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4-Hydroxy-7-oxo-5-heptenoic Acid (HOHA) Lactone is a Potent Inducer of the Complement Pathway in Human Retinal Pigmented Epithelial Cells

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Abstract

We previously discovered that oxidative cleavage of docosahexaenoate (DHA), which is especially abundant in the retinal photoreceptor rod outer segments and retinal pigmented endothelial (RPE) cells, generates 4-hydroxy-7-oxo-5-heptenoate (HOHA) lactone, and that HOHA lactone can enter RPE cells that metabolize it through conjugation with glutathione (GSH). The consequent depletion of GSH results in oxidative stress. We now find that HOHA-lactone induces upregulation of the antioxidant transcription factor Nrf2 in ARPE-19 cells. This leads to expression of GCLM, HO1, and NQO1, three known Nrf2-responsive antioxidant genes. Besides this protective response, HOHA lactone also triggers a countervailing inflammatory activation of innate immunity. Evidence for a contribution of the complement pathway to age-related macular degeneration (AMD) pathology includes the presence of complement proteins in drusen and Bruch's membrane from AMD donor eyes, and the identification of genetic susceptibility loci for AMD in the complement pathway. In eye tissues from a mouse model of AMD, accumulation of complement protein in Bruch's membrane below the RPE suggested that the complement pathway targets this interface where lesions occur in the RPE and photoreceptor rod outer segments. In animal models of AMD, intravenous injection of NaIO₃ to induce oxidative injury selectively destroys the RPE, and causes secretion of factor C3 from the RPE into areas directly adjacent to

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Notes

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sites of RPE damage. However, a molecular level link between oxidative injury and complement activation remained elusive. We now find that submicromolar concentrations of HOHA lactone foster expression of C3, CFB and C5 in ARPE-19 cells, and induce a countervailing upregulation of CD55, an inhibitor of C3 convertase production and complement cascade amplification. Ultimately, HOHA lactone causes membrane attack complex formation on the plasma membrane. Thus, HOHA lactone provides a molecular level connection between free radical-induced oxidative cleavage of DHA and activation of the complement pathway in AMD pathology.

Graphical Abstract

Keywords

oxidative stress; Nrf2; alternative complement pathway; age related macular degeneration; retinal pigment epithelium cells; 4-hydroxy-7-oxo-5-heptenoic acid (HOHA) lactone; membrane attack complex

INTRODUCTION

The pathogenesis of age related macular degeneration (AMD) is a complex multifactorial process, and a molecular level understanding of the etiology of AMD is incomplete. AMD is a progressive loss of central vision resulting from the damage to retinal pigmented epithelium (RPE) and neural retina that affects almost 50 million people worldwide, and its prevalence increases with age.¹ For approximately 90% of individuals with AMD, there is atrophy and degeneration of the RPE (dry AMD), while choroidal neovascularization leads to leakage of blood into and detachment of the neural retina (wet AMD) in about 10 % of patients. The susceptibility of RPE cells to oxidative stress and the prevalence of AMD progressively increases with age as antioxidant defenses deteriorate.

Inappropriate Activation of the Complement Cascade in AMD

The involvement of the complement cascade in AMD pathology is suggested by the presence of complement pathway proteins in drusen and Bruch's membrane from AMD donor eyes.²⁻⁵ Genetic AMD susceptibility loci for the complement pathway, including complement factor H (CFH)^{6, 7}, component 3 (C3)^{8, 9} and factor B (CFB)¹⁰ have been identified that affect regulation of inflammatory responses resulting in a hyperactive complement system. There are three complement pathways – classical, lectin and alternative – that all lead to expression of C3. CFH regulates cleavage of C3, the common complement protein of all three pathways. CFB expression leads to the formation of a C3 convertase of

the alternative pathway that amplifies a proteolytic cascade resulting in C3 cleavage to form C3a and C3b that, in turn, forms the C5 convertase. This promotes formation of C5a and C5b and ultimately formation of the C5b-9 membrane attack complex (MAC). The MAC is found in donor eyes with early (dry) AMD.^{4, 11, 12} In a mouse model of AMD, intravenous injection of NaIO₃ selectively destroys the RPE resulting in patchy RPE loss similar to that seen in dry AMD.^{13–16} In this model, secretion of C3 from the RPE was observed into areas directly adjacent to sites of RPE damage and C3 mRNA expression in the RPE/choroid was maximally elevated 5-fold over control mice 3 days after injection of NaIO₃.¹⁷ Immunolocalization of complement component C3d (Fig. 1) in Bruch's membrane below the RPE in eye tissues from another mouse model of dry AMD¹⁸ suggested that the complement pathway targets this interface where lesions occur in the RPE and photoreceptor rod outer segments. C3d is a degradation product of C3b, a key component in the generation of the C3 and C5 convertases required for complement activation through the classical, lectin, and alternate pathways.¹⁹

Lipid Oxidation in AMD

Docosahexaenoate (DHA) is the most oxidizable fatty acid in the body, and it is highly concentrated in the disc membranes of photoreceptor cells and in RPE cells that endocytose oxidatively damaged rod photoreceptor outer segments.^{20, 21} The combination of high oxygen levels and the focus of environmental light on the retina fosters the generation of free radicals and lipid oxidation that can damage DHA. We discovered that oxidative cleavage of DHA phospholipids generates 4-hydroxy-7-oxo-5-heptenoate (HOHA) phospholipid esters (Scheme 1) that undergo rapid intramolecular transesterification to release HOHA lactone.²² The reaction of HOHA lactone with proteins generates carboxyethylpyrrole (CEP) derivatives of protein lysyl ε-amino groups.²³ CEP accumulates in the rod outer segments and RPE in the retina and is present in AMD eye tissue and in the blood of AMD patients at higher levels than found in age-matched non-AMD tissues.^{24, 25}

Evidence is accumulating that supports the hypothesis that HOHA lactone and CEP are causally involved in initiating an inflammatory response in AMD.^{23, 26–29} Previously we showed that exposure of ARPE-19 cells to >15 μM concentrations of HOHA-lactone is cytotoxic inducing apoptosis, but submicromolar concentrations of HOHA-lactone promote survival. We now find that submicromolar concentrations of HOHA lactone foster expression of C3, at both the mRNA and protein levels, that is required for both classical and alternative complement activation pathways. This is accompanied by an increase in the level of CFB, a component of the alternative pathway of complement activation. CFB is the precursor of complement factor Bb that associates with C3b to form the alternative pathway C3 convertase that amplifies the proteolytic cascade resulting in C3 cleavage and ultimately MAC formation. We now find that submicromolar concentrations of HOHA lactone increase expression of CD59, also known as MAC-inhibitory protein (MAC-IP), that can prevent formation of the complement membrane attack complex (MAC). In contrast, micromolar concentrations of HOHA lactone seem to inhibit the expression of CD59, favoring MAC formation, which can lead to ARPE cell lysis and death.

We previously showed that HOHA lactone causes depletion of glutathione (GSH) and oxidative stress in ARPE cells.³⁰ We now find that HOHA lactone also triggers antioxidant protein expression that counteracts its contribution to oxidative stress. HOHA-lactone elevates hemoxygenase-1 (HO-1) levels in ARPE cells consistent with the conclusion that oxidative stress, induced by HOHA lactone, contributes to activation of the complement pathway.³¹ Furthermore, ARPE cells respond to this HOHA lactone-induced GSH depletion and oxidative stress by upregulating expression of NAD(P)H dehydrogenase quinone 1 (NQO1) and glutamate–cysteine ligase modifier subunit (GCLM), the first rate-limiting enzyme of GSH synthesis. Finally, we discovered a dose-dependent increase in MAC formation on the ARPE-19 plasma membrane with increasing levels of HOHA-lactone.

MATERIALS AND METHODS

Reagents

Dulbecco's modified Eagle's (DMEM)/F12 medium, phosphate buffered saline and fetal bovine serum (FBS) were purchased from Fisher Scientific (Pittsburgh, PA). The lactone of 4-hydroxy-7-oxohept-5-enoate (HOHA-lactone) was synthesized as described elsewhere.^{23, 32} **Antibodies.** Mouse monoclonal antihuman NRF2 (clone 1E9E3; 66504-1-Ig; observed molecular weight: 110 kDa), rabbit polyclonal complement factor B (10170-1-AP; observed molecular weight: 93–100 kDa), rabbit polyclonal complement factor C3 (21337-1-AP; observed molecular weight: 115 kDa) antibodies, rabbit polyclonal CD59 antibodies (10742-1-AP; observed molecular weight: 18–20 kDa) and HRP-conjugated beta-actin mouse monoclonal antibody (cytosolic proteins loading control, HRP-60008) were procured from Proteintech, Rosemont, IL. Mouse monoclonal anti-human C5 antibody (sc-52636) and mouse monoclonal anti-human C5b-9 antibody (SC-58935) and HRP-conjugated laminin B1 (nuclear proteins loading control, sc-374015 HRP) were obtained from Santa Cruz Biotechnology, Dallas, TX. Goat anti-mouse IgG cross-adsorbed secondary antibody with Alexa Fluor 647 (A32728) was from ThermoFisher Scientific, Waltham, MA). Cell lysis buffer (Tissue-PELB) was from Gold Biotechnology (St. Louis, MO). Restore Plus Western blot stripping buffer as well as the enhanced chemiluminescence (ECL) Western blot detection system were from Pierce Biotechnology (Rockford, IL) via Fisher Scientific (Pittsburgh, PA). Reagents and pre-cast gels (4–20% gradient) for SDS–PAGE were purchased from Invitrogen Life Technologies (Carlsbad, CA). The bicinchoninic acid (BCA) assay³³ was used to determine the total amount of protein in the lysates.

Cell Culture

The cell line ARPE-19 (ATCC; CRL-2302) derived from spontaneously arising retinal pigment epithelia of a healthy person³⁴ was obtained from American Type Culture Collection (Manassas, VA). The stock cells were grown on 100-mm dishes in a humidified CO₂ incubator at 37 °C and 5% CO₂ in Ham's F12 medium and Dulbecco's modified Eagle's medium (DMEM) (50:50 ratio), containing L-glutamine and 10% heat-inactivated FBS. Cells were trypsinized and passaged every 2–3 days.

Dose-dependent Effects of Exposing ARPE19 cells to HOHA-lactone

Serum starved monolayers of ARPE19 cells (80–90% confluence) on 60 mm plates in 5% CO₂/95% air at 37 °C were challenged for 2 h with 0–30 µM HOHA-lactone solutions (2 ml) in the respective basal medium. Cells were then scraped with a rubber policeman, transferred to 5-ml conical tubes and centrifuged at 480 *g* at 4 °C for 10 min. The medium was carefully aspirated and the cells were washed three times with ice-cold PBS followed by centrifugation at 480 *g* at 4 °C for 10 min. Cells were disrupted upon incubation with a RIPA lysis buffer, 50 mM HEPES (pH 7.4), 0.5% sodium deoxycholate, 0.1% sodium dodecylsulphate, 1 mM phenylmethanesulfonyl fluoride (PMSF), and 1× HALT protease inhibitor cocktail for 15 min at 4 °C. Cell lysate was further placed on ice and sonicated at 40% power (5 cycles of 5 sec on and 5 sec off). The cell lysate was spun at 14,000 *g* at 4 °C for 20 min and the supernatant was collected and snap-frozen in liquid nitrogen. The BCA assay¹ was used to determine the total amount of protein in the lysates.

Time-course of Effects Induced by Exposing ARPE19 Cells to HOHA Lactone

After 16 h of starvation in the basal medium, ARPE19 cells (80–90% confluence) in 100-mm plates were treated for 0, 15, 30, 60, 90, or 120 min with 20 µM HOHA lactone solution in a basal medium under 5% CO₂/95% air at 37 °C. Handling cells, preparation of the cell lysates and measuring total protein concentration in the cell lysates were carried out in a manner similar to that described in the previous paragraph.

PAGE and Western Blot Analysis

ARPE19 cell extracts (25–30 µg protein/lane) were separated by SDS-PAGE (4–20% gels) and the proteins were electrotransferred onto a PVDF membrane (Bio-Rad, Hercules, CA). The blot was blocked with 5% bovine serum albumin (BSA) in Tris buffered saline containing 0.1% Tween-20 (TBST) for an hour at room temperature. The blots were subsequently probed overnight with the indicated antibody at 4 °C in TBST buffer. After washing with TBST, the membrane was incubated with the appropriate secondary antibody-HRP conjugate at room temperature for one hour. The membrane was again washed with TBST and the immunoblot was developed with the SuperSignal West Pico Chemiluminescent Substrate or SuperSignal West Femto Chemiluminescent Substrate, both from Pierce Biotechnology (Rockford, IL) according to the manufacturer's instructions.

RNA Isolation and Real-time Quantitative PCR

Total RNA was isolated from 60-mm Petri dishes using Rneasy Plus Mini Kit (Qaigen, Hilden, Germany). cDNA was synthesized from total cellular RNA using a reaction mix First Strand cDNA Synthesis Kit (11801-100) from Origene, Rockville, MD according to the manufacturer's protocol. In brief, 750 ng total RNA was reverse transcribed in a 20-µL volume containing 4 µL of cDNA Master Mix and 1 µL of Reverse Transcriptase for 30 minutes at 42 °C. cDNA was then incubated at 85 °C for 5 minutes. Quantitative real-time PCR was performed on a sequence-detection system (LightCycler 1.5; Roche, Basel, Switzerland) using heat-activated *Taq* DNA polymerase (FastStart Essential Green Master, 06402 702001; Roche), according to the manufacturer's protocol. The cycling conditions were as follows: after 10 minutes of pre-incubation at 95 °C, the samples were cycled 45

times at 95 °C for 30 seconds, 55–60 °C depending on the T_m for the primers used for 60 seconds, and 72 °C for 30 seconds. The quantity of mRNA expression was analyzed by standard curve quantification for the target gene and the β -actin mRNA in the same sample using double delta CT analysis. All measurements were performed in triplicate. Controls consisting of bi-distilled H₂O were negative in all runs. Experiments were repeated at least three times. The primer pairs used are listed in Table 1.

Complement Attack and MAC Deposition Assay

ARPE19 cells (10,000 cells/per well) were plated on 8-chamber Nunc Lab-Tek™ II CC2 Chamber Slides in 500 μ L of DMEM/F12 medium supplemented with 10% of heat-inactivated FBS and were grown to near confluence. The cells were starved in basal DMEM/F12 medium for 36 hours and washed with basal DMEM/F12 medium. Solutions of 0, 0.1, 1.0 and 10.0 μ M HOHA-lactone were prepared in basal DMEM/F12 medium and added to the respective wells followed by 2 h incubation in a CO₂ incubator at 37 °C and 5% CO₂ followed by incubation in 10% normal human serum (NHS) or in 10% heat-induced normal human serum for 1 h at 37 °C and 5% CO₂. At the end of the incubation, the chambers were aspirated, washed with PBS and the cells were fixed with cold acetone (–25 °C) for 12 min. The slides were blocked with 3% BSA in PBST for an hour at room temperature, aspirated and incubated with anti-human C5b-9 mouse monoclonal antibody (clone aE11; 1:100 dilution in 3% BSA in PBST) overnight at 4 °C and washed with PBST next day. The slides were treated with Alexa Fluor 647 goat anti-mouse (1:100 dilution; A32728, Invitrogen) antibody in PBST overnight at 4 °C, washed with PBST and aspirated. The slides were incubated with 1:20 diluted in PBS stock solution of CF488A-Phalloidin (0.2 U/ μ L, Biotium, Fremont, CA) in the dark for 30 min at room temperature protected from light. After washing with PBST slides were mounted in the DAPI Fluoromount-G (Southern Biotech, Birmingham, AL) sealed. All images were acquired with a Leica DMI 6000 B inverted fluorescent microscope using a Retiga EXI camera. Image analysis was performed using Metamorph imaging software (Molecular Devices, Downingtown, PA). The images were taken at 20 $\times$ magnification.

LDH Assay

Briefly, ARPE19 cells (20,000 cells/per well) were plated on 48-well plates in 500 μ L of DMEM/F12 medium supplemented with 10% of heat-inactivated FBS and grown to near confluence. The cells were starved in basal DMEM/F12 medium for 48 hours and washed with basal DMEM/F12 medium. Solutions of 0, 0.1, 1.0, 10.0 μ M HOHA-lactone and 1% Triton X-100 were prepared in basal DMEM/F12 medium and added to the respective wells (3 replicate wells were used for each concentration) followed by 2 h incubation in a CO₂ incubator at 37 °C and 5% CO₂. The cells were washed with PBS and incubated in 10%, 25% and 50% normal human serum (NHS) or in 10%, 25% and 50% heat-induced normal human serum, respectively for 2 h at 37 °C and 5% CO₂. Cells were washed with DMEM/F12 basal cell culture medium and incubated for another two hours in a CO₂ incubator at 37 °C and 5% CO₂. Aliquots of the conditioned extracellular cell medium were used to assess cell viability (cells plasma membrane integrity)⁴¹ by the LDH assay according to the manufacturer's instructions.

Quantification of intracellular GSH in ARPE-19 cells

Aliquots (10 μ L) of ARPE-19 cell lysates from time-course and dose-dependence studies were assayed to determine intracellular GSH using a spectrofluorometric microplate method described earlier.⁴² 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 μ L of KPE buffer, GSH standards or samples were added to the corresponding microplate wells, followed by the addition of 120 μ L of a freshly prepared mixture of DTNB (1mg/3ml) and glutathione reductase (1.7U/ml). Then, 60.0 μ L of β -NADPH (2mg/3ml) was added and mixed well after shaking a microplate for 10 min. The plate was immediately placed in a microplate reader (Molecular Devices) and absorbance was measured at $\lambda = 412$ nm.

Statistical Analysis

Statistical analyses were performed by using Student's t test. P value <0.05 is considered as statistically significant. Data are presented as mean $\pm$ SD. For results obtained from qPCR, errors were calculated by propagating standard error associated with the Cq values obtained from qPCR. Cq values that fell outside the standard deviation were not considered in calculating either increase in expression or error. Statistical analyses were performed by using the Student's t test on the differences between the Cq values for each experimental gene and the mean Cq for the reference gene at the experimental time point and the differences between the Cq values for each experimental gene and the mean Cq for the reference gene for the control. For consistency, two Cq values were used for each experimental gene in the Student's t test (one degree of freedom), as in some cases, a Cq value fell outside the standard deviation. In cases where a Cq value was not thrown out, the two values that resulted in the lowest significance were considered.

RESULTS

HOHA lactone upregulates Nrf2 expression in ARPE-19 cells

HOHA-lactone is produced from oxidative damage of DHA, the most abundant PUFA in photoreceptor disc membranes that are endocytosed by RPE cells. We previously showed that HOHA-lactone causes depletion of GSH in ARPE-19 cells²³, concomitant with an almost 8-fold increase in oxidative stress as judged by the data from a DCFH-DA assay.^{23, 32} Oxidative stress is known to induce the expression of GSH synthetic enzymes and their expression is regulated by the Nrf2 transcription factor through the antioxidant response element (ARE), activator protein-1 (AP-1) and nuclear factor κ B (NF κ B).^{43–45} Thus, we hypothesized that HOHA-lactone can promote upregulation of Nrf2. We now report that 10 μ M HOHA lactone causes an almost 2-fold statistically significant upregulation of Nrf2 (2.17 ± 0.35 fold, Figure 2A) within 90 min of exposure and shows a dose-dependent up-regulation in ARPE-19 cells on the translational level (Figure 2B). This coincided with a notable decrease in intracellular GSH (Figure 2C) during the 2 h incubation (from 41.1 ± 0.6 nmoles/mg protein vs 21.5 nmoles/mg protein; up to 49% decrease from the original GSH levels) presumably due to scavenging intracellular reactive oxygen species and the conjugation of HOHA lactone to GSH through Michael addition of its nucleophilic sulfur to the electrophilic C-3 of HOHA lactone in RPE cells that is catalyzed by glutathione S-transferase (GST).²³

On the transcriptional level, ARPE-19 cells respond to the increase in oxidative stress at significantly lower HOHA lactone levels. At 0.1 μ M HOHA lactone within 2 h of incubation along with an almost 2-fold upregulation of Nrf2, HOHA lactone caused statistically significant upregulation of three Nrf2-responsive genes: HO-1 mRNA (1.60 ± 0.03 fold), GCLM mRNA (1.89 ± 0.09 fold), and NQO1 mRNA (1.55 ± 0.15 fold) as detected by RT-qPCR (Figure 3). These responses to HOHA lactone were not associated with cell death. HOHA lactone causes insignificant levels of cytotoxicity to ARPE-19 cells at concentrations lower than 10 μ M.^{23, 32}

HOHA lactone upregulates complement and membrane complement regulatory protein expression in ARPE-19 cells

Previous observations suggested that lipid oxidation products, e.g., HOHA lactone, a product of the oxidative stress in the retina^{23, 32}, might also promote complement protein expression by RPE cells. Cigarette smoke extract induces oxidative stress in ARPE-19 cells leading to induction of Nrf2, and this is accompanied by initiation of the complement pathway.³¹ Furthermore, exposure to submillimolar concentrations of H₂O₂ sensitizes RPE cells to complement-mediated injury.⁴⁶ This suggests that this oxidant facilitates up-regulation of C3 and influences expression of the membrane complement regulatory proteins (mCRPs) including CD46, CD55, CD59, and CR1.⁴⁷

To test the hypothesis that the same lipid oxidation products that induce antioxidant enzyme expression also promote complement protein expression, we examined the effect of HOHA lactone on expression of complement proteins. Concurrently, with the upregulation of Nrf2 and antioxidant enzymes (Figures 2 and 3), we observed upregulation of C3, C5 and CFB complement genes in a time-dependent manner at the transcriptional level (Figure 4). At submicromolar concentrations of HOHA-lactone (0.1 μ M) we observed that C3 mRNA expression reaches its maximum at 105 min of incubation (1.84 ± 0.48 fold, Fig. 4A) which coincides with recovery of cell's initially depleted GSH levels (Figure 2C).

Similar to the C3 complement factor, at 0.1 μ M HOHA lactone we observed CFB gene up-regulation at the transcriptional level (Figure 4C) as well. The highest statistically significant increase in the level of CFB mRNA occurred at 90 min reaching 1.59 ± 0.09 fold. Activation of the alternative complement pathway usually leads to elevation of the levels of C3b proteins, which in turn facilitates the incorporation of extra C3b fragments into the C3 convertase, thus resulting in C3Bb3b complexes that act as a C5 convertase.^{48, 49} Cleavage of C5 by the C5 convertase to C5a and C5b, with C5b initiating assembly of the MAC on the cell. Thus, upregulation of C5 simultaneously with C3 and CFB, formation of C3 and C5 convertases and ultimately generation of the C5b fragment from C5 is the necessary precondition for the MAC to be assembled on the cell's membrane.⁵⁰ On the transcriptional level, C5 up-regulation occurs earlier compared to the C3 and CFB genes (Figure 4B). ARPE-19 cells treated with 0.1 μ M HOHA lactone showed appreciable (2.14 ± 0.21 fold) increase in C5 mRNA after 60 min.

On transcriptional level, and probably as the result of cellular response to the elevation in C3 levels in ARPE-19 cells upon treatment of with 0.1 μ M HOHA lactone we detected up-regulation of mCRPs CD46 and CD55 (Fig. 5).

After exposure of ARPE-19 cells to 0.1 μ M HOHA lactone for 60 min, both mCRPs are up-regulated ahead of C3 complement factor gene up-regulation by this electrophile (Fig. 5A and 5B) reaching 1.55 ± 0.35 fold and 1.93 ± 0.27 fold for CD46 and CD55, respectively. These two mCRPs control the fate of C3 complement factor. CD46 controls the amplification loop of the alternative pathway. It is an intrinsic cofactor for the factor-I-mediated cleavage of C3b and C4b. The cleavage of C3b produces the fragment C3bi, which is unable to support further complement activation because C3b is not deposited onto cell surfaces and thus cannot induce the formation of a MAC.^{51, 52} CD55 (complement decay-accelerating factor, DAF) also recognizes C3b fragments that are created during activation of C3 (alternative pathway). Interaction of DAF with C3b of the alternative pathway interferes with the conversion of factor B to Bb by factor D, thus preventing formation of the C3bBb C3 convertase of the alternative pathway.^{52, 53} Thus, DAF indirectly blocks the formation of the MAC by limiting the amplification convertases of the complement cascade in the early stages of complement activation. In contrast, CD59 responds to the later stages of complement activation. When C5b678 deposits on host cells, CD59 can prevent C9 from polymerizing and forming the MAC.⁵⁴ It may also signal the cell to perform active measures such as endocytosis of the CD59-C9 complex. As expected, treatment of ARPE-19 cells with 0.1 μ M HOHA-lactone does not produce any appreciable up or down-regulation of CD59 on the transcriptional level (Fig. 5C), presumably because the levels of sub-lytic MAC on the cell's plasma membrane are low, which is in a good agreement with the levels of MAC as will be shown below in Fig. 8 (vide infra).

The levels of cytosolic or membrane-bound C3 complement factor on the translational level seem to mirror C3 gene upregulation in ARPE-19 cells challenged with HOHA lactone as detected by Western blot (Figure 6). Even at 0.01 μ M HOHA lactone, C3 complement factor was elevated by 2.37 ± 1.09 -fold ($t < 0.05$) compared to the respective control after 2 hours exposure (Figure 6A). The level of C3 up-regulation increased 3.6-fold upon exposure to 0.1 μ M HOHA lactone (3.66 ± 1.23 fold $t < 0.025$), but decreased at concentrations of HOHA lactone higher than 1.0 μ M, e.g. at 1 μ M of HOHA-lactone it was elevated only 2.29 ± 0.68 -fold ($t < 0.01$) and 2.28 ± 0.75 -fold ($t < 0.05$) at 10 μ M of HOHA lactone. The upregulation of the C3 complement factor was not transient, as we still detected elevated levels of this protein in the cell lysates of ARPE-19 cells after incubation with HOHA lactone for 24 h, e.g. at 0.01 μ M HOHA lactone the level of C3 was still elevated by 1.70 ± 0.37 -fold ($t < 0.01$) and with 5 μ M HOHA lactone the level of C3 was still elevated by 2.19 ± 0.98 -fold ($t < 0.05$) compared to the levels of C3 in unchallenged ARPE-19 cells (Figure 6B).

Formation of an unstable bimolecular complex C3 convertase (C3bBb or C3(H₂O)Bb) from C3⁵⁵ or C3(H₂O)⁵⁶, the central amplification enzyme of the complement cascade, is initiated by the binding of CFB to C3b (or C3(H₂O)) to form the proconvertase, C3bB (C3(H₂O)B). C3bB or C3(H₂O)Bb is subsequently cleaved by factor D (CFD) at a single site in CFB, producing Ba and Bb fragments.^{47, 49, 55, 56} Ba dissociates from the complex, while Bb remains bound to C3b⁴⁹, forming the active alternative pathway convertase, C3bBb and a new C3 convertase molecule that will cleave more C3 thus creating a positive feedback loop.^{47, 49, 57} Thus, upregulation of CFB simultaneously with C3 is the necessary precondition for initiation of the alternative complement pathway.

The levels of CFB (translational level) in cell lysates (2 h) seem to mirror C3 gene upregulation in HOHA lactone-challenged ARPE-19 cells as detected by Western blot (compare Figures 6 and 7). Within 2 h of incubation with 0.01 μ M HOHA lactone, ARPE-19 cells increased CFB biosynthesis by 1.79 ± 0.56 fold ($t < 0.05$) compared to the respective control (Figure 7A). This level of CFB up-regulation was maintained over the range of 0.01 to 10 μ M HOHA lactone, and was statistically significant after another 22 h (Figure 7B). At 24 h, HOHA-lactone at 0.5 μ M induced a 3.88 ± 1.59 fold increase ($t < 0.05$), although at 24 h we observed a CFB downregulation trend at higher concentrations, i.e. 1 to 10 μ M HOHA-lactone from 2.62 ± 0.54 fold increase at 1 μ M HOHA-lactone down to 2.00 ± 0.48 fold increase ($t < 0.025$) at 10 μ M HOHA-lactone, respectively (Figure 7B).

HOHA lactone promotes formation of the membrane attack complex on ARPE-19 cells

Activation of the alternative complement pathway usually leads to elevation of the levels of C3b proteins, which in turn facilitates the incorporation of extra C3b fragments into the C3 convertase, thus resulting in C3Bb3b complexes that act as a C5 convertase.^{48, 49} Cleavage of C5 by the C5 convertase to C5a and C5b, with C5b initiating assembly of the MAC on the cell. Thus, upregulation of C5 simultaneously with C3 and CFB, formation of C3 and C5 convertases and ultimately generation of the C5b fragment from C5 is the necessary precondition for the MAC to be assembled on the cell's membrane.⁵⁰

While we able to detect upregulation of C5 on the transcriptional level (see Fig. 4B), we failed to detect C5 zymogen in the cell lysate for both the 2 h and 24 h time-points (data not shown). On the other hand, we were able to determine a deposition of C5b-9 neoepitope on the plasma membrane of ARPE-19 cells when these cells were incubated with 10% normal human serum, which is indicative that C5 is generated on the translational level (Figure 8). Generation of the C5b fragment and sequential recruitment to C5b of C6, C7, C8, and finally C9, and polymerization to form the MAC^{49, 50} leads to formation of a circular polymer, a pore on the self-membranes that can perforate the cell's plasma membranes.^{50, 58} To verify that the levels of HOHA lactone tested in these studies can cause MAC formation, we exposed ARPE-19 cells for 2 h to 0.1 – 10 μ M concentrations of HOHA lactone (Figure 8) followed by an addition of normal human serum (10 % NHS, upper row) or 10% heat-inactivated (HI) NHS (lower row). The presence of MAC was detected with a C5b-9 antibody. As can be seen, there is a dose-dependent increase in MAC formation on the ARPE-19 plasma membrane with increasing levels of HOHA lactone.

The conditions employed in the experiment presented in Figure 8 were shown to be sub-lytic. The MAC on the ARPE-19 cell plasma membrane can become lytic. According to data acquired by LDH assay, in the presence of 10% NHS, the MAC shown in Figure 8 is not lytic. A trend to become lytic was observed at 50% NHS (data not shown).

Membrane-attack-complex insertion into the host plasma cell membrane is tightly regulated by cells in order to avoid accidental host cell membrane damage.⁶⁰ CD59 complement regulator protein which is expressed on most cell types^{60–62} prevents membrane perforation by C5b-8 and C5b-9^{63, 64} and is considered as one of the key protective responses to sub-lytic complement in the retinal pigment epithelium.^{65, 66} We determined that within 2 h of incubation with 0.01 μ M – 0.5 μ M HOHA lactone, CD59 biosynthesis in ARPE-19 cells

peaked to only a modest, but statistically significant 1.42 ± 0.35 fold increase compared to the respective control (Figure 9A), and exhibited a steady decline in the levels of CD59 at 10 μ M HOHA lactone (0.82 ± 0.11 fold, $t < 0.05$). At 24 h the level of CD59 showed a statistically significant up-regulation at 0.05 μ M and 0.1 μ M HOHA lactone (1.68 ± 0.32 fold and 1.61 ± 0.36 fold, respectively) and showed a steady decline of CD59 at higher HOHA lactone concentrations that was almost indistinguishable from the control (Figure 9B). It should be noted that retina from AMD shows decreased CD59 expression^{65, 66} and increased MAC deposition in sub-RPE drusen and at the RPE-choroid interface.^{65, 67}

DISCUSSION

Two factors are known to play pivotal roles in the development of RPE dysfunction and apoptosis: oxidative damage and activation of the complement pathway.^{11, 68–70} Our previous research showed that HOHA lactone is both produced through free radical-induced oxidative damage of DHA and that it compromises antioxidant defenses by depleting GSH in RPE cells and generating a biologically active reduced GSH adduct 5-glutathionyl-4,7-dihydroxyheptanoic acid (GS-DHHA) lactone (Figure 10).^{22, 30} The present study establishes that HOHA-lactone also (1) promotes a countervailing reinforcement of antioxidant defenses in ARPE-19 cells by upregulating Nrf2 signaling, and (2) triggers activation of the complement pathway culminating in MAC formation on the plasma membrane (Figure 10).

HOHA Lactone Promotes the Release and Nuclear Accumulation of Nrf2

The induction of proteolytic activation and release of Nrf2 from its complex with Keap1 by HOHA lactone and other α,β -unsaturated carbonyl electrophiles, i.e., KOHA and HNE (Figure 11), most likely involves the same Michael addition of thiols as their adduction to GSH. In contrast, ONE apparently causes a decrease in Nrf2 levels. However, ONE uniquely causes the appearance of high molecular weight bands (data not shown) that presumably correspond to crosslinked oligomeric Nrf2. Keap1 incorporates 25 sulfhydryl groups that can form covalent Michael adducts with α,β -unsaturated carbonyl compounds⁷² leading to release of Nrf2 from Keap1.⁷³ Activation and release of Nrf2 induced by 15-deoxy-^{12,14}-prostaglandin J₂ (15d-PGJ₂), an α,β -unsaturated carbonyl compound,⁷⁴ was shown to involve covalent adduct formation between Keap1 and 15d-PGJ₂ leading to activation of Nrf2 and Nrf2-dependent induction of HO1.

HOHA Lactone Upregulates Complement Protein Expression in ARPE-19 Cells

The complement system aids in the maintenance of homeostasis and, defense against immunological challenges^{75, 76} that is especially important for the retina owing to the immune privilege of the eye.⁷⁷ Owing to chronic oxidative insult over decades that increases in level with advancing age, a low level of complement activation contributes to the maintenance of homeostasis in the healthy aging eye. The retinal innate-immune system responds with low levels of activation (parainflammation). Low level complement activation facilitates initiation of humoral defenses against infectious pathogens, and promotes the removal of potentially noxious substances such as extracellular debris and apoptotic cells. However, in individuals with AMD, this parainflammatory response becomes dysregulated

and contributes to damage of photoreceptor and RPE cells, and of the blood retina barrier, resulting in the breach of retinal-immune privilege, and the development of retinal lesions.^{78, 79} We discovered that submicromolar concentrations of HOHA lactone foster expression of C3, CFB and C5 in ARPE-19 cells. HOHA lactone also induces a countervailing upregulation of CD55, an inhibitor of C3 convertase production and complement cascade amplification in the early stages of the alternative pathway. Ultimately, HOHA lactone causes MAC formation on the plasma membrane. Excessive activation of this pathway by HOHA lactone-induced over expression of complement factor genes can become pathological. Individuals with genetic variations associated with AMD are expected to be prone to such over expression in response to oxidative stress-induced production of HOHA lactone. Overexpression of C3 in the retina produces AMD-like pathology.^{80, 81} We discovered a HOHA-lactone dose-dependent increase in C3, CFB and C5 expression and MAC formation on the plasma membrane of ARPE-19 cells. Thus, HOHA lactone provides a molecular level connection between free radical-induced oxidative cleavage of DHA and excessive activation of the complement pathway in AMD pathology.

A mechanistic connection between the induction of complement factor and Nrf2 gene expression by HOHA lactone remains to be determined. In this regard, it is noteworthy that exposure of ARPE-19 cells to cigarette smoke extract (CSE) also causes expression of Nrf2 and antioxidant proteins as well as complement proteins.³¹ Cigarette smoking causes oxidative stress and is a risk factor for AMD. Since knock down of Nrf2 exacerbates the upregulation of complement expression in response to CSE³¹, transcription of complement genes is independent of Nrf2 expression. Furthermore, the upregulation of complement gene expression is significant at HOHA lactone concentrations at least an order of magnitude lower than activation of Nrf2.

CSE causes covalent modification of pure C3 in vitro that abolishes its ability to bind to CFH and this potentiates its ability to activate the alternative pathway of complement in the presence of NHS.⁸² It is possible that covalent modification of C3 involves Michael addition of small molecular weight components of CSE, which include numerous α,β -unsaturated aldehyde and ketone analogues of the Michael acceptors shown in Figure 11: acrolein, crotonaldehyde, methacrolein, butanone, 3-methyl-3-buten-2-one and 4-penten-3-one.⁸³ Treatment with CSE results in significant complement C3d deposition on the apical surface of an ARPE-19 cell monolayer.⁸⁴ CSE triggers the production and release of C3 from RPE cells, which leads to activation of the alternative pathway and subsequent production of C3a.⁸⁴ CSE induces both oxidative stress and complement activation in ARPE-19 cells. Blocking complement activation eliminates CSE-induced oxidative stress and, vice versa, blocking oxidative stress prevents C3 production and release.⁸⁴ CSE-induced oxidation of cellular lipids in ARPE-19 monolayers is expected to generate α,β -unsaturated carbonyl electrophiles, which can covalently modify complement proteins. In contrast with modification of pure C3 by reactive components of CSE to generate a form that can activate the alternative pathway, activation of the alternative pathway in ARPE-19 cells by CSE presumably triggers an oxidative stress-mediated indirect mechanism that involves covalent adduction of lipid oxidation products generated in situ to complement component C3. These observations suggest the possibility that activation of the alternative pathway by HOHA lactone involves covalent adduction to C3 by Michael addition to cysteine sulfhydryl or

histidine imidazole groups. Furthermore, depletion of GSH by HOHA lactone creates oxidative stress leading to the generation of additional bioactive α,β -unsaturated aldehyde and ketone products of lipid oxidation that can amplify the effects of HOHA lactone in a vicious cycle feedback loop.

CONCLUSIONS AND FUTURE PROSPECTS

HOHA Lactone May Directly or Indirectly Activate the Alternative Complement Pathway

Although we have identified electrophilic products of lipid oxidation that induce activation of Nrf2 and of the alternative complement pathway, the molecular mechanisms for these effects remain to be established. Covalent modification of C3 by HOHA lactone is a possible mechanism for the induction of hyperactivity of the complement pathway, e.g., by abolishing the ability of C3 to bind to CFH, an inhibitor of complement activation. However, rather than promoting activation of the complement pathway indirectly by preventing inhibition by CFH, covalent adduction of HOHA lactone to a complement protein may directly promote activation of the complement pathway. A small amount of C3b is constantly generated in blood. It can deposit on membranes where it forms an unstable ($t_{1/2} = 30 - 40$ sec) complex with factor B (Bb), the C3 convertase.⁸⁵ This complex is stabilized ($t_{1/2} = 3 - 4$ min) by binding with properdin, a protein that potentiates activation of the alternative complement pathway by stabilizing the C3 and C5 convertases.¹⁷ Stabilization of the C3 convertase by properdin promotes efficient amplification of the enzyme cascade during complement activation. A histidine residue in C3b, histidine-1431, is involved in its binding with properdin resulting in stabilization of the C3 convertase C3b-Bb complex, that then produces a torrent of C3a and C3b by catalyzing the efficient cleavage of C3 (Figure 12).⁸⁶ Covalent Michael adduct formation by binding of the electrophilic α,β -unsaturated aldehyde functional array in HOHA lactone, or other α,β -unsaturated carbonyls produced by lipid oxidation, with this histidine residue in the C3b of the C3 convertase complex (Figure 12) might mimic the stabilizing effects of properdin.

A Vicious Cycle of Lipid Oxidation-induced Inflammation and Inflammation-induced Lipid Oxidation

Previous studies established that CEP promotes inflammation through T-cell activation leading to IFN γ and IL17 secretion and inflammatory (M1) polarization of macrophages that infiltrate the retina in a CEP-induced mouse model of AMD.²⁶ Furthermore, complement component 5a (C5a) induces the expression of IL-17 and IL-22 by human CD4⁺ T cells, and blood from AMD patients contains higher levels of these cytokines compared to controls.⁸⁷ Depletion of GSH by Michael addition of a precursor of CEP, HOHA lactone, also creates oxidative stress that leads to additional lipid oxidation and the generation of more bioactive α,β -unsaturated aldehyde and ketone products of lipid oxidation. This can amplify the effects of HOHA lactone in a vicious cycle feedback loop.

The present study reveals another molecular-level connection between oxidative damage of DHA and the pathogenesis of dry AMD. HOHA lactone promotes activation of the alternative complement pathway. As suggested above, stabilization of the C3 convertase by covalent adduction of HOHA lactone to a key histidyl residue may promote C3a + C3b

generation. This would lead to formation of the C5 convertase and generation of C5a + C5b (Figure 12). The anaphylatoxins C3a/C5a could stimulate IFN γ and IL17 secretion by CD4⁺ T-cells.⁸⁸ C5b + C6-C9 will assemble the MAC, while C3a/C5a promoted inflammation would lead to the generation of more HOHA-lactone and completion of a vicious cycle of lipid oxidation-induced inflammation and inflammation-induced lipid oxidation. Our future studies will test these hypotheses.

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ABBREVIATIONS

15d-PGJ₂	15-deoxy- ^{12,14} -prostaglandin J ₂
AMD	age-related macular degeneration
AP-1	activator protein-1
ARE	antioxidant response element
BCA	bicinchoninic acid
BSA	bovine serum albumin
CEP	carboxyethylpyrrole
CFB	complement factor B
CFH	complement factor H
CSE	cigarette smoke extract
DAPI	4,6-diamidino-2-phenylindole
DHA	docosahexaenoate
DMEM	Dulbecco's modified Eagle's medium
ECL	enhanced chemiluminescence
FBS	fetal bovine serum
GCLM	glutamate–cysteine ligase modifier
GSH	glutathione
GST	glutathione S-transferase
HO-1	hemoxygenase-1
HOHA	4-hydroxy-7-oxo-5-heptenoate

HRP	horseradish peroxidase
Keap1	Kelch-like ECH-associated protein 1
LDH	lactate dehydrogenase
MAC	membrane attack complex
MAC-IP	MAC-inhibitory protein
NAD(P)H	dehydrogenase quinone 1
NFκB	nuclear factor κB
NHS	normal human serum
Nrf2	nuclear factor erythroid-derived 2-like 2
PBS	phosphate-buffered saline
RPE	retinal pigmented endothelium

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Figure 1. Immunocytochemistry of the mouse outer retina showing the presence of C3d (green) in Bruch's membrane.¹⁸ Bar length represents 10 μ m. Adapted by permission from Springer Molecular Neurobiology, A Hapten Generated from an Oxidation Fragment of Docosahexaenoic Acid Is Sufficient to Initiate Age-Related Macular Degeneration, Joe G. Hollyfield, Victor L. Perez, Robert G. Salomon © 2010

Figure 2.

HOHA-lactone induces upregulation of Nrf2 in RPE cells. Data in panels A and B are presented as fold change over control. (A) Representative Western blot that shows the time-course of Nrf2 accumulation in ARPE-19 cells as the result of HOHA lactone challenge (10 μ M, 2 h). Below is the graph quantifying Nrf2 protein abundance. (B) Representative Western blot that shows a dose-dependent increase in the levels of Nrf2 in ARPE-19 cells challenged with HOHA lactone for two hours under standard conditions. Below is the graph quantifying Nrf2 protein abundance. Panel C shows the time-course of intracellular glutathione level changes in ARPE-19 cells after treatment with 10 μ M HOHA lactone. All panels show a representative immunoblot of three independent experiments. Values represent means $\pm$ S.D., n = 3. *-p<0.05, **-p<0.025, ****-p<0.005 compared to the respective controls.

Figure 3. HOHA-lactone induces upregulation of Nrf2 responsive antioxidant genes HO1, GCLM, and NQO1 upon exposure to 0.1 μ M HOHA lactone within 2 h of incubation as measured by RT-qPCR. Data show the average of three independent experiments that were run in triplicates. * $p < 0.025$, ** $p < 0.005$, *** $p < 0.0005$ compared to the respective controls.

Figure 4. Time-course for upregulation of (A) C3, (B) C5 and (C) CFB genes upon exposure to 0.1 μM HOHA lactone within 2 h of incubation as measured by RT-qPCR. *-p<0.05; **-p<0.025; ***-p<0.01; ****-p<0.005; *****-p<0.001 compared to control. For all assays, three independent experiments run in triplicates were conducted.

Figure 5.

Time-course for up-regulation of complement regulatory proteins: (A) CD46, (B) CD55 and (C) CD59 genes in ARPE-19 cells upon exposure to 0.1 μ M HOHA-lactone within 2 h of incubation as measured by RT-qPCR. *-p<0.05; **-p<0.025; ***-p<0.01; ****-p<0.005 and *****-p<0.001 compared to control. For all assays, three independent experiments run in triplicates were conducted.

Figure 6.

Dose-dependent upregulation of C3 in ARPE-19 cells after (A) 2 h and (B) 24 h incubation with various concentrations of HOHA-lactone by Western blot. Panel A and Panel B show representative immunoblots of C3 protein in the cell lysates after exposure of ARPE-19 cells to HOHA lactone for 2 or 24 h, respectively. Graphs in Panel A and Panel B show the results of four independent experiments. *-p<0.05; **-p<0.025; ***-p<0.01; ****-p<0.005 compared to control.

Figure 7.

Dose-dependent upregulation of CFB in ARPE-19 cells after (A) 2 h and (B) 24 h incubation with various concentrations of HOHA lactone by Western blot. Panel A and Panel B show representative immunoblots of CFB protein in the cell lysates after 2 and 24 h exposure, respectively, of ARPE-19 cells to HOHA lactone. Graphs in Panel A and Panel B summarize the results of four independent experiments. *-p<0.05; **-p<0.025; ***-p<0.01; ****-p<0.005; *****-p<0.001 compared to control.

Figure 8.

HOHA-lactone dose-dependent activation of complement alternative pathway and the formation of sublytic membrane attack complex. Retinal pigmented epithelial cells were pre-incubated for 2 h with (control (A and A'), or without HOHA-lactone (0.1 μ M (B and B'), 1.0 μ M (C and C') and 10 μ M (D and D'), respectively at 37 °C in basal DMEM/F12 cell culture medium and followed by the supplementation with 10% NHS (panels A, B, C, and D') or 1HI-NHS (panels A', B', C' and D') for one hour at 37 °C and subsequently fixed (see Materials and Methods). C5b-9 neoepitope was stained using mouse monoclonal anti-human C5b-9 primary antibody (1°) and goat anti-mouse Alexa-647 conjugated antibody (2°; red), f-actin was stained with CF488A-phalloidin (green)⁵⁹ and the nuclei were stained with DAPI (blue). All images were acquired with a Leica DMI 6000 B inverted fluorescent microscope using a Retiga EXI camera. Image analysis was performed using Metamorph imaging software (Molecular Devices, Downingtown, PA). The images were taken at 20 $\times$ magnification. Scale bars: 50 μ . The figure is representative of three independent experiments that showed very similar results.

Figure 9.

Levels of CD59 after (A) 2 h and (B) 24 h incubation with various concentrations of HOHA lactone by Western blot. Panels A and B show representative immunoblots of CD59 protein in the cell lysates after 2-hours and 24-hours exposure of ARPE-19 cells, respectively. Graphs in Panels A and B show the results of three independent experiments. *-p<0.05; **-p<0.025.

Figure 10.

HOHA-lactone promotes proteolytic activation/release of Nrf2 that translocates to the nucleus of ARPE-1 cells where it binds to the ARE leading to expression of antioxidant genes. Expression of genes for complement factors C3, C5 and CFB are also upregulated by HOHA-lactone resulting in initiation of the alternative pathway and MAC formation that is barely detectable at sublytic concentrations (0.1 μM) but extensive upon exposure to 10 μM HOHA lactone. The graphics in this figure were inspired by a review by Jaramillo and Zhang⁷¹ and the image of the MAC is from figure 1a in Serna, M. et al. Structural basis of complement membrane attack complex formation. *Nat. Commun.* 7:10587 doi: 10.1038/ncomms10587 (2016) both are used in accordance with a Creative Commons license: <http://creativecommons.org/licenses/by/4.0/>

Figure 11.

Elevation of Nrf2 levels in ARPE-19 cells induced by incubation of these cells with various α,β -unsaturated carbonyl electrophiles. Top panel shows a representative immunoblot of Nrf2 protein in the cell lysates after 2-hours and 24-hours exposure of ARPE-19 cells to 20 μ M concentration of various electrophiles. Lower panel shows the structures of the various electrophiles. All the data in the graph were normalized using laminin B1 as the home keeping protein. The graph shows the results of five independent experiments. *-p<0.001.

Figure 12.

Postulated molecular mechanisms for promoting the alternative pathway of complement activation, inflammation and T-cell-dependent retinal atrophy (dry AMD) by Michael addition of a C3 convertase histidyl residue to α,β -unsaturated carbonyl electrophiles. The image of the MAC is a grayscale adaptation of figure 1a from Serna, M. et al. Structural basis of complement membrane attack complex formation. Nat. Commun. 7:10587 doi: 10.1038/ncomms10587 (2016) used in accordance with a Creative Commons license: <http://creativecommons.org/licenses/by/4.0/>

Scheme 1.
Generation of HOHA-lactone and CEP from DHA-PL.

TABLE 1

PCR Primer Sequences and Product Sizes Used for Quantitative Real-Time RT-PCR

Target Gene	Position	Oligonucleotide Sequence (5' → 3')	Product Size (bp)	Gene bank code
C3 ³⁵	313-330	ACTGTGCTGACCCCTGCC (f)	61	NM_000064
	373-356	TGTTGGCTGGGATCGTGA (r)		
C5 ³⁶	3419-3438	AGGGTACTTTGCCTGCTGAA (f)	173	NM_001735
	3745-3726	TGTGAAGGTGCTCTTGGATG (r)		
CFB ³⁷	1393-1411	TCCCTCCTGAAGGCTGGAA (f)	117	NM_001710
	1509-1487	TGTATAGCAAGTCCCGGATCTCA (r)		
CD46 ³⁸	830-851	GCTGCTCCAGAGTGTAAGTGG (f)	399	NM_002389
	1228-1209	AACAATCACAGCAATGACCC (r)		
CD55 ³⁸	1356-1377	TACTACCCGTCTTCTATCTGGG (f)	296	NM_000574
	1651-1630	TTTTCAAGAGGTGTAGGTGTGC (r)		
CD59 ³⁸	226-245	ACTGCAAAACAGCCGTCAT (f)	210	NM_000611
	435-416	AGGATGTCCCACCATTTTCA (r)		
Nrf2 ³⁹	819-838	AGCCCAGCACATCCAGTCAG (f)	99	NM_006164
	917-893	TGCATGCAGTCATCAAAGTACAAAG (r)		
NQ01 ³⁹	280-300	TGAAGAAGAAAGGATGGGAGG (f)	223	NM_000903
	502-483	AGGGGGAAGTGAATATCAC (r)		
HO-1 ³⁹	622-641	TTGCCAGTGCCACCAAGTTC (f)	150	NM_002133
	771-752	TCAGCAGCTCCTGCAACTCC (r)		
GCLM ⁴⁰	608-631	TCAACCCAGATTTGGTCAGGGAGT (f)	196	NM_002061
	803-782	TCCAGCTGTGCAACTCCAAGGA (r)		
β-actin	432-455	CGAGAAGATGACCCAGATCATGTT (f)	155	NM_001101
	586-566	CCTCGTAGATGGGCACAGTGT (r)		

(f) - forward, (r) -reverse