Metabolic products of soluble epoxide hydrolase are essential for monocyte chemotaxis to MCP-1 in vitro and in vivo

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Abstract  Monocyte chemoattractant protein-1 (MCP-1)-induced monocyte chemotaxis is a major event in inflammatory disease. Our prior studies have demonstrated that MCP-1-dependent chemotaxis requires release of arachidonic acid (AA) by activated cytosolic phospholipase A₂ (cPLA₂). Here we investigated the involvement of AA metabolites in chemotaxis. Neither cyclooxygenase nor lipoxygenase pathways were required, whereas pharmacologic inhibitors of both the cytochrome-P450 (CYP) and the soluble epoxide hydrolase (sEH) pathways blocked monocyte chemotaxis to MCP-1. To verify specificity, we demonstrated that the CYP and sEH products epoxyeicosatrienoic acids (EETs) and dihydroxyeicosatrienoic acids (DHETs), respectively, restored chemotaxis in the presence of the inhibitors, indicating that sEH-derived products are essential for MCP-1-driven chemotaxis. Importantly, DHETs also rescued chemotaxis in cPLA₂-deficient monocytes and monocytes with blocked Erk1/2 activity, because Erk controls cPLA₂ activation. The in vitro findings regarding the involvement of CYP/sEH pathways were further validated in vivo using two complementary approaches measuring MCP-1-dependent chemotaxis in mice. These observations reveal the importance of sEH in MCP-1-regulated monocyte chemotaxis and may explain the observed therapeutic value of sEH inhibitors in treatment of inflammatory diseases, cardiovascular diseases, pain, and even carcinogenesis. Their effectiveness, often attributed to increasing EET levels, is probably influenced by the impairment of DHET formation and inhibition of chemotaxis.—Kundu, S., T. Roome, A. Bhattacharjee, K. A. Carnevale, V. P. Yakubenko, R. Zhang, S. H. Hwang, B. D. Hammock, and M. K. Cathcart

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Monocyte migration from peripheral blood into inflammatory sites is a complex phenomenon that occurs during acute and chronic inflammatory diseases such as peritonitis, atherosclerosis, rheumatoid arthritis, and inflammatory bowel disease. Monocyte chemoattractant protein-1 (MCP-1/CCL2) is one of the key chemokines that regulate migration and infiltration of monocytes in response to inflammatory conditions by attracting monocytes toward MCP-1 in a gradient-dependent manner (1, 2). MCP-1 expression has been demonstrated to be elevated in a number of different diseases, including atherosclerosis (3). In vivo studies with MCP-1- or MCP-1 receptor-deficient mice have shown the reduction of atherosclerotic plaque size, supporting the relevance of MCP-1 in atherogenesis (4, 5).

The cellular effect of MCP-1 is mediated through interaction with its receptor, CCR2 (6). Our laboratory has identified several critical signaling pathways that are activated downstream of this receptor and regulate monocyte chemotaxis to MCP-1 both in vitro and in vivo (2, 7, 8).

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Abbreviations: AA, arachidonic acid; BCS, bovine calf serum; COX, cyclooxygenase; CYP, cytochrome P450; DHET, dihydroxyeicosatrienoic acid; EET, epoxyeicosatrienoic acid; iPLA₂, calcium-independent PLA₂; LOX, lipoxygenase; MCP-1, monocyte chemoattractant protein 1; ODN, oligodeoxyribonucleotide; PLA₂, cytosolic phospholipase A₂; PPARα, peroxisome proliferator-activated receptor α; sEH, soluble epoxide hydrolase; SRM, selective reaction monitoring.

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Among these are two different intracellular phospholipase \( A_2 \) (PLA\(_2\)) enzymes, cytosolic PLA\(_2\) (cPLA\(_2\)), and the calcium-independent PLA\(_2\) (iPLA\(_2\)).

PLA\(_2\)s are enzymes that hydrolyze FAs from the sn-2 position of phospholipids, resulting in the release of free FAs and lysophospholipid. Both cPLA\(_2\) and iPLA\(_2\) release two different products, arachidonic acid (AA) and lysophosphatidic acid, respectively. We previously showed that cPLA\(_2\) and iPLA\(_2\) are involved in two different aspects of MCP-1-induced monocyte migration. cPLA\(_2\) regulates the speed of monocyte migration, whereas iPLA\(_2\) regulates both speed and directionality of migration (7). These two phospholipases are differentially located in polarized, migrating monocytes, with iPLA\(_2\) being located at the leading edge and cPLA\(_2\) localizing to the endoplasmic reticulum behind the nucleus and closer to the uropod (7). These observations delineate distinct roles for these two PLA\(_2\)s that are required for monocyte chemotaxis toward MCP-1 both in vitro as well as in vivo.

In the present study, we focused on exploring how cPLA\(_2\)-generated AA regulates monocyte chemotaxis toward MCP-1. We hypothesized that AA regulates MCP-1 chemotaxis via bioactive metabolites. Metabolites are formed through the action of three classes of metabolic enzymes: cyclooxygenase (COX), lipooxygenase (LOX), and cytochrome-P450 (CYP) (9). We made the surprising discovery that CYP and the downstream enzyme soluble epoxide hydrolase (sEH), are both required for AA regulation of monocyte chemotaxis to MCP-1 and that the important products of this pathway are dihydroxyeicosatrienoic acids (DHETs), the corresponding vicinal diol products of sEH action on epoxides of AA, epoxyeicosatrienoic acids (EETs).

MATERIALS AND METHODS

Materials

SKF-525A-HCl (Proadifen) and 17-octadecynoic acid were purchased from Enzo Life Sciences International (Plymouth Meeting, PA). MK886, indomethacin, MS-PPOH, AUDA, CUDA, DHETs, and EETs were purchased from Cayman Chemical Co. (Ann Arbor, MI). PD 98059, U0126, and U0124 were purchased from EMD Millipore (Billerica, MA). The sEH inhibitor (trans-4-[4-[3-(4-trifluoromethoxy-phenyl)-ureido]cyclohexyloxy]-benzoic acid, t-TUCB) was synthesized as described (10). Human MCP-1 was purchased from BD Biosciences and diluted to 50 µg/ml with PBS containing 1 mg/ml BSA as a 1,000-fold stock solution and stored at \(-80^\circ\)C. MCP-1 was used at 50 ng/ml to attract human monocytes. ChemoTx Disposable Chemotaxis System (#106-5) was purchased from Neuro Probe, Inc. (Gaithersburg, MD), and Hema 3 stain Set (#122-911) was from Fisher Scientific (Pittsburgh, PA). PEG-400 was purchased from Sigma-Aldrich (St. Louis, MO), and safflower oil from Spectrum organic products LLC (Petaluma, CA). The rabbit polyclonal cPLA\(_2\) antibody was purchased from Cell Signaling Technology (Beverly, MA). β-Tubulin antibody was from Santa Cruz Biotechnology (Santa Cruz, CA).

Isolation of human monocytes and cell culture

Human monocytes were isolated from freshly drawn human whole blood by sequential centrifugation over a Ficoll-Paque density solution, removal of platelets, and adherence to tissue culture flasks precoated with bovine calf serum (BCS) as previously described (11, 12). After nonadherent cells were removed, the adherent cells were released with 5 mM EDTA, washed twice with PBS, and added to polypropylene tubes at 2 \(\times 10^6\) cells/ml. The isolated monocytes were usually rest for 1 h in DMEM with 10% BCS at 37°C in 10% CO\(_2\) before use. Human monocytes purified by this method were consistently \(\geq 90\%\) CD14+. Studies on human monocytes were approved by the Institutional Review Board of the Cleveland Clinic.

Treatment of monocytes with pharmacological inhibitors and ODN

For inhibitor treatment, monocytes were washed once with PBS and resuspended in DMEM without serum. The cells were treated with the indicated pharmacological inhibitor and incubated for 1 h at 37°C with 10% CO\(_2\) before performing chemotaxis or enzyme activity assays. Solvent controls were included in all cases, and no significant inhibition of MCP-1-dependent chemotaxis was observed [SKF 525A in water; 17-octadecynoic acid (17-ODYA), Nmethylsulfonyl-6-(proparglyoxyphenyl) hexanamide (MS-PPOH), 12-(3-cyclohexyleureido)-dodecanoic acid (CUDA) in ethanol; and 12-(3-adamantan-1-yl-ureido) dodecanoic acid (AUDA) in DMSO].

For experiments employing oligodeoxyribonucleotides (ODNs), human monocytes (0.5 ml of \(2 \times 10^6\) cells/ml in polypropylene tubes) were pretreated with sense or antisense ODNs (5 or 10 µM) for 48 h in DMEM with 10% BCS. Incubation with ODN totaled 48 h with a refeeding of ODN at 24 h before performing the chemotaxis assay. Expression of macrophage markers (CD36 and CD206) was quantified by fluorescence activated cell sorting analysis (13) in freshly isolated monocytes and monocytes incubated for 48 h. No significant differences were observed.

The antisense ODNs used in our studies were derived from our prior publications reporting their efficacy in primary human monocytes (8). The cPLA\(_2\) sequence was complementary to nucleotides from 219 to 238 of cPLA\(_2\), coding for amino acids from 27 to 34 of the protein. The sequence was 5′-CCCCCTTTGTCACTTTGTGTG-3′. The sequence of the cPLA\(_2\) sense ODN was 5′-CACAAAGTGA-CAAAGGGGGG-3′. Phosphorothioate-modified ODNs were used for these studies to limit cellular degradation. All oligonucleotides were HPLC purified (Invitrogen; Carlsbad, CA).

Treatment of cells with DHETs and EETs

For experiments adding back arachidonate metabolites, DHETs and EETs were added to cells after treatment with ODN or pharmacological inhibitors, and incubated for 1 h at 37°C with 10% CO\(_2\) before performing chemotaxis assays.

Chemotaxis assay

Monocyte migration was evaluated using a microchannel technique (14). 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 in a total volume of 29 µl. The cell suspension (50 µl of \(2 \times 10^6\) cells/ml, \(1 \times 10^5\) cells/well) was added to the upper compartment of the chamber. The two compartments were separated by a 5 µm pore size, polycarbonate, polystyrene filter. The chamber was incubated at 37°C with 10% CO\(_2\) 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 the Hema3 stain set. Migrated monocytes were counted in five high-power fields (\(\times 100\)) using a light microscope. All samples were tested in triplicate, and the data are expressed as the mean ± SD.
Western blotting analysis

Monocytes (5 × 10^7 in 2 ml 10% BCS/DMEM) were plated in six-well culture plates. After plating for 2 h, cells were incubated with cPLA2 sense or antisense ODNs for 48 h with one refeed at 24 h as described in Materials and Methods. Cells were harvested and postnuclear lysates were prepared as described previously (15). After determining the protein concentration using the Bio-Rad protein assay reagent (Hercules, CA), lysate proteins (50 µg/well) were resolved by 8% SDS-PAGE, transferred to a polyvinylidene difluoride membrane, blocked with 5% BSA in PBS with 0.1% Tween 20, and subjected to immunoblotting with a rabbit polyclonal cPLA2 antibody (1:1,000 dilution) overnight. This antibody recognizes a distinct cPLA2 protein band at the predicted migration of 110 kDa. The hybridization signal was detected using SuperSignal West Pico Chemiluminescent substrate (Pierce; Rockford, IL). Immunoblots were stripped and reprobed to assess equal loading according to our previously published protocol (15).

Lipid extraction and quantification

Freshly isolated human monocytes (2 × 10^7 cells/2 ml) were treated with or without MCP-1 for 30 min at 37°C with 10% CO₂ in a polypropylene tube. The numbers of MCP-1 molecules per cell were kept constant, as compared with the amounts used in the chemotaxis assays. Two milliliters antioxidant cocktail (mixture of equal proportions of 2 mM diethylenetriamine-pentaacetic acid and 2 mM butylated hydroxytoluene), 2 ng/µl 15-hydroxyeicosatetraenoic acid (HETE)-d₄ (internal standard), and 800 µl 10 M NaOH (saturated with Argon gas) were then added to each group. Each group was incubated at 60°C for 2 h. Then 1.4 ml concentrated hydrochloric acid (to adjust pH ≤3), 5 ml extraction solution (a mixture of 120 ml 2-propanol, 80 ml hexane, and 2 ml 2 M acetic acid), and 4 ml hexane were added to each group after cooling the solution to room temperature. The solution was centrifuged at 2,095 g for 10 min at 4°C after vigorous mixing. The upper layer (hexane) was collected, and the same extraction procedure was repeated one more time. The collected hexane layers were dried under nitrogen. DHETs were quantified using the modified LC/MS/MS method published by Yue et al. (16). In brief, the dried sample was suspended in 200 µl 85% methanol containing 0.2% acetic acid and centrifuged at 12,000 rpm at 4°C for 15 min. Forty microliters of supernatant was injected into a reverse-phase C18 column (2.0 × 150 mm, Prodigy, 5 µm, ODS (2), Phenomenx; Torrance, CA), and different DHETs were resolved using a gradient at a flow rate of 0.2 ml/min driven by a Waters 2690 HPLC module. Mobile phase A consisted of 0.2% acetic acid in water, and mobile phase B consisted of 0.2% acetic acid in methanol. The column was equilibrated with 85% B for 4 min, and a linear gradient was performed from 85% B to 100% B over 6 min and then kept at 100% B for 8 min. The HPLC column effluent was introduced into a triple quadrupole mass spectrometer (Quattro Ultima, Micromass; Manchester, UK). The mass spectrometer was configured with the capillary voltage at 3.0 kV, cone voltage at 35 V, source temperature at 130°C, and a desolvation temperature at 300°C. The nitrogen flow rate was set at 600 l/h for the desolvation and 60 l/h for the core. Collision-induced dissociation was obtained using argon gas. Analyses were performed using electrospray ionization in the negative-ion mode with selective reaction monitoring (SRM) of the precursor and the characteristic product ions specific for each of the DHETs. The SRM transitions monitored are mass-to-charge ratio (m/z) at m/z 337 → 127 for 8,9-DHET, m/z 337 → 145 for 5,6-DHET, m/z 337 → 167 for 11,12-DHET, and m/z 337 → 207 for 14,15-DHET. The internal standard 15(S)-HETE with SRM transition at m/z 327 → 182 was used for quantification of all the DHETs.

Isolation of mouse peripheral blood mononuclear cells

Female mice (BALB/cJ, Jackson Research Laboratory) were used according to the protocol approved by the Cleveland Clinic Institutional Animal Care and Use Committee. Mice were anesthetized with sodium nembutal (5 mg/100 µl per mouse, i.p.). Blood was collected by cardiac puncture in a 1 ml syringe containing 50 µl of EDTA (100 mM). Blood was diluted with PBS (1:1, v/v) and subjected to centrifugation at 220 g for 7 min at room temperature with no brake to separate platelets. Platelet-rich plasma was removed, and blood was layered onto the Histopaque surface, followed by centrifugation at 400 g for 30 min at 20°C with no brake. The upper layer was removed, and the interface containing mononuclear cells was collected and washed with PBS at 250 g for 10 min with low brake. Cells were resuspended in PBS and counted. Isolated mononuclear cells were comprised of ≈10% monocytes as quantified by fluorescent-activated cell-sorting analysis after staining the cells with FITC-conjugated anti-mouse CD14 and CD11b.

Fluorescent labeling of mouse mononuclear cells with PKH26

Cells were labeled according to the manufacturer’s instructions (2 × 10^6 M dye for 20 × 10^6 cells in a total volume of 2 ml Diluent C). After staining, cells were washed three times with PBS containing 0.5% EDTA and 1% BSA at 524 g for 10 min. Cells were finally suspended in DMEM and counted.

Treatment of mouse mononuclear cells with pharmacological inhibitors

Mouse mononuclear cells (2 × 10^6/ml; PKH26-labeled) were pretreated with different concentrations of AUDA and MS-PPOH (5, 15, and 30 µM) or vehicle control (0.15% DMSO) at 37°C in 10% CO₂ for 1 h, and washed in PBS before adoptive transfer.

Adoptive transfer of mouse mononuclear cells and initiation of peritonitis

Tail veins of recipient mice (n = 3–5 in each group) were dilated with warm water (45°C) and cleaned with 95% ethanol. The labeled mononuclear cells (3.0 × 10^7 million per animal) were injected into the tail vein. Injected cells represented approximately 15–20% of total peripheral blood mononuclear cells of recipient animals. Peritoneal inflammation was induced by thioglycollate injection (1 ml, 4% in purified water, autoclaved). After 24 h, peritoneal cells were harvested in 10 ml of ice-cold PBS containing 5 mM EDTA, washed twice and resuspended in PBS (1 ml), and counted for total leukocyte migration in response to thioglycollate. For each experiment, cells (100,000/0.1 ml) were centrifuged at 72 g for 4 min using cytospin and fixed using formaldehyde (1% for 20 min), washed with PBS, and mounted in Vectashield 4’,6-diamidino-2-phenylindole (DAPI) to stain the nuclei of infiltrated cells. PKH26-positive cells were counted on an upright fluorescent microscope (model DMR, Leica) using a Texas Red filter.

Oral treatment of sEH inhibitor and thioglycollate-induced peritonitis in mice

BALB/cj female mice weighing 30–35 g were treated orally with the sEH inhibitor E-TUCB or vehicle (10% PEG 400 in safflower oil) using a gavage feeding tube without anesthetizing the animal, because this feeding procedure does not cause discomfort to mice. E-TUCB was delivered at a dose of 10 mg/kg per day for 3 days (with an interval of 24 h).

In this study, spontaneous motor activity, sedation, abdominal constriction, and flaccidity in mice were observed by analyzing various autonomic and behavioral responses after every treatment for 3 days. These responses were noted up to 4 h with an interval


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of 30 min and then after 8 h of treatment (17, 18), comparing with the group of vehicle control (10% PEG 400 in safflower oil) or mice treated with water orally for normal behavior.

Abdominal constriction (wriths) was characterized by abdominal contortions, together with stretching of hind limbs (19). Each mouse was observed for such responses, and numbers of writhes were counted for 20 min at 1/2 h, 2 h, 4 h, and 8 h of treatment.

Spontaneous responses such as respiration rate by gasping for breath, heart rate, licking activity, apathy, salivation, and urination were monitored every 30 min for 4 h. In addition, sedative behavior was noted by corneal reflexes or immobility against vibration. Flaccidity is related to lack of motor coordination of muscles and was noted by reduced locomotor behavior and movements.

Peritoneal inflammation was initiated by thioglycollate (1 ml, 4% in purified water) after 8 h of the first day of drug treatment. After almost 70 h of thioglycollate treatment, the mice were euthanized by inhalation of CO₂, and peritoneal cells were harvested in 10 ml ice-cold PBS containing 5 mM EDTA, washed twice, resuspended in PBS (1 ml), and counted for total migration of leukocytes in the absence and presence of sEH inhibitor. Furthermore, peritoneal cells were stained with phycoerythrin anti-mouse F4/80 to quantify macrophages using flow cytometry.

**RESULTS**

We have previously shown that the phospholipase cPLA₂ liberates AA from phospholipid pools and that AA release is critical for human monocyte chemotaxis to MCP-1 (20). We hypothesized that an oxidative metabolite of AA is the likely regulator of monocyte chemotaxis. To test this hypothesis we set out to perform a series of experiments to evaluate the contributions of these various metabolic pathways by using selective pharmacologic inhibitors (Scheme 1).

The nonselective COX inhibitor (indomethacin) and the 5-lipoxygenase-activating protein (FLAP) inhibitor (MK886) did not affect human monocyte chemotaxis to MCP-1 (Fig. 1A, B) at the doses that significantly inhibit the activity of these enzymes in monocytes. In contrast, CYP inhibitors dramatically and significantly decreased human monocyte chemotaxis (Fig. 2). We tested the effect of three different CYP inhibitors, SKF 525A (Fig. 2A), 17-ODYA (Fig. 2B), and MS-PPOH (Fig. 2C), at various concentrations (1, 3, 10, and 30 µM) on human monocyte chemotaxis to MCP-1. All three CYP inhibitors showed significant inhibition of MCP-1-directed chemotaxis in a dose-dependent manner.

**Scheme 1.** Metabolism of arachidonic acid.

**Table 1.** Metabolism of arachidonic acid.

<table>
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<tr>
<th>Prostaglandins</th>
<th>Cyclooxygenase</th>
<th>Epoxydienes</th>
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<td>Thromboxanes</td>
<td>Cox1, Cox2</td>
<td>Epoxides</td>
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<td>Lipoxigenase</td>
<td>12/15</td>
<td>EpoxyEicosatetraenoic Acids (EETs)</td>
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<td>Lipoxins</td>
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AA is oxidized to four different regioisomers of EETs by CYP. These four EETs can be further hydrolyzed to their corresponding diol, DHETs by the sEH (Scheme 2).

Inhibition of sEH raises EET levels and amplifies their biologic effects (21). We therefore examined the effect of two sEH-selective inhibitors, AUDA and CUDA, on monocyte chemotaxis, expecting enhanced migration or no change, if, indeed, EETs are important effectors of chemotaxis. Surprisingly, these sEH inhibitors also significantly blocked human monocyte chemotaxis to MCP-1 in a dose-dependent manner (Fig. 3A, B).

We appreciate that pharmacological inhibitors of an enzyme may cause inhibition due to off-target effects. Therefore, to support our hypothesis that both CYP and downstream sEH are critical for human monocyte chemotaxis to MCP-1, we examined whether addition of CYP and/or sEH products could restore chemotaxis in monocytes with blocked CYP or sEH enzymatic activity, respectively. CYP products, either separate (data not shown) or mixed (8,9-EETs, 11,12-EETs, and 14,15-EETs) were added to monocytes that had been preincubated for 1 h with the CYP inhibitor (SKF 525A, 10 µM), and then after another hour, MCP-1-dependent chemotaxis was evaluated. Results, shown in Fig. 4A, indicate that the addition of CYP products fully restored monocyte chemotaxis. sEH products were similarly tested for their ability to restore migration in monocytes that had been pretreated with AUDA. The DHETs (5,6-DHETs, 8,9-DHETs, 11,12-DHETs, and 14,15-DHETs) added either separately (data not shown) or as a mixture of four were all capable of restoring human monocyte chemotaxis to MCP-1 (Fig. 4A). EETs showed partial restoration of AUDA-inhibited human monocyte migration, but to a significantly lesser extent than did DHETs. It is important to note that none of these metabolites...
induced migration above background in the absence of MCP-1. They are therefore not chemokinetic (data not shown).

Because cPLA₂ is responsible for liberating AA that is oxidatively metabolized by CYP and then further hydrolyzed by sEH, we predicted that products of CYP as well as sEH should restore monocyte chemotaxis in cPLA₂-deficient monocytes. To test this concept and to examine the dose-dependent effects of both of the mixtures of EETs and DHETs, we performed a full dose-response experiment. We treated human monocytes with 10 µM cPLA₂ antisense ODN for 48 h to decrease protein expression of cPLA₂ and then added back mixtures of either CYP or sEH products. Our results indicate that addition of either EETs or DHETs to cPLA₂-deficient monocytes restores chemotaxis in a dose-dependent manner (Fig. 4B). Furthermore, we demonstrate that both of the products achieve maximum recovery at 3 µM concentration. At the highest dose (3 µM), DHETs show a greater restorative effect (96%), as compared with EETs (67%) (Fig. 4B). To extend our prior work and to investigate whether any specific positional isomer of DHET (5,6-DHET, 8,9-DHET, 11,12-DHET, or 14,15-DHET) is responsible for this restoration effect, we added each individual isomer (3 µM each) separately to cPLA₂-deficient monocytes (Fig. 4C). Our results reveal that all of the positional isomers of DHETs show a similar ability to restore MCP-1-induced chemotaxis in these cPLA₂-deficient monocytes (Fig. 4C). To confirm that cPLA₂ antisense ODN treatment created cPLA₂-deficient cells, we performed a Western blot for cPLA₂ protein (Fig. 4D).

The blot was stripped and reprobed with β tubulin. Densitometric quantitation of the Western blot data indicate that antisense ODN treatment at 10 µM significantly reduced the cPLA₂ protein expression by ~60%, compared with the sense ODN-treated control.

To further substantiate that the lipid mediators, EETs and DHETs, play a major role in controlling MCP-1-induced monocyte chemotaxis, we attempted to measure the EET and DHET levels in monocytes in the presence and absence of MCP-1. Our LC/MS/MS analysis showed that the levels of all of the positional isomers of DHET were increased in human monocytes by 2.5–5-fold in the presence of MCP-1 (Fig. 5). Furthermore, these MCP-1-enhanced levels of DHETs were substantially inhibited by treatment with the sEH inhibitor AUDA (data not shown). In our experiments, the levels of EETs were below detection by this method because the method was 10-fold less sensitive in detecting EETs.

To evaluate the relevance of these in vitro findings in vivo, we utilized our recently described adoptive transfer mouse model of peritonitis, which relies on the prior observation that monocyte migration to the peritoneum in response to thioglycollate is dependent on MCP-1 (7). In this model, adoptively transferred mouse mononuclear cells are tracked in vivo for their extravasation from the blood into the peritoneum in response to thioglycollate. This model therefore assesses chemotaxis to MCP-1 in vivo. For these experiments, freshly isolated mouse mononuclear cells were first treated in vitro with slowly reversible pharmacological inhibitors of either CYP (MS-PPOH) or

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**Scheme 2.** Arachidonate metabolite regulation of monocyte chemotaxis

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Epoxide hydrolase products control monocyte chemotaxis

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in monocyte/macrophage migration to the peritoneum in response to thioglycollate. Additionally, the migration of all leukocytes was significantly inhibited (Fig. 7).

Previously published reports demonstrated that MCP-1 induced activation of ERK1/2 MAPK in both human monocytes and the human monocytic cell line THP-1 (22, 23). Furthermore, these reports revealed that inhibitors of MEK activation (upstream, activating kinase of ERK1/2 MAPK) not only abrogated ERK1/2 MAPK activity but also substantially inhibited MCP1-mediated chemotaxis (22, 23). To explore whether EETs and DHETs can exert their effects via the ERK1/2 MAPK signaling pathway, we first examined the dose-dependent effects of the MEK inhibitors PD98059 and U0126 on human monocyte chemotaxis in response to MCP-1 and then investigated the effects of add-back of EETs and DHETs to monocytes that were preincubated with the optimal doses of both of the MEK inhibitors.

Our results indicated a significant dose-dependent inhibition of MCP-1-induced monocyte chemotaxis by both PD98059 and U0126, and the maximum inhibitory effect was achieved at 20 \( \mu M \) for both inhibitors (Fig. 8A, B). In contrast, U0124 (a structural analog of U0126) at 20 \( \mu M \) showed no inhibition of MCP-1-mediated chemotaxis, further confirming the specificity of the drug inhibition for the MAPK pathway (Fig. 8B). We next pretreated monocytes with 20 \( \mu M \) PD98059 and U0126 and then added a mixture of EETs or DHETs to examine their ability to rescue monocyte chemotaxis in the presence of these ERK1/2 inhibitors. Our results showed substantial...
Fig. 4. A: EETs restore CYP-inhibited (SKF525A) and sEH-inhibited (AUDA), and DHETs restore sEH-inhibited monocyte chemotaxis. Monocytes were preincubated with 10 µM SKF525A and 10 µM AUDA in serum-free DMEM for 1 h, then 3 µM EET mixture (8,9-EET, 11,12-EET, and 14,15-EET) or 3 µM DHET mixture (5,6-DHET, 8,9-DHET, 11,12-DHET, and 14,15-DHET) was added for another 1 h. Monocyte chemotaxis across a polycarbonate filter in response to MCP-1 (50 ng/ml) was then measured. All experiments were performed in triplicate. The results are expressed as the mean number of migrated monocytes in five high-power fields ± SD (400× light microscope) and are representative of three similar experiments. ** P < 0.005 from one-way ANOVA. B: Dose response of DHETs and EETs for restoring chemotaxis in cPLA2-deficient monocytes. Monocytes were pretreated with cPLA2 sense or antisense ODN (10 nM) as indicated in Materials and Methods. Different concentrations (0.1, 0.3, 1.0, and 3.0 µM) of DHETs and EETs were added to the antisense ODN-treated group 1 h before the chemotaxis assay. Monocyte chemotaxis across a polycarbonate filter in response to MCP-1 (50 ng/ml) was then measured. All experiments were performed in triplicate. The results are expressed as the mean number of migrated monocytes in five high-power fields ± SD (400× light microscope) and are representative of three similar experiments. * P < 0.05 from one-way ANOVA. C: DHETs restore chemotaxis in cPLA2-deficient monocytes. Monocytes were pretreated with cPLA2 sense or antisense ODN (10 µM) as indicated in Materials and Methods. Of each DHET, 3 µM (5,6-DHET, 8,9-DHET, 11,12-DHET, and 14,15-DHET) was added to the antisense ODN-treated group 1 h before the chemotaxis assay. All experiments were performed in triplicate. The results are expressed as the mean number of migrated monocytes in five high-power fields ± SD (400× light microscope) and are representative of three similar experiments. ** P < 0.005 from one-way ANOVA. D: cPLA2 AS-ODNs downregulate cPLA2 protein expression. Monocytes were treated with cPLA2 AS- or S-ODN as described in Materials and Methods. The cells were then lysed, and the postnuclear extracts were resolved by SDS-PAGE gels and analyzed by Western blot analysis. cPLA2 protein was detected using a rabbit polyclonal cPLA2 antibody (upper panel). The same blot was then stripped and reprobed with β-tubulin antibody to assess equal loading (lower panel). Arrows indicate the predicted positions of cPLA2 and β-tubulin as determined by the molecular mass markers in adjacent lanes. Densitometric analysis of cPLA2 protein expression after AS- or S-ODN treatment is shown in the adjacent panel (left) as determined by the ratio of expression of cPLA2 and β-tubulin proteins. Data are mean ± SD (n = 3). ** P < 0.005 vs. S-ODN-treated control.
Fig. 5. MCP-1 induces generation of DHETs in human monocytes. Monocytes were incubated in the presence or absence of MCP-1 for 30 min. DHETs were extracted and measured (by LC/MS/MS) as described in Materials and Methods. All experiments were performed in triplicate. The mean ± SD of each data set is shown.

Fig. 6. Pharmacological inhibition of cytochrome P450 epoxygenase and soluble epoxide hydrolase reduces monocytes chemotaxis to MCP-1 in vivo. Mouse mononuclear cells without drug treatment (control; 0.15% DMSO) and with MS-PPOH (30 µM for 1 h) and AUDA (30 µM for 1 h) were analyzed for migration to peritoneum in response to thioglycollate-induced peritonitis. Cells were counted for adoptively transferred PKH26-labeled mouse monocytes (A) and total leukocytes (B). Data are mean ± SEM (n = 3–5). **P < 0.005 vs. control. Statistics were not performed for the other groups, where n < 3.

Restoration of MCP-1-induced chemotaxis upon addition of either EETs or DHETs (Fig. 8C). These results thus show that MEK and related ERK1/2 activities are upstream of CYP and sEH regulation of monocyte chemotaxis and suggest a focus for future studies.

DISCUSSION

The main focus of this paper was to explore whether cPLA₂-derived AA regulates monocyte chemotaxis by generating bioactive metabolites. Our studies revealed that only the CYP/sEH-mediated AA metabolic pathway regulates MCP-1-induced monocyte chemotaxis. Involvement of COX and LOX pathways in cell migration has been reported by others (24, 25); however, in our experimental system, we showed that neither the COX nor the LOX pathway is involved in MCP-1-mediated monocyte migration.

CYP metabolizes AA to several biologically active eicosanoids by a group of enzymes called CYP epoxygenases that convert AA to four regioisomeric EETs (5,6-, 8,9-, 11,12-, and 14,15-EETs) (26). We used three different CYP inhibitors: SKF 525A (27), 17-ODYA (28, 29), and MS-PPOH (30). MS-PPOH reduces cell migration of the prostate cancer cell PC-3 (24). 17-ODYA inhibits cell migration in a different cancer cell line (25). MS-PPOH also inhibits endothelial cell (HUVEC) migration (30). Interestingly, all three CYP inhibitors showed significant inhibition in monocyte chemotaxis toward MCP-1. We decided next to investigate enzymes further downstream of this pathway.

EETs, which are CYP epoxygenase products, are further hydrated by sEH to their corresponding DHETs (5,6-, 8,9-, 11,12-, and 14,15-DHETs) (30). We used two highly selective sEH inhibitors, AUDA and CUDA, and found that both inhibited MCP-1-induced monocyte migration. We also showed that MCP-1 chemotaxis can be restored in CYP epoxygenase and sEH-inhibited monocytes by their products, EETs and DHETs, respectively. The restoration of chemotaxis with the products reinforced the importance of CYP epoxygenase and sEH in regulating MCP-1-induced human monocyte chemotaxis. Reduction of monocyte chemotaxis to MCP-1 with AUDA and CUDA (sEH inhibitors) indicates that increased concentrations of EETs are not sufficient for monocyte migration and that DHETs are required. Moreover, less restoration of AUDA-inhibited human monocyte migration by EETs as compared with DHETs indicates that EETs may have some capacity to promote chemotaxis but that DHETs are more-powerful components of this pathway. Alternatively, because sEH inhibition is not complete, addition of EETs may merely lead to elevations in DHETs from the remaining sEH activity.

cPLA₂ plays a major role in the development of several inflammatory diseases (32). AA is believed to be the critical mediator for cPLA₂-regulated inflammation. cPLA₂ accelerates inflammation through its selective release of AA from membrane phospholipids, and contributes to MCP-1-induced monocyte chemotaxis by liberating AA (20). AA is further metabolized to EETs and then DHETs. This indicates the possibility of involvement of EETs as well as DHETs in the cPLA₂-mediated inflammatory pathway.

EETs are recognized as biologically active AA metabolites, sEH inhibitors, which prevent conversion of EETs to DHETs, have shown potential as therapeutic agents in cardiovascular disease (33), hypertension (34), cerebral damage following stroke (35), inflammatory and neuropathic pain (36), weight loss (37), and even carcinogenesis (38). Most of these studies were performed in vivo. In these studies, investigators suggest that EETs play a major role
constricted with a thromboxane mimetic (46, 47). 14, 15-DHET at concentrations between 3 \( \mu \text{M} \) and 10 \( \mu \text{M} \) activated peroxisome proliferator-activated receptor \( \alpha \) (PPAR\( \alpha \))-mediated gene expression in transfected COS-7 cells (48), and all of the DHET isomers at a concentration of 5 \( \mu \text{M} \) inhibited the activation of PPAR\( \alpha \) by rosiglitazone in transfected endothelial cells (49). Thus, the bioactive concentrations of DHETs observed in our studies are consistent with those observed by others.

DHETs have been reported to be involved in signaling pathways related to large conductance calcium-activated potassium channels (BK\( \text{Ca} \)) in smooth muscle cells (50), and that sEH inhibition is required because it inhibits the conversion of EETs to DHETs and thereby elevates EETs. Our results also showed that EETs are biologically active, but importantly, DHETs also play a major role in MCP-1-induced monocyte migration. cPLA\( \text{2} \)-deficient monocyte migration was restored by individual DHET regioisomers, as well as a mixture of regioisomers, which suggests that DHETs are biologically active and required for MCP-1-induced monocyte chemotaxis. This finding indicates that the effectiveness of sEH inhibitors in blocking monocyte chemotaxis is due to decreasing DHET levels rather than elevating EET levels. The data support the further hypothesis that sEH inhibitors work in some cases by stabilizing anti-inflammatory FA epoxides and decreasing their proinflammatory diols.

It is very difficult to determine the actual physiological concentrations of these AA metabolites that are required at specific subcellular locations for regulating migration. Inefficient lipid delivery from aqueous media across cell membranes to subcellular compartments and loss of activity during the treatment usually demands addition of significantly higher concentrations, as compared with the actual bioactive concentrations required for cellular action. Normal human plasma levels of EETs are reported to be approximately 0.3 \( \mu \text{M} \) (39). The intracellular concentration of EET after exposure of platelets to thrombin has been estimated to reach levels as high as 1 \( \mu \text{M} \) (40). Levels would be predicted to be greater at sites of inflammation. The doses that are required for bioactivity in our experiments are consistent with those observed in many published studies (40–44). DHETs at a concentration of 1 \( \mu \text{M} \) inhibited the hydroosmotic effect of vasopression (45), and at concentrations between 1 \( \mu \text{M} \) and 5 \( \mu \text{M} \), they produced relaxation of porcine coronary artery rings...
as well as PPARα and PPARγ (48, 49). We explored the involvement of each of these pathways in our system, but ruled out their involvement (data not shown).

We did not observe a strong regioisomer preference in either MCP-1-induced formation (Fig. 5) or for mediating the restoration of chemotaxis (Fig. 4). MCP-1 induced all four DHETs with somewhat more formation of 11,12 and 14,15 DHETs. The lack of regioisomer preference suggests that perhaps the contribution of DHET formation is one involving modifications in membrane structure/function, but this remains to be determined.

cPLA₂ has been shown to be a substrate for Erk1/2 at its Ser505 activation site. Erk1/2 has been observed to regulate cPLA₂ activity in monocytic cell lines, mouse macrophages, and primary human monocytes (51–53). Our results confirm that Erk1/2 activity is required for MCP-1-induced monocyte chemotaxis, and further extend this observation to demonstrate that DHET formation through sEH is the bioactive product controlling chemotaxis downstream of Erk1/2 activation (Fig. 8), as well as downstream of cPLA₂ and CYP (Figs. 4, 6, 7). Thus the critical downstream mediator of Erk1/2, cPLA₂, and CYP regulation of MCP-1 chemotaxis is DHET.

Leukotoxin is a CYP-derived epoxide product of linoleic acid that when hydrolyzed by sEH to leukotoxin diol becomes a potent cytotoxin and a mediator of perivascular acid that when hydrolyzed by sEH to leukotoxin diol becomes a potent cytotoxin and a mediator of perivascular and alveolar edema. Thus precedence exists for sEH hydrolysis contributing to the pathologic bioactivity of lipid metabolites (54, 55). These data prompted us to extrapolate our in vitro findings to in vivo settings to explore the therapeutic value of sEH inhibitors and DHETs in inflammation.

We found that inhibition of monocyte CYP and sEH activities significantly reduced MCP-1-dependent peritoneal migration of adoptively transferred, drug-treated mouse monocytes in vivo, thus confirming our in vitro observations. To take this finding to the next level, we sought orally active sEH inhibitors for in vivo testing. Several sEH inhibitors are available for use in animal models. Commercial compounds such as AUDA are very potent, but are readily metabolized. Compounds like AEPU and APAU have less potency but higher oral bioavailability and improved physicochemical properties. Compounds such as TPU and eTUCB, have higher potency and much longer half-lives in vivo (56). eTUCB is a tight-binding but kinetically slowly-reversible inhibitor. Thus, by the time one could isolate the sEH enzyme, the inhibitor would have diffused away from the catalytic site; however, the 10 mg/kg/day dose used in this study results in blood levels above the IC₅₀ for eTUCB, and eTUCB increases the ratio of epoxides to diols for several oxylipin chemical mediators and reduces inflammatory cytokines in the plasma even at 3 mg/kg in mice. These plasma epoxide-to-diol ratios have been demonstrated to be dose-related indicators of in vivo sEH target engagement in a number of species, including mice (57–59), rats (60, 61), dogs (62), and monkeys (63).

Using this particular compound, we observed inhibition of monocyte migration in our acute peritoneal inflammation in vivo. These in vivo observations provide us with new insight into how sEH inhibitors work in controlling different inflammatory conditions (29, 34–38). The reduction of inflammation and pain by sEH inhibitors was widely believed to be due to increased concentration of EETs (36).

Regulation of inflammation is critical for survival, and a balance between inflammatory and anti-inflammatory eicosanoids seems essential for the rapid onset of inflammation and its rapid resolution. In this case, the conversion of an inflammatory class of chemical mediators to a pro-inflammatory class by a single enzyme provides exquisite regulation. The EETs in the absence of sEH are relatively stable compounds. Owing to their lipophilicity, they tend to remain in cells. In contrast, the polar DHETs are rapidly metabolized to glucuronides and other products, and are rapidly secreted. Their polar nature allows their rapid movement out of cells and very short and tightly controlled action. Our in vitro and in vivo results support an alternative or additional interpretation recognizing DHETs as active and regulatory metabolites of AA involved in inflammatory cascades and perhaps pain.}

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REFERENCES


