Pixel intensities were kept in the range of the digital image camera. Optical sections through the samples were taken with increments of 0.2–0.5 μm depending on magnification. The images were deconvolved and examined either section by section or volume views were generated by combining areas of maximal intensity of each optical section with SoftWorx programs. Data Inspector application was used to quantitatively analyze the images. Adobe Photoshop 6.0 software was used to design figures.

Flow Cytometry—The relative content of F-actin in macrophages active in phagocytosis was assessed by image cytometry as described in Ref. 16 with some modification. In brief, at the end of incubation of the plated macrophages with apoptotic thymocytes, 1 volume of the solution containing 1.6 μM FITC-phallolidin, 18% paraformaldehyde, and 0.8% saponin (all from Sigma) was added to 3 volumes of the culture medium and incubated for an additional 10 min. Cells were then washed, scraped from the plate, filtered through a Nitek nylon mesh (Sefar America), and analyzed on a FACSscan (Becton Dickinson).

To examine the expression of cell-specific CD markers, the cells attached to the plate were gently scraped, incubated in suspension for 30 min with either a FITC-conjugated anti-CD80 antibody, a FITC-conjugated anti-CD3 antibody, or a phycoerythrin-conjugated anti-CD19 antibody (all from PharMingen), washed, and analyzed on the FACSscan.

Actin Polymerization—Assays were performed as described previously (17). This assay is based on the measurement of fluorescence intensity of pyrene covalently linked to actin, which increases when actin polymerizes. In brief, unlabeled and pyrene-labeled monomeric G-actin from rabbit muscle (kindly provided by K. Aman from the Salk Institute) at the ratio of 95:5 were diluted in G-buffer (2 mM Tris, pH 8.0, 0.2 mM ATP, 0.1 mM CaCl₂, and 0.5 mM dithiothreitol) and then converted to Mg-actin by adding 0.1 volume of 10 mM EGTA and 1 mM MgCl₂. Polymerization was initiated by addition of either macrophage lysates or 0.1 volume of 10× KMEI (500 mM KCl, 10 mM MgCl₂, 10 mM EGTA, and 100 mM imidazole, pH 7.0). Lysates were prepared from macrophages scraped from the plate in a lysis buffer (2 mM Tris, pH 8.0, 1 mM EGTA, 0.2 mM MgCl₂, and protease inhibitors mixture (Sigma)) by sonication and centrifugation at 10,000 x g for 30 min. Protein concentration was measured using a BCA kit from Pierce. Spectra and time courses of pyrene fluorescence were measured on an LS50B luminescence spectrophotometer (PerkinElmer Life Sciences).

RESULTS

12/15-LO Expression and Phagocytic Activity of Elicited Macrophages—When non-septic inflammation in mice is induced by intraperitoneal injection of thioglycollate, many monocytes are recruited to the peritoneum, where they differentiate into macrophages. Initially, these newly recruited, “elicited” macrophages express very little 12/15-LO. This was evident from Western blots of cell lysates made from elicited and resident macrophages. Relative to total cell protein, there was 10–15-fold less 12/15-LO expressed in the elicited macrophages as compared with the enzyme content in resident macrophages (data not shown). Immunocytochemical examination of the elicited macrophage population revealed two cell populations, either positive or negative for 12/15-LO staining (Fig. 1a). The 12/15-LO-positive cells (less than 10% of total) presumably originated from resident macrophages. The 12/15-LO-negative cells were probably newly recruited monocyte-macrophages that did not yet express the enzyme. This observation is in agreement with an earlier report on heterogeneity of elicited macrophages from immunodeficient mice (6).

To ensure that the thioglycollate-elicited cells that attached to the plate overnight were indeed macrophages and not other cell types, these cells were analyzed by flow cytometry for the presence of cell-specific markers. Ninety eight percent of the attached cells were positive for the macrophage marker CD80 and negative for the T-cell marker CD3 and the B-cell marker CD19. (Splenocytes, a mixed population of all the three cell types, were used as positive controls.) Thus, the majority of the plate-attached cells harvested from peritoneum were macrophages, and the difference in the 12/15-LO expression is probably a function of the stage of macrophage differentiation.

When apoptotic thymocytes were incubated with the elicited macrophage population, we noted a striking difference between 12/15-LO-positive and -negative cells in the ability to bind and engulf apoptotic thymocytes (Fig. 1a). Counting multiple microscopic fields confirmed that following a 15-min incubation, the 12/15-LO-expressing macrophages bound 20 times more apoptotic cells than the 12/15-LO-negative cells (cross-hatched columns in Fig. 1b). This ability of the 12/15-LO-positive cells to bind apoptotic cells was significantly reduced when the elicited macrophages were pretreated with the specific 12/15-LO inhibitor PD 146176 (18). The effect of PD 146176 was dose-dependent. A statistically significant decrease in apoptotic cells binding to 12/15-LO-positive elicited macrophages was observed already at 0.5 μM PD 146176. This result corresponds well to the previously reported IC₅₀ values of 0.8 μM for the PD 146176 inhibition of 15-LO activity in cell culture (19). Following a 1-h incubation, when most of the apoptotic thymocytes were already engulfed by the macrophages, the same tendency was observed (black columns in Fig. 1b). The correlation between the phagocytic function of elicited macrophages and the 12/15-LO activity suggests a role for 12/15-LO in phagocytosis.

Translocation of 12/15-LO in Phagocytosing Macrophages—To explore further a potential relationship of 12/15-LO to phagocytosis, we examined the localization of 12/15-LO and F-actin in elicited macrophages, resting or phagocytosing apoptotic thymocytes. Nearly all (more than 95%) resident macrophages expressed 12/15-LO. In resident macrophages not exposed to apoptotic cells (Fig. 2a, a volume view, Fig. 2b, a 2-fold magnified optical section), 12/15-LO protein (red) was evenly distributed throughout the cytosol and did not colocalize with F-actin (green) at the cell surface. The three-dimensional intensity graphs below Fig. 2b document the very different distributions of 12/15-LO and F-actin in the highlighted area. In contrast, in resident macrophages exposed to apoptotic thymocytes, 12/15-LO concentrated on the cell surfaces in general, and this
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was greatly enhanced at the sites where apoptotic cells were bound (Fig. 2, c and d). To show that the translocation of 12/15-LO toward the bound apoptotic cell at the periphery of the macrophage was specific and not just following general movement of cytosol, we labeled cytosol green with CellTracker, a dye that evenly binds to thiol groups in the cell (20). The presence of 12/15-LO on the cell surface (red color) close to attached apoptotic thymocytes but the absence of the yellow color (that would have represented colocalization with CellTracker) suggest the specificity of the 12/15-LO translocation (Fig. 2, c and d). Accordingly, the intensity graphs below Fig. 2d show a different distribution of 12/15-LO and CellTracker in the highlighted area.

Fig. 3 presents another example in which a resident macrophage is in close or partial contact with four different apoptotic cells simultaneously. Fig. 3b demonstrates staining for 12/15-LO (red) and Fig. 3c staining for F-actin (green). Fig. 3a is a merged view where the yellow color demonstrates 12/15-LO colocalization with the sites of actin polymerization. The yellow color is clearly more heavily concentrated in the vicinity of bound apoptotic cells. Quantification of the effect is provided by intensity maps of the whole cell (derived from the volume view) for each color. In general, the concentrations of 12/15-LO and F-actin on the surface of macrophagophagocytosing apoptotic cells were 3–6-fold higher than in resting macrophages (compare intensity scales in Figs. 2 and 3). This supports the generalized movement of both to the cell periphery. However, the intensity graph for 12/15-LO (below Fig. 3b) also demonstrates increased 12/15-LO concentration at the sites of apoptotic cell binding, as compared with either cytosol or even other sites of the periphery of the cell. Specific 12/15-LO intensity in the areas of cell-cell contact was 3.11 ± 0.54/voxel as compared with 1.49 ± 0.73/voxel in all the rest of the cell perimeter (p < 0.001). The intensity maps derived from the highlighted area of a specific focal plane (below Fig. 3, e and f) show nearly identical patterns of 12/15-LO and F-actin distribution in the area of contact with an apoptotic cell, confirming their specific colocalization.

12/15-LO Translocation in Macrophages with Disrupted F-actin—We next examined the relationship between activity of 12/15-LO and F-actin function. To approach this question, we inhibited either the activity of 12/15-LO or the process of actin polymerization. Formation of F-actin was inhibited by treating macrophages with either cytochalasin D or latrunculin A, toxins that bind monomeric G-actin, thereby preventing formation of filamentous F-actin (21, 22). The images in Fig. 4, a–c show a non-treated resident macrophage caught in the process of phagocytosis of an apoptotic thymocyte. 12/15-LO has translocated to the surface where it appears to be interacting with the apoptotic cell. This same site has also been greatly enriched by 12/15-LO (see black-white images in b and c, and intensity graphs below). Again, although there is clear translocation of the 12/15-LO to the periphery of the cell, a formal analysis of the intensity maps shows 12/15-LO-specific intensity of 5.40 ± 0.73/voxel at the site of apoptotic cell binding versus 2.40 ± 1.16/voxel in the rest of the cell perimeter (p < 0.001) providing additional evidence that 12/15-LO translocation is concentrated at sites of contact with apoptotic cells.

The cytochalasin D treatment partially disrupted the actin polymerization (Fig. 4, d and f). Nevertheless, 12/15-LO translocation toward the bound apoptotic thymocyte did not seem to be impaired (Fig. 4, d and e). Latrunculin A treatment almost completely disrupted F-actin formation (Fig. 4, g and i), and even under these severe conditions 12/15-LO translocation to sites of apoptotic cell binding occurred (Fig. 4, g and h). These data suggest that actin polymerization is not a prerequisite for 12/15-LO translocation.

Actin Polymerization in Macrophages with Inhibited or Disrupted 12/15-LO—Can 12/15-LO activity in turn affect the process of actin polymerization? In order to assess the level of polymerized actin in individual cells, we used a flow cytometry assay as described under “Experimental Procedures.” A shift of the cell distribution histogram to the area of higher fluorescence intensity (e.g. to the right) reflects an increase in the level of F-actin. Such a shift was observed in resident macrophages in response to incubation with apoptotic thymocytes (Fig. 5, a and c). It was not due to the F-actin of internalized apoptotic thymocytes because the latter did not show any F-actin signal by flow cytometry (green histograms barely seen in left bottom corners of Fig. 5, a and c), and no F-actin staining was observed.
microscopically in the apoptotic cells (Figs. 1–4). The F-actin response usually reached its peak in 10–20 min and then disappeared 40–60 min after the start of incubation (data not shown). Macrophages harvested from Swiss Webster mice (Fig. 5a) generally responded with a higher level of polymerized actin than macrophages from C57BL/6 mice (compare Fig. 5, a

**Fig. 2. Translocation of 12/15-LO in phagocytosing resident macrophages.** Images in a and c represent volume views generated by combining areas of maximal intensity of each optical section, whereas those in b and d are representative focal planes from the middle of the cells (zoom × 2). a and b, resident macrophage without any treatment. c and d, resident macrophages incubated for 15 min with apoptotic thymocytes. Cells were fixed and stained to visualize 12/15-LO (red), nuclei (blue), and either F-actin (green) (a and b) or overall cytosol with the CellTracker label (green) (c and d). Bar, 5 μm. The image areas enclosed in white rectangular frames were quantitatively analyzed separately for 12/15-LO and either F-actin or CellTracker staining and shown below the images. The color spectrum chart from blue to red shows change in the intensity of each fluorophore from low to high. Note an order of magnitude difference in the intensity of 12/15-LO staining in the cytosol of the resting macrophage (below b) and on the surface of the macrophage with bound apoptotic cells (below d).
FIG. 3. Colocalization of 12/15-LO and F-actin in phagocytosing resident macrophages. Resident macrophages were incubated for 15 min with apoptotic thymocytes. The upper row of images (a–c) represents a volume view, and the lower row (d–f) shows a focal plane, as explained in the legend to Fig. 2. Merged red (12/15-LO), green (F-actin), and blue (nuclei) colors are shown in a and d. The spots of red and green voxels colocalizing in the volume view and pixels in the focal plane appear as yellow on the images. Separated red (b and e) and green (c and f) colors show specific concentration of 12/15-LO and F-actin, respectively, in the vicinity of bound apoptotic cells. Three-dimensional intensity maps below the upper row of images quantify overall distribution of nucleic acid, 12/15-LO, and F-actin in the cell. The color spectrum charts from blue to red show change in the intensity of each fluorophore from low to high. Intensity maps at the bottom of the figure show intensities of nucleic acid, 12/15-LO, and F-actin staining in the highlighted area of the focal plane. Similarity in the intensity maps under e and f for 12/15-LO and F-actin, respectively, represents their colocalization. Note also a 3–4-fold difference in the intensity of F-actin staining in the resting macrophage (Fig. 2, below b) and the macrophage with bound apoptotic cells (Fig. 3, below c and f).
versus c). Treatment of the resident macrophages with the 12/15-LO inhibitor PD 146176 prior to addition of the apoptotic cells blocked actin polymerization in the macrophages (Fig. 5b). Finally, in macrophages harvested from 12/15-LO knockout mice (12/15-LO−/−), no change in the F-actin content in response to addition of apoptotic cells was observed (Fig. 5d).

Effect of Macrophage Lysates and 12/15-LO Products on Actin Polymerization in Vitro—The next set of experiments directly examined the effect of the products of 12/15-LO on actin polymerization, using an in vitro polymerization assay in the presence or absence of cell lysates. In the first set of experiments, actin polymerization was initiated by addition of cell lysates (Fig. 6a). Whole cell lysates prepared from the 12/15-LO knockout macrophages had a limited ability to promote in vitro polymerization of G-actin (Fig. 6a, dotted line). In contrast, lysates from wild type macrophage had much higher nucleating and elongating activities as seen by a shorter lag phase before the start of elongation and an increased rate of elongation (a 130 ± 27% increase, p < 0.001; Fig. 6a, solid line). Remarkably, addition of 13(S)-HODE (the oxidation product of linoleic acid) to the 12/15-LO−/− lysates significantly increased the elongation rate but did not affect the lag phase, indicating that 13(S)-

**Fig. 4.** Cellular localization of 12/15-LO in macrophages with disrupted actin polymerization. Resident macrophages incubated with apoptotic thymocytes for 15 min without prior treatment (a–c) or with pretreatment with either 150 nM cytochalasin D for 1 h (d–f) or 10 μM latrunculin A for 15 min (g–i). Cells were fixed and stained for 12/15-LO (red), nuclei (blue), and F-actin (green). Merged three-colored volume view images are shown in a, d, and g. Black and white images in b, e, and h represent staining for 12/15-LO only, and the images in c, f, and i show staining for F-actin only. Bar, 5 μm. Intensity maps below the 1st row of images quantify cell distribution of nucleic acid, 12/15-LO, and F-actin. The color spectrum charts from blue to red show change in the intensity of each fluorophore from low to high. Note that an intense nucleic acid staining of an apoptotic cell on the left-hand side of the intensity map ends at the 7–8th mesh quadrangle, the place where an intense macrophage staining for 12/15-LO and F-actin begins.
HODE does not have a nucleating activity (Fig. 6a, dashed line). The same positive effect of 13(S)-HODE on actin elongation but not nucleation was also observed in an in vitro assay conducted in the absence of cell lysates, when actin polymerization was initiated by addition of KMEI instead of cell lysate (a 50% increase, p < 0.01; Fig. 6b, solid line). Preincubation of G-actin with non-oxidized linoleic acid did not show any sizable difference from the ethanol vehicle (Fig. 6b, dashed and dotted lines). Because light scattering from cell lysates could have interfered with fluorescence measurements, emission spectra were recorded at the beginning and the end of the time courses. A multipeak analysis of a difference spectrum (Fig. 6a, inset) shows a peak of 405.5 nm, which is fairly close to the peak of pyrene emission (406.8 nm) from F-actin in lysate-free samples (Fig. 6b, inset). Similar results were also observed in experiments with the products of 12/15-LO oxidation of arachidonic acid, 15(S)-HETE and 12(S)-HETE, although the effects were not as pronounced (data not shown).

Spreading of 12/15-LO Overexpressing Fibroblasts—To determine if the relationship between LO activity and F-actin formation could also be observed in another cell type, we also examined spreading of fibroblasts, another function dependent on actin polymerization. For these studies we used murine cell lines stably overexpressing either human 15-LO (clone 12) or β-galactosidase (LacZ, control cells) (12). Plated clone 12 cells spread much faster than LacZ cells, and the presence of the 15-LO inhibitor PD 146176 in the medium delayed spreading of the clone 12 cells (Fig. 7). These observations complement the hypothesis that 12/15-LO stimulates actin polymerization and that the functions of 12/15-LO may be more pleiotropic than only assisting macrophage phagocytic function.

FIG. 6. Effect of macrophage lysates and 13(S)-HODE on in vitro actin polymerization. Actin polymerization was registered as an increase in fluorescence intensity of pyrene-actin at excitation/emission wavelengths of 365/407 nm. a, lysates of either wild type (WT) or 12/15-LO knockout (KO) macrophages were added at the final protein concentration of 50 μg/ml to 2.5 mM of G-actin (5% pyrene-actin). In some experiments, KO lysates were preincubated with 6 μM 13(S)-HODE on ice for 30 min and then mixed with G-actin. Lysis buffer replacing cell lysates served as a control. Inset to a shows a difference emission spectrum (excitation at 365 nm) of F-actin at the end of the time course relative to G-actin at its beginning in the KO lysate supplemented with 13(S)-HODE. Multipeak analysis (dashed lines) reveals a longer wavelength emission maximum of 405.6 nm. b, polymerization of 3 μM actin (5% pyrene-actin) preincubated with either 0.7% ethanol (vehicle), 6 μM 13(S)-HODE or 6 μM linoleic acid was initiated by addition of KMEI (see “Experimental Procedures”). Control shows a reaction without addition of KMEI. Inset to b shows emission spectra of the samples with 13(S)-HODE (trace 1) and ethanol (trace 2) at the end of the time course and the spectrum of G-actin (trace 3) prior to addition of KMEI. Representative spectra and time courses from three experiments are shown.

DISCUSSION

Many lines of evidence suggest an important role for 15-LO (and its 12/15-LO homologue in mice) in atherogenesis (7, 8, 12,
move pro-inflammatory oxidized lipids and cellular debris from the extracellular space), one might speculate that normally 12/15-LO acts as anti-inflammatory enzyme, both by supplying activating ligands for PPAR-γ and by promoting actin polymerization and phagocytosis.

We assume that the 12/15-LO involvement in phagocytosis requires the enzyme translocation. Binding of apoptotic cells to macrophages induces translocation of 12/15-LO from cytosol to the cell membrane and an enhanced concentration at sites where apoptotic cells are bound (Figs. 2 and 3). 12/15-LO is generally considered to be a cytosolic enzyme (30). It lacks obvious membrane binding domains. At the same time, a substrate for 12/15-LO is fatty acids and phospholipids, the components of lipoproteins and membranes. A recently presented three-dimensional structure of rabbit 15-LO (31) revealed the presence of an N-terminal β-barrel domain that has a high homology with that of lipases. A function of this domain in both enzymes is probably binding to their lipophilic substrates. A similar N-terminal β-barrel domain in 5-LO has been reported to bind calcium and mediate calcium stimulation of enzyme activity (32, 33). The translocation of 15-LO from cytosol to intracellular and plasma membranes has been also described in reticulocytes and human monocytes and also shown to be dependent on Ca\(^{2+}\) concentration (34). Moreover, the membrane-bound 15-LO was more active than the cytosolic enzyme. Indeed, a Ca\(^{2+}\) influx has been observed during the process of macrophage phagocytosis (35). It is possible that one mechanism by which Ca\(^{2+}\) influx affects phagocytosis is by mediating 12/15-LO translocation and its effect on actin polymerization.

The data presented in Figs. 1–3 were reproducible in independent experiments.

FIG. 7. Spreading of clone 12 and LacZ fibroblasts in culture. a and b, in this example 15-LO-overexpressing clone 12 (a) and β-galactosidase-overexpressing LacZ (b) fibroblasts were fixed and stained for F-actin 90 min after plating. Bar, 30 μm. c, a 10-h time course of cells spreading. ■, clone 12; ○, LacZ; ▲, clone 12 in the presence of 1 μM of the 15-LO inhibitor PD 146176 (PD). Data are mean ± S.D. of three independent experiments.

18, 19, 23–26). Inhibitors of 12/15-LO decrease the ability of murine macrophages to oxidize LDL (14, 23), and murine fibroblasts transfected with human 15-LO have a greatly enhanced ability to initiate oxidation of LDL (12). Both human and rabbit atherosclerotic lesions express 15-LO mRNA, protein, and enzymatic activity (7, 24–26). Importantly, inhibitors of 15-LO decreased atherogenesis in rabbits (18, 19), and deleting 12/15-LO activity in apo E\(^{-/-}\) mice dramatically decreased atherogenesis (8). These data support an important pathophysiological role for 12/15-LO in atherogenesis. However, the physiological role of 15-LO remains unclear. In reticulocytes it appears to play an important role in degradation of organelles as the cell matures, although no abnormality in red blood cell development was noted in the 12/15-LO \(^{-/-}\) mice (27). Its physiological role in macrophages has remained equally obscure. Recently, our laboratory demonstrated an important role for 12/15-LO in providing activating ligands for nuclear receptor PPAR-γ (3), which appears to be an anti-inflammatory and anti-atherogenic factor (28, 29).

The present work demonstrates another novel function of 12/15-LO in macrophages, namely its ability to regulate actin polymerization and presumably phagocytosis of apoptotic cells. In the heterogeneous population of elicited macrophages, only 12/15-LO-expressing cells were efficient in binding and engulfment of apoptotic cells (Fig. 1), and this was associated with translocation of 12/15-LO from cytosol to the periphery of the cell where it concentrated at sites of binding of apoptotic cells (Figs. 2–4). These data suggest that receptor-mediated signaling leads to 12/15-LO translocation and involvement in actin polymerization, in preparation for phagocytosis. It is conceivable that the presence of 12/15-LO in the filopodia could contribute to the oxidation of unsaturated lipids in particles that are being ingested, leading to the engagement of various scavenger receptors and a more efficient phagocytosis. As well, initial steps of the degradation of the ingested particle may be initiated. Because phagocytosis of apoptotic cells and debris (as well as oxidized LDL) is very likely to be a normal homeostatic mechanism to dampen an inflammatory response (e.g. to re-
increase the filament elongation rate (Fig. 6b), in the same manner as it was observed in lysate-free polymerization experiments (Fig. 7).

It has been reported that spreading of HeLa cells depends on lipoxygenase products of arachidonic acid (38). Our data showing a higher rate of spreading of 15-LO-overexpressing fibroblasts as compared with control β-galactosidase-expressing fibroblasts (Fig. 7) support that report and also suggest that 12/15-LO plays a more general function in cytoskeleton regulation in many cell types rather than only in activated macrophages. Indeed cytoskeletal rearrangement of B16a melanoma cells also has been suggested to be dependent on 12(S)-HETE (11). Addition of exogenous 12(S)-HETE induced actin polymerization in B16a melanoma cells responding to chemotractants (10). The authors believe that the effect is mediated by protein kinases. Indeed, preliminary data from our laboratory show that the addition of exogenous 12(S)-HETE can activate endogenous lipoxygenase in murine macrophages and further promote actin polymerization (data not shown). These data would be compatible with the known ability of fatty acid hydroperoxides to activate 12/15-LO (39). Thus, the present studies support a possible signaling role for endogenous 12/15-LO. The concentration of the 12/15-LO itself at the sites that are targets for actin polymerization suggests that the enzyme or its fatty acid hydroperoxide products might be involved in actin signaling. A contemporary view on the filopodia that are targets for actin polymerization (data not shown). These data show that the addition of exogenous 12(S)-HETE in vitro increases the filament elongation rate (Fig. 6b), in the same manner as it was observed in lysate-free polymerization experiments (Fig. 7).

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