Mono (S) hydroxy fatty acids: novel ligands for cytosolic actin

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Abstract The ubiquitous hydroxylated fatty acids derived from arachidonic acid (HETEs) or linoleic acid (HODEs) exhibit diverse biological effects including chemotaxis, cell proliferation, and modulation of several enzymatic pathways, including the 5-lipoxygenase leading to the inflammatory leukotrienes. It was observed that 12(S)- and 15(S)-HETE and 13(S)-HODE (12- and 15-lipoxygenase-derived metabolites, respectively) inhibited the 5-lipoxygenase present in rat basophilic leukemia (RBL-1) cell homogenates whereas the 15(R) chiral enantiomer and the nonhydroxylated linoleic, oleic, and stearic acids were either less potent or ineffective. In examining the mechanism of this inhibition, the relative effectiveness of several fatty acids in displacing [3H]15-HETE bound to cytosol preparations were compared and the results indicated that these (S) hydroxy fatty acids and 5(S)-HETE were significantly more potent than either the 15(R) enantiomer, 15(S)-HETE methyl ester, arachidonic acid, or prostaglandin F2α. In order to identify the protein(s) that specifically binds HETEs, 15(S)-HETE biotin hydrazide was used as a probe to detect any HETE–protein complexes as this compound both inhibited the 5-lipoxygenase and interfered with the binding of [3H]15-HETE to cytosol preparations. SDS-PAGE analysis and chemiluminescent detection revealed that the major cytosolic proteins that bound this biotinylated probe had molecular masses of 43 and 51 kD. Fatty acid competition experiments indicated that the order of effectiveness in displacing this probe from these proteins was 13(S)-HODE < 5(S)-HETE < arachidonic acid = 15(R)-HETE. Amino acid sequence analysis showed that the 43 kD protein was actin. These findings suggest the possibility that actin may play a major role in the biological effects of monohydroxylated metabolites derived from cellular 5-, 12-, and 15-lipoxygenases.—Kang, L-T., and J. Y. Vanderhoek. Mono (S) hydroxy fatty acids: novel ligands for cytosolic actin. J. Lipid Res. 1998. 39: 1476–1482.

Supplementary key words 5-lipoxygenase • inhibition • binding • chemiluminescence

Hydroxylated fatty acids are formed ubiquitously by both animals and plants. Many of these fatty acids are lipoxygenase products derived from polyunsaturated fatty acids and the most common are HETEs, formed from arachidonic acid, and HODEs which are produced from linoleic acid. The HETEs and HODEs exhibit a wide spectrum of biological activities and are involved in the regulation of chemotaxis, ion transport, hormone secretion, cell proliferation, and other inflammatory responses (1). For example, 5-HETE has been shown to stimulate human neutrophil degranulation, the isomeric 12-HETE has been reported to enhance tumor cell surface expression of αIIbβ3 integrin, both 12- and 15-HETE were determined to increase mucus secretion, and 13-HODE was characterized as the vessel wall chemorepellent factor (2–5).

A variety of reports indicate that HETEs are involved in the regulation of several enzymatic pathways. 5-, 12-, and 15-HETE inhibited the platelet and neutrophil phospholipase A2 (6) and we have observed that 15-HETE, the major product of the 15-lipoxygenase enzyme, inhibits the platelet 12-lipoxygenase as well as the neutrophil 5-lipoxygenase, the initial enzyme involved in the production of the inflammatory leukotrienes (7, 8).

In examining the mechanism of the 15-HETE-induced inhibition of the 5-lipoxygenase in RBL-1 cells, we have previously presented evidence that specific 15-HETE binding sites may mediate this inhibitory action (9). Furthermore, we determined that the cytosol contained about 30% of the specific 15-HETE binding activity. In this report, we have further characterized this specific 15-HETE binding activity and using a novel biotinylated 15-HETE probe, we have identified the major protein responsible for this activity.

MATERIALS AND METHODS

Arachidonic acid was obtained from NuChek Prep (Ely, MN), 5(S)-HETE, 12(S)-HETE, 15(R)-HETE, 13(S)-HODE, and PGF2α were purchased from Cayman Chemical (Ann Arbor, MI).

Abbreviations: HETE, hydroxyeicosatetraenoic acid; HODE, hydroxyoctadecadienoic acid; PGF, prostaglandin F; RBL-1, rat basophilic leukemia; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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Linoleic acid, oleic acid, stearic acid, A23187, phenylsulfonyl fluoride, and bacitracin were bought from Sigma (St. Louis, MO). 15(S)-HETE, 15(S)-HETE methyl ester, and 15(S)-HETE biotin hydrazide were prepared as previously described (7, 10, 11). [3H]arachidonic acid and [3H]15(S)-HETE were purchased from DuPont-NEN (Boston, MA) and MagaCell anti-biotin beads were obtained from Cortex Biochem (San Leandro, CA). Biotinylated molecular weight markers, pH 3.5–10 and pH 4–6 amphotolyte solutions were bought from Bio-Rad (Richmond, CA) and neutravidin-HRP conjugate and the SuperSignal CL-HRP substrate system were from Pierce Chemical (Rockford, IL).

The preparation of cytosol fractions of RBL-1 cells, the 5-lipoxygenase inhibition assay utilizing RBL-1 homogenates and the binding studies involving cytosolic fractions, fatty acids, and [3H]15(S)-HETE were carried out by previously described procedures (9).

To assess the binding of 15-HETE biotin hydrazide to cytosolic proteins, cytosolic fractions and 15-HETE biotin hydrazide were incubated for 45 min at 4°C. In some experiments, samples were preincubated with HETE isomers or other fatty acids for 10 min prior to the addition of 15-HETE biotin hydrazide and in other experiments, cytosolic fractions were treated with biotin hydrazide instead of 15-HETE biotin hydrazide. Samples were subjected to SDS-PAGE analysis on a 12% gel using a Hoefer model SE6000 electrophoresis unit run at 48 volts overnight at 4°C. After transferring the gel to nitrocellulose membranes, the blots were blocked with 5% nonfat dry milk for at least 1 h and then treated with neutravidin-HRP (1 μg/ml 10 mm sodium phosphate, 0.15 m NaCl buffer, pH 6.8, final concentration) for 1.5–2 h on a rocker. The blot was washed several times with Trisbuffered saline containing 0.005% Tween 20, and then incubated with SuperSignal reagent for 5–10 min. The membrane was then exposed to autoradiography film and developed.

To determine the pI value of the 43 kD protein, a native 5% T and 33% C IEF gel was used which was prepared by mixing 2 ml acrylamide/bis (30%), 48 μl amphotolyte solution, pH 3.5–10, 240 μl amphotolyte solution, pH 4–6, 50 μl ammonium persulfate (10%), 20 μl TEMED, and 9.7 ml of water. The protein samples were mixed with an equal volume of 2× native gel sample buffer which consisted of 3 ml glycerol and 200 μl amphotolytes (pH 4–6/pH 3.5–10, 5:1) and 1.8 ml of water. The gel was run at room temperature for 1.5 h at 200 volts (constant voltage) and then for 1.5 h at 400 volts.

The immunopurification of the 43 kD protein was carried out as follows. After incubating cytosolic fractions (2 mg in 0.75 ml) with 150–300 μM (final concentration) 15-HETE biotin hydrazide at 4°C for 45 min, the mixture was treated with goat anti-biotin IgG particles (0.6 ml at 10 mg/ml (w/v)); approx. 107 particles/ml) for 1 h. Next, a magnetic field of one lambda strength was applied and the beads were separated from the supernatant. The beads were washed three times with phosphate-buffered saline and then treated with 200 μl citric acid (pH 2) followed by removal of the beads. The resulting supernatant protein solution was neutralized and analyzed by SDS-PAGE and Western blotting as previously described (11). The 43 kD protein was then excised and collected for amino acid sequence analysis.

Band intensity analysis of chemiluminescent-stained autoradiographic film was carried out using a Personal Densitometer SI (Molecular Dynamics, Sunnyvale, CA) with ImageQuant v4.2 software.

For the [3H]15-HETE binding studies, duplicate samples were tested and the values are expressed as the mean ± SEM. For the 5-lipoxygenase inhibition studies and the band intensity analyses, the paired two-tailed Student’s t test was used for statistical analysis and P values < 0.05 were taken to be statistically significant.

### RESULTS

Our earlier reports examined the inhibitory effects of several 15-HETE derivatives on the 5-lipoxygenase present in PMNs and RBL-1 cells (8–10). We have now evaluated the effects of several isomeric hydroxylated polyenoic acids derived from arachidonic acid (i.e., 12-HETE and 15-HETE) and linoleic acid (i.e., 13-HODE) as well as nonhydroxylated fatty acids on the 5-lipoxygenase present in RBL-1 homogenates. IC50 values were determined from dose–response curves (not shown) and the results shown in Table 1 indicate that the inhibitory potencies of the isomeric 12-HETE and 15-HETE and 13-HODE were comparable (IC50 s = 6–7 μM) and that the nonhydroxylated fatty acids tested were ineffective. Furthermore, the 15(S)-HETE enantiomer was twice as effective as the 15(R)-HETE enantiomer.

In continuing our investigations into the mechanism of this HETE-induced inhibition of the RBL-1 5-lipoxygenase, we had previously established that about one third of [3H]15-HETE was specifically associated with the RBL-1 120,000 g cytosolic fraction (9). We have examined the nature of this interaction further. Specific [3H]15-HETE binding to the cytosol preparation was found to increase linearly for 60 min and the affinity and density of [3H]15-HETE binding for 30 min at 4°C yielded a Kd of 266 ± 8.3 nM with a Bmax of 9.7 ± 1.9 nM (n = 4 separate experiments, results not shown). We then tested the effects of 15-HETE and various isomers and analogs on the binding of [3H]15-HETE to the cytosolic fraction. As shown in Table 2, no statistically significant differences were observed among the (S) hydroxylated fatty acids tested. The 3-fold difference in competitive effectiveness between the (S) and (R) enantiomers was statistically significant (P < 0.05). The 15(S)-HETE methyl ester derivative, arachidonic acid and PGF2α (which also contains a 15-hydroxy group) were also at least 3-fold less effective than 15(S)-HETE.

To further characterize cytosolic proteins that interact

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<tr>
<th>Inhibitor</th>
<th>IC50 μM</th>
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<tr>
<td>15(S)-HETE</td>
<td>7.0 ± 3.1</td>
<td>5</td>
</tr>
<tr>
<td>12(S)-HETE</td>
<td>6.0 ± 1.1</td>
<td>3</td>
</tr>
<tr>
<td>15(R)-HETE</td>
<td>13.4 ± 2.1b</td>
<td>3</td>
</tr>
<tr>
<td>13(S)-HODE</td>
<td>6.1 ± 2.4</td>
<td>3</td>
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<tr>
<td>PGE2α</td>
<td>&gt;&gt;30b</td>
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<td>Linoleic acid</td>
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<td>Oleic acid</td>
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<td>Searic acid</td>
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RBL-1 cell homogenates were incubated with A23187 (7.8 μM) for 1 min at 37°C, followed by the addition of 15(S)-HETE, 15(S)-HETE isomers, analogs, or other fatty acids. One minute later. 179–280 pM [3H] arachidonic acid plus 15 μM unlabeled arachidonic acid was added. The 5-lipoxygenase activity was determined as [3H]5-HETE formation and was assayed as described in Materials and Methods. All IC50 values are given as the mean ± SEM, n, number of separate experiments.

b Concentration of inhibitor required for half-maximal inhibition.

By guest, on August 27, 2017
with 15(S)-HETE, we used a novel 15-HETE derivative, i.e., 15(S)-HETE biotin hydrazide, whose synthesis we recently reported (11). We have now determined that this compound inhibited the 5-lipoxygenase activity in RBL-1 cytosol-bound [3H]15-HETE to RBL-1 cytosolic preparations by 15(S)-HETE and 15(S)-HETE biotin hydrazide. RBL-1 cytosolic preparations (60 μg protein/ml) were incubated with 232–310 pm [3H]15-HETE and unlabeled 15(S)-HETE, 15-HETE isomers, analogs, or other fatty acids (0.1–30 μm) at 4°C for 30 min. Specific cytosol-bound [3H]15-HETE was determined by filtrations and liquid scintillation counting as described in Materials and Methods. The data are presented as the I50 values of each fatty acid relative to that of the I50 of 15(S)-HETE. All values of I50 are given as the mean ± SEM; n, number of separate experiments.

Table 3 summarizes the band intensity analyses of all fatty acids examined and shows that the competitive effectiveness of the (S)-mono-hydroxylated fatty acids to the 43 and 51 kD proteins was significantly greater than the other fatty acids tested.

In order to isolate and characterize these cytosolic proteins, it was decided to utilize anti-biotin antibodies. After incubating a cytosol preparation with 15-HETE biotin hydrazide, the mixture was treated with goat anti-biotin IgG that had been coupled to magnetizable cellulose/iron oxide particles. After applying a magnetic field and separating the particles, proteins were extracted from the beads. SDS-PAGE separation, Western blotting, and chemiluminescent analysis of this protein solution showed a major band at 43 kD and several minor bands (Fig. 4, lane 5). When this procedure was carried out in the presence of unlabeled 15(S)-HETE, the intensity of the 43 kD band was reduced by more than 90% (Fig. 4, lane 5). In this and other experiments, bands corresponding to 51 and 66 kD proteins were also observed but the band intensities were much weaker than the 43 kD band indicating
Fig. 2. 15-HETE dose response on the binding of 15(S)-HETE biotin hydrazide to cytosolic RBL-1 proteins. RBL-1 cytosol preparations (60 µg protein) were incubated with 150 µm (final concentration) 15(S)-HETE biotin hydrazide at 4°C for 45 min and then treated with increasing concentrations of 15(S)-HETE for 30 min. The samples were subjected to SDS-PAGE, transferred to nitrocellulose membranes by Western blotting, and analyzed by chemiluminescent detection as described in Materials and Methods. Left: Western blot. Biotinylated molecular weight markers (lane 1), cytosol treated with 15(S)-HETE biotin hydrazide alone (lane 7), in the presence of 0.15 mM (lane 6), of 0.45 mM (lane 5), of 1.5 mM (lane 4), and of 4.5 mM 15(S)-HETE (lane 3). Lane 2 is from a sample of cytosol incubated with 150 mM biotin hydrazide. Right: The intensities of several selected bands were analyzed with a densitometer as described in Materials and Methods. The blot shown is representative of six separate experiments.

Fig. 3. Effects of isomeric HETEs on the binding of 15(S)-HETE biotin hydrazide to RBL-1 cytosolic proteins. RBL-1 cytosol preparations (30 µg) were incubated with 150 µM 15(S)-HETE biotin hydrazide in the presence or absence of 1 mM HETE isomers at 4°C for 45 min. The samples were subjected to SDS-PAGE, transferred to nitrocellulose membranes by Western blotting, and analyzed by chemiluminescent detection as described in Materials and Methods. Left: Western blot. Cytosol treated with 15(S)-HETE biotin hydrazide alone (lane 1), in the presence of 15(S)-HETE (lane 2), of 5(S)-HETE (lane 3), of 15(R)-HETE (lane 4). Lane 5 is from a sample of cytosol incubated with 150 µM biotin hydrazide. Right: The intensities of several selected bands were analyzed with a densitometer as described in Materials and Methods. The blot shown is representative of three separate experiments.
that the 43 kD protein is the major protein isolated via this procedure.

The 43 kD protein was removed from the blot and microsequenced. Figure 5 shows the amino acid sequence obtained from a digested peptide which showed that there is 100% homology between the 43 kD protein and amino acids 241–257 of human smooth muscle cell actin (12). Isoelectric focusing of the 43 kD protein indicated a pI of 5.0 ± 0.5 (n = 3).

### DISCUSSION

Several recent reports have examined the mode of inhibition of 15-HETE on the 5-lipoxygenase pathway present in different cells by testing various HETE analogs (13, 14). Although these structure–activity studies revealed that multiple regions in the HETE molecule are important for this inhibitory characteristic, several differences between these findings and the present study are noteworthy. In our experiments, the inhibitory potency of 13(S)-HODE was comparable to that of 15(S)-HETE (I50s were 6.1 and 7.0 μM, respectively) whereas Haviv and coworkers (13) found that 15(S)-HETE was about 10 times more potent and the report by Petrich et al. (14) indicated that 15(S)-HETE was at least 7-fold more effective than the racemic 13-HODE. In addition, we found that the 15(S)-HETE was twice as potent as the R enantiomer which was not observed by Petrich and coworkers (14). Possible explanations for these differences are the use of different cell types (Petrich’s group (14) used intact human PMNs) and/or cell preparations as we used a cell homogenate and ionophore A23187 and Haviv and coworkers (13) used a 20,000 g RBL-1 supernatant and no ionophore. Our findings that 1) the isomeric 5(S)-HETE (10) and 12(S)-HETE (12) were also effective inhibitors, and 2) neither PGF2α, a cyclic 15-hydroxy fatty acid, nor linoleic, oleic, or stearic acids exhibited inhibitory properties (at the concentrations tested) suggested that a monohydroxy (or other oxygenated functionality), acyclic C18 or C20 polyunsaturated fatty acid structure is sufficient to inhibit the 5-lipoxygenase.

It does not appear to be necessary that the inhibitory fatty acid be a substrate for the 5-lipoxygenase as the inhibitory effectiveness of both 13(S)-HODE and 5(S)-HETE (present study and reference 10) is quite similar to that of 15(S)-HETE. In view of this and our recent report that there are specific subcellular binding sites for 15-HETE on RBL-1 cells (9), we decided to further examine the nature of the cytosolic binding sites. The results shown in Table 2 indicate that the relative competitive effectiveness of the monohydroxy (S) unsaturated fatty acid [5(S)-HETE, 12(S)-HETE, 15(S)-HETE, and 13(S)-HODE] in inhibiting [3H]15-HETE binding to cytosolic preparations is about the same (≤ a 2-fold range) whereas the chiral 15(R) enantiomer, the 15(S)-HETE methyl ester, and arachidonic acid are significantly less potent. These results differ from those reported with several cyto
colic fatty acid binding proteins (15–17). Thus, brain, epidermal, and keratinocyte fatty acid binding proteins were shown to have a higher affinity for nonhydroxylated fatty acids such as oleic or arachidonic acid than for either 5-, 12-, or 15-HETE.

In order to characterize cytosolic proteins that specifically interact with 15-HETE, we synthesized and tested the effectiveness of 15(S)-HETE biotin hydrazide. This biotinylated 15(S)-HETE derivative was less potent than 15(S)-HETE in inhibiting both the 5-lipoxygenase (about 25-fold) and the binding of [3H]15-HETE to cytosolic preparations (about 240-fold). This is not an unexpected
result. For example, Goldman and coworkers (18) reported that the aminopropyl amide derivative of leukotriene B₄ was two orders of magnitude less effective than leukotriene B₄ in binding to leukotriene B₄ receptors.

Treating RBL-1 cytosolic fractions with 15(S)-HETE biotin hydrazide followed by SDS-PAGE, Western blotting, and chemiluminescent detection revealed the presence of several protein bands that had complexed with the biotinylated 15-HETE. The two major protein bands at 43 and 51 kD represented about 50% of the total specific binding of the 15(S)-HETE biotin hydrazide to the cytosolic preparations. This finding is quite different from the observation by Herbertsson and Hammarstrom (19) who reported the presence of a 667 kD protein in cytosolic Lewis carcinoma cells that specifically binds to 12(S)-HETE. The results from the competition experiments (Fig. 2 and Table 3) indicated that 13(S)-HODE, 5(S)-HETE, and 15(S)-HETE were most potent in displacing the 15(S)-HETE biotin hydrazide from the 43 and 51 kD proteins, whereas the 15(R) enantiomer and arachidonic and stearic acids were much less effective. These findings and the identification of the 43 kD proteins, whereas the 15(R) enantiomer and arachidonic and stearic acids were much less effective. These findings and the identification of the 43 kD protein as actin indicate that the (S)-HETEs and 13(S)-HODE bind directly to actin itself. This observation differs from other studies on the interaction of HETEs with the actin system which report that 5(S)-HETE and 12(S)-HETE modulated actin polymerization presumably by affecting actin-binding sites on rat basophilic leukemia cells. Biochim. Biophys. Acta. 1256: 297–304.

These studies on 1) the potency of several HETE isomers, analogs, and fatty acids in inhibiting the 5-lipoxygenase, and 2) the relative effectiveness of these fatty acids in displacing [³H]15-HETE bound to the cytosol and 15(S)-HETE biotin hydrazide bound to actin yielded similar results. This suggests the possibility that the association of the cytosolic actin with (S)-HETE or (S)-HODE, rather than the HETE (or HODE) alone, might yield a complex that is crucial in inhibiting the 5-lipoxygenase pathway. However, the exact role of actin in the diverse actions of these hydroxy fatty acids remains to be determined.

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REFERENCES


