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Metabolism of 4-Hydroxy-7-oxo-5-heptenoic Acid (HOHA) Lactone by Retinal Pigmented Epithelial Cells

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Abstract

4-Hydroxy-7-oxo-5-heptenoic acid (HOHA)-lactone is a biologically active oxidative truncation product released ($t_{1/2} = 30$ min at 37 °C) by non-enzymatic transesterification/deacylation from docosahexaenoate lipids. We now report that HOHA-lactone readily diffuses into retinal pigmented epithelial (RPE) cells where it is metabolized. A reduced glutathione (GSH) Michael adduct of HOHA-lactone is the most prominent metabolite detected by LC-MS in both the extracellular medium and cell lysates. This molecule appeared inside of ARPE-19 cells within seconds after exposure to HOHA-lactone. The intracellular level reached a maximum concentration at 30 min and then decreased with concomitant increases in its level in the extracellular medium, thus revealing a unidirectional export of the reduced GSH-HOHA-lactone adduct from the cytosol to extracellular medium. This metabolism is likely to modulate the involvement of HOHA-lactone in the pathogenesis of human diseases. HOHA-lactone is biologically active, e.g., low concentrations (0.1–1 μ M) induce secretion of vascular endothelial growth factor (VEGF) from ARPE-19 cells. HOHA-lactone is also a precursor of 2-(ω -carboxyethyl)pyrrole (CEP) derivatives of primary amino groups in proteins and ethanolamine phospholipids that have significant pathological and physiological relevance to age-related macular degeneration (AMD), cancer and wound healing. Both HOHA-lactone and the derived CEP can contribute to the angiogenesis that defines the neovascular “wet” form of AMD and that promotes the growth of tumors. While GSH depletion can increase the lethality of radiotherapy, because it will impair the metabolism of HOHA-lactone, the present study suggests that GSH depletion will also increase levels of HOHA-lactone and CEP that may promote recurrence of tumor growth.

INTRODUCTION

Vision is the consequence of light induced activation of rhodopsin, a membrane-bound protein that resides in a stack of discs located within photoreceptor cells. Photoreceptor disc membrane phospholipids are extraordinarily rich in docosahexaenoate. Previously we showed that oxidative truncation of docosahexaenoate-containing phosphatidylcholine generates 1-palmityl-2-(4-hydroxy-7-oxo-5-heptenoyl)-*sn*-glycero-3-phosphatidylcholine (HOHA-PC) that rapidly releases HOHA-lactone ($t_{1/2} = 30$ min at 37 °C) by non-enzymatic transesterification/deacylation (Figure 1).¹ While HOHA esterified to phospholipids is

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membrane bound, HOHA-lactone is only slightly lipophobic (CLogP = -1.02) and is expected to be comparable to cortisone (CLogP = -0.93) in its ability to diffuse across cell membranes. Previously, we showed that micromolar levels of HOHA-lactone are toxic to retinal pigmented epithelial (RPE) cells that are adjacent to photoreceptor cells (Figure 1).² Consequently, protecting cells from the cascade of HOHA-lactone released from photoreceptor disc membranes owing to oxidative damage is important for the viability of cells in the retina

HOHA-lactone not only is biologically active, but also undergoes covalent adduction with biological nucleophiles, such as proteins and ethanolamine phospholipids. HOHA-lactone readily modifies primary amino groups in proteins and ethanolamine phospholipids in cell membranes to produce 2-(ω -carboxyethyl)pyrrole (CEP) derivatives (Figure 1).² Previously, we discovered that CEP immunoreactivity is localized in mouse retina that exhibits prominent anti-CEP staining in the photoreceptor rod outer segments and retinal pigmented epithelium.³ Western blot analysis of PAGE gels of protein extracts from the choroid/RPE/rod outer segment complex of AMD retinas show remarkably elevated levels of CEP immunoreactivity compared with protein extracts from age-matched control retinas with no disease.⁴ CEP induces migration and tube formation by human umbilical vein endothelial cells resulting in angiogenesis through a Toll-like receptor (TLR)2 mediated pathway^{5, 6} that can contribute to choroidal neovascularization⁷, the uncontrolled blood vessel growth into the neural retina, that characterizes the advanced “wet” form of age-related macular degeneration (AMD).⁸

The human retinal pigment epithelial cells cooperate with adjacent rod photoreceptor cells in performing the multistep contrathermodynamic *trans* to *cis* isomerization of retinal in the visual cycle. It is also responsible for phagocytosis of oxidatively damaged discs that accumulate in photoreceptor cell outer segment tips that are endocytosed by adjacent RPE cells.⁹ Owing to high oxygen tension and photoinitiation of free radical-induced oxidative damage in the retina, the entire stack of photoreceptor discs is replaced every ten days.^{8, 10, 11} Defects in RPE function, whether through chronic dysfunction or age-related decline, are associated with retinal degenerative diseases including age-related macular degeneration (AMD).¹² We postulated that, besides their roles in the visual cycle and the disposal of damaged photoreceptor discs, RPE cells might also participate in detoxification of HOHA-lactone released from photoreceptor disc membranes. We now report that RPE cells metabolize HOHA-lactone. Upon exposure to 10 mM HOHA-lactone, its Michael adduct with glutathione begins to appear intracellularly within seconds, but this adduct is only detected in the extracellular medium when the cells are exposed to higher concentrations of HOHA-lactone. Instead, the aldehyde group of the Michael adduct is reduced and the reduced Michael adduct is secreted.

EXPERIMENTAL PROCEDURES

Materials

Reduced L-glutathione, sodium borohydride, 2-methyl-2-butene solution (2.0 M in THF), sodium chlorite, tert-butanol, glutathione reductase from baker's yeast (250 units/ml. Sigma catalogue #G3664), 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), and NADPH were

purchased from Sigma-Aldrich (St. Louis, MO). HOHA-lactone (**1**) was prepared as described previously.¹ N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was obtained from Acros Organics (Thermo Fisher, New Jersey). Dulbecco's modified Eagle's medium, high glucose (DMEM/F12), fetal bovine serum, penicillin–streptomycin and L-glutamine were obtained from Gibco (Life Technologies, Grand Island, NY). ARPE-19 cells were obtained from American Type Culture Collection (ATCC, Rockville, MD). The hRPE cells were obtained from Lonza Walkersville, Inc., Walkersville, MD, catalog # 00194987, lot # 0000424472. According to the manufacturer, they exhibited pan-cytokeratin staining (95%), anti-fibroblast staining (0%) and ZO-1 staining (strongly positive), and they have viability >70%, average doubling time 22 hrs. Retinal pigmented epithelial cell basal medium, an optimized mixture of growth factors and supplements for primary hRPE cells (SingleQuots™ Kit), was obtained from Lonza (Allendale, NJ).

(E)-5-(3-Hydroxyprop-1-en-1-yl)dihydrofuran-2(3H)-one (2)

To a solution of HOHA-lactone, (**1**, 1.4 mg, 10 μ mol) in PBS (pH = 7, 0.5 mL) at 0 °C, a solution of sodium borohydride (10 mM in PBS, 0.5 mL) was slowly added. The mixture was stirred on ice for 1 h. Then the reaction mixture was extracted three times with 2 mL of chloroform. The organic layers were then combined and rotary evaporated to dryness under reduced pressure. The residue was purified by flash silica gel chromatography (chloroform/methanol = 20:1, R_f = 0.20) to give pure **2** (0.8 mg, 57%); ¹H NMR (400 MHz, CDCl₃) δ 5.99 – 5.80 (m, 1H), 5.80 – 5.66 (m, 1H), 5.01 – 4.81 (m, 1H), 4.15 (s, 2H), 2.55 – 2.44 (m, 2H), 2.42 – 2.28 (m, 1H), 2.01 – 1.89 (m, 1H). HRMS (EI): m/z calcd for C₇H₁₀O₃ (M⁺), 142.0630; found, 142.0631.

(E)-3-(5-Oxotetrahydrofuran-2-yl)acrylic acid (3)

To a solution of HOHA-lactone (**1**, 1.4 mg, 10 μ mol) in *t*-BuOH-H₂O (5:1, v/v, 0.3 mL) was added NaH₂PO₄ (2.1 mg, 15 μ mol), 2-methyl-2-butene (50 μ L, 0.10 mmol, 2M solution in THF) and sodium chlorite (2.8 mg, 30 μ mol). The resulting mixture was stirred for 2 h at room temperature under argon. After the solution was concentrated to about 50 μ L by evaporation of solvent under a stream of nitrogen gas, the mixture was diluted to 1 mL with water, and then the pH was adjusted to 2. The solution was extracted three times with 2 mL of chloroform. Then the organic layers were combined, and then rotary evaporated to dryness under reduced pressure. The crude product was purified by flash chromatography on a silica gel column (chloroform: methanol = 15:1, R_f = 0.22) to give pure **3** (1.3 mg, 83%); ¹H NMR (400 MHz, CDCl₃) δ 6.95 (dd, J = 15.6, 4.4 Hz, 1H), 6.07 (dd, J = 15.7, 1.7 Hz, 1H), 5.08 (m, 1H), 2.50 (m, 3H), 2.21 – 1.88 (m, 2H). HRMS (EI): m/z calcd for C₇H₈O₄ (M⁺), 156.0423; found, 156.0421.

Glutathione-HOHA-lactone adduct 4

HOHA-lactone (**1**, 1.4 mg, 10 μ mol) in water (0.5 mL) was mixed with reduced L-glutathione (GSH, 6.14 mg, 20 μ mol). The reaction mixture was stirred at room temperature for 2 h. The resulting mixture was then extracted three times with chloroform to remove unreacted HOHA-lactone. Then the remaining aqueous solution was separated by HPLC. Reverse-phase HPLC was conducted using a Shimadzu UFLC system equipped with a 5 μ m

4.6 × 250 mm Phenomenex Luna C₁₈ column with a water-methanol supplemented with 0.1% formic acid gradient elution system at a flow rate of 1.0 mL/min, with effluent monitoring at 220 nm. Mobile phase A consisted of HPLC grade water containing 0.1% formic acid. Mobile phase B was HPLC grade methanol containing 0.1% formic acid. For purification of **4**, 100 µL of reaction mixture was loaded onto the HPLC column for separation. HPLC gradient steps were as follows: 0-2.5 min, isocratic at 2% solvent B; 2.5-15 min, linear gradient from 2 to 100% solvent B; 15-20 min, isocratic at 100% solvent B; 20-21 min, linear gradient from 100 to 2% solvent B; 21-30 min, isocratic at 2% solvent B to give pure **4** (2 mg, 4.5 µmol, 45%). A representative HPLC chromatogram for purification of compound **4** is shown in Figure 2. The fraction between 11.00 to 13:00 min was collected.

Reduced glutathione-HOHA-lactone adduct **5**

GSH-HOHA-lactone adduct **4** (90.0 µg, 0.2 µmol) in PBS buffer (pH = 7.4, 0.1 mL) was mixed with sodium borohydride (10 mM solution in PBS, 0.2 µmol). The reaction mixture was stirred on ice for 1 h. Then excess NaBH₄ was destroyed by adding 1 µL of neat formic acid. The resulting mixture was loaded onto a reverse phase SPE column (Strata-X 33µ polymeric reverse phase, 100 mg/3ml), which was preconditioned first with 3 mL of methanol then 3 mL of 0.1% formic acid in water. The SPE column was further washed with 3 mL of 0.1% formic acid in water to remove salts and then the compound **5** was eluted by 1 mL of 50% acetonitrile in water.

Cell culture

2 × 10⁶ of ARPE-19 cells were cultured in DMEM/F12 containing 10% fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine at 37 °C, 5% CO₂. 1.5 × 10⁶ of hRPE cells were grown in 60 mm culture dishes and washed with retinal pigmented epithelial cell basal medium. For studies of dose-dependence, 3 mL of basal medium containing various concentrations of HOHA-lactone (0-100 µM) were added and the mixture was incubated for 2 h at 37 °C, 5% CO₂. For time course studies, 3 mL of basal medium containing 10 µM of HOHA-lactone were added and the mixture was incubated for up to 2 h at 37 °C, 5% CO₂. At the end of incubation, the supernatants were collected and designated the “extracellular medium” (ECM). Then the cell dishes were washed three times with the corresponding basal medium, followed by the addition of 400 µL of basal medium. The cells were scraped and collected, and then subjected to sonication at 4 °C and designated the “cell lysate” (CL). The ECM and CL were kept at -20 °C until analysis. The medium was changed every 2 days and all studies were conducted by using confluent cells of passages 20-40 following a 12 h quiescence in serum-free medium. All the experiments were repeated three times (*n* = 3) for measurements of metabolites in cell lysates and extracellular medium by either LC-MS or GC-MS.

High performance liquid chromatography/mass spectrometry

Chromatographic separation was carried out with a Surveyor LC system equipped with a Luna C18(2) column (2.0 mm i.d. × 150 mm length, 5 µm, Phenomenex). Mobile phase A consisted of HPLC grade water containing 0.1% formic acid. Mobile phase B was HPLC

grade methanol containing 0.1% formic acid. ESI mass spectrometry was performed with Thermo Finnigan LCQ Deca XP instrument in the positive ion mode using nitrogen as the sheath and auxiliary gas. The heated capillary temperature was 300 °C, the source voltage was 4.5 kV, and the capillary voltage was 31.00 V. All data were processed with Qual browser in Xcalibur software.

Before analysis, the cell lysates and extracellular medium samples were centrifuged at 10,000 g for 5 min. Then the supernatant was transferred to a new vial and stored on ice. Sample solution (20 µL) was directly injected into the LC-MS for analysis. For the analysis of reduced or unreduced GSH Michael adducts of HOHA-lactone, metabolites **4** and **5**, an isocratic HPLC method with 40% solvent B was used. The total run time was 10 min and the flow rate was 200 µL/min. Full scan spectra were acquired in a scan range from m/z 400 to 500 (scan rate 0.5 scans/s). MS/MS experiments were performed by selecting an ion with an isolation width of 2 m/z and collision energy at 30%.

Derivatization with BSTFA of metabolites **2** and **3** extracted from extracellular media

ECM (500 µL) from ARPE-19 cells that were challenged with 10 µM of HOHA-lactone for up to 2 h was collected. Then it was carefully acidified to pH = 2 by 1 N HCl aqueous solution, and then extracted three times with 1 mL of chloroform. The organic layers were combined, dried under a stream of nitrogen gas, followed by derivatization with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA, 80 µL) at 60 °C for 30 min. The final preparation was transferred into a glass insert and sealed into GC-MS vials. Authentic compounds **2** and **3** at various concentrations were derivatized with BSTFA to generate calibration curves.

Gas chromatography-mass spectrometry (GC-MS) analysis

GC-MS analysis was performed using a Focus DSQ II single quadrupole GC-MS (Thermo Electron Corp. New Jersey) instrument with 70 eV electron ionization. The compounds were separated on a stationary methylpolysiloxane phase capillary column (18 m × 0.25 mm ID × 0.25 µm film thickness from Supelco, Sigma-Aldrich). The GC injection port and interface temperature were 250 °C, with helium as carrier gas maintained at 80 psi. Injections of 1 µL were made with split mode in 1:40 ratio. The GC oven temperature was held initially at 100 °C for 1 min and then ramped at a rate of 20 °C /min to 250 °C, which was held for another 3 min.

Quantification of intracellular GSH in hRPE and ARPE-19 cells

Aliquots (10 µL) of hRPE or ARPE cell lysates from time-course and dose-dependence studies were assayed to determine intracellular GSH using a spectrofluoremetric microplate method described earlier.¹³ In this experiment, all the reagents were prepared in 0.1M potassium phosphate buffer with 5 mM EDTA disodium salt, pH 7.5 (KPE buffer). Briefly, 10 µL of KPE buffer, GSH standards or samples were added to the corresponding microplate wells, followed by the addition of 120 µL of a freshly prepared mixture of DTNB (1 mg/3 ml) and glutathione reductase (5 U/3 ml). Then, 60.0 µL of NADPH (2 mg/3 ml) was added and mixed well. The plate was immediately placed in a microplate reader (Molecular

Devices) and absorbance was measured at a wavelength of 412 nm after mixing for 2 min or 10 min for ARPE-19 or hRPE cells, respectively.

RESULTS

Synthesis of putative HOHA-lactone metabolites

Because HOHA-lactone (**1**) is a structural analog of 4-HNE, it was expected to be detoxified in living cells by oxidative, reductive and conjugative pathways.¹⁴ Guided by analogy with the reported metabolism of 4-HNE in cultured keratinocytes and PC-12 cells,^{15, 16} we postulated that detoxification of HOHA-lactone by living cells would be promoted by glutathione S-transferase, alcohol dehydrogenase and aldehyde dehydrogenase, to generate metabolites **2-5** in ARPE-19 and hRPE cells as shown in Scheme 1.

LC-MS analysis of the extracellular medium (ECM) identified the reduced HOHA-lactone-GSH Michael adduct **5** as a major metabolite in ARPE-19 cells

ARPE-19 cells were treated with either 0 μ M (control) or 10 μ M HOHA-lactone for 2 h. Then 20 μ L of each ECM was directly injected into an LC-ESI-MS and analysis was performed in the positive ion mode monitoring the mass range from m/z 400 to 500. Total ion chromatograms (TIC) and two-dimensional plots indicated that a peak eluting at 4.41 min is found in ECM from cells treated with 10 μ M HOHA-lactone but not in control ECM (Figure 3). The mass spectrum at 4.41 min shows two significant peaks at m/z 450 and 472, which correspond to the m/z value of $[M + H]^+$ and $[M + Na]^+$ ions of **5**, the product from reduction of the aldehyde of the Michael adduct **4** of glutathione with HOHA-lactone. The adduct formation and its reduction presumably involves catalysis by glutathione S-transferase (GST) and alcohol dehydrogenase (ADH).

To confirm that the metabolite produced by ARPE-19 cells treated with HOHA-lactone is compound **5**, we synthesized authentic samples of the glutathione Michael adduct **4** and the reduced HOHA-lactone-GSH adduct **5**, through unambiguous chemical syntheses, and then applied LC-MS/MS to compare the metabolite with the product of chemical synthesis. A MS/MS scan of collision-induced dissociation (CID) of the m/z 450.4 spectrum was recorded from m/z 200 to 400 using a MS/MS collision energy at 35%. The metabolite detected in the ECM has the same retention time and MS/MS fragmentation pattern as authentic reduced HOHA-lactone-GSH Michael adduct **5**. The intracellular presence of this metabolite was detected in the lysate of cells that had been treated with 10 μ M HOHA-lactone (Figure 4). The proposed structures of the major fragments in the MS2 spectrum of **5** are shown in Scheme 2.

Time course of the formation of metabolite **5** in ARPE-19 cells

The kinetics of the appearance of metabolite **5** in ARPE-19 cells was examined using LC-MS/MS analysis of both intracellular (cell lysate) and extracellular (ECM) levels after various incubation time periods. The MS/MS m/z 450.4 $\rightarrow$ 321.0 transition was monitored because the daughter ion m/z 321.0 is the strongest CID induced fragment ion in the mass spectrum of **5**. The peak area obtained from the peak with the same retention time as authentic **5** (i.e. 4.10 $\pm$ 0.20 min) was used to determine the concentration of **5** in these

samples. Figure 5 shows representative LC-MS/MS data for the formation of the reduced Michael adduct **5** in the time course study. Traces of metabolite **5** were formed immediately in the cell, as it was clearly detectable in the lysate from cells that were treated with HOHA-lactone for only a few seconds. The rapid formation of metabolite **5** in the cell lysate demonstrates that HOHA-lactone readily diffuses into ARPE-19 cells. The complete time course study shows that this molecule accumulated inside of cells starting immediately upon addition of lactone **1** to the cells. It reached a maximum concentration at 30 min. Then the intracellular level of **5** decreased with concomitant increases in the level of **5** in the extracellular medium. This shows that there is a unidirectional export of the reduced GSH-HOHA-lactone Michael adduct **5** from the cytosol to the ECM.

The unreduced HOHA-lactone-GSH Michael adduct **4 is detectable in cell lysates and ECM from incubation of ARPE-19 cells with high concentrations of HOHA-lactone**

The unreduced HOHA-lactone-GSH Michael adduct **4** was detected simultaneously by a LC-MS/MS method developed with the chemically synthesized authentic standard **4**. In contrast to the reduced HOHA-lactone-GSH adduct **5**, compound **4** was not detectable in the extracellular medium from cells treated with 10 μ M HOHA-lactone. It was only detectable in the cell lysates during the first 30 min of incubation, reaching the highest level at 5 min of incubation. Presumably, cooperation between GST and ADH in ARPE-19 cells efficiently detoxifies HOHA-lactone by conversion to the unreactive metabolite **5**. However, **4** was detectable in the extracellular medium of cells treated with higher concentrations ($> 25 \mu$ M) of HOHA-lactone (Figure 6).

In a concentration dependence study, we incubated ARPE-19 cells with 0 to 200 μ M HOHA-lactone for 2 h. The metabolites **4** and **5** were analyzed simultaneously by LC-MS/MS (Figure 7). The level of the reduced glutathione Michael adduct **5** in the extracellular medium increased in a concentration-dependent manner in the HOHA-lactone concentration range from 0-25 μ M, then its level slowly decreased when the cells were treated with higher levels (25-200 μ M) of HOHA-lactone.

At low concentrations of HOHA-lactone (0-10 μ M), the extracellular level of the unreduced HOHA-lactone Michael adduct **4** was very low. However, the level of metabolite **4** was significantly increased in the extracellular medium from cells treated with high levels of HOHA-lactone. There is nearly a 10 fold increase in total amount of metabolite **4** in the ECM for cells treated with 25 μ M compared to 10 μ M HOHA-lactone (Figure 7B). These observations, along with the fact that the level of metabolite **5** decreases in cells treated with high concentrations of HOHA-lactone, indicate that ARPE-19 cells were not able to efficiently metabolize high levels of HOHA-lactone to completely convert it into the non-toxic metabolite **5**, but rather reactive metabolite **4** persisted. This may be because of either cell death, dysfunction of ADH enzymes or depletion of reducing capacity, leading to the failure to efficiently reduce the aldehyde carbonyl in **4** resulting in higher the levels of **4** and lower levels of **5**.

Formation and export of the glutathione conjugates of HOHA-lactone in hRPE cells

We also evaluated the capacity of human primary retinal pigmented epithelial cells (hRPE), to eliminate HOHA-lactone. After treating hRPE cells with 10 μ M HOHA-lactone, 20 μ L of both intracellular (cell lysate, CL) and extra-cellular medium (ECM), presumably containing GSH metabolites of HOHA-lactone, was analyzed by LC-MS/MS as described above for ARPE-19 cells.

The GSH metabolites aldehyde **4** and alcohol **5** of HOHA-lactone in both CL and ECM from hRPE cells were quantitated by LC-MS/MS analysis over the time course of treatment and expressed in pmol/ 10^6 cells. MS/MS analysis was performed in the positive ion mode monitoring the transition 448.4 $\rightarrow$ 301 for aldehyde **4** and 450.4 $\rightarrow$ 321 for alcohol **5**, respectively. As shown in Figure 8, treatment of RPE cells with HOHA-lactone engendered a similar behavior to ARPE-19 cells, i.e., a time-dependent increase of HOHA-lactone-GSH levels. The reduced GSH metabolite, alcohol **5**, of HOHA-lactone appeared rapidly in the intracellular fraction achieving concentrations of 13.4 ± 3.8 pmol per 10^6 hRPE cells $^{-1}$ at 5 min and then was efficiently eliminated, coinciding with a rise in the levels of alcohol **5** in the extracellular medium.

Effect of HOHA-lactone on intracellular GSH levels in RPE cells

Addition of 10 μ M HOHA-lactone to ARPE-19 or hRPE cells resulted in a notable drop in intracellular GSH (Figure 9) coincident with rapid production of HOHA-lactone-GSH adducts. Presumably, conjugation with GSH through Michael addition of its nucleophilic sulfur to the electrophilic C-3 of HOHA-lactone in RPE cells is catalyzed by glutathione S-transferase (GST). Following rapid depletion of cellular GSH, its level remained constant, only increasing 120 min after exposure to HOHA-lactone, especially in hRPE cells.

The carboxylic acid **3** from oxidation of HOHA-lactone, is the major non GSH-adducted metabolite from HOHA-lactone that appears in the extracellular medium

Pure samples of two potential metabolites of HOHA-lactone, the alcohol **2** and the carboxylic acid **3**, were prepared by chemical synthesis (Scheme 3). These two compounds could be detected as TMS derivatives by gas chromatography-mass spectrometry (GC-MS) with an electron ionization (EI) source. Since both compounds contain polar hydrogen bonding groups, i.e., hydroxyl or carboxyl, which are not adequately volatile for GC, they were derivatized with BSTFA to generate volatile TMS derivatives.¹⁷

GC-MS analysis of the TMS derivatives of authentic **2** and **3** standards by the scan mode in the range of m/z 50 to 300 showed only single peaks for each standard, with retention times of 4.37 and 4.86 min, respectively (Figure 10). The chloroform extracts, from 500 μ L of extracellular medium that were taken at various times from ARPE-19 cells treated with 10 μ M HOHA-lactone, were derivatized by treatment with BSTFA and then analyzed by GC-MS in the selected ion monitoring mode (SIM). The level of the carboxylic acid metabolite **3** in the ECM was found to increase in a time-dependent manner (Figure 11). In contrast, the putative alcohol metabolite **2** was not detected by GC-MS. (data not shown). This observation suggests that, oxidative metabolism of HOHA-lactone (in 10 μ M) by ALDH,

rather than reductive metabolism by ADH, is the predominant pathway for the non-GSH-adducted HOHA-lactone.

DISCUSSION

HOHA-lactone diffuses into RPE cells that metabolize it and secrete the metabolites

HOHA-lactone is a product of non-enzymatic free radical-induced oxidation of docosahexaenoate-rich phospholipids that are especially abundant in photoreceptor disc membranes. Based on their similar hydrophobicity, we postulated that HOHA-lactone (CLogP: -1.02) diffuses across cell membranes as readily as cortisone (CLogP: -0.93). Consequently, HOHA-lactone released from oxidatively damaged photoreceptor disc membranes can diffuse from photoreceptor cells into adjacent RPE cells. A possible alternative route for the transport of HOHA-lactone from oxidatively damaged photoreceptor cell disc membranes into RPE cells is through endocytosis of photoreceptor rod outer segment tips¹⁸ followed by diffusion of HOHA-lactone out of the endosome and into the RPE cell cytosol. Figure 12 depicts the putative generation HOHA-lactone in a retinal rod cell, and its diffusion into an adjacent RPE cell where it is metabolized and from which the metabolites are secreted into the extracellular matrix. While drug-efflux transporters of GSH conjugated drugs, i.e., multi-drug resistance proteins, are well known¹⁹ and expressed in RPE,²⁰ the identity of the transporter(s) involved in the metabolism of HOHA-lactone remain(s) unknown. The possibility that HOHA-lactone or its GSH conjugates induce expression of the transporter(s) or, by covalent modification, decrease or abolish the activity of the transporter(s) are also important questions for further investigation.

LC-MS and GC-MS analyses of cell lysates and extracellular medium from RPE cells treated with 10 μ M HOHA-lactone confirmed that HOHA-lactone readily diffuses into RPE cells. In photoreceptor and RPE cells, HOHA-lactone can generate CEP through modification of primary amino groups of proteins and ethanolamine phospholipids. The present study defines pathways by which RPE cells metabolize HOHA-lactone. Metabolites of HOHA-lactone produced in the RPE cell cytosol are transported to the ECM. The reduced Michael adduct **5** of HOHA-lactone with glutathione is the most prominent metabolite detected by LC-MS in both the extracellular medium and cell lysates. The generation of **5** in RPE cells presumably involves the co-operation of both GST and ADH. The unreduced Michael adduct of HOHA-lactone **4** was only detected in small amounts in cell lysates at an early stage of incubation with 10 μ M HOHA-lactone. However, high levels were found in both cell lysates and extracellular medium from ARPE-19 cells treated with a high concentration of HOHA-lactone (25 μ M). The carboxylic acid **3** produced by oxidation of HOHA-lactone, presumably through an ALDH mediated pathway, is the major non-GSH adducted metabolite in the extracellular medium.

Metabolic detoxification of HOHA-lactone prevents pathological sequelae of oxidative stress

HOHA-lactone can play a variety of roles in oxidative stress-induced retinal diseases. Previously, we showed that HOHA-lactone is cytotoxic to ARPE-19 cells at high concentrations (LD₅₀ 38.6 μ M).² Under conditions of oxidative stress, where concentrations

of GSH might be depressed, protection from the cytotoxicity of HOHA-lactone might be compromised contributing to the global retinal atrophy of “dry” AMD. As expected, because metabolism of HOHA-lactone consumes GSH (Figure 9), this resource, needed by RPE cells to combat oxidative stress, is depleted making them vulnerable to further oxidative damage.

In spite of the fact, now established, that HOHA-lactone is rapidly metabolized by RPE cells, HOHA-lactone is still able to trigger cell signaling pathways that exert paracrine effects on RPE cells. Thus, we previously found that low concentrations of HOHA-lactone (0.1-1 μM) induce secretion of vascular endothelial growth factor (VEGF) from ARPE-19 cells.² The adduction of HOHA-lactone to primary amino groups of proteins and ethanolamine phospholipids generates CEP derivatives.^{2, 21} Therefore, HOHA-lactone is an important precursor for CEP derivatives that accumulate in rod photoreceptor and RPE cells in retinas of individuals with age-related macular degeneration.⁸ Both VEGF and CEP are angiogenic.^{2, 7} Therefore, HOHA-lactone can also contribute to the neovascularization of “wet” AMD not only through the VEGF pathway, but also through a VEGF-independent TLR2-dependent pathway involving the formation of CEP.⁵⁻⁷

HOHA-lactone-dependent generation of VEGF and CEP might also contribute to the angiogenesis that promotes tumor growth.⁵ Since docosahexaenoate is especially abundant in brain, the biological sequelae of HOHA-lactone and consequent CEP generation may be especially important in promoting growth of the highly vascularized glioblastoma multiforme (GBM) tumors in the brain. Notably, after a year or less, anti-VEGF therapy loses efficacy for inhibiting the progression of GBM tumors. The possibility that such “resistance” of GBM tumors to anti-VEGF therapy is conferred by HOHA-lactone and the derived CEP deserves consideration. This possibility highlights a conundrum for the use of GSH depleting measures to potentiate the destruction of GBM tumors by radiotherapy.²² GBMs are aggressive brain tumors that always recur after radiotherapy. While GSH depletion can increase the lethality of radiotherapy by increasing the levels of reactive oxygen species, it also is expected to increase levels of HOHA-lactone and CEP that can promote tumor angiogenesis and growth. Careful attention to restoring or boosting GSH levels after radiotherapy-induced tumor cell killing has transpired may stave off recurrence by efficient metabolic detoxification of HOHA-lactone. Notably, treatment of GBM with the anti-VEGF drug bevacizumab (Avastin) also causes depletion in GSH levels suggesting that the treatment causes oxidative stress in the tumors.²³ Here too, boosting GSH levels may prevent the consequent increases in HOHA-lactone and CEP levels that can favor tumor progression by promoting tumor angiogenesis in a TLR2-dependent manner that circumvents blockage of the VEGF pathway by bevacizumab.⁵

Diffusion of HOHA-lactone from the retina may lead to CEP production in the blood

Passive diffusion of HOHA-lactone through photoreceptor, RPE and vascular cell membranes in the retina may allow it to enter the circulation. In the blood it can modify proteins and ethanolamine phospholipids and thereby contribute to the generation of elevated levels of CEP that are found in blood from AMD patients compared to individuals with no disease.³ Oxidative damage, even in retinas from healthy individuals, necessitates replacement of the entire stack of photoreceptor discs every ten days. The consequent

continuous cascade of HOHA-lactone may account for basal levels of CEP detected in blood from individuals with no eye disease. Accumulation of CEP in photoreceptor and RPE cells in retinas and in the blood of individuals with AMD may indicate elevated levels of oxidative damage. Alternatively, deficient metabolic detoxification of HOHA-lactone may account for elevated levels of CEP in the blood of individuals with AMD.

CONCLUSIONS

The present study establishes that HOHA-lactone readily diffuses into RPE cells. This provides presumptive evidence that HOHA-lactone can diffuse from photoreceptor disc membranes, where it is produced, and enter the adjacent RPE cells. In RPE cells it can be metabolized by Michael addition of GSH. Subsequent reduction of this aldehyde prevents Michael elimination assuring stability of the detoxified GSH derivative that is transported unidirectionally out of the RPE cell. Under conditions of chronic oxidative stress and the concomitant failure of metabolic detoxification, HOHA-lactone can modify proteins and ethanolamine phospholipids to generate CEP. Both HOHA-lactone and the derived CEP can contribute to the angiogenesis that defines the neovascular “wet” form of AMD and promotes the growth of highly vascularized tumors, e.g. GBM, through VEGF and VEGF-independent TLR2-dependent pathways respectively.⁵ A clear understanding of the metabolic fate of HOHA-lactone in cells may eventually facilitate the discovery of novel methods to prevent pathogenesis induced by HOHA-lactone or CEP derivatives of proteins and ethanolaminephospholipids derived from HOHA-lactone.

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Abbreviations

ADH	alcohol dehydrogenase
ALDH	aldehyde dehydrogenase
AMD	age-related macular degeneration
BSTFA	N,O-bis(trimethylsilyl)trifluoroacetamide
CEP	2-(ω -carboxyethyl)pyrrole
CID	collision-induced dissociation
DHA	docosahexaenoic acid
DHA-PC	1-palmityl-2-docosahexaenoyl-sn-glycero-3-phosphocholine
ECM	extracellular medium
EI	electron ionization

GSH	reduced glutathione
GST	glutathione S-transferase
HOHA	4-hydroxy-7-oxo-hept-5-enoic acid
HOHA-PC	1-palmityl-2-(4-hydroxy-7-oxo-5-heptenoyl)- <i>sn</i> -glycero-3-phosphatidylcholine
RPE	retinal pigmented endothelium
SIM	selected ion monitoring
TIC	total ion chromatogram

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Figure 1.
HOHA-lactone production and protein adduction in rod photoreceptor cells.

Figure 2.
HPLC purification of compound 4.

Figure 3.

LC-MS analysis of extracellular media from ARPE-19 cells incubated with HOHA-lactone (0 or 10 μ M). Top: Positive ion monitoring (m/z 400-500), TIC chromatograms of control cell ECM (*Left*) and HOHA-lactone treated cell ECM (*Right*). Inserts: Mass spectrum at retention time 4.41 min. Bottom: Two-dimensional plots (m/z values vs retention times) of control cell ECM (*Left*) and HOHA-lactone treated cell ECM (*Right*).

Figure 4. LC-MS and LC-MS/MS analysis of the reduced HOHA-lactone-GSH adduct **5**. *Left:* Selected ion monitoring (SIM) chromatograms of m/z 450.4; *Right:* MS/MS spectrum of fragmentation ions from the CID of m/z 450.4. (A) Authentic standard **5**. (B) ECM of ARPE-19 cells after incubation with 10 μ M HOHA-lactone for 2 h. (C) Cell lysate from ARPE-19 cells after incubation with 10 μ M HOHA-lactone for 2 h.

Figure 5.

(A) LC-MS/MS analysis of metabolite **5** in cell lysates (*Left*) and ECM (*Right*) of APE-19 cells. Selected ion recording at m/z 321.0 from CID fragmentation of m/z 450.4 in the positive ion mode was used to specifically identify compound **5**. (B) Time course of appearance of **5** (in % yield) in (closed square) cell lysates and (open square) ECM of ARPE-19 cells and reported as means $\pm$ S.D. of three independent experiments. (C) Calibration curve of metabolite **5** in LC-MS analysis.

Figure 6.

LC-MS/MS analysis of metabolite **4** production in HOHA-lactone treated ARPE-19 cells.

Left: LC chromatograms showing the MS2 transition m/z 448.4 $\rightarrow$ 301.0; *Right:* MS/MS spectrum of fragmentation ions from CID of m/z 448.4. (A) Authentic Standards **4**. (B) cell lysate of ARPE-19 cells incubated with 10 μ M HOHA-lactone for 5 min. (C) ECM of ARPE-19 cells incubated with 25 μ M HOHA-lactone for 2 h. (D) Calibration curve of metabolite **4** in LC-MS analysis. (E) Time course of the intracellular appearance of **4** (% yield) in cell lysates from cells treated with 10 μ M HOHA-lactone. (F) Proposed structures of fragmentation ions in the MS/MS spectrum of **4**.

Figure 7.

LC-MS/MS analysis of metabolites **4** and **5** formed in APRE-19 cells treated with HOHA-lactone in a concentration dependence study. (A) Total amount of **5** (in % yield) detected in the (open square) extracellular media and (closed square) cell lysates after 2 h incubation. (B) Total amount of **4** in (open circle) extracellular media and (closed circle) cell lysates after 2 h incubation. Results are reported as means $\pm$ S.D. of three independent experiments.

Figure 8.

Time course and quantification of HOHA-lactone-derived GSH conjugates in hRPE cells after treatment with 10 μ M HOHA-lactone. Quantitation of alcohol **5** in the extra-cellular medium (open square) and in cell lysates (closed circle). Values represent means $\pm$ S.D. of three independent experiments.

Figure 9.

Time course of intracellular glutathione levels in (A) ARPE-19 cells and (B) hRPE cells after treatment with 10 μ M HOHA-lactone. Values represent means $\pm$ S.D. of three independent experiments.

Figure 10.

GC-EI⁺/MS analysis of TMS derivatives of authentic standard 2 and 3. (A) & (B) GC-MS spectrum of 2 standard; (C) & (D) GC-MS spectrum of 3 standard.

Figure 11.

Appearance of metabolite **3** in the extracellular medium from ARPE-19 cells treated with 10 μM HOHA-lactone. ECM was extracted with chloroform, then the chloroform layer was dried, and the residue was derivatized by treatment with BSTFA. GC-MS was done in the SIM mode with $m/z = 213$ (TMS-M-CH_3)⁺. (A) GC-MS spectrum of ECM collected at 0 h. (B) GC-MS spectrum of ECM collected at 2 h. (C) Calibration curve of compound **3**. (D) Evolution of metabolite **3** in ECM ($n = 3$).

Figure 12.

Metabolic processing in RPE cells of HOHA-lactone released from oxidatively damaged rod photoreceptor disc membranes. *ALDH* = aldehyde dehydrogenase; *GST* = glutathione S-transferase; *ADH* = alcohol dehydrogenase.

Scheme 1.

Putative metabolites of HOHA-lactone in ARPE-19 cells.

Scheme 2.

Proposed structures of CID fragments of m/z 450 in mass spectrometry.

Scheme 3.

Chemical synthesis and TMS derivatization of putative metabolites **2** and **3**.