Calcium-Independent Phospholipase A2 Is Required for Human Monocyte Chemotaxis to Monocyte Chemoattractant Protein 1

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Monocyte migration into the intima of an arterial wall is thought to be one of the key initial steps in atherogenesis. Recruitment of monocytes from the peripheral blood is a multistep process in which locally produced chemokines are believed to play a crucial role (1). Monocyte chemoattractant protein 1 (MCP-1) is a member of the CC or β chemokine subfamily and exhibits its most potent chemotactic activity toward monocytes. MCP-1 binds to the CCR2 and recently identified CCR11 receptors (2). It plays an important role in inflammation and the inflammatory response. MCP-1 has also been associated with a series of human inflammatory diseases including rheumatoid arthritis, viral meningitis, psoriasis, and inflammatory bowel disease (3).

The importance of MCP-1 in inflammation has been determined by numerous knockout and transgenic studies focusing on the inflammatory response and infection in mice (4–10). CCR2-deficient mice have a severe reduction in leukocyte adhesion and extravasation (4–6). Conversely, MCP-1-transgenic mice with tissue-specific promoters developed considerable increases in monocyte infiltration into tissues in which MCP-1 expression was detected (7, 8). MCP-1-deficient mice or mice overexpressing MCP-1 have also been shown to have altered Th1 and/or Th2 responses (11–13). These in vivo studies demonstrate how MCP-1 and its receptor are important in the inflammatory response involving monocytes and T lymphocytes.

Furthermore, there is growing evidence that MCP-1 plays an important role in atherogenesis (9, 10, 14, 15). Two well-documented atherogenic stimuli such as modified low-density lipoprotein (16) and fluid shear stress (17) induce MCP-1 expression. The cellular components of the arterial wall, including endothelial cells and smooth muscle cells, secrete MCP-1 (18–20), and MCP-1 is up-regulated in human atherosclerotic plaques (21). These studies indirectly support a role for MCP-1 in atherogenesis, however, direct evidence was recently obtained with double-knockout mice fed Western diets. Low density lipoprotein receptor/MCP-1-deficient mice fed a Western diet had 83% less plaque formation throughout the aorta as compared with controls (9). Mice lacking apolipoprotein E and the CCR2 receptor also showed significant reduction in the size of atherosclerotic lesions covering the aorta after 13 wk on a Western diet (10). Finally, apolipoprotein E-deficient mice with macrophages overexpressing the MCP-1 transgene developed increased atherosclerotic lesions in their aorta and aortic valve regions (22). These in vivo studies provide strong direct evidence that MCP-1 is important in atherogenesis.

CCR2 is one of numerous serpentine receptors characterized by seven-transmembrane domains and coupled to a GTP binding protein (23). When MCP-1 binds to CCR2 it induces a rapid (15 s) and transient (15 min) release of \[^{3}H\]arachidonic acid (AA) from radiolabeled human monocytes (24, 25). This effect was inhibited by Bordetella pertussis toxin treatment, was dependent on the influx of extracellular calcium, and was increased in a synergistic fashion by platelet-activating factor. Also, general pharmacological inhibitors of PLA2 such as mepacrine, p-bromophenacyl bromide, and...
manoiide inhibited monocyte chemotaxis to MCP-1 (24). In ad-
tion, human monocyte chemotaxis to MCP-1 and AA release were 
inhibited by an antisense oligonucleotide specific for cyto-
olic phospholipase A2 (cPLA2) (26). These results suggest a role 
for PLA2 products, AA, and/or lysolipids, as second messengers in 
monocyte migration to MCP-1.

PLA2 enzymes hydrolyze the fatty acyl group from the sn-2 
position of phospholipid with the concomitant production of 
lysophospholipid. Cells of monocyte lineage have three differ-
ent PLA2: 1) secretory, referred to as sPLA2; 2) cPLA2; and 3) 
calcium independent (iPLA2). sPLA2 is a low-molecular-mass (14 
kd) secreted enzyme that requires millimolar concentrations of 
calcium for its catalytic activity and does not show a selectivity 
for fatty acid esterification at the sn-2 position (27, 28). cPLA2 is an 
85-kDa protein that requires nanomolar to micromolar concentra-
tions of calcium, is the only PLA2 identified that has selectivity 
for sn-2 AA, and plays an important role in agonist-induced AA re-
lease (29, 30). iPLA2 is an 85-kDa protein that requires no calcium 
for its catalytic activity. iPLA2 has no acyl specificity and has 
been suggested to function in the steady-state remodeling of phospho-
lipid fatty acyl groups (31).

In this paper we report that selective pharmacologic inhibition of 
iPLA2 substantially and significantly decreased the chemotactic 
response of human monocytes to MCP-1. With the use of specific 
antisense oligodeoxynucleotides (ODN), we were able to spe-
cifically inhibit expression of iPLA2, and confirmed its importance 
in this monocyte chemotactic response. Our results indicate re-
quirements for both PLA2, and cPLA2 enzymes. To evaluate 
in this monocyte chemotactic response, our results indicate req-
tions of calcium, is the only PLA2 identified that has selectivity 
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lipid fatty acyl groups (31).

Materials and Methods

Materials

Aristolochic acid, ONO- RS-082 (2-p-arylcinnamamoyl)amin o-4-chloro-
benzoic acid), AAOCEF (arachidonyl trifluoroacetyl ketone), and BEL (6-
(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one) were 
 purchased from Biomol (Plymouth Meeting, PA). Each of these reagents was 
diluted to 50 μg/ml with Dulbecco’s PBS containing 1 mg/ml BSA as a 
1000-fold stock solution and stored at −80°C before use. Human MCP-1 obtained from BD PharMingen (San Diego, CA) was 
diluted to 50 μg/ml with Dulbecco’s PBS containing 1 mg/ml BSA as a 
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1000-fold stock solution and stored at −80°C before use.
The reaction was conducted as previously described (36). Cell lysates (200 μg) were mixed with substrate and 0.8 mM ATP and brought up to a final volume of 500 μl. The samples were then incubated at 40°C for 1 h. After incubation, the assay was quenched by adding 1 ml of chloroform/methanol/acetic acid (2:4:1, v/v/v), followed by the addition of 0.5 ml of chloroform and 0.5 ml of water. The chloroform layer was dried under a stream of N₂, resuspended in chloroform/methanol (2:1, v/v), and spotted onto a Silica Gel G TLC plate. The lipids were separated by a solvent composed of chloroform/methanol/acetic acid/water (25:15:4:2, v/v/v/v). The lipids were visualized with iodine vapor, and the zones corresponding to fatty acid and dipalmitoyl phosphatidylcholine (as determined by the migration of purified standards) were scraped and counted. Normalized activity represents cpm of free fatty acid divided by the cpm of free fatty acid plus the cpm of the dipalmitoyl phosphatidylcholine times the total added counts (300,000 cpm). This calculation was performed to correct for any variability in the efficiency of lipid extraction between samples.

cPLA₂ activity assay

This assay was performed according to published protocols with the exception that the sonicated lysate was not centrifuged before assay performance (34).

Chemotaxis assay

Monocyte migration was evaluated using a microchamber technique (37). Human recombinant MCP-1 (50 ng/ml) in DMEM with 0.1% BSA was added to the lower compartment of the disposable 96-well chemotaxis chamber (NeuroProbe, Gaithersburg, MD) in a volume totaling 29 μl. The cell suspension (50 μl of 2 × 10⁶ cells/ml; 1 × 10⁵ cells/well) was added to the upper compartment of the chamber that had been precoated with BCS for 2 h. The two compartments were separated by a 5-μm pore size, polycarbonate, polyvinylpyrrolidone-free filter. The chamber was incubated at 37°C in air with 10% CO₂ for 90 min. At the end of the incubation, the filter facing the upper compartment was scraped with a sponge and rinsed gently with PBS to remove all nonmigrated cells. The side of the filter with the migrated cells was fixed and stained with Hema 3 Stain Set (Biochemical Science, distributed by Fisher Scientific, Pittsburgh, PA). Migrated monocytes were counted in five high-power fields (×400) using a light microscope. All samples were tested in triplicate, and the data are expressed as the mean ± SD.

Flow cytometry

After isolation, 2 × 10⁶ suspended human monocytes were washed in 1 ml of PBS with 3% BSA. The PBS was removed and the cells were resuspended in 10 μl of PE-labeled monoclonal mouse anti-human CCR2 IgG2b Ab (R&D Systems, Minneapolis, MN) and incubated for 30 min at room temperature. PE-labeled anti-human CD20 and CD3 IgG2b Abs (BD PharMingen) were used as a negative control. After incubation, the cells were washed three times in PBS and centrifuged at 200 × g. Labeled cells were detected using FACScan (BD Biosciences, San Jose, CA) in which 10,000 cells were analyzed in each gated event using CellQuest software.

Results

Aristolochic acid and ONO-RS-082, general PLA₂ inhibitors, both inhibited migration of human monocytes to MCP-1 (Fig. 1). Aristolochic acid showed significant inhibition at all three doses (Fig. 1A) with the greatest being 80.7% at 100 μM (Fig. 1A). ONO-RS-082 inhibition was significant and dose-dependent with 88.9% inhibition at 10 μM (Fig. 1B). We next tested more selective inhibitors that have not previously been examined for their effects on monocyte chemotaxis to MCP-1. AACCOCF₃ has been reported to inhibit both cPLA₂ and iPLA₂, but not sPLA₂ (38, 39). AACCOCF₃ caused significant, dose-dependent inhibition of monocyte migration to MCP-1 with complete inhibition of migration at 50 μM (Fig. 1C).

BEL is a selective inhibitor of iPLA₂ that does not affect cPLA₂ or sPLA₂ activities (38–40). BEL significantly inhibited iPLA₂ activity by 66 and 94.8% at concentrations of 0.1 and 1 μM, respectively (Fig. 2A). At these concentrations, BEL also significantly suppressed MCP-1-stimulated migration by 63.5 and 87.1%, respectively (Fig. 2B). It should be noted that the level of activity in the no lysate control is due to contaminating free fatty acid in the substrate and does not represent phospholipase activity.

Because BEL caused inhibition of chemotaxis, this strongly suggested that iPLA₂ was playing a role in monocyte migration to MCP-1. To test this more rigorously, antisense ODN to iPLA₂ was evaluated for effects on MCP-1-stimulated monocyte migration. iPLA₂ antisense ODN reduced iPLA₂ protein by ~90% (Fig. 3A, top). Compiled data from five experiments indicated a range of inhibition between 75 and 100% and a mean percent inhibition of 91% as determined by densitometry (data not shown). This level of protein inhibition is greater than that observed in the original report by Balsinde et al. (35) using this conserved antisense ODN in murine cells. The blots were stripped and reprobed with cPLA₂ Ab. Antisense ODN to iPLA₂ did not inhibit the expression of cPLA₂ protein (Fig. 3A, bottom). Thus the antisense ODN inhibition was selective for iPLA₂.

The effect of inhibition of iPLA₂ expression on iPLA₂ activity assays was also assessed. iPLA₂ antisense ODN significantly reduced iPLA₂ activity by greater than 95% at 5 μM (Fig. 3B). Again, it should be noted that the level of activity in the no lysate control is due to contaminating free fatty acid in the substrate.
preparation. iPLA₂ activity, which was significant in monocytes in the absence of MCP-1 (see “Con” group in Fig. 3B), was induced 2-fold by exposure to MCP-1 (Fig. 4A). Treatment with iPLA₂ antisense inhibited both basal and induced iPLA₂ activity while having no inhibitory effect on the activity of cPLA₂ (Fig. 4A). Thus the decrease in protein expression correlates with decreased enzymatic activity.

iPLA₂ antisense ODN was next evaluated for its effect on monocyte chemotaxis to MCP-1. Doses of antisense ODN that were effective in inhibiting protein expression and enzymatic activity were also found to totally inhibit MCP-1-stimulated monocyte chemotaxis, whereas iPLA₂ sense ODN caused no inhibition (Fig. 3C).

We next evaluated whether certain products of PLA₂ activity could restore monocyte migration when iPLA₂ activity was inhibited by antisense ODN treatment. Numerous fatty acids and lysophospholipids were tested. Addition of AA did not influence the chemotactic response of iPLA₂-deficient monocytes (Fig. 5A), however, LPA restored the MCP-1-stimulated chemotactic response of iPLA₂-deficient monocytes at 50 μM and above (Fig. 5B). Importantly, LPA did not induce migration on its own. Addition of other free fatty acids, linoleic and palmitic, or addition of LPC or PA did not restore the chemotactic response of iPLA₂-deficient monocytes (data not shown). LPA was the only lipid tested that restored monocyte migration to MCP-1 in monocytes with diminished iPLA₂ expression.

Because cPLA₂ had been previously reported to participate in monocyte chemotaxis to MCP-1, we designed an experiment to confirm this finding and then to extend the prior work by examining the ability of cPLA₂ products to restore migration in the cPLA₂-deficient monocytes. First we confirmed that we could create cPLA₂-deficient cells using antisense ODN. Antisense ODN treatment resulted in decreasing cPLA₂ protein expression by greater than 90% at 10 μM (Fig. 6A, top). The blot was stripped and reprobed with iPLA₂ Ab to demonstrate the specificity of the inhibition. iPLA₂ protein was not reduced when monocytes were treated with 10 μM of cPLA₂ antisense ODN (Fig. 6A, bottom).

cPLA₂ antisense ODN was also evaluated for its effect on PLA₂ activities (Fig. 4). We have previously demonstrated that cPLA₂ antisense ODN effectively inhibits cPLA₂ activity (34). Basal levels of cPLA₂ activity are extremely low; however, activity was stimulated 25-fold by exposure of the monocytes to MCP-1. Our
cytes; it fully restored chemotaxis at 3/10 is restored in DMEM with 10% BCS in the presence of 5/10 antisense ODN. Monocytes were either treated with 50 ng/ml MCP1 (+) or untreated (−) for each group. iPLA2 activity (A) and cPLA2 activity (B) were assayed in duplicate samples of cell lysates. Substrate incubated without lysate is labeled NL.

results confirm that cPLA2 antisense ODN treatment caused substantial inhibition of cPLA2 enzyme activity (Fig. 4B). We also examined the effect on iPLA2 activity and found that cPLA2 antisense ODN treatment had little or no effect on iPLA2 activity (Fig. 4A). cPLA2 antisense ODN also inhibited monocyte chemotaxis to MCP-1 at 5 and 10 μM showing 75.3 and 100% inhibition, respectively (Fig. 6B), whereas cPLA2 sense ODN had little to no inhibitory effect at these concentrations (Fig. 6B). In contrast to results obtained with iPLA2-deficient monocytes, when AA was added back at various concentrations (0.3, 1.5, and 3 μM) to see whether it would restore the chemotaxis of cPLA2-deficient monocytes; it fully restored chemotaxis at 3 μM (Fig. 7A). Furthermore, LPA had no effect, even at 100 μM (Fig. 7B). Adding back other lipids such as LPC, PA, linoleic acid, and palmitic acid had no effect on restoring the chemotactic response of cPLA2-deficient monocytes (data not shown).

One possible mechanism for inhibiting chemotaxis to MCP-1 might be through the inhibition of CCR2 expression. We measured CCR2 expression on monocytes exposed to each of the antisense and sense ODN reagents, AACOCF3 and BEL. CCR2 expression was detected in human monocytes, and expression was not substantially modulated by any of these treatments (Fig. 8).

Discussion
Relatively little is known about the regulation of monocyte chemotaxis to MCP-1. The majority of studies have used pharmacologic inhibitors to understand possible pathways that may be involved. Some pathways that have been implicated as regulators of monocyte chemotaxis to MCP-1 include phosphatidylinositol 3 kinase, mitogen-activated protein kinases, protein kinase C, and PL2 (23, 41–43). Although studies from knockout mice indicate the importance of phosphatidylinositol 3 kinase in macrophage chemotaxis to a variety of stimuli, chemotaxis to MCP-1 was not evaluated (44). One protein that has been specifically inhibited using antisense oligonucleotides and evaluated for its effect on MCP-1-induced monocyte chemotaxis is cPLA2 (26). The results from our experiments are the first report that iPLA2 is a key regulator of monocyte chemotaxis to MCP-1. Therefore, based on prior studies and our results presented here, two PL2 enzymes are required for this important inflammatory event (Figs. 3C and 6B). The expression of CCR2 does not appear to be regulated by either of these enzymes, thus alternative regulatory pathways should be considered (Fig. 8).

It has been suggested that iPLA2 plays an important role in phospholipid remodeling of membranes (31). In the remodeling pathway, preformed diacylglycerol-phosphatides (phospholipids) are acted on by iPLA2 to produce their respective monoacylglycerol-phosphatides (lyso phospholipids) (45). Inhibition of iPLA2 expression by antisense ODN has been reported to decrease both steady-state levels of lyso phosphatidylcholine and the capacity of the cell to incorporate AA into membrane phospholipids, whereas...
were incubated with no ODN, 10 μM cPLA₂ sense ODN, or 10 μM antisense ODN at 37°C for 24 h. Total cytoplasmic protein (100 μg) was evaluated by Western blot for cPLA₂ protein (top). The same blot was stripped and reprobed with iPLA₂ Ab (bottom). B, Monocytes were cultured in DMEM with 10% BCS and 5 and 10 μM of cPLA₂ sense (hatched bars) or antisense (cross-hatched bars) ODN for 24 h. Monocyte chemotaxis across a polycarbonate filter in response to MCP-1 (50 ng/ml) was then measured. All samples were run in triplicate. The results are expressed as the mean number of migrated monocytes ± SEM in five high-power fields (×400 light microscope) and are compiled results from three similar experiments. Percent inhibition is shown in parenthesis. *, p < 0.05; **, p < 0.005 from paired t tests.

iPLA₂ antisense had no effect on platelet-activating factor-stimulated AA release in P388D₁ macrophages (35). In contrast, induction of AA release in zymosan-stimulated P388D₁ macrophages was in part due to iPLA₂ (46). Therefore, iPLA₂ is thought to be involved in phospholipid remodeling in membranes and also AA release in certain situations.

LPA but not AA fully restored the MCP-1-stimulated chemotaxis of iPLA₂-deficient monocytes (Fig. 5). We hypothesize that phospholipid remodeling of membranes may be important for chemotaxis in which iPLA₂ generation of LPA is playing a key role. LPA has been reported to enhance migration of a variety of cell types (47, 48); however, under the conditions of our experiments, LPA alone did not induce monocyte migration nor modify the MCP-1-induced chemotaxis of untreated monocytes. LPA has also been reported to be a chemotactic agent and to mediate a haptotactic response (49–51). We do not believe that this activity is contributing to our results because the LPA was present on the side of the filter with the monocytes and not on the side with the MCP-1. Therefore, if LPA was serving as a potent chemotactic agent it might have been expected to inhibit migration by keeping monocytes on the side of the filter where they were seeded. Additional evidence that the LPA effect is not due to its haptotactic activity is derived from the observation that PA was shown to cause equal haptotaxis (51), whereas PA was without effect in any of our chemotaxis experiments. Further evidence for specificity is that concentrations of LPA, severalfold higher than those used in our experiments to restore chemotaxis, have been shown to have no untoward morphologic changes on leukocytes as determined by transmission electron microscopy (50). We examined our cells by trypan blue exclusion and found no toxicity by these concentrations of any of the reagents used in these experiments. The exact monocyte function requiring iPLA₂ that is essential for monocyte chemotaxis to MCP-1 deserves further investigation. Interestingly, iPLA₂ has recently been implicated in regulating the actin cytoskeleton in preadipocytes. In these studies, BEL totally inhibited α₂-adrenergic induced spreading of preadipocytes, and this inhibition was completely restored by LPA but not AA or other fatty acids (52).

It has previously been demonstrated that AA release and chemotaxis of human monocytes to MCP-1 are dependent on cPLA₂ (26). We confirmed that cPLA₂ is required for human monocyte chemotaxis to MCP-1. In our system cPLA₂ antisense ODN inhibited cPLA₂ protein by greater than 90% (Fig. 6A) and showed total inhibition of chemotaxis at this dose (Fig. 6B). This level of inhibition is consistent with our prior observations using this antisense ODN (34). Our novel finding presented in this manuscript is that addition of AA could completely circumvent the inhibitory effects of cPLA₂ antisense ODN (Fig. 7A) thus suggesting that cPLA₂ production of AA is essential in human monocyte migration to MCP-1. Remarkably, LPA had no effect on restoring the chemotactic response of cPLA₂-deficient monocytes (Fig. 7B).
Other fatty acids or phospholipids that were tested also had no effect on restoring this chemotactic response. Because eicosanoids are potent modulators of chemotaxis (53–56), we hypothesized that cPLA₂ may mobilize AA for eicosanoid production and thereby regulate chemotaxis; however, the role of eicosanoids in the process of chemotaxis of human monocytes to MCP-1 remains unknown.

The complementary yet distinct roles of these two phospholipases in regulating monocyte chemotaxis suggest that iPLA₂ and cPLA₂ may be located in different cellular compartments and controlling unique aspects of the monocyte response to MCP-1. Localization studies have been difficult to conduct because the location of the active enzymes, not inactive enzymes, is the critical issue. Alternatively, these two enzymes may have distinct substrate preferences allowing for discrete product formation. The activity assays reported in Fig. 4 suggest that these two enzymes work in parallel and not in sequence, and thereby appear to represent key enzymes in a bifurcated signal transduction pathway. Future studies will focus on the unique contributions of the two lipid products that we have shown to provide essential regulatory functions in monocyte chemotaxis.

In summary, our studies are the first to find a critical role for iPLA₂ in the chemotactic response of monocytes to MCP-1. Because LPA restored chemotaxis to iPLA₂-deficient monocytes and AA restored chemotaxis to cPLA₂-deficient monocytes, the requisite roles for these two enzymes appear to be substantially different. Our results indicate that iPLA₂ and cPLA₂ are separately and uniquely required for human monocyte chemotaxis to MCP-1. These findings suggest that iPLA₂, as well as cPLA₂, may serve as novel therapeutic targets in certain disease conditions where MCP-1 and monocyte migration are involved in the pathogenesis.

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