Published in final edited form as:

Prostaglandins Other Lipid Mediat. 2016 March ; 123: 33–39. doi:10.1016/j.prostaglandins.2016.04.001.

(5Z,11Z,15R)-15-Hydroxyeicosa-5,11-dien-13-ynoic acid: A stable isomer of 15(S)-HETE that retains key vasoconstrictive and antiproliferative activity

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Abstract

15(S)-Hydroxyeicosa-(5Z,8Z,11Z,13E)-tetraenoic acid (15(S)-HETE) is a metabolite of arachidonic acid that elicits a number of biological effects including vasoconstriction and angiogenesis. (5Z,11Z,15R)-15-Hydroxyeicosa-5,11-dien-13-ynoic acid (HETE analog **1**) is a synthetic isomer of 15(S)-HETE that is much more stable to autoxidation. Using isometric recording of isolated pulmonary arteries from male and female rabbits, HETE analog **1** and 15(S)-HETE were found to elicit concentration-dependent contractions that were slightly greater in females compared to males. The maximal response in females was greater with 15(S)-HETE. HETE analog **1** and 15(S)-HETE increased [³H]-thymidine incorporation in vascular smooth muscle cells cultured from male rabbit pulmonary arteries; both the maximal response and potency were greater with 15(S)-HETE. In contrast, HETE analog **1** produced a concentration-dependent inhibition in proliferation and migration of human hormone-independent prostate carcinoma PC-3 cells. The protocol for synthesis of HETE analog **1** is reported. The stability of this substance and its similar biological profile to 15(S)-HETE support future studies in eicosanoid research.

Keywords

Arachidonic acid; 15-lipoxygenase; 15-HETE; vasoconstriction; proliferation

INTRODUCTION

Arachidonic acid (5Z,8Z,11Z,14Z-eicosatetraenoic acid) is a 20-carbon polyunsaturated fatty acid stored in the phospholipids of the cell membrane. A variety of physiological and pathological stimuli release arachidonic acid from membrane phospholipids. Free arachidonic acid is metabolized by at least three different enzyme systems including cyclooxygenases, lipoxygenases and cytochrome P450 epoxygenases to a variety of

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metabolites with a range of biological actions [1]. Of particular interest are the vascular effects of 15-lipoxygenase-derived arachidonic acid metabolites. An oxygenation reaction catalyzed by a specific 15-lipoxygenase converts arachidonic acid to 15-hydroperoxyeicosatetraenoic acid (15-HPETE). 15-HPETE is a short-acting compound and is further metabolized to 15-hydroxyeicosatetraenoic acid (HETE). 15-HPETE and 15-HETE have distinct biological properties depending on the target cell. For example, 15-HETE is a potent vasoconstrictor in various vascular tissues [2]. In previous studies it was found that female pulmonary arteries have enhanced 15-HETE-induced contractions compared to males, identifying for the first time sex-specific differences in 15-HETE-induced contractions [3]. 15-HETE is also reported to be a pro-atherogenic metabolite because of its ability to stimulate vascular cell proliferation [4]. However, these mitogenic effects appear to be cell-specific because other studies in cancer cells reported that 15-HETE reduced cell proliferation [5–9].

The natural product 15(S)-HETE has been shown to stimulate ocular mucin secretion in vitro [10, 11]. Research at Alcon demonstrated that 15(S)-HETE protects the cornea in a rabbit model of desiccation-induced injury [12]. These observations led to the investigation at Alcon of 15(S)-HETE as a therapeutic agent for treating dry eye disorders [13]. Early studies along these lines were hampered by the susceptibility of 15(S)-HETE to autoxidation; however, a surprising increase in stability was realized by converting this carboxylic acid to the sodium or ammonium salt [14, 15]. In a quest to further improve stability, a program of chemical synthesis was then initiated. Analogs were prepared in which the labile skipped polyene array in 15(S)-HETE was replaced with less reactive functional groups [16] [17]. (5Z,11Z,15R)-15-Hydroxyeicosa-5,11-dien-13-ynoic acid (**1**), its S enantiomer and the racemic mixture were among the synthetic compounds selected for study in various biological assays. In the present report, we disclose the effects of 15(S)-HETE and HETE analog **1** on vascular contractions in female and male rabbit pulmonary artery, and on proliferation of both vascular smooth muscle and prostate cancer cells.

MATERIALS AND METHODS

Animals

Animal protocol was approved by the Institutional Animal Care and Use Committee of the Medical College of Wisconsin, and procedures were performed in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (1996). Six to eight week old female and male New Zealand White (NZW) rabbits were obtained from Kuiper Rabbit Ranch (Gary, IN). The animals were housed in the Medical College of Wisconsin Biomedical Research Facilities and maintained on a standard rabbit chow diet and given tap water *ad libitum*.

Vascular Reactivity

Pulmonary artery tissue was isolated as previously described [18]. Briefly, female and male NZW rabbits were sacrificed (sodium pentobarbital, 120 mg/kg, iv), the heart and lungs removed as a unit, and placed immediately in a Krebs's bicarbonate buffer of the following composition (mmol/L): NaCl 118, KCl 4, CaCl₂ 3.3, NaHCO₃ 24, KH₂PO₄ 1.4, MgSO₄ 1.2

and glucose 11, pH 7.4. The main pulmonary artery was identified at its origin from the right ventricle, and both left and right pulmonary arteries were dissected to their most distal end. The pulmonary artery distal to the first branching of the left or right pulmonary artery was used, and this is referred to as the intrapulmonary artery. After dissection, the tissue was cleaned of adherent lung parenchyma and connective tissue using care not to disturb the endothelial layer. Rings of intrapulmonary artery were obtained (2–3 mm) and mounted in a four-chamber myograph (model 610M, Danish Myo Technology) containing Kreb's bicarbonate buffer (warmed to 37° C) and continuously aerated with a 95% O₂-5% CO₂ mixture. The resting tension was 1.0 g and was not different between females and males. Vessels equilibrated at resting tension for 1 hr. Contractions were produced by raising the KCl concentration to 40 mmol/L. After vessels had reproducible, stable contractions to KCl, then cumulative concentration-response curves to 15(S)-HETE (10⁻⁹–5×10⁻⁶ mol/L) or HETE analog **1** (10⁻⁹–10⁻⁵ mol/L) were determined. Experimental preparations were randomly assigned to receive either 15(S)-HETE or HETE analog **1** in order to circumvent any desensitization effects due to multiple concentration-response curves in same vessel. To standardize for any minor differences in tissue size, contractile responses were expressed as a percentage of the maximal response to 40 mmol/L KCl. Maximal KCl contractions were not different between females and males (1.06 ± 0.07 gr vs 0.92 ± 0.09 gr; female vs male).

Measurement of [³H]-thymidine Incorporation

Vascular smooth muscle cells were isolated and cultured from male rabbit pulmonary arteries using previously described methods [19]. After collection of endothelial cells, strips of denuded vessels were placed into gelatin coated flasks with M199 medium containing 10% fetal calf serum with L-glutamine (1%) and antibiotics (1% antibiotic/antimycotic solution; 10,000 units penicillin, 10 mg streptomycin, 25 ng amphotericin B (Sigma)). Smooth muscle cells migrated to the flasks within three to five days. Once growth was established on the coated flasks, then the vessels were removed and the cells resuspended in M199 medium containing 20% fetal calf serum. Purity of smooth muscle cells was confirmed by positive staining for smooth muscle cell α-actin. Human hormone-independent prostate carcinoma PC-3 cells were obtained from the American Type Culture Collection (Manassas, VA). PC-3 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, l-glutamine (2 mM), streptomycin (100 µg/mL), and penicillin (100 U/mL) at 37 °C in 5% CO₂. To measure [³H]-thymidine incorporation, pulmonary artery vascular smooth muscle cells or PC-3 cells were grown to 60% confluency in 24-well plates. The cells were exposed to Dulbecco's modified Eagle's medium (DMEM) containing antibiotics (1% antibiotic/antimycotic solution) and 0.5% rabbit serum or fetal bovine serum to arrest cell growth. After 48 hours, new DMEM was added containing 0.5 % appropriate serum, and the cells were stimulated with increasing concentrations of 15(S)-HETE or HETE analog **1** for 24 hours. [³H]-Thymidine (1 µCi/ well) was added during the final 4 hours. The medium was removed, and cells were washed with ice cold PBS, followed by 0.3 mL of ice cold perchloric acid (0.3 M) and PBS. In order to solubilize the cells, 0.5 mL of 0.1% SDS/0.1 N NaOH was added to each well. Radioactivity in the solubilized monolayer was determined by liquid scintillation spectrometry.

Cell Migration

For cell migration measurement by X-celligence system, PC3 cells (30,000 cells/well) were plated in the upper chamber (8.0 μ m filter pores without Matrigel) of the Migration CIM-Plate of the Real Time Cell Analyzer DP instrument (Roche Applied Science) in the serum-free media [20]. Media containing 10% fetal bovine serum was placed in the bottom chamber. Varying concentrations of HETE analog **1** were added in the upper chamber and the system was equilibrated for 30 min in the incubator at 37 °C. Then cell migration was monitored and recorded every 15 min for up to 72 h. Four wells were used and averaged for each treatment concentration.

Stability Assay

To estimate relative storage stability, 15(S)-HETE and HETE analog compounds were subjected to a heat-accelerated autoxidation assay. Each HETE analog was dissolved in 100% ethanol (about 3300 μ g/mL) as a stock standard (SS). Thirty microliters (30 μ L) of SS was added to 3.0 mL of diluent solution (D.S.), 50% EtOH/H₂O, v/v, to be used as reference standard. Another thirty microliters (30 μ L) of SS was dried with nitrogen gas at room temperature and then stressed in an oven in air at 75°C for either 15 minutes, three hours, one week, two weeks or three weeks. When a sample was removed from the oven, it was cooled in an ice/water bath, then the residue was redissolved with 3.0 mL D.S. Each heat-stressed sample was then analyzed against its unstressed reference standard using HPLC.

HETE Analog Synthesis

A synthetic route suitable for producing HETE analog **1** on multigram scale was developed. To summarize, 6-heptynoic acid was converted [21] to iodo acid **2** and thence to amide **3** (70% yield). Hydroboration [22] of **3** afforded cis iodo alkene **4** (96%). Coupling [23] of **4** with (R)-1-octyn-3-ol [24] yielded enynol **5** (80%), which was silylated to give **6** (91%). Hydride reduction of **6** gave aldehyde **7** (88%), which upon Wittig reaction with 4-carboxybutyltriphenylphosphane followed by desilylation provided **1** (89%). The synthetic scheme is shown in Figure 1.

7-Iodo-6-heptynoic acid (2)—Potassium hydroxide (85%, 32 g, 0.49 mol) was dissolved in a stirred solution of 6-heptynoic acid (18 g, 0.14 mol) in 145 mL of MeOH and 72 mL of H₂O under N₂. The solution was cooled in ice. Iodine (44 g, 0.17 mol) was added and the flask was wrapped with Al foil to exclude light. The temperature was allowed to rise to 23 °C over several hours. After 3 days the colorless mixture was poured into excess 1 M aq NaHSO₄ whereupon excess I₂ was liberated. The product was extracted into EtOAc and the brown color of I₂ was discharged with NaHSO₃. The organic solution was extracted with water (2x) and brine, dried (MgSO₄), filtered and concentrated to give 37.4 g of **2** as a tan solid. This material was used in the following step without purification.

N,O-Dimethyl-7-iodo-6-heptynohydroxamate (3)—N-3-(Dimethylamino)propyl-N'-ethylcarbodiimide hydrochloride (55 g, 0.29 mol) was added to a stirred solution of compound **2** (37.4 g, ~ 0.15 mol), N,O-dimethylhydroxylamine hydrochloride (28.8 g, 0.295 mol), *i*-Pr₂NEt (51 mL, 0.30 mol) and 4-DMAP (3.6 g, 0.030 mol) in 350 mL of dry DMF

under N₂. The mixture was stirred for 16 h, then quenched with water and stirred for a further 20 min. The product was extracted into EtOAc and the organic solution was washed with water (2x), sat. aq. KH₂PO₄ (to pH 4.5), water and brine, dried (MgSO₄), filtered and concentrated. The residue was purified by chromatography on 300 g of silica, eluting with 40% → 50% EtOAc/hexane, to give 30.4 g of a 94:6 mixture of **3** (70% over two steps) and desido-**3**. ¹H NMR (CDCl₃) δ 1.57 (pent, *J* = 7, 2H), 1.74 (pent, *J* = 7, 2H), 2.40 (t, *J* = 7, 2H), 2.44 (br t, *J* = 7, 2H), 3.18 (s, 3H), 3.69 (s, 3H). LC/MS (+APCI) 296 (M+H)⁺.

N,O-Dimethyl-(Z)-7-iodo-6-heptenohydroxamate (4)—Borane·Me₂S (1.0 mL, 10 mmol) was added to a stirred, ice-cooled solution of cyclohexene (2.1 mL, 21 mmol) in 8.5 mL of dry diethyl ether under N₂. The mixture was allowed to warm to 23 °C, resulting in the precipitation of dicyclohexylborane, and was then re-cooled in ice. A solution of iodo alkyne **3** (2.45 g, 94% pure, 8.0 mmol) in 10 mL of dry diethyl ether was added over 3 min. The mixture was allowed to warm to 23 °C whereupon the solid dissolved, then re-cooled in ice. Acetic acid (0.57 mL, 10 mmol) was added, the solution was allowed to warm to 23 °C, then re-cooled in ice. Ethanolamine (1.3 mL, 22 mmol) was added and the solution was allowed to warm to 23 °C whereupon a granular solid precipitated. Stirring was continued for 16 h. The mixture was eluted through a pad of silica with Et₂O and concentrated to give 2.76 g of an oil. This material was purified by chromatography on 125 g of silica, eluting with 33% → 40% → 50% EtOAc/hexane, to give 2.28 g (96%) of **4** as an oil. ¹H NMR (C₆D₆) δ 0.18 (s, 3H), 0.28 (s, 3H), 0.86 (t, *J* = 7, 3H), 1.03 (s, 9H), 1.27 (m, 4H), 1.42 (pent, *J* = 7, 2H), 1.55 (m, 2H), 1.81 (sept, *J* = 7, 4H), 2.32 (t, *J* = 7, 2H), 2.42 (q, *J* = 7, 2H), 2.88 (s, 3H), 3.05 (s, 3H), 4.60 (t, *J* = 6.5, 1H), 5.50 (d, 1H, *J* = 11), 5.67 (m, 1H). LC/MS (+APCI) 427 (M+NH₄)⁺, 410 (M+H)⁺, 278 (M+H-TBSOH)⁺.

N,O-Dimethyl-(6Z,10R)-10-hydroxypentadec-6-en-8-ynohydroxamate (5)—Under N₂, iodo alkene **4** (1.96 g, 6.61 mmol) was dissolved in 45 mL of diethylamine (dried over 3A molecular sieves and deoxygenated with a stream of N₂). (Ph₃P)₂PdCl₂ (0.13 g, 0.18 mmol, 2.7 mol %) was added, followed by CuI (Alfa, 99.999%) (73 mg, 0.38 mmol, 5.8 mol %). After 10 min the mixture was purged with a stream of H₂ for 2 min and then placed under an atmosphere of ~1:1 H₂-N₂ (balloon). R-(+)-1-Octyn-3-ol (Aldrich, 99%) (1.00 mL, 6.8 mmol) was then added in 0.1-mL portions over 2 min. After 1.7 h, the mixture was partitioned between diethyl ether and water. The organic extract was washed with 1 M aqueous Na₂S₂O₃ and then eluted through a pad of Florisil with EtOAc. The eluate was dried (Na₂SO₄), filtered and concentrated. The residue was purified by chromatography on 100 g of silica, eluting with 45% → 50% EtOAc/hexane, to give 1.57 g (80%) of **5** as an oil. ¹H NMR (C₆D₆) δ 0.86 (t, *J* = 7, 3H), 1.23 (m, 4H), 1.42 (pent, *J* = 7, 2H), 1.54 (br pent, *J* = 7, 2H), 1.77 (sept, *J* = 7, 4H), 2.32 (br t, *J* = 7, 2H), 2.36 (q, *J* = 7, 2H), 2.84 (s, 3H), 2.95 (br d, *J* = 6.5, 1H, OH), 3.00 (s, 3H), 4.53 (q, *J* = 6.5, 1H), 5.53 (d, *J* = 11, 1H), 5.71 (m, 1H). LC/MS (+APCI) 296 (M+H)⁺, 278 (M+H-H₂O)⁺.

N,O-Dimethyl-(6Z,10R)-10-(tert-butyldimethylsiloxy)pentadec-6-en-8-ynohydroxamate (6)—*tert*-Butyldimethylchlorosilane (1.6 g, 11 mmol) was added to a stirred solution of enynol **5** (1.55 g, 5.25 mmol) and imidazole (1.4 g, 20 mmol) in 10 mL of dry DMF under N₂. After 1.5 h, the flask and contents were transferred to a cold room

(5 °C) overnight. The mixture was then quenched with water, allowed to warm to 23 °C, and stirred for 2 h. Diethyl ether was added and the mixture was washed with water (2x), brine, dried (Na₂SO₄), filtered and concentrated to give 2.19 g of an oil. This material was purified by chromatography on 130 g of silica, eluting with 20%→25% EtOAc/hexane, to give 1.96 g (91%) of **6** as an oil. ¹H NMR (C₆D₆) δ 0.18 (s, 3H), 0.28 (s, 3H), 0.86 (t, *J* = 7, 3H), 1.03 (s, 9H), 1.27 (m, 4H), 1.42 (pent, *J* = 7, 2H), 1.55 (m, 2H), 1.81 (sept, *J* = 7, 4H), 2.32 (t, *J* = 7, 2H), 2.42 (q, *J* = 7, 2H), 2.88 (s, 3H), 3.05 (s, 3H), 4.60 (t, *J* = 6.5, 1H), 5.50 (d, 1H, *J* = 11), 5.67 (m, 1H). LC/MS (+APCI) 427 (M+NH₄)⁺, 410 (M+H)⁺, 278 (M+H-TBSOH)⁺.

(6Z,10R)-10-(tert-Butyldimethylsiloxy)pentadec-6-en-8-ynal (7)—

Diisobutylaluminum hydride (5.4 mL of a 1.0 M solution in toluene) was added dropwise to a stirred, cooled (−78 °C bath) solution of compound **6** (1.76 g, 4.30 mmol) in 38 mL of dry toluene under N₂. After 15 min, the reaction was quenched by dropwise addition of MeOH (2 mL) and then allowed to warm to 23 °C. Diethyl ether and sat. aq. Rochelle's salt were added and the mixture was stirred vigorously for 15 min, then separated. The organic solution was washed with water (2x), brine, dried (MgSO₄), filtered and concentrated to give 1.50 g of an oil. This material was purified by chromatography on 100 g of silica, eluting with 12% EtOAc/hexane, to give 1.32 g (88%) of **7** as an oil which was used without delay in the next step.

(5Z,11Z,15R)-15-Hydroxyeicosa-5,11-dien-13-ynoic acid (1)—Potassium *tert*-

butoxide (12 mL of a 1.0 M solution in THF) was added to a stirred suspension of 4-carboxybutyltriphenylphosphonium bromide (2.5 g, 5.6 mmol, dried *in vacuo* at 65 °C) in 20 mL of dry THF under N₂. After 15 min the orange-red mixture was cooled in a −78 °C bath. A solution of aldehyde **7** (1.32 g, 3.77 mmol) in 20 mL of dry toluene was added over 15 min, keeping the internal temperature between −68 and −71 °C. The mixture was allowed to warm to 23 °C, then quenched with sat. aq. KH₂PO₄ and stirred for 16 h. The biphasic mixture (pH of aqueous phase = 4.5) was extracted twice with diethyl ether. The combined organic extract was dried (Na₂SO₄), eluted through a silica gel pad with diethyl ether and concentrated to give 1.54 g of an oil. To a stirred solution of this material in 20 mL of THF under N₂ was added tetrabutylammonium fluoride (1.0 M in THF, 20 mL) in two equal portions 16 h apart. After a further 4 h, the mixture was diluted with half-saturated brine and extracted with three portions of EtOAc. The combined organic extract was dried (Na₂SO₄), filtered and concentrated to give 1.7 g of an oil. This material was purified by chromatography on 125 g of silica, eluting with 50% EtOAc/hexane, to give 1.08 g (89%) of **1** as an oil. The ratio of 5Z to 5E isomers was determined to be 94.4 : 5.6 by ¹H NMR. ¹H NMR (C₆D₆) δ 0.87 (t, *J* = 7, 3H), 0.93 (s, 1H, OH), 1.22 (m, 4H), 1.35 (m, 4H), 1.46 (br t, *J* = 7, 2H), 1.57 (br t, *J* = 7, 2H), 1.71 (m, 2H), 2.00 (br q, *J* = 7, 4H), 2.16 (t, *J* = 7, 2H), 2.38 (br q, *J* = 6.5, 2H), 4.45 (br t, *J* = 6.5, 1H), 5.27 (m, 1H), 5.41 (m, 1H), 5.52 (d, *J* = 11, 1H), 5.70 (m, 1H). ¹³C NMR (C₆D₆) δ 14.2, 22.9, 24.8, 25.3, 26.6, 27.2, 28.6, 29.5, 30.3, 31.8, 33.1, 38.3, 63.2, 81.8, 95.5, 109.4, 129.0, 131.2, 144.0, 178.8. LC/MS (+ESI) 303 (M+H-H₂O)⁺; (−ESI) 319 (M−H)[−].

Statistical Analysis

Data are expressed as the mean $\pm$ SEM. Statistical analysis of the vascular reactivity data was performed by a two-way ANOVA followed by Bonferroni's posttest. The cell proliferation and cell migration studies were analyzed using Student's t-test. Values were considered significant at $P < 0.05$.

Materials

15(S)-HETE was obtained from Cayman Chemical (Ann Arbor, MI). [^3H]-Thymidine was obtained from Du Pont NEN (Boston, MA). Cell culture reagents were obtained from Invitrogen (Carlsbad, CA). Flasks used in cell culture were obtained from Corning (Corning, NY). Chemicals used in the synthesis of HETE analog **1** were obtained from Sigma-Aldrich (Milwaukee, WI). All other chemicals were of reagent grade.

RESULTS

Stability assays at 75°C indicated that 15(S)-HETE decomposed to the extent of $78 \pm 2\%$ in 15 minutes, while HETE analog **1** (racemic form) showed only $1.6 \pm 0.4\%$ decomposition after 3 hours (Table 1). Thus HETE analog **1** proved suitable for extended storage and periodic sampling in various forms (neat, solution, formulation) with no special precautions other than routine refrigeration at 5 °C under nitrogen.

The ability of 15(S)-HETE and HETE analog **1** to contract pulmonary arteries from female and male rabbits is shown in Figure 2. Both 15(S)-HETE and HETE analog **1** produced a concentration-dependent vasoconstriction that was significantly greater in females compared to males. The EC_{50} values were not different between 15(S)-HETE and HETE analog **1** (see Table 2). While both compounds had a greater effect in females, the maximal response was greater with 15(S)-HETE compared to HETE analog **1** (maximal contraction in females; $49.7 \pm 4.1\%$ vs $27.3 \pm 4.4\%$; 15(S)-HETE vs HETE analog **1**). In males the maximal contraction to the two compounds was not different (maximal contraction in males; $19.3 \pm 2.8\%$ vs $24.1 \pm 2.3\%$; 15(S)-HETE vs HETE analog **1**).

The effect of 15(S)-HETE and HETE analog **1** on vascular smooth muscle cell proliferation is shown in Figure 3. Both compounds increased [^3H]-thymidine incorporation but the effect was greater with 15(S)-HETE (top panel) compared to HETE analog **1**. Results indicated that 15(S)-HETE was more potent than HETE analog **1**, as no effect on proliferation was seen with 10 μM HETE analog **1** but the same concentration of 15(S)-HETE caused a 2-fold increase compared to control cells (* $p < .05$). In the PC-3 cells, HETE analog **1** had opposite effects to those seen in the vascular smooth cells. A concentration-dependent decrease in [^3H]-thymidine incorporation was observed (Figure 4). Table 3 compares the EC_{50} for compounds in vascular smooth muscle and PC3 cells on cell proliferation. In a separate study, HETE analog **1** caused a concentration-dependent decrease in the migration of cells through the filter pores of Boyden chamber as measured by X-celligence and compared to control cells (Figure 5). The top panel shows an example of cell migration through the filters in the PC3 cells and the bottom panel is the averaged migration results after 24 hours.

DISCUSSION

In this study, the biological characterization of HETE analog **1** showed that it contracted rabbit pulmonary artery. However, it was less potent and efficacious than the naturally occurring metabolite 15(S)-HETE. An interesting observation was that 15(S)-HETE showed a greater sex-specific difference than did HETE analog **1**. While the vasoconstrictor mechanisms of many arachidonic acid metabolites have been well defined, less is known about 15-HETE. Early studies reported that 15-HETE constricted isolated blood vessels, including pulmonary arteries [25–27]. This contrasts with other work showing that 15-HETE relaxes precontracted vessels [28, 29]. The exact cellular processes that contribute to 15-HETE vasoconstriction were addressed primarily by the work of Zhu and colleagues [30–32]. In pulmonary vascular smooth muscle cells of neonatal rabbits exposed to hypoxia or normoxia, 15-HETE decreased whole cell Kv currents. With rat pulmonary artery vascular smooth muscle cells, 15-HETE acted at Kv 1.5, Kv 1.2 and Kv 3.4 channels [33]. PKC inhibition also blocked 15-HETE-induced vasoconstriction in the isolated rat aortas. It is not known whether 15-HETE activates different vasoconstrictor mechanisms in females compared to males. It also remains unresolved whether 15-HETE acts on a specific membrane receptor like many other biologically active arachidonic acid metabolites. The relaxations to 15-HETE noted by other studies happened in vessels precontracted with either the thromboxane mimetic U46619 or prostaglandin PGF_{2α} raising the possibility that the mechanism of effect is due to non-specific blockade of thromboxane or PGF receptors. HETE analog **1** will be an important tool for aiding in the delineation of cellular mechanisms that contribute to sex-specific differences in 15-HETE-induced alterations in vascular tone.

Many studies have shown that 15-HETE increases vascular smooth muscle cell proliferation and this was confirmed by our studies using rabbit pulmonary artery smooth muscle cells. HETE analog **1** similarly increased proliferation, although the analog was less potent and maximal effect was reduced when compared to 15(S)-HETE. This effect of 15-HETE and HETE analog **1** was not unexpected, as 15-HETE has been shown to have mitogenic effects in other vascular cells including endothelial cells and fibroblasts. Atherosclerosis is associated with increased expression of 15-lipoxygenase and 15-HETE. Numerous studies have reported a role for 15-HETE in angiogenesis that contributes to progression of atherosclerosis [34–37].

In contrast to the vascular cells, the role of 15-lipoxygenase and 15(S)-HETE in cancer is unclear and controversial [8]. To review, two mammalian forms of 15-lipoxygenase are known to exist, 15-lipoxygenase-1 and 15-lipoxygenase-2. 15-lipoxygenase-1 utilizes arachidonic acid to form 15(S)-HETE and to a lesser extent, 12(S)-HETE. 15-lipoxygenase-1 also utilizes linoleic acid to form 13(S)-hydroxyoctadecadienoic acid (13-HODE). The preferred substrate for 15-lipoxygenase-2 is arachidonic acid which is converted to 15(S)-HETE. In prostate cancer, 12(S)-HETE is proangiogenic while 15(S)-HETE is antiangiogenic [38, 39]. Using PC3 cells, Shappel and coworkers [6] showed that exogenous 15(S)-HETE caused a concentration-dependent inhibition of proliferation with the soft agar colony formation assay (IC₅₀ of ~ μ30 M). We tested HETE analog **1** on [³H]-thymidine incorporation in PC3 cells and also found it decreased cell proliferation in a

concentration-dependent manner with an IC₅₀ of ~ 2 μ M. The ability of cancer cells to undergo migration and invasion contribute to metastatic characteristic of various cancers. A migration assay with the PC3 cells showed that HETE analog **1** decreased cell migration over a 72 hour period. These results support the experimental evidence in studies that have reported that overexpression of 15-lipoxygenase-2 in PC3 cells causes these cells to stop proliferating and become senescent [39]. Because of the gaps in knowledge on the precise contribution of 15-lipoxygenase metabolites to cancer progression, this area of investigation remains an active research focus and benefits from the availability of a stable HETE analog.

CONCLUSIONS

The present studies confirm that 15(S)-HETE has a role in both pulmonary artery vasoconstriction and cellular proliferation. These effects combined with previous work by others suggest that 15(S)-HETE could potentially contribute to a variety of diseases like hypertension, atherosclerosis and cancer. Furthermore, 15-lipoxygenase metabolites have been shown to contribute to many other conditions, including obesity, diabetes and respiratory disease [40–42]. However, many details of 15(S)-HETE cell signal transduction mechanisms are not understood. An important contribution of the current work is the synthesis and biological characterization of the stable HETE analog **1**. This analog will be an integral tool in future studies to further elucidate the mechanism of endogenous 15(S)-HETE. Knowledge of these mechanisms will advance eicosanoid research and may ultimately translate to improved pharmacological treatments of disease.

Acknowledgments

The authors thank Dr. John Liao, Ms. Thivashnee Pillay, and Mr. Adam Pfeiffer for their technical assistance, and Ms. Gretchen Barg for her secretarial assistance. Studies at Medical College of Wisconsin were supported by grants from the National Heart, Lung and Blood Institute (HL093181) and American Heart Association (0151421Z).

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HIGHLIGHTS

Pulmonary artery vasoconstriction in rabbits is produced by 15(S)-HETE and by HETE analog **1**. Response to 15(S)-HETE is greater and sex-specific when compared to HETE analog **1**. Both 15(S)-HETE and HETE analog **1** inhibit pulmonary artery vascular smooth muscle cell proliferation, with a more potent effect observed with 15(S)-HETE. In human hormone-independent prostate carcinoma PC-3 cells, HETE analog **1** inhibits proliferation and migration. HETE analog **1** is a stable compound that will be a useful tool in future eicosanoid research.

Figure 1.
Chemical Synthesis of HETE analog **1**

Figure 2.

Contractile responses to 15(S)-HETE (top panel) and HETE analog **1** (bottom panel) in female and male rabbit pulmonary arteries. Results are expressed as percent KCl (40 mM) contractions, and data points are the mean $\pm$ SEM for $n = 8-12$. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; female vs male.

Figure 3.

15(S)-HETE- (top panel) and HETE analog **1** (bottom panel) stimulated [³H]thymidine incorporation in rabbit pulmonary artery vascular smooth muscle cells. The values are normalized to [³H]thymidine uptake by control cells under basal conditions. Results are the mean ± SEM from six independent experiments conducted with quadruplicate samples in each experiment. Symbols above a column indicate a statistical comparison between the indicated sample and the control cells (* p < 0.01).

Figure 4.

Effect of HETE analog **1** on [³H]thymidine incorporation in PC3 cells. The values are normalized to [³H]thymidine uptake by control cells under basal conditions. Results are the mean ± SEM from six independent experiments conducted with quadruplicate samples in each experiment. Symbols above a column indicate a statistical comparison between the indicated sample and the control cells (* p < 0.01).

Figure 5.

Effect of HETE analog **1** on cell migration in PC3 cells. Top panel is representative example of cell migration through filters over the 72 hour collection period and bottom panel is summarized results of cell migration at the 24 hour time point. Results are the mean $\pm$ SEM ($n = 4$). Symbols above a column indicate a statistical comparison between the specified sample and the control cells (* $p < 0.05$).

TABLE 1

PERCENT DECOMPOSITION AT 75°C

	0.25 hours	3.0 hours	330 hours
HETE analog 1	0.0 ± 0.0	1.6 ± 0.4	94 ± 1
15(S)-HETE	78 ± 1	ND	ND

Data presented as mean ± SEM. ND, not determined.

TABLE 2

COMPARISONS OF RESPONSES TO 15(S)-HETE AND HETE ANALOG **1** IN FEMALE AND MALE PULMONARY ARTERY

		15(S)-HETE	HETE ANALOG 1
FEMALE	EC ₅₀ (μM)	542.5 ± 203.0	953.7 ± 326.7
	maximal contraction (% of KCl)	49.7 ± 4.1	27.3 ± 4.4
MALE	EC ₅₀ (μM)	618.1 ± 272.6	1250.0 ± 406.1
	maximal contraction (% of KCl)	19.3 ± 2.8	24.1 ± 2.3

Values are the mean ± SEM EC₅₀, concentration of agonist yielding 50% of the maximal response. EC₅₀ values were calculated using the nonlinear regression sigmoid curve fitting program of PRISM.

TABLE 3

COMPARISONS OF RESPONSES TO 15(S)-HETE AND HETE ANALOG 1 IN VASCULAR SMOOTH MUSCLE AND PC3 CELLS

		15(S)-HETE	HETE ANALOG 1
VSMC	EC ₅₀ (μM)	5.6 ± 3.5	634 ± 64.9 *
PC3	EC ₅₀ (μM)	N.D.	1.5 ± 0.9

Values are the mean ± SEM EC₅₀, concentration of agonist yielding 50% of the maximal response. EC₅₀ values were calculated using the nonlinear regression sigmoid curve fitting.

* P < 0.05 HETE ANALOG 1 VS 15-HETE. ND, not determined.