Published in final edited form as:

Free Radic Biol Med. 2009 October 1; 47(7): 953–961. doi:10.1016/j.freeradbiomed.2009.06.035.

A 4-Oxo-2(*E*)-Nonenal-Derived Glutathione-Adduct from 15-Lipoxygenase-1-Mediated Oxidation of Cytosolic and Esterified Arachidonic Acid[§]

Peijuan Zhu, Wenying Jian, and Ian A. Blair^{*}

Centers for Cancer Pharmacology and Excellence in Environmental Toxicology, University of Pennsylvania School of Medicine, 854 BRB II/III, 421 Curie Boulevard, Philadelphia, PA 19104-6160

Abstract

15(*S*)-hydroperoxy-[5*Z*,8*Z*,11*Z*,13*E*]-eicosatetraenoic acid (15(*S*)-HpETE) undergoes homolytic decomposition to bifunctional electrophiles such as 4-oxo-2(*E*)-nonenal. 4-Oxo-2(*E*)-nonenal reacts with glutathione to form thiadiazabicyclo-4-oxo-2(*E*)-nonenal-glutathione-adduct (TOG). Therefore, this endogenous glutathione-adduct can serve as a specific biomarker of lipid-hydroperoxide-mediated 4-oxo-2(*E*)-nonenal formation. A monocyte/macrophage cell line was generated to constitutively express human 15-lipoxygenase-1. In these cells, TOG was formed from 15(*S*)-HpETE-derived 4-Oxo-2(*E*)-nonenal in a non-linear dose-dependent manner upon arachidonic acid treatment. The lipoxygenase inhibitor, cinnamyl-3,4-dihydroxy- α -cyanocinnamate, abolished arachidonic acid-mediated TOG formation. Calcium ionophore A23187 was also used to induce the formation of 15(*S*)-HpETE from esterified arachidonic acid present in the membrane lipids. In the 15-lipoxygenase-1-expressing cells, calcium ionophore A23187 significantly increased TOG levels compared with the mock transfected cells. This was due to the 15-lipoxygenase-mediated formation of 15(*S*)-HpETE in esterified lipids, which was subsequently converted to 4-oxo-2(*E*)-nonenal. The increase in TOG formation was again abrogated by pre-treatment with cinnamyl-3, 4-dihydroxy- α -cyanocinnamate. Only 8.7 % 15(*S*)-HETE (both the free fatty acid and its esterified form in the cell membrane) was formed after ionophore A23187 stimulation when compared with that formed after the addition of arachidonic acid. In contrast, the TOG levels after treatment with ionophore A23187 or arachidonic acid were comparable. Thus, it is likely that esterified 15(*S*)-HpETE underwent homolytic decomposition to 4-oxo-2(*E*)-nonenal more efficiently than the free 15(*S*)-HpETE that was formed in the cytosol.

Keywords

Lipid peroxidation; arachidonic acid; 4-oxo-2-nonenal; glutathione adduct; monocyte/macrophage

[§]This project was supported by NIH grants RO1CA091016 and P30ES013508.

^{*}To whom correspondence should be addressed: Ian A. Blair, Center for Cancer Pharmacology, University of Pennsylvania School of Medicine, 854 BRB II/III, 421 Curie Boulevard, Philadelphia, PA 19104-6160.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Introduction

Mammalian reticulocyte-type 15-lipoxygenase (LO)-1 constitutes a family of enzymes with molecular weights of approximately 75 kDa [1]. Their enzymatic activity results in the stereospecific insertion of an oxygen molecule at C-15 of arachidonic acid to form the lipid hydroperoxide 15(*S*)-HpETE, which is reduced in cells to the lipid hydroxide 15(*S*)-HETE. Although free radical-mediated oxidation of arachidonic can also produce 15-HpETE and 15-HETE together with many other products, the HPETEs and HETEs are racemic with equal amounts of *S* and *R* enantiomers [2]. Thus the amount of 15(*S*)-HETE in excess over the 15(*R*)-HETE can be quantified as an index of 15-LO activity [3]. Although the exact biological role of 15-LO-1 is still not clear, it has been frequently linked to inflammation and atherosclerosis [4-8]. 15-LO-1 is up-regulated in human peripheral monocytes and alveolar macrophages cultured in the presence of interleukin-4 or interleukin-13, both of which are inflammatory cytokines [4,9,10]. Kühn and colleagues showed that arachidonic acid esterified into phospholipids or low-density lipoprotein could be directly oxidized by rabbit 15-LO-1 or recombinant human 15-LO-1 [11-13]. In addition, *ex vivo* cultured primary murine peritoneal macrophages expressing 12/15-LO had a much higher capacity to oxidize low density lipoprotein, when compared with non-12/15-LO expressing cells [14]. *In vivo* studies also showed that 12/15-LO/apoE double knockout mice had a pronounced delay and attenuation of atherosclerotic lesion development when compared to apoE deficient mice expressing one or two gene copies of 12/15-LO [6]. Furthermore, it was shown by Folcik *et al.* that 15-LO contributes to the formation of oxidized lipids in human atherosclerotic lesions [15]. All of the above studies demonstrate a pro-atherogenic role of 15-LO. However, other studies have suggested an anti-atherogenic effect of 12/15-LO [16,17].

We reasoned that when intracellular reducing pathways were compromised, 15(*S*)-HpETE might accumulate and decompose to bifunctional electrophiles such as 4-oxo-2(*E*)-nonenal and 4-hydroxy-2-nonenal [17-20]. 4-Oxo-2(*E*)-nonenal forms DNA-adducts from 15(*S*)-HpETE [21] through COX-2-mediated oxidation of arachidonic acid in cultured cells [22] and in the small intestines of *Min* mice [23]. It also forms adducts with RNA [24] and proteins [25,26]. Major pathways involved in the detoxification of lipid hydroperoxide-derived bifunctional electrophiles include glutathione *S*-transferase-mediated addition of glutathione and aldo-keto reductase-mediated reduction of the terminal aldehyde [27-29]. The glutathione-adducts are subsequently released from cells and then hydrolyzed and acetylated to form N-acetyl-cysteine-adducts also known as mercapturic acid derivatives [29].

TOG ((2*S*,7*R*)-7-[N-(carboxymethyl) carbamoyl]-5-oxo-12-pentyl-9-thia-1,6-diazabicyclo [8.2.1]trideca-10(13),11-diene-2-carboxylic acid) was first detected when 4-oxo-2(*E*)-nonenal was added to EA.hy 926 endothelial cells in culture [30]. The structure of TOG was subsequently characterized as an unusual thiadiazabicyclo derivative that arose from the glutathione *S*-transferase-mediated reaction of 4-oxo-2(*E*)-nonenal with glutathione [31] (Scheme 1). Initial nucleophilic attack of glutathione through its γ -glutamate α -amino moiety is thought to occur at the C-1 aldehyde of 4-oxo-2(*E*)-nonenal to form a Schiff base. glutathione *S*-transferase then facilitates a Michael addition of the cysteine thiol to give an α , β -unsaturated ketone intermediate. Another nucleophilic attack takes place from Schiff base nitrogen to the ketone of 4-oxo-2(*E*)-nonenal. Finally, the loss of one water molecule leads to the formation of the pyrrole moiety of TOG (Scheme 1) [31]. Interestingly, the γ -glutamate residue is not accessible to γ -glutamyltranspeptidase so TOG is resistant to the hydrolysis observed with other glutathione-adducts [29]. Intracellular TOG was detected when endothelial cells were subjected to Fe^{II}-induced reactive oxygen species-mediated oxidative stress [31]. However, it was not clear whether TOG could form under more physiologically relevant conditions such as induction of lipid peroxidase 15-LO-1 by interleukin-4 and interleukin-13 [4,9,10]. The present study used the murine macrophage/monocyte RAW 267.4 cell line transfected with

human 15-LO-1 (R15LO) as a model system to determine whether 4-oxo-2(*E*)-nonenal-dependent TOG formation could also arise from endogenous lipid peroxidation of free and esterified arachidonic acid by 15-LO-1. Control experiments were conducted with mock transfected RAW 267.4 (RMock) cells.

Materials and Methods

Materials

Dulbecco's Modified Eagle's Medium (DMEM), phosphate buffer saline, Lipofectamine 2000 and geneticine were supplied by Invitrogen (Carlsbad, CA). The bicinchoninic acid (BCA) assay kit was obtained from Pierce (Rockford, IL). Arachidonic acid, 15(*R*)-HETE, 15(*S*)-HETE and [²H₈]-15(*S*)-HETE were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). Cinnamyl-3, 4-dihydroxy- α -cyanocinnamate (CDC) was obtained from BioMol (Plymouth Meeting, PA). High performance liquid chromatography grade water, acetonitrile, hexane, methanol and isopropanol were obtained from Fisher Scientific Co. (Fair Lawn, NJ). glutathione, fetal bovine serum, calcium ionophore A23187, formic acid, trifluoroacetic acid, sodium borohydride, ethylenediaminetetraacetic acid (ETDA) disodium salt, diisopropylethylamine and 2,3,4,5,6-pentafluorobenzyl bromide were acquired from SigmaAldrich (St. Louis, MO). Gases were supplied by BOC Gases (Lebanon, NJ).

Mass Spectrometry

The quantitative analysis of TOG was conducted using a triple-quadrupole TSQ Quantum Ultra AM spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with an electrospray ionization source in the positive ion mode. Operating conditions were: spray voltage, 5 kV; heated capillary temperature, 250 °C. Nitrogen was used for the sheath gas and auxiliary gas set at 35 and 10 (in arbitrary units), respectively. Collision-induced dissociation was performed using argon as the collision gas at 1.5 milliTor in the second quadrupole. Multiple reaction monitoring (MRM) analysis was conducted at unit resolution for both parent and product ions. The following MRM transitions were monitored: TOG (m/z 426 $\rightarrow$ m/z 280), [²H₃]-TOG (m/z 429 $\rightarrow$ m/z 283) and [¹³C₉]-TOG (m/z 435 $\rightarrow$ m/z 289). Quantitative analyses of 15-HETEs were also conducted on a TSQ Quantum Ultra AM spectrometer (Thermo Fisher Scientific). However, it was equipped with an atmospheric pressure chemical ionization source and used in the electron capture negative ion mode [32]. Operating conditions were as follows: vaporizer temperature at 450 °C, ion transfer tube temperature at 250 °C, with the corona discharge needle set at 28 μ A, the source collision-induced dissociation was set at 10 V. The sheath gas (nitrogen) and auxiliary gas (nitrogen) pressures were 25 psi and 3 psi, respectively. Collision-induced dissociation was again performed using argon as the collision gas at 1.5 mTorr and the collision energy was set at 18 eV. MRM analysis was conducted at unit resolution for both parent and product ions. The following MRM transitions were monitored: 15-HETEs (m/z 319 $\rightarrow$ m/z 219) and [²H₈]-15(*S*)-HETE (m/z 327 $\rightarrow$ m/z 227).

Liquid Chromatography

Liquid chromatography (LC)-MRM/mass spectrometry (MS) for quantitative TOG analysis was conducted using gradient systems 1 and 2 on a Hitachi L-2130 Intelligent Pump (Hitachi, San Jose, CA). Separations were conducted using a Phenomenex Jupiter 5- μ m C18 column (150 $\times$ 2 mm internal diameter, 5 μ m; Phenomenex, Torrance, CA) at ambient temperature. Solvent A was 0.1 % formic acid (v/v) in water, and solvent B was acetonitrile. Gradient system 1 was as follows; 2 % B at 0 min, 11 % B at 2 min, 11 % B at 20 min, 25 % B at 21 min, 30 % B at 25 min, 40 % B at 27 min, 60 % B at 35 min, 82 % B at 36 min, 2 % B at 38 min, and 2 % B at 46 min. The flow rate was 0.3 mL/min. Gradient system 2 was as follows: 15 % B at 0 min, 60 % B at 20 min, 80 % B at 21 min, 80% B at 24 min, 15 % B at 25 min, and 15 % B at 32 min. The flow rate was 0.2 mL/min. Purification of TOG and [²H₃]-TOG were carried

out using gradient system 3. Separations were conducted using a 250×10.0 mm internal diameter, $5 \mu\text{m}$ Phenomenex Jupiter C18 column. Solvent A was 0.1 % formic acid (v/v) and 0.01 % trifluoroacetic acid (v/v) in water and solvent B was acetonitrile. For gradient 3, a linear gradient was employed from 20 % B at 0 min to 80 % B at 27 min at a flow rate of 3 mL/min. LC-MRM/MS chromatography for quantitative analyses of 15-HETEs was conducted using gradient system 4 on a Waters Alliance 2690 HPLC system (Waters, Milford, MA). The separation employed a Chiralpak AD-H column (250×4.6 mm internal diameter, $5 \mu\text{m}$; Chiral Technologies, Inc., West Chester, PA) at ambient temperature with a flow rate of 1 mL/min. Solvent A was hexane and solvent B was methanol/isopropanol (1:1, v/v). The gradient system 4 was as follows: 2 % B at 0 min, 2 % B at 3 min, 3.6 % B at 11 min, 8 % B at 15 min, 8 % B at 27 min, 50 % B at 30 min, 50 % B at 35 min, 2 % B at 37 min and 2 % B at 45 min. A post-column infusion of methanol at 0.75 mL/min was applied to prevent carbon film formation on the atmospheric pressure chemical ionization discharge needle.

Culture, Transfection, and Clone Selection of R15LO and RMock cells

RAW 267.4 cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were grown on Corning 10 cm plastic dishes in DMEM with 10 % fetal bovine serum, 4,500 mg/L D-glucose, and 100,000 units/L streptomycin. The pcDNA3 plasmid containing the human 15-LO-1 gene and the empty pcDNA3 plasmid were generous gifts from Dr Colin Funk (Queen's University, Canada). For cell transfection, RAW 267.4 cells were seeded into six well plates and grown to 90 % confluence. Plasmid DNA was transfected into the RAW 267.4 cells using Lipofectamine 2000 according to the manufacturer's instructions. Cells were gently scraped off the plate and suspended in DMEM with 10 % fetal bovine serum, 4,500 mg/L D-glucose, and 0.8 g/L geneticin 24 hours after transfection and seeded into 60-mm-diameter culture dishes. Isolated single clones were picked, cultured and expanded to give the R15LO and RMock cell lines. Specific 15-LO-1 expression in the R15LO cells was confirmed by Western blot analysis.

R15LO and RMock Cell Incubations and Volume Measurements

Before cell treatment, the culture media was replaced with serum-free DMEM. CDC in DMSO was added to the media to reach a final concentration of $20 \mu\text{M}$ and incubated for 40 min. Arachidonic acid ($50 \mu\text{M}$) or calcium ionophore A23187 ($5 \mu\text{M}$) was then added and cells were incubated for another 30 min. The media and cells were then harvested for analysis. Cell volumes were measured by volume displacement. Briefly, one plate of cells was counted using a hemocytometer and then centrifuged at $2500 \times g$. The cell pellet was then suspended in 100 μL phosphate-buffered saline buffer, and the final volume was measured by a 200 μL pipette. The extra volume above 100 μL was the volume of the cell pellet. The calculated total pellet volume was determined to be $10 \mu\text{L}/10^7$ cells. Assuming that 50 % of this volume was accounted by the interstitial fluid, nuclei, organelles etc, and in general, 2×10^7 cells contained 3 mg protein as measured using the BCA assay kit, the final volume of the cytosol was determined to be $3.5 \mu\text{L}/\text{mg}$ protein for both RMock and R15LO cells.

Western blot Analysis

Transfected RAW 267.4 cells were collected, washed with phosphate-buffered saline and lysed in buffer containing 50 mM Tris pH 8.0, 5mM ethylenediaminetetraacetic acid, 150 mM sodium chloride, 0.5 % NP40 and $1 \times$ Halt protease inhibitor cocktail (Fisher Scientific). Protein concentrations were determined by the BCA assay. 20 μg of protein was loaded onto 4-12 % bis-tris gel (Invitrogen, Carlsbad, CA) and subsequently transferred onto a nitrocellulose membrane at 30 V for 2 h. The membrane was first probed with 1:5000 diluted anti-human 15-LO-1 polyclonal antibody that also cross-reacts with the murine 12/15-LO (Cayman Chemical) and then with 1:10,000 diluted horseradish peroxidase conjugated anti-goat IgG

(Cayman Chemical, Ann Arbor, MI). The 15-LO-1 band was visualized with ECL plus Western blotting detection system (Amersham Bioscience, Piscataway, NJ).

Synthesis of 4-oxo-2(E)-nonenal

4-Hydroxy-2(E)-nonenal diethyl acetal was oxidized with activated MnO₂. The 4-oxo-2(E)-nonenal diethyl acetal was then hydrolyzed by citric acid/HCl as described previously [33]. The crude 4-oxo-2(E)-nonenal was purified using a flash column and the purity was estimated to be > 99 % by ¹H nuclear magnetic resonance spectroscopy. [²H₃]-4-oxo-2(E)-nonenal was prepared from 4-hydroxy-2-nonyl diethylacetal as described previously [25].

Synthesis of TOG and [²H₃]-TOG

4-Oxo-2(E)-nonenal (100 mM) in 10 μL ethanol was added to glutathione (1 mM) in 100 mM potassium phosphate buffer with 1 mM ethylenediaminetetraacetic acid (pH 6.5) and incubated in the presence of equine glutathione S-transferase (80 units/mL) at 37 °C for 1 h. The reaction mixture was centrifuged at 14,500 × g through Amicon Ultra-4 5,000 molecular weight cut-off filters at the completion of the reaction. The resulting TOG was isolated by preparative HPLC using gradient system 3. The reaction product was fraction-collected, combined, and concentrated under nitrogen to give pure TOG. The stable isotope labeled internal standard [²H₃]-TOG was synthesized in a similar manner using [²H₃]-4-oxo-2(E)-nonenal.

Quantification of TOG in cell lysates

TOG quantification was performed using the method published previously [31]. R15LO or RMock cells were gently scraped off the plate, collected in 15 mL Nunc tubes and washed twice with phosphate-buffered saline. Cells were suspended in 300 μL phosphate-buffered saline, the internal standard [²H₃]-TOG (10 ng) was added to each sample, and cells were lysed with a sonic dismembrator (Thermo Fisher Scientific) on ice. Protein concentrations were determined using a BCA assay kit. The cell lysates were centrifuged at 14,500 × g through Amicon Ultra-4 MWCO 5000 filters and 40 μL of the flowthrough was analyzed by LC-MRM/MS. A calibration curve was generated by spiking known amounts of TOG (final amounts were 0.2, 0.5, 1.0, 2.0, 5.0, 10.0, 20.0 and 50.0 ng/mL) and internal standard [²H₃]-TOG (10 ng) into 500 μL phosphate-buffered saline buffer and analyzing by LC-MRM/MS. TOG concentrations were calculated based on the calibration curve using the peak area ratios between TOG and the internal standard [²H₃]-TOG. The equation of a typical regression line was $y = 0.065x + 0.0045$ ($r^2 = 0.9999$). Replicate determinations (n=5) of quality control samples were conducted at the lower limit of quantitation (0.2 ng/mL), low concentration (0.5 ng/mL), mid-range concentration (5.0 ng/mL), and high concentration (20 ng/mL) on three separate days. The intra- and inter-day accuracy of all quality control samples was within 100 ± 15 % of theoretical with a precision (coefficient of variation) better than 15 %. Final TOG concentrations were normalized relative to the amount of cellular protein.

Extraction, Derivatization and Quantitation of HETEs

15(R)- and 15(S)-HETE were quantified using the method by Lee and Blair [32]. Glass tubes containing cell culture medium (3 mL) were spiked with the following amounts of 15(R)- and 15(S)-HETE: 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, and 1 ng to make the standard samples. Internal standard ([²H₈]-15(S)-HETE, 1 ng) was added to both unknown samples and standard samples. All samples were adjusted to pH 3 with 2.5 N hydrochloric acid. Lipids were extracted with 4 mL diethyl ether and the organic layer was then evaporated to dryness under nitrogen. 100 μL acetonitrile, 100 μL of 2,3,4,5,6-pentafluorobenzyl bromide in acetonitrile (1:19, v/v) followed by 100 μL of diisopropylethylamine in acetonitrile (1:9, v/v) was added to each sample and the solution was heated at 60 °C for 60 min. The solution was allowed to cool down, evaporated to dryness under nitrogen at room temperature, dissolved in 100 μL of

hexane/ethanol (97:3, v/v) and an aliquot of 20 μ L was used for chiral LC-electron capture atmospheric pressure chemical ionization/MS analysis. Calibration curves were obtained with linear regression lines of analyte to internal standard peak-area ratios (15(*R*)- and 15(*S*)-HETEs/[2 H $_8$]-15(*S*)-HETE) against analyte concentrations [32]. Concentrations of 15(*R*)- and 15(*S*)-HETE in the medium were calculated by interpolation from the calibration curves.

Quantification of 15(*R*)- and 15(*S*)-HETE in cell membrane extracts

The cells were harvested as described above and suspended in 1 mL of phosphate-buffered saline. An aliquot of 350 μ L cell suspension was used for protein concentration measurement. The remaining 650 μ L was used for membrane lipid analysis. The membrane lipid extraction was performed using the Folch extraction procedure [34]. Briefly, in a glass tube, the internal standard ([2 H $_8$]-15(*S*)-HETE, 1 ng) was added and 5 mL of chloroform and methanol (2:1, v/v) was added to the cell suspension (typically 10^7 cells) and shaken for 15 min, then centrifuged at $1500 \times g$ for 5 min. The lower organic phase was transferred to a new tube and washed with 1 mL of 0.9 % sodium chloride solution followed by another centrifugation. The lower organic phase was transferred to a new tube and evaporated to dryness under nitrogen. Hydrolysis of esterified lipids was performed in 850 μ L methanol and chloroform (8:1, v/v) with the addition of 0.1 M sodium borohydride in 40 % KOH (wt/v, 150 μ L). The purpose of adding sodium borohydride was to reduce the lipid hydroperoxides to the lipid hydroxide, as well as to prevent auto-oxidation of arachidonic acid during hydrolysis. The tubes were sparged with argon and hydrolysis was conducted at 60 $^{\circ}$ C for 30 min. At the end of hydrolysis, 50 mM phosphate buffer (pH 7.4, 700 μ L) was added and the pH of reaction mixture was adjusted with 150 μ L of formic acid to pH 3-4. The hydrolyzed lipids were then extracted with diethyl ether and hexane (1:1, v/v, 4 mL) and the organic layer was transferred to a new tube and evaporated to dryness under nitrogen. The samples were then derivatized with 2,3,4,5,6-pentafluorobenzyl bromide and analyzed by normal-phase chiral LC-MS analysis, as described above. A calibration curve was constructed with each set of analyses by spiking known amount of 15(*R*)- and 15(*S*)-HETEs (0.01, 0.02, 0.05, 0.1, 0.2, 0.5, and 1 ng) and internal standard ([2 H $_8$]-15(*S*)-HETE, 1 ng) into 650 μ L of phosphate-buffered saline. Samples were processed in an identical manner to the study samples. Final 15(*R*)- and 15(*S*)-HETE concentrations were calculated based on the calibration curve and measured peak area ratios between 15(*R*)- and 15(*S*)-HETE and the internal standard [2 H $_8$]-15(*S*)-HETE.

Results

R15LO and RMock Cells

Western blot analysis showed human 15-LO-1 (MW 75 kDa) expression in R15LO cells and none in RMock cells. Expression of 15-LO-1 in the R15LO cells was stable for more than 10 passages in culture. No COX-2 or murine 12/15-LO was detected in either R15LO or RMock cells (data not shown). Both R15LO and RMock cells had similar cell proliferation rates as the parental cells.

TOG Production in R15LO Cells

R15LO cells were treated with 50 μ M arachidonic acid in DMEM or blank DMEM for 30 min and the intracellular TOG concentrations were analyzed. LC-MRM/MS chromatograms showed that TOG was below the detection limit in untreated R15LO cells (Figure 1A). After arachidonic acid treatment, a significant amount of TOG was observed in the cells (Figure 1B). Pre-treating R15LO cells with the 20 μ M CDC for 40 min completely abolished TOG formation (Figure 1C). The amount of TOG after arachidonic acid treatment was calculated to be 0.17 ± 0.07 pmol/mg protein (Figure 2A). This was reduced to < 0.01 pmol/mg protein by pre-treatment with 20 μ M CDC. R15LO cells were treated with 5 μ M calcium ionophore A23187 for 30 min. LC-MRM/MS chromatograms showed that TOG was below the detection limit in

the untreated R15LO cells (Figure 2A). After calcium ionophore A23187 treatment, 0.10 ± 0.03 pmol/mg of TOG was observed in the cells (Figures 2A). Pre-treating R15LO cells with the 20 μ M CDC for 40 min before ionophore A23187 treatment reduced TOG to < 0.01 pmol/mg protein (Figure 2A). Neither arachidonic acid (Figure 2B) nor calcium ionophore A23187 treatment (Figure 2B) could induce TOG formation in RMock cells.

15(S)-HETE released into the cell culture medium

After arachidonic acid treatment, R15LO cells produced significantly higher levels of 15(S)-HETE (119.3 ± 32.1 pmol/mg protein) compared to untreated cells (< 2 pmol/mg protein) (Figure 3A). The amount of 15(S)-HETE was approximately three-fold that of 15(R)-HETE (37.3 ± 7.4 pmol/mg protein), indicating that some 52 % (82 pmol/mg protein) of the total 15-HETE was produced by 15-LO-1, while the rest was generated non-enzymatically (75 pmol/mg protein) (Figure 3A). Pretreatment with CDC significantly reduced 15(S)-HETE generated from the 15-LO-1 pathway. (Figure 3A) For R15LO cells treated with calcium ionophore A23187, the level of 15(S)-HETE increased more than 5-fold from 0.8 ± 0.3 pmol/mg protein (no treatment) to 4.2 ± 0.9 pmol/mg protein (Figure 3B). The trace amount of 15(R)-HETE could not be quantified accurately due to the presence of interfering substances and lack of a heavy isotope internal standard for this enantiomer. R15LO cells that had been pretreated with the LO inhibitor CDC released significantly less 15(S)-HETE upon calcium ionophore A23187 stimulation than cells not treated with CDC. (Figure 3B) For RMock cells, only trace amounts of released racemic 15-HETEs were detected under all treatment conditions (Figure 3C).

Esterified 15(S)-HETE formation upon calcium ionophore A23187 treatment

In the untreated R15LO cells, esterified 15(S)-HETE levels were determined to be 2.3 ± 0.3 pmol/mg protein (Figure 4). This was approximately three-fold higher than the 15(R)-HETE levels. After calcium ionophore A23187 treatment, esterified 15(S)-HETE levels increased almost three-fold to 6.2 ± 0.7 pmol/mg protein, while the esterified 15(R)-HETE levels were similar to those in the untreated cells (Figure 4). In RMock cells, the esterified 15-HETEs were racemic at 0.7 ± 0.1 pmol/mg protein and the levels did not change with calcium ionophore A23187 treatment (Figure 4).

Intracellular TOG Formation

R15LO cells were treated with increasing concentrations of arachidonic acid: 0 μ M, 10 μ M, 20 μ M, 40 μ M and 100 μ M in DMEM for 30 min. Intracellular TOG levels were found to be elevated in a non-linear dose-dependent manner (Figure 5). There was an over 10-fold increase in TOG from 0.05 ± 0.02 pmol/mg protein to 0.63 ± 0.04 pmol/mg protein when arachidonic acid was increased from 20 μ M to 40 μ M.

Treatment of R15LO Cells with [$^{13}\text{C}_{20}$]-Arachidonic Acid

R15LO cells were treated with 20 μ M [$^{13}\text{C}_{20}$]-arachidonic acid and the intracellular TOG was monitored by LC-MRM/MS. TOG (0.04 pmol/mg protein) was detected in the MRM channel corresponding to [$^{13}\text{C}_9$]-TOG, not in the channel corresponding to unlabeled [$^{12}\text{C}_9$]-TOG (Figure 6).

Discussion

The 15-LO-1-derived arachidonic acid metabolite 15(S)-HpETE is rapidly reduced to 15(S)-HETE in cells [35] and so this eicosanoid was used as an index of 15-LO activity [32]. When R15LO cells were treated with arachidonic acid, approximately half of the released 15-HETE was the 15(S)-enantiomer produced by 15-LO-1, while the other half was racemic and arose through non-enzymatic oxidation of arachidonic acid. The final ratio of 15(S)-HETE to 15

(*R*)-HETE was 3:1 (Figure 3A). 12(*S*)- and 12(*R*)- HETEs were also observed, with 12(*S*)-HETE being the predominant form and at a concentration that was about an order of magnitude lower than the 15(*S*)-HETEs (data not shown). These results are in keeping with previous studies, which showed that the relative regioselectivity of recombinant human 15-LO-1 to form the 15(*S*)- and 12(*S*)- HETEs was 12:1 [11].

15(*S*)-HpETE is known to readily undergo homolytic decomposition to 4-oxo-2(*E*)-nonenal [36]. However, it was not clear whether this could occur in a cellular context where there are numerous protective mechanisms that could eliminate 15(*S*)-HpETE efficiently. Because 4-oxo-2(*E*)-nonenal is removed by glutathione *S*-transferase-mediated reaction with glutathione [31] and by reaction with cellular macromolecules [21,26], it cannot be detected in cells. Some evidence has been obtained showing that the lipid hydroperoxide decomposition to 4-oxo-2(*E*)-nonenal does occur in cells through the detection of 4-oxo-2(*E*)-nonenal-derived DNA-adducts. For example, Lee *et al.* showed that heptanone-etheno-2'-deoxyguanosine was increased in COX-2 over-expressing rat intestinal epithelial cells upon vitamin C treatment [22,37]. The formation of TOG in R15LO cells after the addition of arachidonic acid (Figure 3A) and [¹³C₉]-TOG after the addition of [¹³C₂₀]-arachidonic acid (Figure 6) has provided additional compelling evidence that 15(*S*)-HpETE can undergo homolytic decomposition to 4-oxo-2(*E*)-nonenal in cells.

TOG was almost undetectable in untreated R15LO cells at < 0.01 pmol/mg protein. Upon arachidonic acid treatment, the intracellular TOG levels in R15LO cells increased ~50 fold to 0.17 ± 0.07 pmol/mg protein. Given that 1 mg protein corresponded to 3.5 μ L cell cytosol volume, the intracellular concentration of TOG in arachidonic acid-treated R15LO cells would be approximately 50 nM. Calcium ionophore A23187, which stimulates translocation of 15-LO-1 to the cell membrane, also significantly increased TOG levels to 0.10 ± 0.03 pmol/mg protein in R15LO cells (Figure 2A). CDC pretreatment abolished TOG formation in R15LO cells after treatment with arachidonic acid or calcium ionophore A23187 (Figure 2A). RMock cells produced almost undetectable levels of TOG (< 0.01 pmol/mg protein) under all treatment conditions showing that lipid peroxidation and subsequent TOG formation did not occur by the artifactual generation of reactive oxygen species during sample work-up (Figure 2B). Therefore, TOG formation observed in the R15LO cells is clearly dependent upon 15-LO-1 activity.

15(*S*)-HETE concentrations in the R15LO cells increased approximately 100-fold after treatment with 50 μ M arachidonic acid when compared with untreated cells (Figure 3A). This resulted in a 50-fold increase in TOG concentrations (Figure 2A). When cells were pretreated with the LO inhibitor CDC, the amount of 15(*S*)-HETE was reduced by approximately 70 % (Figure 3A). In contrast, the TOG levels were reduced to almost undetectable levels (Figure 2A). Furthermore, there was a non-linear increase in TOG levels in the R15LO cells with increasing concentrations of arachidonic acid (Figure 5). A particularly striking 10-fold increase in TOG levels was observed when the concentration of arachidonic acid was increased from 20 μ M to 40 μ M. These findings suggest that once the 15(*S*)-HpETE has accumulated past a certain threshold, reducing pathways are compromised [35] and the homolytic decomposition pathway becomes more prominent [21].

When R15LO cells were treated with calcium ionophore A23187, released 15(*S*)-HETE increased only 5-fold compared with untreated cells to give a final concentration of 4.2 ± 0.9 pmol/mg protein (Figure 3B). This was only 3.5 % of that observed with arachidonic acid treatment (119.3 ± 32.1 pmol/mg protein, Figure 3A). Brinckmann *et al.* reported that 15-LO-1 translocated to plasma membrane in a calcium-dependent manner in rabbit hematopoietic cells and that the translocation activated the oxygenase activity of the enzyme [38]. In addition, it is known that 15-LO-1 has a broad substrate specificity so that it can oxidize esterified lipids

as well as lipid-protein assemblies [39,40]. Furthermore, a recent publication by Maskrey *et al.* showed that when interleukin-4-treated monocytes were stimulated with A23187, they generated predominantly esterified 15-HETE, with many fold less free 15-HETE [41]. These results combined with our observation suggested that calcium ionophore A23187 treatment of R15LO cells resulted in translocation of 15-LO-1 to the plasma membrane so that it was no longer present in the cytosol. 15(*S*)-HpETE was then formed primarily on esterified arachidonic acid rather than from free arachidonic acid released by ionophore A23187-mediated translocation of cytoplasmic phospholipase A₂ to the membrane [42,43]. The resulting esterified 15(*S*)-HpETE in the membrane then either underwent homolytic decomposition to 4-oxo-2(*E*)-nonenal [36] or was reduced to 15(*S*)-HETE and a significant amount then released by cytoplasmic phospholipase A₂-mediated hydrolysis of the esterified 15(*S*)-HETE and released from the cells as suggested previously by Chaitidis *et al.* [44].

15-HETEs were analyzed in the cell membranes after Folch extraction [34] and hydrolysis in order to confirm that ionophore A23187-mediated activation of 15-LO-1 had stimulated oxidation of esterified arachidonic acid. The amount of cytosolic free 15(*S*)-HETE was negligible (data not shown), so the 15(*S*)-HETE recovered by Folch extraction was present in an esterified form in the cell membranes. After calcium ionophore A23187 treatment, esterified 15(*S*)-HETE increased almost 3-fold from 2.3 ± 0.3 pmol/mg protein to 6.2 ± 0.7 pmol/mg protein, whereas the level of esterified 15(*R*)-HETE was unchanged (Figure 4). The increase of esterified 15(*S*)-HETE upon calcium ionophore A23187 treatment was 15-LO-1-dependent because it was not observed in RMock cells (Figure 4). This confirmed that 15-LO-1-mediated oxidation of esterified arachidonic acid to 15(*S*)-HpETE was the major source of released 15(*S*)-HETE. The amount of 15(*S*)-HpETE determined by analysis of released and esterified membrane 15(*S*)-HETE after calcium ionophore A23187 treatment (10.4 ± 1.6 pmol/mg protein) was only 8.7 % of the released 15(*S*)-HETE produced after arachidonic acid treatment (119.3 ± 32.1 pmol/mg protein). However, a comparable level of TOG formation was observed (Figure 2). One possible explanation for these results is that cytosolic 15(*S*)-HpETE is reduced to 15(*S*)-HETE much more efficiently than esterified 15(*S*)-HpETE. Therefore, less 4-oxo-2(*E*)-nonenal was formed from cytosolic 15(*S*)-HpETE than from esterified forms in membrane lipids. The homolytic decomposition of esterified 15(*S*)-HpETE to 4-oxo-2(*E*)-nonenal will result in the concomitant formation of a free aldehyde esterified in the plasma membrane (Scheme 1). This oxidized lipid could then be involved in cell-cell signaling as suggested by the Hazen group [45].

It is known that most glutathione-adducts, such as those arising from drug metabolism, are actively secreted by membrane transporters [46,47]. We have shown previously that when EA.hy 926 endothelial cells were treated with 4-oxo-2(*E*)-nonenal, its glutathione-adduct (subsequently characterized as TOG) could be detected in both the intracellular and extracellular compartments [30]. This suggested that TOG would be released by the R15LO cells. Surprisingly, in the present experiments TOG was not observed in the media of R15LO cells after treatment with arachidonic acid or calcium ionophore A23187 (data not shown). This means that either the released TOG was below the detection limit of our assay or that the R15LO cells could not transport TOG into the extracellular compartment as readily as the EA.hy 926 endothelial cells.

As a potential next-generation biomarker for oxidative stress, TOG exhibits many advantages over other existing biomarkers such as lipid hydroxide and DNA and RNA- adducts. Unlike lipid hydroxide such as 15-HETEs and 12-HETEs, which only indicate the extent of lipid peroxidation, TOG can serve as a direct barometer for the extent of lipid peroxide degradation. Since 4-oxo-2(*E*)-nonenal contains α,β -unsaturated aldehyde moiety, it is chemically more reactive, and thus hypothetically more toxic to cells than lipid hydroperoxides. TOG as a direct product from 4-oxo-2(*E*)-nonenal, could provide valuable information as to the extent of

cellular damage caused by lipid peroxide degradation following both enzymatic and non-enzymatic lipid peroxidation. DNA and RNA adducts derived from 4-oxo-2(*E*)-nonenal can also serve as biomarkers for this purpose. However, the procedures for quantitation of DNA and RNA adducts are time-consuming, labor intensive (involving multiple steps of DNA/RNA isolation, hydrolysis, concentration, etc) and susceptible to interference by transition metal contamination [22,23]. In comparison, quantitation of TOG is much easier and faster.

In summary, 4-oxo-2(*E*)-nonenal can be generated non-enzymatically by reactive oxygen species-mediated lipid peroxidation [31] or enzymatically through lipid peroxidation arising from oxygenases such as COXs [48] and LOs [49]. 4-oxo-2(*E*)-nonenal is efficiently converted to TOG, which can be readily quantified using stable isotope dilution LC-MRM/MS. Thus, the analysis of TOG offers a new way to monitor oxidative stress induced by both non-enzymatic [31] or enzymatic [48,49] lipid peroxidation as a complement to isoprostane and DNA-adduct measurements [21,50,51].

Acknowledgments

We thank Dr. Colin D. Funk (Queen's University, Kingston, ON) for the pcDNA3 plasmid containing the human 15-LO-1 gene.

Abbreviations

CDC	cinnamyl-3, 4-dihydroxy- α -cyanocinnamate
DMEM	Dulbecco's Modified Eagle's Medium
HETE	hydroxy-[5Z,8Z,11Z,13E]-eicosatetraenoic acid
HpETE	hydroperoxy-[5Z,8Z,11Z,13E]-eicosatetraenoic acid
LC	liquid chromatography
LO	lipoxygenase
MRM	multiple reaction monitoring
MS	mass spectrometry
TOG	thiadiazabicyclo-4-oxo-2(<i>E</i>)-nonenal-glutathione-adduct [(2 <i>S</i> ,7 <i>R</i>)-7-[N-(carboxymethyl) carbamoyl]-5-oxo-12-pentyl-9-thia-1,6-diazabicyclo[8.2.1]trideca-10(13),11-diene-2-carboxylic acid)]

References

1. Kühn H, Walther M, Kuban RJ. Prostaglandins Other Lipid Mediat 2002;68-69:263–290. [PubMed: 12432923]
2. Fridovich SE, Porter NA. J Biol Chem 1981;256:260–265. [PubMed: 6256348]
3. Levy BD, Romano M, Chapman HA, Reilly JJ, Drazen J, Serhan CN. J Clin Invest 1993;92:1572–1579. [PubMed: 8376607]
4. Conrad DJ, Kühn H, Mulkins M, Highland E, Sigal E. Proc Natl Acad Sci U S A 1992;89:217–221. [PubMed: 1729692]
5. Cornicelli JA, Trivedi BK. Curr Pharm Des 1999;5:11–20. [PubMed: 10066881]
6. Cyrus T, Witztum JL, Rader DJ, Tangirala R, Fazio S, Linton MF, Funk CD. J Clin Invest 1999;103:1597–1604. [PubMed: 10359569]
7. George J, Afek A, Shaish A, Levkovitz H, Bloom N, Cyrus T, Zhao L, Funk CD, Sigal E, Harats D. Circulation 2001;104:1646–1650. [PubMed: 11581143]

8. Zhao L, Funk CD. Trends Cardiovasc Med 2004;14:191–195. [PubMed: 15261891]
9. Nassar GM, Morrow JD, Roberts LJ, Lakkis FG, Badr KF. J Biol Chem 1994;269:27631–27634. [PubMed: 7961680]
10. Heydeck D, Thomas L, Schnurr K, Trebus F, Thierfelder WE, Ihle JN, Kühn H. Blood 1998;92:2503–2510. [PubMed: 9746791]
11. Kühn H, Barnett J, Grunberger D, Baecker P, Chow J, Nguyen B, Bursztyn-Pettegrew H, Chan H, Sigal E. Biochim Biophys Acta 1993;1169:80–89. [PubMed: 8334154]
12. Belkner J, Wiesner R, Rathman J, Barnett J, Sigal E, Kuhn H. Eur J Biochem 1993;213:251–261. [PubMed: 8477699]
13. Walther M, Wiesner R, Kühn HJ. Biol Chem 2004;279:3717–3725.
14. Sun D, Funk CD. J Biol Chem 1996;271:24055–24062. [PubMed: 8798642]
15. Folcik VA, Nivar-Aristy RA, Krajewski LP, Cathcart MK. J Clin Invest 1995;96:504–510. [PubMed: 7615823]
16. Shen J, Herderick E, Cornhill JF, Zsigmond E, Kim HS, Kuhn H, Guevara NV, Chan L. J Clin Invest 1996;98:2201–2208. [PubMed: 8941635]
17. Funk CD, Cyrus T. Trends Cardiovasc Med 2001;11:116–124. [PubMed: 11686000]
18. Lee SH, Blair IA. Trends Cardiovasc Med 2001;11:148–155. [PubMed: 11686005]
19. Blair IA. Exp Gerontol 2001;36:1473–1481. [PubMed: 11525870]
20. Lee SH, Blair IA. Chem Res Toxicol 2000;13:698–702. [PubMed: 10956056]
21. Blair IA. J Biol Chem 2008;283:15545–15549. [PubMed: 18285329]
22. Lee SH, Williams MV, Dubois RN, Blair IA. J Biol Chem 2005;280:28337–28346. [PubMed: 15964853]
23. Williams MV, Lee SH, Pollack M, Blair IA. J Biol Chem 2006;281:10127–10133. [PubMed: 16449227]
24. Zhu P, Lee SH, Wehrli S, Blair IA. Chem Res Toxicol 2006;19:809–817. [PubMed: 16780360]
25. Oe T, Arora JS, Lee SH, Blair IA. J Biol Chem 2003;278:42098–42105. [PubMed: 12930824]
26. Sayre LM, Lin D, Yuan Q, Zhu X, Tang X. Drug Metab Rev 2006;38:651–675. [PubMed: 17145694]
27. Burczynski ME, Sridhar GR, Palackal NT, Penning TM. J Biol Chem 2001;276:2890–2897. [PubMed: 11060293]
28. Alary J, Gueraud F, Cravedi JP. Mol Aspects Med 2003;24:177–187. [PubMed: 12892995]
29. Blair IA. Curr Drug Metab 2006;7:853–872. [PubMed: 17168687]
30. Jian W, Arora JS, Oe T, Shuvaev VV, Blair IA. Free Radic Biol Med 2005;39:1162–1176. [PubMed: 16214032]
31. Jian W, Lee SH, Mesaros C, Oe T, Silva Elipse MV, Blair IA. Chem Res Toxicol 2007;20:1008–1018. [PubMed: 17550273]
32. Lee SH, Blair IA. Methods Enzymol 2007;453:159–174.
33. Rindgen D, Lee SH, Nakajima M, Blair IA. Chem Res Toxicol 2000;13:846–852. [PubMed: 10995257]
34. Folch J, Lees M, Sloane Stanley GH. J Biol Chem 1957;226:497–509. [PubMed: 13428781]
35. Kühn H, Borchert A. Free Radic Biol Med 2002;33:154–172. [PubMed: 12106812]
36. Williams MV, Lee SH, Blair IA. Rapid Commun Mass Spectrom 2005;19:849–858. [PubMed: 15723435]
37. Lee SH, Oe T, Blair IA. Science 2001;292:2083–2086. [PubMed: 11408659]
38. Brinckmann R, Schnurr K, Heydeck D, Rosenbach T, Kolde G, Kuhn H. Blood 1998;91:64–74. [PubMed: 9414270]
39. Rapoport SM, Schewe T, Wiesner R, Halangk W, Ludwig P, Janicke-Hohne M, Tannert C, Hiebsch C, Klatt D. Eur J Biochem 1979;96:545–561. [PubMed: 111925]
40. Schewe T, Rapoport SM, Kuhn H. Adv Enzymol Relat Areas Mol Biol 1986;58:191–272. [PubMed: 3087141]
41. Maskrey BH, Bermúdez-Fajardo A, Morgan AH, Stewart-Jones E, Kioszeghy V, Taylor GW, Baker PRS, Coles Barbara, Coffey MJ, Kühn H. J Biol Chem 2007;282:20151–20163. [PubMed: 17519227]

42. Gerritsen ME, Nganele DM, Rodrigues AM. J Pharmacol Exp Ther 1987;240:837–846. [PubMed: 3104581]
43. Ganss M, Seemann D, Furstenberger G, Marks F. FEBS Lett 1982;142:54–58. [PubMed: 6809493]
44. Chaitidis P, Schewe T, Sutherland M, Kuhn H, Nigam S. FEBS Lett 1998;434:437–441. [PubMed: 9742970]
45. Hazen SL. J Biol Chem 2008;283:15527–15531. [PubMed: 18285328]
46. Ritter CA, Jedlitschky G, Meyer Zu SH, Grube M, Kock K, Kroemer HK. Drug Metab Rev 2005;37:253–278. [PubMed: 15747503]
47. Ballatori N, Hammond CL, Cunningham JB, Krance SM, Marchan R. Toxicol Appl Pharmacol 2005;204:238–255. [PubMed: 15845416]
48. Lee SH, Rangiah K, Williams MV, Wehr AY, Dubois RN, Blair IA. Chem Res Toxicol 2007;20:665–1675.
49. Zhu P, Oe T, Blair IA. Rapid Commun Mass Spectrom 2008;22:432–440. [PubMed: 18215009]
50. Pratico D, Rokach J, Lawson J, Fitzgerald GA. Chem Phys Lipids 2004;128:165–171. [PubMed: 15037161]
51. Milne GL, Morrow JD. Antioxid Redox Signal 2006;8:1379–1384. [PubMed: 16910785]

Figure 1.

LC-electrospray ionization/MRM/MS analysis of TOG and its corresponding internal standard [2H₃]-TOG in R15LO cells with arachidonic acid treatment in the presence or absence of LO inhibitor CDC. (A) no treatment (< 0.01 pmol/mg protein), (B) arachidonic acid 50 μM for 30 min (0.17 pmol/mg protein), (C) pretreatment with CDC 20 μM for 40 min, then arachidonic acid 50 μM for 30 min (< 0.01 pmol/mg protein). Ion chromatograms for TOG (*m/z* 426 → *m/z* 280) and [2H₃]-TOG (*m/z* 429 → *m/z* 283).

Figure 2.

Intracellular levels of TOG in R15LO and RMock cells with arachidonic acid or calcium ionophore A23187 treatment in the presence or absence of LO inhibitor CDC. (A) R15LO cells were pretreated with 20 μ M CDC in DMEM media or DMEM media alone for 40 min, then 50 μ M of arachidonic acid or 5 μ M of calcium ionophore A23187 was added to the media and the cells were incubated for another 30 min. Cells were harvested for intracellular TOG analysis. (B) RMock cells were pre-incubated in DMEM media for 40 min, then 50 μ M of arachidonic acid or 5 μ M of calcium ionophore A23187 was added to the media and the cells were incubated for another 30 min. Cells were harvested for intracellular TOG analysis. (Mean $\pm$ SD, n = 3). ** p < 0.01. AA: arachidonic acid, CI: calcium ionophore A23187.

Figure 3.

Amounts of 15-HETEs formed in the media by R15LO and RMock cells with arachidonic acid or calcium ionophore A23187 treatment in the presence or absence of LO inhibitor CDC. (A, B) R15LO cells were pretreated with 20 μ M CDC in DMEM media or DMEM media alone for 40 min, then 50 μ M of arachidonic acid or 5 μ M of calcium ionophore A23187 was added to the media and cells were incubated for another 30 min. The media were harvested for 15-HETE analysis. (C) RMock cells were pre-incubated in DMEM media for 40 min, then 50 μ M of arachidonic acid or 5 μ M of calcium ionophore A23187 was added to the media and cells were incubated for another 30 min. The media were harvested for 15-HETE analysis using stable isotope dilution chiral LC-electron capture atmospheric pressure chemical ionization/MS. (Mean $\pm$ SD, n = 3). * p < 0.05, ** p < 0.01, *** p < 0.001. AA: arachidonic acid, CI: calcium ionophore A23187.

Figure 4.

Amounts of 15-HETEs in membrane fractions of R15LO and RMock cells with or without calcium ionophore A23187 treatment. R15LO or RMock cells were treated with 5 μ M of calcium ionophore A23187 for 30 min. The membrane fractions were extracted and hydrolyzed for 15-HETE measurements using stable isotope dilution chiral LC-electron capture atmospheric pressure chemical ionization/MS. (Mean $\pm$ SD, n = 3). *** p < 0.001. CI: calcium ionophore A23187.

Figure 5. Amounts of intracellular TOG in R15LO cells with increasing concentrations of arachidonic acid in the media. Analyses were conducted in duplicates by stable isotope dilution LC-electrospray ionization/MRM/MS method. (Mean $\pm$ SD, n = 2).

Figure 6.

LC- electrospray ionization/MRM/MS analysis of TOG and the $[^{13}\text{C}_9]$ -TOG in R15LO cells with $20\ \mu\text{M}$ $[^{13}\text{C}_{20}]$ -arachidonic acid treatment. (A) TOG, (B) $[^2\text{H}_3]$ -TOG, (C) $[^{13}\text{C}_9]$ -TOG. Ion chromatograms for TOG ($m/z\ 426 \rightarrow m/z\ 280$), $[^2\text{H}_3]$ -TOG ($m/z\ 429 \rightarrow m/z\ 283$) and $[^{13}\text{C}_9]$ -TOG ($m/z\ 435 \rightarrow m/z\ 289$). The amount of $[^{13}\text{C}_9]$ -TOG corresponded to $0.04\ \text{pmol/mg}$ protein. TOG from endogenous arachidonic acid was not detected ($< 0.01\ \text{pmol/mg}$ protein).

Scheme 1.
Mechanism of TOG formation in R15LO cells.