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A Stable Aspirin-Triggered Lipoxin A₄ Analog Blocks Phosphorylation of Leukocyte-Specific Protein 1 in Human Neutrophils

Taisuke Ohira, Gerard Bannenberg, Makoto Arita, Minoru Takahashi, Qingyan Ge, Thomas E. Van Dyke, Gregory L. Stahl, Charles N. Serhan, and John A. Badwey

Lipoxins and their aspirin-triggered 15-epimers are endogenous anti-inflammatory agents that block neutrophil chemotaxis in vitro and inhibit neutrophil influx in several models of acute inflammation. In this study, we examined the effects of 15-epi-16-(p-fluoro)-phenoxy-lipoxin A₄ methyl ester, an aspirin-triggered lipoxin A₄-stable analog (ATLa), on the protein phosphorylation pattern of human neutrophils. Neutrophils stimulated with the chemoattractant fMLP were found to exhibit intense phosphorylation of a 55-kDa protein that was blocked by ATLa (10–50 nM). This 55-kDa protein was identified as leukocyte-specific protein 1, a downstream component of the p38-MAPK cascade in neutrophils, by mass spectrometry, Western blotting, and immuno-precipitation experiments. ATLa (50 nM) also reduced phosphorylation/activation of several components of the p38-MAPK pathway in these cells (MAPK kinase 3/MAPK kinase 6, p38-MAPK, MAPK-activated protein kinase-2). These results indicate that ATLa exerts its anti-inflammatory effects, at least in part, by blocking activation of the p38-MAPK cascade in neutrophils, which is known to promote chemotaxis and other proinflammatory responses by these cells. The Journal of Immunology, 2004, 173: 2091–2098.

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Lipoxins are endogenous anti-inflammatory lipid mediators that promote the resolution of inflammation. Biosynthesis of these lipid mediators in humans requires cell-cell interactions and is highly influenced by the presence of certain cytokines and aspirin (reviewed in Refs. 1–3). Monocytes, eosinophils, and airway-epithelial cells can convert arachidonate into 15S-hydroxycicosatetraenoic acid (15S-HETE) by a 15-lipoxygenase catalyzed reaction. 15S-HETE is rapidly taken up by neutrophils and converted to lipoxin A₄ (LXA₄) by a 5-lipoxygenase catalyzed reaction. Of interest, acetylation of cyclooxygenase 2 by aspirin alters the activity of this enzyme to catalyze conversion of arachidonate to 15R-HETE (1). 15R-HETE can also be converted by neutrophils and other cells to 15-epi-LXA₄, LXA₄ and 15-epi LXA₄ are rapidly generated in response to specific signals, act locally, and then are quickly inactivated by conversion to 15-oxo-containing metabolites in monocytes (1–3). A series of metabolically resistant LXA₄ analogues (e.g., 15-epi-16-(p-fluoro)-phenoxy-LXA₄-methyl ester or aspirin-triggered LXA₄-stable analog (ATLa)) were designed and synthesized to prevent this rapid inactivation (4). Lipoxins and their stable analogues potently block many of the functional responses of neutrophils in vitro (chemotaxis, transmigration, degranulation, superoxide production, IL-1β release) (4–8) and prevent leukocyte influx/recruitment in acute inflammation (8–12). For example, topical application of ATLa dramatically blocks leukocyte infiltration and tissue destruction in a rabbit model of periodontal disease (9).

Specific G-protein coupled receptors for lipoxins have been identified on a variety of cells (13–15), and some of the biochemical pathways/second messenger systems that become activated when these receptors are occupied have been described (16–20). Protein phosphorylation reactions are critically involved in most, if not all, of the functional responses of stimulated neutrophils (21). At this juncture, little is known about how lipoxins might impact protein phosphorylation in human neutrophils. In this manuscript, we report that ATLa blocks phosphorylation of leukocyte-specific protein 1 (LSP1) and alters phosphorylation/activation of components of the p38-MAPK cascade in chemoattractant-stimulated human neutrophils. The significance of the p38-MAPK cascade as a “target” for lipoxins is consistent with the proinflammatory nature of this pathway and the anti-inflammatory actions of ATLa.

Materials and Methods

Materials

Affinity-purified rabbit phosphospecific Abs (pAbs) that recognize phospho-Ser/Thr in proteins preceded by two or more Lys/Arg residues at the −2 to −5 positions (pAkt(S) Ab), MAPK-activated protein kinase-2 (MAPKAP-K2) when phosphorylated at Thr323 (pMAPKAP-K2(Thr323) Ab), MAPK kinase 3 (pMKK3) when phosphorylated at Ser186 and Ser197 (pMKK3(Ser186/197) Ab), p44/42-MAPK (ERK1/2) when phosphorylated at both Thr202 and Tyr204 (p44/42(Thr202 and Tyr204) Ab), and p38-MAPK when phosphorylated at both Thr180 and

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Tyr182 (p38-MAPK/Thr180 and Tyr182) Ab) were obtained from Cell Signaling Technology (Beverly, MA). Active MAPKAP-K2 (recombinant protein expressed in Escherichia coli, residues 46–400 of the human enzyme) and a monomolecular pAb that only recognized heat shock protein 27 (Hsp27) when phosphorylated at Ser15 (Hsp27/Ser15) Ab) were purchased from Calbiochem (La Jolla, CA). Glutathione Sepharose 4B beads and the GST gene fusion vector (GEX4T2) vector were obtained from Amersham Biosciences (Uppsala, Sweden). The methyl ester and free acid of the stable (ATLa) 15-epi-16-(fluoro)-phenoxy-LXA4 were prepared by isolation of GST-LSP1 fusion proteins.

Vectors for expression and isolation of GST-LSP1 fusion proteins.

Expression and isolation of GST-LSP1 fusion proteins were described previously (24). This procedure resulted in cell suspensions that contained >95% neutrophils with viabilities always >90%. These experiments were performed as described previously (24). This procedure resulted in cell suspensions that contained >95% neutrophils with viabilities always >90%. Experiments described herein were performed in compliance with all institutional policies and federal guidelines governing the use of humans in research. Blood donors signed an informed consent form after the nature and possible consequences of our investigations were fully explained.

Cell stimulation and Western blotting.

Neutrophils (5.0 × 10^6/ml) were stimulated in disposable 1-cm plastic cuvettes at 37°C. The standard assay mixture consisted of modified Dulbecco’s PBS medium (135 mM NaCl, 2.7 mM KCl, 16.2 mM Na2PO4, 1.47 mM KH2PO4, 0.90 mM CaCl2, and 0.50 mM MgCl2; (pH 7.35)) containing 7.5 mM D-glucose. Cells were generally incubated in this medium for 15 min with or without inhibitors before stimulation. Concentrations of agonists and stimulation times are presented in the figure legends. At the appropriate time, the cells were rapidly lysed by adding 0.25 ml of 5% concentrated “solubilization buffer” (SDS-B) to 1 ml of the reaction mixture, and the samples were boiled for 4 min. The final composition of SDS-B after mixing was 2.3% (w/v) SDS, 62.5 mM Tris-HCl (pH 6.8), 5.0 mM EDTA, 10.0% (v/v) glycerol, 5.0% (v/v) 2-ME, and 0.002% (v/v) bromophenol blue. Aliquots of these samples were separated by SDS-PAGE (17 μg/lane) on 8.0% (w/v) polyacrylamide gels (22). Western blotting of the separated proteins was performed exactly as described by Ohira et al. (21). Membranes were incubated with the appropriate primary Abs overnight and proteins/Ags were visualized with a lumino-ECLE detection system (Pierce) (21).

At the end of these experiments, both the immunodetection system and the bound Abs were removed from the blot by incubating the membrane with ImmunoPure elution buffer (Pierce) for 30–60 min at room temperature followed by two washes in TBST (20 mM Tris-HCl (pH 7.5) containing 150 mM NaCl and 0.10% (v/v) Tween 20). The blots were then stained with an Ab that recognized both the phosphorylated and nonphosphorylated forms of the ERK1/2 or LSP1 to confirm that equal amounts of protein were present in each lane of the gels. The concentrations of inhibitors and conditions used in these studies did not affect cell viability as measured by the exclusion of trypan blue or by the release of lactate dehydrogenase (21, 23).

Immunoprecipitation experiments. These experiments were performed exactly as described by Ohira et al. (21), except that the goat Ab used to immunoprecipitate LSP1 (LSP1(N-20) Ab) was used at a concentration of 4 μg/ml.

Protein analysis by liquid chromatography (LC)-nanospray-mass spectrometry (MS)-MS and peptide mapping.

Lysates of 1MLP-stimulated neutrophils were subjected to SDS-PAGE on a 7.0% (w/v) gel. The gel was cut in half, and one portion was either stained directly for protein with Coomassie blue or was transferred to an Immobilon-P membrane and then stained with Coomassie blue. Proteins in the remaining portion were transferred to an Immobilon-P membrane and then stained for phosphorylated p55 with the pAkt(S) Ab. Phosphorylated p55 was found to correspond with a discrete protein band when the Coomassie-stained gel/membrane and the Western blot were aligned.

Proteins in gel pieces corresponding to p55 were in-gel digested overnight at 28°C using sequencing-grade modified trypsin (Promega, Madison, WI) at 300 ng of trypsin per 100 μl of solution as described by Rosenfeld et al. (25). In other experiments, p55 was excised from a Coomassie-stained Immobilon-P membrane, placed in an Eppendorf tube, and extracted by incubation in 70% acetonitrile containing 1% trifluoroacetic acid at 42°C for 18 h. The membrane was rinsed once with the same solution, combined with the first extract, and dried under nitrogen gas. The protein was digested in 150 μl of 50 mM NH4HCO3 containing 500 ng of trypsin (sequencing-grade modified trypsin; Promega) for 14 h at 28°C. Both gel- and Immobilon-P membrane-derived protein samples were then extracted by driedness under reduced pressure by vacuum centrifugation (SpeedVac, Savant, Farmingdale, NY) and stored at −80°C until mass spectrometric analysis.

Tryptic peptides were separated by reversed phase LC using a capillary column (PepMap; 15-cm length, 75-μm internal diameter, particle size 5 μm; LC Packings, Amsterdam, The Netherlands) at a flow rate of 200 nl/min. This flow rate was achieved using an Accurate flow splitter (LC Packings) reducing a 400 μl/min flow delivered by an HPLC pump (series 1100; Agilent, Wilmington, DE). The following mobile phases were used: mobile phase A, 9.7% water (Burdick & Jackson, Muskegon, MI), 2% acetonitrile (Burdick & Jackson), and 0.1% formic acid (Merck, Darmstadt, Germany) (v/v/v); mobile phase B, 99.7% water, 0.4% acetonitrile and 0.1% formic acid (v/v/v). The following mobile phase gradient was used: 0–10 min, 0–25%; 10–180 min, 25–60% B. Tryptic peptides were injected onto a 2-μg capacity peptide trap (Michrom Bioresources, Auburn, CA) placed in the loading loop position of an injector port (7725i; Rheodyne, Rohnert Park, CA). The bound peptides were washed with 5 μl of mobile phase A and eluted off the trap onto the capillary column during LC.

The mass of the eluting peptides was determined with an Advantage nano-electrospray ionization trap mass spectrometer (ThermoFinnigan, San Jose, CA). Eluting peptides were ionized using end-coated nanospray needles (outer diameter, 360 μm; inner diameter, 75 μm; tip orifice, 15 μm; New Objective, Woburn, MA) at a spray voltage of 1.8 kV. The nanospray needle orifice was held at a distance of ~2 mm from the mass spectrometer inlet capillary, which was kept at 160°C. The capillary voltage was set at 5 V, and peptide ions were monitored in the positive ion mode. For each peptide mass with intensity greater than 2 × 105, zoom-scans (5 atomic mass units above and below the mass charge (m/z) of the monitored peptide ion) and collision-induced dissociation tandem mass spectra were automatically acquired using dynamic exclusion (26). The mass spectrometer was regularly tuned with synthetic angiotensin II (Sigma-Aldrich, St. Louis, MO). Mass spectrometric data were analyzed using Xcalibur software (ThermoFinnigan). Protein identification was performed by peptide mapping using TurboSequest in the BioWorks software package (version 3.1; ThermoFinnigan) (26, 27). Peptide masses and tandem mass spectra were compared with the nr fasta protein database (April 2003) downloaded from the National Center for Biotechnology Information. Peptide matches were considered taking into account that a preceding tryptic cleavage site had to be present, that methionine residues could be oxidized, and that at least one of a minimum of two peptide matches to a particular protein displayed a cross-correlation score (Xcorr) of 2.5 (27).

Expression and isolation of GST-LSP1 fusion proteins.

Vectors for expression of LSP1 in E. coli were constructed by insertion of cDNA encoding full-length or N-terminal LSP1 (residues 1–178) into GST gene fusion vector (GEX4T2). E. coli strain BL21 (DE3) cells (Strategene, La Jolla, CA) were transformed with the constructed plasmids and grown overnight in Luria-Bertani medium containing ampicillin (100 μg/ml). Bacteria were diluted in 400 ml of fresh Luria-Bertani medium and expression of LSP1 was induced with 0.50 mM isopropyl thiogalactosidase for 3 h (28). Bacteria were collected by centrifugation, frozen at −80°C, thawed quickly in a warm water bath, resuspended in 40 ml of PBS containing protease inhibitor mixture (Sigma-Aldrich), and sonicated. Insoluble debris was removed from the cell lysate by centrifugation at 12,000 × g for 30 min following by filtration. The cleared supernatant (20 ml) was incubated with 2.0 ml of glutathione Sepharose beads for 30 min at 4°C with gentle shaking, and the beads were then washed three times
with PBS (10 ml). rLSP1 was eluted from the beads by three washes (3 ml/wash) with elution buffer (50 mM Tris-HCl with 10 mM reduced glutathione (pH 8.0)) (28). Protease inhibitor mixture (0.1 ml) was added to each of the eluted fractions (3 ml), and the recombinant protein was stored in small aliquots at −80°C. The concentration of recombinant protein was estimated by densitometry after SDS-PAGE/Coomassie blue staining using BSA as the standard.

Phosphorylation of rLSP1 with MAPKAP-K2. A kit from Upstate Biotechnology that measures the ability of MAPKAP-K2 to catalyze phosphorylation of Hsp27 in vitro was used to assay the phosphorylation of rLSP1 and Hsp27 in vitro. The reaction mixture (40 μl) contained recombinant active human MAPKAP-K2 (25 ng), GST-LSP1 (500 ng) or GST-Hsp27 (500 ng), 100 μM ATP, 15.0 mM MgCl₂, 20 mM MOPS (pH 7.2), 7.5 mM β-glycerophosphate, 1.0 mM sodium orthovanadate, 5 mM EGTA and 1 mM DTT. The reaction was run for 20 min at 30°C and then was terminated by adding 10 μl of 5X concentrated sample buffer followed by boiling the samples for 4 min. Phosphorylation of GST-LSP1 and GST-Hsp27 was monitored by Western blotting as described above with the pAkt(S) Ab or pHsp27(Ser⁷⁸) Ab, respectively.

Analysis of data

Unless otherwise noted, all of the autoradiographic observations were confirmed in at least three separate experiments performed on different cell preparations. The number of observations (n) are based on different cell preparations.

Results

ATLa alters the protein phosphorylation pattern of human neutrophils

Neutrophils stimulated with the chemotactrant fMLP exhibit intense phosphorylation of a 55-kDa protein (p55) that can be monitored by Western blotting with the pAkt(S) Ab (21). Optimal phosphorylation of this protein occurred within 3–5 min after cell stimulation (Fig. 1A). Although the pAkt(S) Ab was originally developed to detect products of Akt, it also recognizes products of a variety of “Arg/Lys-directed kinases” that have consensus sequences with two or more basic residues in the N-terminal region of the phosphorylation site (e.g., protein kinase C (PKC), PKA, p21-activated kinase, MAPKAP-K2) (21, 29).

Treatment of neutrophils with 50 nM ATLa for 15 min at 37°C before stimulation of the cells with 100 nM fMLP substantially reduced phosphorylation of p55 (Fig. 1A, arrow; another example of this is shown in Fig. 6D). In contrast, ATLa did not appear to impact phosphorylation of a 130-kDa protein (filled arrowhead) or dephosphorylation of a minor 80-kDa protein (open arrowhead) in stimulated neutrophils (Fig. 1A). Thus, the inhibitory actions of ATLa on neutrophils are selective for certain proteins/kinases. ATLa was also capable of significantly inhibiting the phosphorylation of p55 in fMLP-stimulated neutrophils at 10 nM (Fig. 1B).

Phosphorylation of p55 was also observed in neutrophils stimulated with 1 μM platelet activating factor or TNF-α (100 ng/ml) (21), and this reaction was blocked by 50 nM ATLa (our unpublished data). These results indicate that ATLa does not inhibit the phosphorylation of p55.

FIGURE 1. Effects of ATLa on protein phosphorylation in neutrophils.
A, Neutrophils were incubated with and without 50 nM ATLa for 15 min at 37°C and then were stimulated with 100 nM fMLP for the indicated time periods. The positions of the 130-, 80-, and 55-kDa phosphoproteins are indicated by a filled arrowhead, open arrowhead, and arrow, respectively. B, Bar graph summarizes the percent phosphorylation of the 55-kDa protein in neutrophils treated with and without 50 or 50 nM ATLa as described above. Cells were stimulated with 100 nM fMLP for 3 min. Results represent the mean values ± SD for three separate donors. Values were estimated by densitometry with the 100% value being the amount of phosphorylated p55 in stimulated neutrophils not treated with ATLa. Protein phosphorylation was monitored in neutrophils by Western blotting with the pAkt(S) Ab as described in Materials and Methods.

Lymphocyte-specific protein 1 [Merogeton] g12194347776jg14a@18965

1 MAAEASSSGAEELLEEEILGLPAVWDEVEEDWDDHELRRQGLAQAOEDIEEGGHPYRKP
21 DNLLELKLPSAPELEDSEDEEDFOVAGQEGRQEEOAGOEGTLDQIGDPOGFRCPEEGEDGRRP
121 GLHRVLEEDDEESELLEPLIEKSESDGFPECDEVIDGIAQAGENEDRHCEEDQPRFPLV
241 LDYDDEQSSPLPSFTPKLDIRTELELRSEKSDNGKQGPDPSIKQGQVDLGQYDQTSET
301 GSGKSTIESKEEERYYKVFVATHQKYEKRVEGHHGPAP

FIGURE 2. Tryptic peptide mapping of p55LSP1 by MS. Peptides A–O were analyzed by LC-nanospray tandem MS and matched to sequences in human LSP1 using the nr.fasta protein database from the National Center for Biotechnology Information. The sequences of peptides A–O are shown along with the total protein coverage of LSP1 (bold type). M* is an oxidized methionine residue.
phosphorylation of p55 in fMLP-stimulated cells by simply blocking binding to the fMLP receptor. The inability of ATLa to alter phosphorylation of the 130- and 80-kDa proteins in fMLP-stimulated neutrophils is also consistent with ATLa affecting a site other than the fMLP receptor (Fig. 1A).

Identification of p55 as LSP1

A Coomassie-stained protein band that corresponded with phosphorylated p55 was treated with trypsin, and the resulting peptides were separated by capillary liquid chromatography and subjected to MS as described in Materials and Methods. Fifteen tryptic peptides (designated A through O) were identified by peptide mapping that matched sequences in human leukocyte (formerly lymphocyte) specific protein 1, which amounted to a total protein coverage of ~30% (Fig. 2, peptides A–O). The presence of both the phosphorylated peptides (peptides J and O) as well as the corresponding unphosphorylated peptides (peptides D and H) indicate that Ser252 is a phosphorylation site for LSP1 in vivo. Fig. 3 compares the measured tandem mass spectrum for phosphorylated peptide J with the theoretically predicted spectrum for this sequence with 17 fragments of a theoretical 25 b and y ions being matched (matches displayed by red and blue values; see Ref. 26 for detailed explanation of these spectra). The fragment ion at m/z 766 observed in the tandem mass spectrum corresponds with the measured doubly charged parent ion of peptide J (m/z 815.2) with a neutral loss of 49 atomic mass units (H₃PO₄/2).

p55 was also identified as LSP1 by analysis of the 55-kDa protein band obtained from an Immobilon-P membrane in certain experiments. On-membrane protein digestion of this Coomassie-stained band with trypsin followed by peptide mapping by LC-MS-MS and TurboSequest peptide mapping identified yet another three tryptic peptides not shown in Fig. 2 that also belong to human LSP1 (data not shown), with no evidence for the presence of other human proteins in this band. The N-terminal sequence of phosphorylated Ser252 in LSP1 is similar to the recognition motif of the pAkt(S) Ab (21).

Both Western blotting (mobility shift) and immunoprecipitation experiments were undertaken to confirm that p55 was LSP1 (Fig. 4). Extracts obtained from human neutrophils stimulated with 100 nM fMLP for 1–5 min exhibited a distinct “mobility shift” during Western blotting with a rabbit Ab to human LSP1 (Fig. 4A). The shifted band exhibited a mass identical with that of p55, and the time course of this shift was very similar to that for phosphorylation of p55 (Fig. 1A). Importantly, treatment of neutrophils with 50 nM ATLa reduced this shift (Fig. 4A, g–i). These results are consistent with LSP1 undergoing phosphorylation in stimulated neutrophils and this phosphorylation reaction being reduced by ATLa. In addition, immunoprecipitates obtained from lysed fMLP-stimulated neutrophils with a goat Ab to LSP1 contained two bands that reacted with the pAkt(S) Ab, and these bands exhibited mobilities similar to those of phosphorylated p55 and LSP1 in neutrophil lysates (Fig. 4B). In contrast, only the lower band was...
observed in immunoprecipitates obtained from unstimulated neutrophils, and this band was significantly less reactive toward the pAkt(S) Ab (Fig. 4Be). These results are also consistent with p55 being LSP1 and undergoing a mobility shift during cell stimulation due to phosphorylation.

Effects of various inhibitors on the phosphorylation of LSP1 in human neutrophils

Phosphorylation of LSP1 in vitro can be catalyzed by PKC and MAPKAP-K2 (30–32). A variety of inhibitors were used to determine which kinases were active at the phosphorylation sites in LSP1 recognized by the pAkt(S) Ab (Fig. 5). Phosphorylation/activation of MAPKAP-K2, p38-MAPK, and p42/p44-ERK in neutrophils was also investigated in these experiments with a panel of pAbs that only recognize these kinases as described in Materials and Methods.

Phosphoinositide 3-kinase-dependent stimulatory pathways for p38-MAPK, whereas higher concentrations of this drug (50 μM) also block activation of p42/p44-ERK (Ref. 23 and Fig. 5, h and i). p38-MAPK catalyzes the phosphorylation/activation of MAPKAP-K2 (Ref. 33; see Fig. 8). SB 203580 at 10 μM completely blocked phosphorylation of LSP1 (Fig. 5Ai). In contrast, PD 98059 (50 μM), which blocks activation of p42/p44-ERK in neutrophils (23), did not affect the phosphorylation of either MAPKAP-K2 or LSP1 (Fig. 5Bi).

A recent study has reported that SB 203580 can also inhibit 3-phosphoinositide-dependent kinase-1 in fMLP-stimulated human neutrophils at 10 μM but not at 3 or 0.3 μM (35). Concentrations of SB 230580 of 3 and 0.30 μM were as effective as 10 μM in completely blocking phosphorylation of MAPKAP-K2 and LSP1 in our experiments (data not shown). These data are consistent with LSP1 being a substrate for MAPKAP-K2 in neutrophils (30). Moreover, the ability of SB 203580 to completely inhibit phosphorylation of LSP1 in stimulated neutrophils monitored with the pAkt(S) Ab indicates that this pAb only recognizes the MAPKAP-K2-sensitive sites in this protein. The sequence surrounding Ser252 in LSP1 conforms to the optimal consensus sequence recognized by MAPKAP-K2 (X-Hyd-X-R-X-S-X; Hyd-hydrophobic amino acid) (30).

ATLa reduces phosphorylation of components of the p38-MAPK cascade in stimulated neutrophils

Studies were undertaken to determine which steps in the p38-MAPK/MAPKAP-K2 pathway were sensitive to ATLa (Fig. 6) (this pathway is shown in Fig. 8). MKK3/MKK6 are closely related 41-kDa kinases that catalyze the phosphorylation/activation of p38-MAPK (33). Phosphorylation/activation of MKK3/MKK6 in neutrophils was monitored with a pAb that only reacts with these kinases.

FIGURE 4. Confirmation that p55 is LSP1 by Western blotting/mobility shift (A) and immunoprecipitation experiments (B). A, Neutrophils were incubated with and without 50 nM ATLa for 15 min at 37°C and were stimulated with 100 nM fMLP for the time periods indicated. The mobility of LSP1 during Western blotting was monitored with a rabbit Ab to this protein. The positions of unshifted and shifted LSP1 are indicated by a solid arrow and broken arrow, respectively. B, Immunoprecipitates derived from neutrophils with a goat Ab to LSP1 were blotted with the pAkt(S) Ab (e and f) and the mobility of the immunoprecipitated proteins compared with those of p55 (c and d) and LSP1 (a and b) in neutrophil lysates. Lysates were derived from neutrophils treated for 3 min with 0.25% (v/v) DMSO (unstimulated cells; −) or 100 nM fMLP (+). Conditions for cell stimulation, immunoprecipitation of LSP1, and Western blotting are described in Materials and Methods.

FIGURE 5. Effects of various inhibitors on the phosphorylation of LSP1 (A) and the phosphorylation/activation of MAPKAP-K2 (B), p38-MAPK (C), and p42/p44-ERK (D). Neutrophils were treated for 15 min at 37°C with 200 nM wortmannin (c), 200 μM H-7 (d), 200 μM HA1004 (e), 1 μM Bim I (f), 50 μM PD 98059 (g), 50 μM SB 203580 (h), or 10 μM SB 203580 (i) and then were stimulated with 100 nM fMLP for 3 min. Lane a is for unstimulated cells and lanes b1 and b2 are two different samples of stimulated cells not treated with inhibitors. Phosphorylation of LSP1 was monitored by Western blotting with the pAkt(S) Ab. Phosphorylation of MAPKAP-K2, p38-MAPK, and p42/p44 ERK was monitored by Western blotting with specific pAbs to each of these kinases as described in Materials and Methods.
Finally, we investigated whether MAPKAP-K2 could catalyze the phosphorylation of LSP1 in vitro to yield products recognized by the pAkt(S) Ab and whether ATLa or its free acid directly inhibit the catalytic activity of this kinase (Fig. 7). A GST fusion protein containing full-length LSP1 (GST-B-LSP1), but not the N-terminal region of LSP1 (GST-N-LSP1), reacted with the pAkt(S) Ab after treatment with MAPKAP-K2 in a kinase assay (Fig. 7A). Neither 50 nM ATLa nor its carbonyl free acid inhibited the phosphorylation of GST-B-LSP1 or GST-Hsp27 in this assay \((n = 3; \text{Fig. 7C). GST-Hsp27 is a selective substrate for MAPKAP-K2 (23, 33). These results establish that the pAkt(S) Ab recognizes LSP1 after this protein is phosphorylated in its C-terminal region in a MAPKAP-K2 catalyzed reaction and that neither the free carbonyl acid nor methyl ester of ATLa directly block the catalytic activity of this kinase.}

**Discussion**

In this paper, we report that ATLa (10–50 nM) blocks phosphorylation of LSP1 and reduces phosphorylation/activation of components of the p38-MAPK cascade in chemoattractant-stimulated neutrophils (MKK3/MKK6, p38-MAPK, MAPKAP-K2; Figs. 1 and 6). This pathway is shown in Fig. 8. The concentrations of this lipid mediator that were effective against LSP1 were similar to those that block neutrophil chemotaxis and trans-epithelial/endothelial migration (4, 8). Thus, we have identified a “target” site of action for lipoxins in human cells that may explain some of the physiological effects of this mediator. The significance of these and other novel observations reported in this paper are developed below.

Neutrophils from lsp<sup>−/−</sup> mice exhibit an increase in chemotaxis and superoxide release in vitro in response to certain agonists (37–39). Lsp<sup>−/−</sup> mice exhibit an increased influx of neutrophils into the peritoneal cavity at 4 and 8 h in response to thioglycolate when compared with normal mice (37). Moreover, neutrophils from patients with neutrophil action dysfunction 47/89 overexpress LSP1.
and exhibit a profound defect in chemotaxis (40). These data strongly suggest that LSP1 exerts a negative regulatory role or “brake” on chemotaxis (37). LSP1 contains at least three binding sites for F-actin (41), and these binding sites contain potential phosphorylation sites for MAPKAP-K2 (Ser^{394} and Ser^{352}) and PKC (Ser^{214}, Ser^{392}, and Ser^{384}) (30, 32). The actin binding regions of LSP1 contain clusters of basic residues (41) where phosphorylation may have a disruptive effect by introducing negative charges. Perhaps phosphorylation of LSP1 during neutrophil stimulation reduces its binding to F-actin and “overrides” the brake LSP1 exerts on chemotaxis. Such a scenario would have ATLa inhibiting neutrophil chemotaxis, at least in part, by blocking phosphorylation of LSP1.

Phosphorylation of LSP1 was assayed in our studies with a pAb that has the potential to recognize products of a variety of Arg/Lys-directed kinases (21). However, inhibitor studies were consistent with the pAkt(S) Ab only detecting the MAPKAP-K2-sensitive sites in LSP1 (Fig. 5). Results presented in Fig. 7 also show that the pAkt(S) Ab reacts with LSP1 phosphorylated in vitro with purified MAPKAP-K2. Whether LSP1 actually undergoes phosphorylation in chemoattractant-stimulated neutrophils at the putative PKC sites described above and whether ATLa can prevent phosphorylation at those sites is of interest. As noted above, p38-MAPK catalyzes the phosphorylation/activation of MAPKAP-K2 in stimulated neutrophils and other cell types (23, 33, 34). p38-MAPK is a “stress-activated” protein kinase that was first identified and cloned as the target of a novel class of highly potent anti-inflammatory drugs such as SB 203580 (42). Four isoforms of p38-MAPK have been identified (α, β, γ, and δ), but only the α- and β-isomers are sensitive to SB 203580 (33). SB 203580 blocks chemotaxis, superoxide release, chemokine production, and degranulation in human neutrophils (43–45) and is highly effective in reducing inflammation in a variety of animal models (33, 46). As noted above, nearly identical actions are observed when human neutrophils and animal models are treated with either ATLa or LXA₄ (Refs. 4–12; Fig. 8, inset). The similar anti-inflammatory effects of SB 203580 and LXA₄/ATLa on isolated neutrophils and in various animal models are consistent with lipoxins targeting the p38-MAPK pathway/cascade. Recent studies with an inhibitory peptide indicate that MAPKAP-K2 mediates many of the p38-MAPK-dependent responses of neutrophils (45). LXA₄ or its aspirin-triggered analogues also block LTβ₄ synthesis in isolated neutrophils and PGE₂ production in inflammatory exudates (47, 48). Thus, lipoxins are capable of blocking multiple key reactions in the inflammatory response.

In addition to p38-MAPK, neutrophils stimulated with fMLP also exhibit a pronounced activation of p42/p44-ERK with kinetics nearly identical with those observed for p38-MAPK (23, 34). p38-MAPK provides a proapoptotic/“death” signal for neutrophils that can be overridden by a survival signal generated by p42/p44-ERK (49). Treatment of human T cells with 10 nM ATLa for 6 h blocks activation of ERK and release of TNF-α upon subsequent stimulation of these cells with an anti-CD3 Ab (50). These data are consistent with ATLa blocking the proinflammatory effects of a variety of immune cells by targeting MAPKs. Given the ability of p42/p44-ERK to override the effects of p38-MAPK under certain circumstances (49), it will be important to determine whether ATLa also affects the p42/p44 pathway in neutrophils.

How might ATLa block phosphorylation of LSP1 and reduce phosphorylation of MKK3/MKK6, p38-MAPK, and MAPKAP-K2 in neutrophils? The complex kinetcs of this inhibition do not offer a simple explanation for these actions (Fig. 6). Human neutrophils contain LXA₄ receptors (51), which bind ATLa with high affinity (13, 52). However, we have not yet determined whether the effects of ATLa on the p38-MAPK/LSP1 pathway are receptor mediated or due to a direct interaction between ATLa and components of this pathway. The availability of a variety of diverse ligands that bind to the LXA₄ receptor should prove useful in sorting out these possibilities (13, 52). Immunoprecipitation experiments indicate that p38-MAPK in neutrophils exists in a complex with MAPKAP-K2, Atk/PKB, and Hsp27 (34, 35). Activation of phospholipase D (PLD) is required for the activation of p38-MAPK in neutrophil-like HL-60 cells stimulated with fMLP (53). Intracellular presqualene diphosphate accumulates rapidly in ATLa-treated neutrophils stimulated with a chemoattractant and blocks both the activation and activity of PLD (20). Whether ATLa blocks phosphorylation of LSP1 through inhibition of PLD and/or alters the stability of the p38-MAPK phospho-relay complex in neutrophils is currently under investigation.

References
Lipoxins Alter Protein Phosphorylation in Neutrophils


