Part I: BIOLOGICAL ACTIVITIES AND CELLULAR METABOLISM OF 4-HYDROXY-7-OXOHEPT-5-ENOATE AND 5-HYDROXY-8-OXO-6-OCTENOATE LACTONES

Part II: CARBOXYALKYLPYRROLE, PENTYLPYRROLE AND 4-OXO-HEPTANEDIOIC AMIDE DERIVATIVES OF ETHANOLAMINE PHOSPHOLIPIDS AND PROTEINS

by

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Submitted in partial fulfillment of the requirements for the degree of

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* We also certify that written approval has been obtained for any proprietary material contained therein
This thesis is dedicated to my parents, my husband and my first baby.
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# LIST OF ABBREVIATIONS AND ACRONYMS

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</tr>
<tr>
<td>BSTFA</td>
<td>N,O-bis(trimethylsilyl) trifluoroacetamide</td>
</tr>
<tr>
<td>CAP</td>
<td>carboxyalkylpyrrole</td>
</tr>
<tr>
<td>CAT</td>
<td>catalase</td>
</tr>
<tr>
<td>CD36</td>
<td>cluster of differentiation 36</td>
</tr>
<tr>
<td>CEF-PC</td>
<td>1-palmitoyl-2-(3-(2-furyl)propanoyl)-sn-glycero-3-phosphatidylcholine</td>
</tr>
<tr>
<td>CEP</td>
<td>2-(ω-carboxyethyl)pyrrole</td>
</tr>
<tr>
<td>CHP</td>
<td>2-(ω-carboxyheptyl)pyrrole</td>
</tr>
<tr>
<td>CID</td>
<td>collision-induced dissociation</td>
</tr>
<tr>
<td>CL</td>
<td>cell lysate</td>
</tr>
<tr>
<td>CM</td>
<td>conditioned medium</td>
</tr>
<tr>
<td>CNV</td>
<td>choroidal neovascularization</td>
</tr>
</tbody>
</table>

xxi
CPP 2-(ω-carboxypropyl)pyrrole
DAPI 4',6-diamidino-2-phenylindole
DBU 1,8-diazabicyclo-[5,4,0]undec-7-ene
dicyclohexylcarbodiimide
DCFDA 2′,7′-dichlorofluorescence diacetate
DCF 2′,7′-dichlorofluorescein
DCFH 2′,7′-dichlorofluorescein
docosahexaenoic acid
dihydroxy-2-nonene
N, N-diisopropylethylamine
N,N-dimethylaminopyridine
Dulbecco’s modified Eagle’s medium
dimethylsulfoxide
deoxyribonucleic acid
4,7-dioxoheptanoic acid
9H-fluoren-9-ylmethyl ester of DOHA
Dulbecco’s phosphate buffered saline
1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine
5,5’-dithio-bis(2-nitrobenzoic acid)
ethyl acetate
extracellular medium
ethylenediaminetetraacetic acid
ELISA  enzyme-linked immunosorbant assay
EP    ethanolamine phospholipids
ER    endoplasmic reticulum
ESI   electrospray ionization
ETN   ethanolamine
FBS   fetal bovine serum
Fmoc  fluorenylmethyloxycarbonyl
GA    geographic atrophy
GAPDH glyceraldehyde 3-phosphate dehydrogenase
GC    gas chromatography
GFR   growth factor-reduced
Gly   glycine
GPx   glutathione peroxidase
GR    glutathione reductase
GSH   reduced L-glutathione
GSSG  glutathione disulfide
GST   glutathione S-transferase
HBSS  Hank’s balanced salt solution
4-HHE 4-hydroxy-2-hexenal
4-HNE 4-hydroxy-2-nonenal
HODA-PC 2-(9-hydroxy-12-oxododec-10-enoyl) phosphatidylcholine
HODA-PL 2-(9-hydroxy-12-oxododec-10-enoyl) phospholipid
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>HOHA</td>
<td>4-hydroxy-7-oxohept-5-enoic acid</td>
</tr>
<tr>
<td>HOHA-lactone</td>
<td>4-hydroxy-7-oxohept-5-enoic acid lactone</td>
</tr>
<tr>
<td>HOHA-PC</td>
<td>2-(4-hydroxy-7-oxohept-5-enoyl) phosphatidylcholine</td>
</tr>
<tr>
<td>HOHA-PL</td>
<td>2-(4-hydroxy-7-oxohept-5-enoyl) phospholipid</td>
</tr>
<tr>
<td>HOOA</td>
<td>5-hydroxy-8-oxo-6-octenoic acid</td>
</tr>
<tr>
<td>HOOA-lactone</td>
<td>5-hydroxy-8-oxo-6-octenoic acid lactone</td>
</tr>
<tr>
<td>HOOA-PC</td>
<td>2-(5-hydroxy-8-oxo-6-octenoyl) phosphatidylcholine</td>
</tr>
<tr>
<td>HOOA-PL</td>
<td>2-(5-hydroxy-8-oxo-6-octenoyl) phospholipid</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>HRP</td>
<td>horseraddish peroxidase</td>
</tr>
<tr>
<td>hRPE</td>
<td>primary human RPE cells</td>
</tr>
<tr>
<td>HSA</td>
<td>human serum albumin</td>
</tr>
<tr>
<td>HUVEC</td>
<td>human umbilical vein endothelial cell</td>
</tr>
<tr>
<td>Hz</td>
<td>Hertz</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>IS</td>
<td>internal standard</td>
</tr>
<tr>
<td>J</td>
<td>hyperfine coupling constant</td>
</tr>
<tr>
<td>JNK</td>
<td>c-jun N-terminal protein kinase</td>
</tr>
<tr>
<td>KLH</td>
<td>keyhole limpet hemocyanine</td>
</tr>
<tr>
<td>KODA-PL</td>
<td>2-(9-keto-12-oxododec-10-enoyl)-phospholipid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>---------------</td>
<td>-----------------------------------------------------------------</td>
</tr>
<tr>
<td>KOHA-PC</td>
<td>2-(4-oxo-7-oxohept-5-enoyl)-phosphatidylcholine</td>
</tr>
<tr>
<td>KOHA-PL</td>
<td>2-(4-oxo-7-oxohept-5-enoyl)-phospholipid</td>
</tr>
<tr>
<td>KOOA-PL</td>
<td>2-(5-keto-8-oxooct-6-enoyl)-phospholipid</td>
</tr>
<tr>
<td>LA</td>
<td>linoleic acid</td>
</tr>
<tr>
<td>LC</td>
<td>liquid chromatography</td>
</tr>
<tr>
<td>LC$_{50}$</td>
<td>inhibitor concentration at the 50% absorbance value</td>
</tr>
<tr>
<td>LDL</td>
<td>low-density lipoprotein</td>
</tr>
<tr>
<td>LDH</td>
<td>lactate dehydrogenase</td>
</tr>
<tr>
<td>LPO</td>
<td>lipoxidation</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>Lys</td>
<td>lysine</td>
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<td>Lyso-PC</td>
<td>2-lysophosphatidylcholine</td>
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<td>MDA</td>
<td>malonaldehyde</td>
</tr>
<tr>
<td>MDM2</td>
<td>mouse double minute</td>
</tr>
<tr>
<td>MES</td>
<td>2-[morpholino]ethanesulfonic acid</td>
</tr>
<tr>
<td>MPO</td>
<td>myeloperoxidase</td>
</tr>
<tr>
<td>MRM</td>
<td>multiple reaction monitoring</td>
</tr>
<tr>
<td>MS/MS</td>
<td>tandem mass spectroscopy</td>
</tr>
<tr>
<td>MSA</td>
<td>mouse serum albumin</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide</td>
</tr>
<tr>
<td>NaBH$_4$</td>
<td>sodium borohydride</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide-phosphate</td>
</tr>
<tr>
<td>NBS</td>
<td>N-bromosuccinimide</td>
</tr>
<tr>
<td>NGS</td>
<td>normal goat serum</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>OMe</td>
<td>methoxy</td>
</tr>
<tr>
<td>4-OHE</td>
<td>4-oxo-2-hexenal</td>
</tr>
<tr>
<td>OHdiA</td>
<td>4-oxo-heptanedioic amide</td>
</tr>
<tr>
<td>4-ONE</td>
<td>4-oxo-2-nonenal</td>
</tr>
<tr>
<td>oxPL</td>
<td>oxidatively truncated phospholipids</td>
</tr>
<tr>
<td>PA</td>
<td>phosphatidic acid</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>phosphatidylcholine</td>
</tr>
<tr>
<td>PE</td>
<td>phosphatidylethanolamine</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>PFB-Br</td>
<td>pentafluorobenzyl bromide</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>PLs</td>
<td>phospholipids</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethanesulfonyl fluoride</td>
</tr>
<tr>
<td>PP</td>
<td>2-pentylypyrrole</td>
</tr>
<tr>
<td>PS</td>
<td>phosphatidylserine</td>
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<tr>
<td>PFB-HA</td>
<td>pentofluorobenzyl hydroxyamine</td>
</tr>
<tr>
<td>PLA2</td>
<td>phospholipase A2</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
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<tr>
<td>PLD</td>
<td>phospholipase D</td>
</tr>
<tr>
<td>ppm</td>
<td>parts per million</td>
</tr>
<tr>
<td>PUFAs</td>
<td>polyunsaturated fatty acid(s)</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RPE</td>
<td>retinal pigmented epithelium</td>
</tr>
<tr>
<td>RPE BM</td>
<td>retinal pigment epithelial cell basal medium</td>
</tr>
<tr>
<td>SCD</td>
<td>sickle cell disease</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>Ser</td>
<td>serine</td>
</tr>
<tr>
<td>SIM</td>
<td>selected ion monitoring</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>SPE</td>
<td>solid phase extraction</td>
</tr>
<tr>
<td>SUV</td>
<td>small unilamellar vesicles</td>
</tr>
<tr>
<td>TAG</td>
<td>triacylglycerol</td>
</tr>
<tr>
<td>TBDMS</td>
<td>tert-butyldimethylchlorosilane</td>
</tr>
<tr>
<td>TBST</td>
<td>tris buffered saline with 0.1% Tween-20</td>
</tr>
<tr>
<td>TEA</td>
<td>triethylamine</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>Thr</td>
<td>threonine</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
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<tr>
<td>TLR</td>
<td>toll-like receptor</td>
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<tr>
<td>TMS</td>
<td>trimethyl silyl</td>
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xxvii
<table>
<thead>
<tr>
<th>TNB</th>
<th>5′-thio-2-nitrobenzoic acid</th>
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<tbody>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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</table>
Part I: Biological Activities and Cellular Metabolism of 4-Hydroxy-7-oxohept-5-enoate and 5-Hydroxy-8-oxo-6-octenoate Lactones

Part II: Carboxyalkylpyrrole, Pentylpyrrole and 4-Oxoheptanedioic Amide Derivatives of Ethanolamine Phospholipids and Proteins

Abstract

By

JUNHONG GUO

Under oxidative stress, phospholipids (PLs) containing polyunsaturated fatty acids (PUFAs) such as docosahexaenoic acid (DHA), arachidonic acid (AA) and linoleic acid (LA), undergo oxidation and truncation to generate a multitude of reactive aldehydes including γ-hydroxy-α,β-unsaturated aldehyde-PLs such as 4-hydroxy-7-oxo-hept-5-enoyl phospholipids (HOHA-PLs), 5-hydroxy-8-oxooct-6-enoyl phospholipids (HOOA-PLs) and 9-hydroxy-12-oxododec-10-enoyl phospholipids (HODA-PLs), and the corresponding γ-oxo-α,β-unsaturated aldehyde-PLs such as 4-keto-7-oxohept-5-enoyl phospholipids (KOHA-PLs), 5-keto-8-oxooct-6-enoyl phospholipids (KOOA-PLs) and 9-keto-12-oxododec-10-enoyl phospholipids (KODA-PLs) from DHA-PLs, AA-PLs and LA-PLs, respectively. Recently, HOHA-PLs and HOOA-PLs were found to undergo spontaneous deacylation via an intramolecular transesterification mechanism to generate the corresponding HOHA-lactone and HOOA-lactone under physiological conditions. More recently, HOHA-lactone was found to be a precursor for the generation of 2-(ω-carboxyethyl)pyrrole (CEP) derivatives of proteins and ethanolamine phospholipids. The
present thesis documents that CEP derivatives accumulate in RPE cells either by exogenous addition of HOHA-lactone or by HOHA-lactone generated endogenously in RPE cells exposed to oxidative or inflammatory insults. In addition, HOHA-lactone was found to exhibit hermetic effects on RPE cellular activity: low levels of HOHA-lactone induce proliferation and cell growth of RPEs while high levels of HOHA-lactone induce apoptosis. At high concentrations, HOHA-lactone induces RPE cell death by activating caspase-3 apoptotic signaling, where p53 may be involved in this apoptotic process, indicated by the induction and phosphorylation of p53, nuclear accumulation of p53, and the degradation of MDM2. Moreover, it has also been confirmed that HOHA-lactone can readily diffuse into RPE cells where it is detoxified by conjugation with GSH in the cytosol, forming an aldehyde adduct HOHA-lactone-GSH (=O) and an alcohol HOHA-lactone-GSH (-OH), which are then transported from cytosol to extracellular medium. At low levels, HOHA-lactone induces the secretion of VEGF in ARPE-19 cells, which correlates well with an increase in intracellular ROS and a decrease in intracellular GSH. VEGF secreted into the media showed angiogenic properties as indicated by increased migration and tube formation of HUVECs in matrigel when grown in media from ARPE-19 cells treated with HOHA-lactone. Wound healing and tube formation assays showed that HOHA-lactone-GSH conjugates have pro-angiogenic effects. The results of these studies show for the first time, that HOHA-lactone causes angiogenesis in HUVECs by more than one molecular pathway. In an indirect mechanism, HOHA-lactone induces the secretion of VEGF by RPE cells and VEGF can promote angiogenesis. In two other molecular mechanisms HOHA-lactone reacts with GSH or with the primary amino groups of biomolecules to form the corresponding GSH conjugates or CEPs that, in turn, also
promote angiogenesis. A fourth angiogenesis pathway induced by HOHA-lactone may involve the formation of HOHA-lactone-GSH (=O), which then produces CEP derivatives by reaction with primary amino groups of biomolecules and CEP then promotes angiogenesis.

In analogy with the generation of CEP from HOHA-lactone, HOOA-lactone was found to serve as a precursor of CPP modifications both in vitro and in vivo. In analogy with the biological activity of HOHA-lactone, HOOA-lactone was demonstrated to be capable of inducing intracellular oxidative stress and of causing apoptosis of ARPE-19 cells at high concentrations via activation of caspase-3. In view of the toxic potential of HOOA-lactone to ARPE-19 cells, the detoxification of HOOA-lactone in this cell type was also studied and the results showed that HOOA-lactone can easily diffuse through the cell membrane into ARPE-19 cells where it is detoxified by conjugation with GSH to form HOOA-lactone-GSH (=O) and HOOA-lactone-GSH (-OH).

Recently, KODA-PLs, one of the LPO products from LA-PLs, were found to form the 4-ketoamide derivatives of the Lys residues of proteins. KOHA-PLs were expected to modify biomolecules to give similar stable 4-ketoamide adducts: 4-oxo-heptanedioic amide (OHdiA) derivatives. Using LC-MS/MS, KOHA-PL was found not only modify the primary ε-amino group of protein Lys residues but also those of the ethanolamine headgroup of ethanolamine phospholipids (EPs) to produce OHdiA derivatives in vitro. In addition, OHdiA derivatives were also detected in vivo, evident by the presence of OHdiA derivatives in blood from both SCD patients and healthy individuals. Moreover, OHdiA adducts were shown to have pro-angiogenic effects and this OHdiA-driven angiogenesis was shown to be TLR2 dependent similar to angiogenesis promoted by CEP but different
from VEGF promoted angiogenesis. Furthermore, anti-OHdiA antibody was found to exhibit significant cross-reactivity with CEP-HSA while anti-CEP antibody showed high structural specificity and did not show cross-reactivity with OHdiA-HSA.

While γ-hydroxy-α,β-unsaturated aldehyde-PLs like HOHA-PLs, HOOA-PLs and HODA-PLs were previously shown to react with primary amino groups of proteins to produce the corresponding carboxyethylpyrroles (CEPs), carboxypropylpyrroles (CPPs) and carboxyheptylpyrroles (CHPs), respectively, the extent of the formation of analogous derivatives of the primary amino groups of EPs in vivo and the biological activities of those modified EPs remain poorly characterized. In the current study, an LC-MS/MS assay that allows simultaneous quantification of global CAP- and PP-modified EPs was developed by measuring levels of CAP- and PP-ETN released through hydrolysis of lipid extracts under catalysis by PLD from *Streptomyces chromofuscus*. The presence of CAP-EPs and PP-EPs in vivo was established. A small pilot study revealed that levels of CAP-EPs and PP-EPs, except CHP-EPs, are significantly elevated in plasma samples from clinical SCD patients compared to those of hospitalized SCD patients.
CHAPTER 1

INTRODUCTION
1.1 Oxidative stress.

Reactive oxygen species (ROS) encompass a variety of diverse chemical species including oxygen related free radicals such as superoxide anion (O$_2^-$•), hydroxyl radical (HO•), hydroperoxyl radical (HO$_2$•), nitric monoxide (NO•), which are molecules or molecular fragments that contain one or more unpaired electrons in their outermost orbits, and other reactive oxygen species such as singlet oxygen ($^1$O$_2$), hydrogen peroxide (H$_2$O$_2$), nitric oxide (NO), hypochlorous acid (HOCl), and peroxynitrite (ONO$O^-$), which contain full complement of electrons, but in an unstable or reactive state.\textsuperscript{1-6} ROS can either be generated exogenously or produced endogenously from several different sources.\textsuperscript{1, 5, 7} Exogenously, ROS are generated as a result of many exogenous sources such as ultraviolet light, ionizing radiation, chemotherapeutics, inflammatory cytokines and environmental toxins.\textsuperscript{7} Potential endogenous sources include mitochondria, cytochrome P450 metabolism, peroxisomes, lipoxygenase, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and inflammatory cell activation.\textsuperscript{2, 7} Being products of normal cellular metabolism, ROS are well recognized for playing a dual role as both deleterious and beneficial species. ROS generated at low concentrations by the cells may act as signaling molecules in intracellular signaling cascades, promoting cell proliferation and survival. However, high levels of ROS are detrimental and induce cell apoptosis or necrosis.\textsuperscript{2, 3, 6, 8} Therefore, maintaining ROS homeostasis is crucial for normal cell growth and survival.

Under physiological conditions, ROS are rapidly neutralized by an effective enzymatic and non-enzymatic antioxidant defense system, contributing to the maintenance of ROS homeostasis. The antioxidant defense actions include: removal of free radicals,
scavenging ROS or their precursors, inhibition of ROS formation, binding of metal ions needed for the catalysis of ROS generation.\textsuperscript{4,9} The most efficient enzymatic antioxidants involve superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) along with some supporting enzymes. SOD, one of the most effective intracellular enzymatic antioxidants, removes the superoxide anion by converting it into the less-reactive species $\text{H}_2\text{O}_2$. Catalase is located in the peroxisome, where it very efficiently promotes the dismutation of $\text{H}_2\text{O}_2$ to water and molecular oxygen. GPx promotes the reduction of $\text{H}_2\text{O}_2$ and hydroperoxides to water and alcohols, respectively.\textsuperscript{2,7,10} Non-enzymatic antioxidants, which can be classified as direct-acting (e.g., scavengers and chain breaking antioxidants) or indirect-acting compounds (e.g., chelating agents), include ascorbic acid (Vitamin C), tocopheroles (Vitamin E), retinoic acid (Vitamin A), carotenoids, thiol antioxidants (glutathione, thioredoxin and lipoic acid), natural flavonoids, as well as melatonin a hormonal product of the pineal gland, and other compounds.\textsuperscript{2,4,9} The direct-acting antioxidants, which come from a number of both endogenous and exogenous sources, are extremely important in defense against ROS. A majority of these direct-acting antioxidants such as ascorbic acid, lipoic acid, polyphenols, and carotenoids, are derived from dietary sources. The cell itself synthesizes only a minority of these antioxidants such as glutathione and NADPH. Indirectly acting antioxidants mostly include chelating agents such as ethylenediaminetetraacetic acid (EDTA) and lactoferrin, which bind to redox metals, disfavoring the redox cycling and, consequently, preventing free radical generation.\textsuperscript{4,9}

Oxidative stress occurs when there is an imbalance between pro- and antioxidant systems, which results from overproduction or incorporation of ROS from environment
to living system, or insufficient activity of antioxidant defense systems or both, giving rise to an excess of ROS.\textsuperscript{5, 6, 11} Oxidative stress results in modification of biomolecules such as nucleic acids, lipids, proteins and structural carbohydrates, which cause damage and genomic instability, alteration of membrane permeability, impaired enzymatic activities, and chromosomal aberrations, leading to cellular dysfunction and even cell death.\textsuperscript{1, 11, 12} Oxidative damage is believed to contribute to the pathogenesis of many chronic diseases such as cancer, atherosclerosis, arthritis, diabetes, post-ischemic perfusion injury, myocardial infarction, cardiovascular diseases, chronic inflammation, stroke and septic shock, aging and other degenerative diseases in humans.\textsuperscript{1, 2, 4, 13}

1.2 Lipid peroxidation.

Although oxidative stress can cause damage to all cellular macromolecules, polyunsaturated fatty acids (PUFAs) can exhibit a high sensitivity to oxidative damage due to the presence of methylene groups located between their conjugated double bonds (bisallylic methylene groups).\textsuperscript{1, 2} For each PUFA, there is a carboxyl group at one end and a methyl group at the other end, referred to as the carboxyl end and methyl end, respectively.\textsuperscript{6} Usually, PUFAs have an even numbered carbon chain possessing two or more double bonds between carbon atoms (C=C).\textsuperscript{14} PUFAs can be designated by indicating the number of carbons, the number of double bonds, and the position of the first C=C double bond counting from the methyl end of the acyl chain.\textsuperscript{14} For example, linoleic acid (LA) is represented as C18:2 ω-6 or C18:2 n-6, indicating that LA contains a carbon chain length of 18 with 2 double bonds and the first double bond located six carbon atoms from the methyl end. Depending on the location of the first double bond from the methyl terminus, PUFAs in nature can be classified into two families: ω-3 (n-3)
and ω-6 (n-6). The major long chain PUFAs such as arachidonic acid (AA; C20:4 ω-6 or C20:4 n-6) and docosahexaenoic acid (DHA; C22:6 ω-3 or C22:6 n-3), which play important roles in a variety of biological functions, belong to ω-6 (n-6) family and ω-3 (n-3) family, respectively. PUFAs are essential lipid components in the structure of the plasmatic/cellular membranes that influence membrane structure, flexibility and selective permeability of the lipid bilayer. In the body, long chain PUFAs are stored mainly as esterified complexes in the sn-2 position of phospholipids (PLs) or triacylglycerols. PLs, polar molecules with a hydrophilic phosphate head group and two hydrophobic fatty acid tails on a glycerol backbone, comprise an abundant class of lipids. Membrane lipids, mainly PLs, are especially vulnerable to free radical-initiated oxidation not only due to their high concentration of PUFAs but also because of their association in the cell membrane with non-enzymatic and enzymatic systems capable of generating prooxidative-free radical species.

The lipid peroxidation of PUFAs can be enzymatic and non-enzymatic. Lipid peroxidation is generally thought to be a major mechanism of cell injury because lipid peroxidation products can further contribute to free radical chain reactions that amplify damage to biomolecules. Non-enzymatic lipid peroxidation is a free radical driven chain reaction, which is a complex process consisting of three stages: initiation, propagation, and termination. Such a chain reaction is initiated by a free radical (R•) that abstracts a hydrogen atom from a doubly allylic methylene (–CH2–) group of a PUFA (LH), which is characterized by weakened hydrogen-carbon bonds (C–H). This produces R-H and leaves an unpaired electron on carbon (–•CH–), giving rise to a carbon-centered lipid radical (L•). The resulting lipid radical can rapidly react with
molecular oxygen (O$_2$) to form a lipid peroxyl radical (LOO•). Being an unstable species, the lipid peroxyl radical can abstract hydrogen from an adjacent free fatty acid (LH) to produce a lipid hydroperoxide (LOOH) and a second lipid radical (L•).$^{6, 11, 15}$ Thus, additional cycles of peroxidation are initiated.$^{17}$ The lipid hydroperoxide if formed in the presence of reduced redox active metal ions or ascorbate can give rise to a great diversity of products, including short and long chain aldehydes and oxidatively-truncated phospholipids.$^{11, 15}$ Lipid hydroperoxide can also in turn suffer reductive cleavage via iron or copper-catalyzed Fenton-like reactions to produce reactive lipid alkoxy radical (LO•) and hydroxyl radical (•OH), which can propagate and branch the chain reaction of lipid peroxidation by abstracting additional hydrogen atoms.$^{17}$ This harmful process can be terminated either by the reaction of two radicals to produce a non-radical species or by antioxidants to trap free radicals.$^6$ The process can be schematically depicted as shown in Scheme 1.1.$^{6, 11, 15}$

\[
\begin{align*}
\text{LH} + \text{R}^\cdot & \rightarrow \text{L}^\cdot + \text{RH} \\
\text{L}^\cdot + \text{O}_2 & \rightarrow \text{LOO}^\cdot \\
\text{LH} + \text{LOO}^\cdot & \rightarrow \text{L}^\cdot + \text{LOOH} \\
\text{LOOH} & \rightarrow \text{LO}^\cdot + \cdot\text{OH}
\end{align*}
\]

**Scheme 1.1** Lipid peroxidation pathways.

1.3 **Reactive α,β-unsaturated aldehydes generated during lipid peroxidation and their covalent protein adducts.**

Under oxidative stress, PUFAs in lipids undergo peroxidation, leading to two broad outcomes. One outcome is the alteration and modification of the cellular membrane,
resulting in cellular dysfunction. Another outcome is the formation of a variety of lipid byproducts, some of which are highly reactive, exerting adverse and/or beneficial biological effects.\textsuperscript{15} Peroxidation of membrane PLs containing essential PUFAs such as LA, AA and DHA in response to oxidative stress generates a variety of corresponding unsaturated lipid hydroperoxides, which further undergo oxidative fragmentation to produce a diversity of carbonyl-containing products called lipoxidation (LPO) products.\textsuperscript{15, 19} These LPO products, especially $\alpha,\beta$-unsaturated aldehydes, are highly reactive and can function as second toxic messengers, that disseminate and increase initial free radical events.\textsuperscript{15} On the basis of their structural features, $\alpha,\beta$-unsaturated aldehydes can be classified as: (1) aldehydes that contain the carboxyl terminus of the acyl chain; or (2) aldehydes that contain the methyl terminus (Scheme 1.2, see next page).\textsuperscript{19} The carboxyl-terminating aldehydes generated from peroxidation of LA-PL, AA-PL and DHA-PL include $\gamma$-hydroxy-$\alpha,\beta$-unsaturated aldehyde-PLs such as 9-hydroxy-12-oxododec-10-enoic acid (HODA)-PL, 5-hydroxy-8-oxooct-6-enoic acid (HOOA)-PL and 4-hydroxy-7-oxohept-5-enoic acid (HOHA)-PL, and the corresponding $\gamma$-oxo-$\alpha,\beta$-unsaturated aldehyde-PLs such as 9-keto-12-oxododec-10-enoic acid (KODA)-PL, 5-keto-8-oxooct-6-enoic acid (KOOA)-PL and 4-keto-7-oxohept-5-enoic acid (KOHA)-PL.\textsuperscript{10, 17, 20-22} The methyl-terminating aldehydes generated from peroxidation of LA-PL, AA-PL and DHA-PL include $\gamma$-hydroxy-$\alpha,\beta$-unsaturated aldehydes, such as 4-hydroxy-2-nonenal (4-HNE) and 4-hydroxy-2-hexenal (4-HHE), and their corresponding $\gamma$-oxo-$\alpha,\beta$-unsaturated aldehydes such as 4-oxo-2-nonenal (4-ONE) and 4-oxo-2-hexenal (4-OHE) (Scheme 1.2, see next page).\textsuperscript{11, 15, 18, 19, 23}
Free radicals produced during lipid peroxidation are usually characterized by instability and short life, which restrict them to locations where they are generated. However, the fragmented aldehydes from lipid hydroperoxides are relatively stable and have prolonged half-life, which allow them to diffuse within or even escape from the cell to attack remote targets, thus serving as “oxidative stress messengers.” These α,β-unsaturated aldehydes readily react with biomolecules, such as such as proteins, DNA and ethanolamine phospholipids, generating a variety of biologically relevant covalent adducts. Protein modification by α,β-unsaturated aldehydes, especially γ-hydroxy-α,β-unsaturated aldehydes, has been extensively studied. 4-HNE, one of the most
abundant bioactive species formed during lipid peroxidation of \( \omega-6 \) PUFAs, such as LA and AA, was found to incorporate with the \( \varepsilon \)-amino group of protein lysyl residues, forming covalent 2-pentylpyrrole (PP) protein derivatives.\(^{10, 20, 25}\) Analogous reactions of the carboxy-terminating \( \gamma \)-hydroxy-\( \alpha, \beta \)-unsaturated aldehyde-PLs such as HODA-PL, HOOA-PL and HOHA-PL with proteins, followed by lipolysis of intermediate PL adducts, produce the corresponding \( \omega \)-carboxyalkylpyrrole (CAP) modifications of proteins: carboxyheptylpyrrole (CHP), carboxypropylpyrrole (CPP), and carboxyethylpyrrole (CEP) derivatives, respectively (Scheme 1.3, see next page).\(^{6, 20, 21, 26}\)

In addition, 4-ONE, the 4-keto cousin of 4-HNE, modifies protein lysyl residues to form stable advanced lipoxidation end products: Lys-derived keto-amides.\(^{27, 28}\) In analogy with the chemistry of 4-ONE, KODA-PL, the carboxy-terminating 4-ONE-like product of oxidation of LA-PL, forms similar 4-ketoamide derivatives of the Lys residues of proteins after hydrolysis of these PL esters through lipolysis by phospholipase A\(_2\) (Scheme 1.4, see next page).\(^{29}\)
Scheme 1.3 The formation of alkyl- and carboxyalky pyrrole derivatives of proteins from α, β- unsaturated aldehydes produced from peroxidation of LA-PL, AA-PL and DHA-PL.

Scheme 1.4 The formation of protein 4-ketoamide derivatives from 4-ONE and KODA-PL.

Modifications of proteins by these aldehydes have been demonstrated in a wide range of physiological and pathological conditions and are believed to contribute to protein
dysfunction or tissue damage in the progression of many diseases. For example, accumulation of PP modified proteins are found in the blood of individuals with atherosclerosis and in brain neurons of patients with Alzheimer’s disease.\textsuperscript{25, 30} Immunological evidence also revealed the presence of CHPs and CPPs in oxidized low-density lipoprotein (oxLDL). Elevated levels of CHP are found in plasma from patients with renal failure and atherosclerosis compared with healthy volunteers.\textsuperscript{21} CEPs are more abundant in Bruch’s membrane/RPE/choroid tissues from eyes with age-related macular degeneration (AMD) than in those from normal eyes.\textsuperscript{31} In addition, elevated levels of CEP adducts are found in human plasma from AMD donors relative to normal healthy donors.\textsuperscript{32} Evidence is accumulating that CEP derivatives accumulate in retina and tumors, and promote choroidal neovascularization, wound healing and tumor growth in a toll-like receptor 2 (TLR2)-dependent manner.\textsuperscript{10, 33}

Recently, the HOHA and HOOA esters of 2-lyso-phosphatidylcholine (HOHA-PC and HOOA-PC), respectively, were found to undergo spontaneous deacylation via an intramolecular transesterification mechanism that generates 2-lyso-PC and the corresponding HOHA-lactone and HOOA-lactone under physiological conditions, that is, 37 °C and pH 7.4.\textsuperscript{34} More recently, it was reported that HOHA-lactone is a biologically active precursor for the generation of CEP derivatives, demonstrated by the formation of CEP adducts upon reaction of HOHA-lactone with biomolecules such as Ac-Gly-Lys-OMe, proteins and ethanolamine phospholipids (Scheme 1.5, see next page).\textsuperscript{35}
Scheme 1.5 The spontaneous deacylation of HOHA-PC and HOOA-PC generates 2-lyso-PC and the corresponding HOHA-lactone and HOOA-lactone. HOHA-lactone can react with the amino group of biomolecules to generate CEP adducts.

1.4 Modification of ethanolamine phospholipids by LPO-derived aldehydes.

In mammalian, plant and yeast cells PCs are the most abundant PLs and ethanolamine phospholipids (EPs) are the second most abundant. However, with few exceptions, in prokaryotes no PC is made and EPs are usually the most abundant PLs. In eukaryotic cells, EPs account for approximately 20% of total PLs. EPs are not equally abundant in membranes of all types of mammalian cells or tissues. About 25% of mammalian PLs are EPs. In the brain EPs are especially abundant, accounting for about 45% of total PLs. In addition, EPs are not symmetrically distributed across the two leaflets of the membrane bilayer. In various eukaryotic plasma membranes, most of EPs are located mainly in the inner leaflet whereas choline-containing PLs such as PC and sphingomyelin are enriched in the outer leaflet. Moreover, EPs have a tendency to form nonbilayer hexagonal
structures that not only have been shown to activate various membrane-bound enzymes such as protein kinase C, calcium pump, and phospholipase D, but also facilitates rapid phospholipid trans-bilayer movement leading to membrane fusion in model membranes.\textsuperscript{37, 38} Compared to other membranes, mitochondria membranes, particularly the inner membranes, are enriched in EPs and evidence is accumulating that EPs play an important role in regulating mitochondrial function and morphology.\textsuperscript{36, 39} Furthermore, EPs are precursors for the synthesis of other compounds.\textsuperscript{40} EPs are also sources of the ethanolamine that can covalently modify several proteins.\textsuperscript{36} Thus, covalent modification of EPs by LPO-derived aldehydes may alter the membrane PL distribution and change its fluidity, resulting in disruption of membrane structure and dysfunction of membrane proteins.\textsuperscript{38, 41}

Oxidative damage has been strongly implicated in the pathogenesis of numerous diseases.\textsuperscript{24} LPO-derived aldehydes serve as key mediators of oxidant injury due to their capacity to covalently modify biomolecules such as proteins, DNA, and lipids.\textsuperscript{41} Although protein and DNA modification by lipid aldehydes have been extensively studied, EP modification by these aldehydes is still poorly understood in spite of the high potential of EPs to rapidly react with these aldehydes.\textsuperscript{42} Furthermore, the amino group of EPs is very close to the original generation site of LPO-derived aldehydes and the amphipathic nature of EPs may both trap the small aldehydes in the core of their vesicles and facilitate the reaction.\textsuperscript{42} Modification of the ethanolamine head group of EPs by LPO-derived aldehydes including 4-HNE, levuglandins/isoketals, acrolein, malondialdehyde (MDA), HOHA and HOHA-lactone has been reported.\textsuperscript{35, 38, 41, 43-45} Modification of PEs by 4-HNE, was proposed to account for the formation of fluorescent chromolipids
upon oxidation of rat liver microsomes or mitochondria. Levels of isolevuglandin-modified EPs were found to be elevated in plasma from patients with AMD as well as in the liver of ethanol-fed mice. In addition to their potential as clinically useful biomarkers of oxidative injury, isolevuglandins-PE also induces endoplasmic reticulum stress and inflammation. CEP-EPs derived from HOHA or HOHA-lactone are not only present in human blood at 4.6-fold higher levels in AMD plasma than in normal plasma, but also exhibit TLR2-dependent proangiogenic activity similar to that exhibited by CEP-peptide and CEP-protein derivatives. Thus, aldehyde-PE adducts have important biological activities and may be involved in the pathogenesis of diseases associated with oxidative injury.

1.5 Age-related macular degeneration (AMD).

Age-related macular degeneration (AMD), a degenerative condition of the central retinal cone-rich zone called the macula, is the most common cause of vision loss in the western world, and its prevalence is rising with increasing longevity. Stages of AMD are categorized as early and late. Early AMD, in which visual symptoms are inconspicuous, is characterized by soft drusen, choroidal or outer retinal hyperpigmentation associated with drusen or depigmentation of the retinal pigment epithelium (RPE). Late AMD, in which severe loss of vision is usual, has ‘‘dry’’ or ‘‘wet’’ forms. Dry AMD, also called geographic atrophy (GA), refers to any sharply demarcated area of hypopigmentation, depigmentation, or RPE death in the macular area. Wet AMD, also called choroidal neovascularization (CNV), is characterized by abnormal blood vessels sprouting from the choriocapillaris through the RPE into the neural retina, typically resulting in hemorrhage, exudation, scarring, and or serous retinal
Approximately 90% of patients with late AMD have the atrophic form which is associated with drusen and defined by the absence of neovascular ingrowth, while 10% of those have the neovascular form in which choroidal neovascularization invades through the Bruch’s Membrane resulting in macular edema and rapid central vision loss.\textsuperscript{14, 31, 49}

The pathophysiology of AMD is complex and epidemiological studies of diet, environmental, and behavioral risk factors suggest that oxidative stress contributes to the development of AMD.\textsuperscript{6, 50} The retina, due to its structural and physiological features, is particularly susceptible to oxidative stress.\textsuperscript{1, 6, 50} The retina, a light-sensitive layer of tissue, lies between the choroid and the vitreous.\textsuperscript{48, 51} The outer retina that is adjacent to the choroid includes the photoreceptors (rods and cones), the RPE, and Bruch’s membrane.\textsuperscript{48} Photoreceptor cells contain two distinct compartments, the inner and outer segments, that are embedded in the interphotoreceptor matrix, in close contact with the RPE.\textsuperscript{48, 51} Between the interphotoreceptor matrix and Bruch’s membrane is the RPE.\textsuperscript{48} Bruch’s membrane is surrounded by the RPE and the choriocapillaris.\textsuperscript{48, 52} The vascular system that feeds the outer retina is the capillary layer of the choroid, the choriocapillaris, (Scheme 1.6, see next page).\textsuperscript{48} The RPE, Bruch’s membrane and the choriocapillaris are called Ruysch’s complex, that provides an optimal environment for retinal function - light capture and transduction.\textsuperscript{48}
Several retinal characteristics facilitate disequilibrium of cellular redox balance to favor oxidation. First, oxygen consumption necessary to support the metabolic needs of the photoreceptors is much greater than required by any other tissue. Second, the photoreceptors and RPE contain an abundance of photosensitizers. Third, the retina is subjected to high level exposure to cumulative irradiation. Fourth, photoreceptors are rich in PUFAs, with DHA accounting for more than 80% of PUFAs in photoreceptor disk membranes. Since DHA contains six C-C double bonds and the number of double bonds is directly related to susceptibility of PUFAs to oxidation, DHA is readily oxidized and initiates a cytotoxic chain-reaction, making the retina particularly susceptible to damage by ROS and by lipid-derived oxidative protein modifications. Finally, the process of phagocytosis of photoreceptor outer segments by the RPE is itself an oxidative stress and results in the generation of endogenous ROS. To protect against oxidative damage, the retina has

Scheme 1.6 Schematic diagram of normal outer retina.
developed an effective intricate antioxidant defense system, evident by its particular richness in non-enzymatic antioxidants such as glutathione, vitamin E and ascorbate, and enzymatic antioxidants such as catalase, superoxide dismutase and glutathione-S-transferases.\textsuperscript{1, 13} However, the anti-oxidative capability of retina seems reduced with increasing age, which possibly makes the aging retina more susceptible to oxidative damage.\textsuperscript{13}

The RPE has a crucial role in the physiology and pathophysiology of the retina, which has its rationale in the fact that RPE, situated between the photoreceptors and Bruch’s membrane-choroid complex, serves a variety of metabolic and supportive functions in the maintenance of the normal functions of the retina.\textsuperscript{6, 53, 54} The RPE functions as the out blood-retinal barrier, mediating the exchange between the choriocapillaris and the photoreceptors.\textsuperscript{13, 55} Another function of RPE is phagocytosis and degradation of constantly shed photoreceptor outer segment tips.\textsuperscript{6, 13, 56} In addition, the RPE also takes part in the maintenance of the visual cycle of retinal (uptake, processing, transport, release of vitamin A derivatives).\textsuperscript{6, 53, 56} Moreover, the RPE secretes a variety of growth factors such as vascular endothelial growth factor (VEGF) to uphold the choroidal endothelium and photoreceptors.\textsuperscript{55, 56} Although the vision loss of AMD results from photoreceptor damage in the outer retina, damage to the RPE is considered to be a crucial early event in the molecular pathways leading to photoreceptor degeneration and clinically relevant AMD.\textsuperscript{6, 13, 50} Geographic atrophy, the widespread death of the RPE cells, causes severe vision loss in AMD patients.\textsuperscript{57} This apoptotic cell death is a key mechanism of RPE loss during AMD,\textsuperscript{50, 58} and RPE apoptosis is a key factor in AMD pathogenesis.\textsuperscript{59}
As mentioned above, CNV, the major cause of total vision loss (blindness) from AMD, is characterized by abnormal blood vessels originating from the choriocapillaris, breaking through the Bruch’s membrane into the RPE. The pathological mechanisms underlying the formation and progression of CNV are still poorly understood, however, recent evidence suggests a central role of VEGF-A in the development of CNV. In the normal eye, a paracrine relation between RPE cells and the choriocapillaris exists, evident by the facts that normal RPEs produce VEGF-A towards their basal (choriocapillaris) side where all three VEGF receptors are localized on the side of the choriocapillaris endothelium facing RPE cells. However, this paracrine relation is disturbed by increasing lipid deposits in Bruch’s membrane with age, which may interfere with the exchange of water-soluble compounds such as VEGFs between choriocapillaris and RPE. In this case, VEGF-A secreted from RPE is unable to reach the choriocapillaris to support it, thus causing choriocapillaris atrophy. Consequently, hypoxia may develop in the outer retina, resulting in accumulation of VEGF-A at the RPE side and contributing to CNV. It has been shown that VEGF levels are significantly increased in retinas and plasma of AMD subjects. In addition, the successful clinical applications of the anti-VEGF compounds bevacizumab, ranibizumab, and pegaptanib in inhibiting neovascular AMD strongly support the importance of VEGF involvement in its progression.

1.6 Sickle cell disease (SCD).

Sickle cell disease (SCD), the first inherited disease to be characterized at the molecular level, is a class of hemoglobinopathy affecting approximately 5% of the world’s population. This disease results from a single point mutation in the genetic
code inducing the substitution of valine (Val) for glutamic acid (Glu) at the sixth amino acid position in the β-globin chain of hemoglobin (Hb). The abnormal sickle hemoglobin (Hb S) of sickle cell disease differs from normal adult hemoglobin (Hb A) by this single substitution. The replacement of hydrophilic glutamate of Hb A with hydrophobic valine in Hb S promotes aggregation/polymerization of hemoglobin in the deoxygenated state, in which HbS molecules alter their configuration, exposing the hydrophobic valine that can bind to a complementary hydrophobic site on a β subunit of another hemoglobin tetramer. The aggregation/polymerization of deoxygenated Hb S generates elongated rope-like fibers, which form a fascicle that damages the RBC membrane and cytoskeleton and alters RBC biochemical properties, transforming the normal biconcave disc shape of red blood cells (RBCs) into classic rigid crescent or sickle shapes. Polymerization of Hb S is reversible, and Hb S can undergo innumerable cycles of deoxygenation-induced polymerization and reoxygenation-induced depolymerization. After a finite number of these cycles, the sickle erythrocyte membrane is irreversibly injured. Compared to normal erythrocytes, the deformed and rigid sickle RBCs have a shorter lifespan and are more easily trapped in capillaries, thereby causing vaso-occlusion leading to tissue ischemia. The consequent cascade of harmful pathological events includes hemolysis, endothelial dysfunction, inflammation, hypercoagulability, oxidative stress, reperfusion injury and hypoxemia. In turn these processes further induce chronic organ damage, resulting in a malicious cycle contributing to SCD progression.

Oxidative stress is increased in SCD owing to several factors. These include elevated levels of cell-free hemoglobin, recurrent ischemia-reperfusion injury, and elevated
autoxidation of sickle hemoglobin (Hb S), and chronic inflammation. Continuous intravascular hemolysis in sickle cell patients results in steady state increases in plasma levels of cell-free hemoglobin, which is generally considered to be deleterious, particularly with respect to nitric oxide (NO) depletion and elevated cell-free heme production. By inactivating NO, cell-free hemoglobin reduces the NO bioavailability, resulting in endothelial dysfunction and vasoconstriction. Increased cell-free hemoglobin leads to increased hydrophobic heme, which is cytotoxic and proinflammatory and rapidly intercalates into the plasma membrane of endothelial cells where it releases its iron. Iron catalyzes the generation of ROS by the Fenton reaction. Ischemia-reperfusion injury refers to the vessel and tissue damage when oxygen-rich blood flow is restored after a period of ischemia, leading to an increase in the concentration of radical species. An ischemic or hypoxic environment is produced by the cessation of blood flow to tissues. Under conditions of ischemia, hypoxanthine and xanthine oxidase (XO) are generated from adenosine triphosphate and xanthine dehydrogenase, respectively. After restitution of oxygen-rich blood flow, the XO generated during ischemia can lead to deleterious effects by catalyzing the conversion of xanthine or hypoxanthine into superoxide and uric acid. In the present of iron, the superoxide radical is ultimately converted to hydroxyl radical, a highly reactive free radical species. Although both normal Hb A and sickle Hb S have a tendency to autoxidize into methemoglobin and superoxide, some studies showed that the instable sickle Hb S has a higher rate of autoxidation compared with normal Hb A. The accelerated Hb S autoxidation causes increased amounts of superoxide production, which is converted to hydrogen peroxide by superoxide dismutase. The exposure of formed
hydrogen peroxide to methemoglobin decomposes hemoglobin and releases iron.\textsuperscript{82} In the presence of iron, the remaining hydrogen peroxide is further transformed to hydroxyl radical.\textsuperscript{69} Consequently, sickle cells ultimately generate great quantities of superoxide, hydrogen peroxide, and hydroxyl radical.\textsuperscript{69, 78} A chronic proinflammatory state is characteristic of sickle cell patients.\textsuperscript{83} Elevated numbers of activated polymorphonuclear neutrophils (PMNs), which produce ROS in an NADPH oxidase mediated respiratory burst, is observed in sickle cell patients.\textsuperscript{73, 79} In addition to elevated generation of pro-oxidants in SCD, protective mechanisms such as antioxidants are decreased, which may be a result of excessive antioxidant consumption or inactivation of the protective mechanisms by the overabundance of oxidative stress in SCD.\textsuperscript{69, 74, 84}

As noted above, oxidative stress is an important feature of SCD and might play a significant role in pathophysiology of SCD.\textsuperscript{73, 77, 85} Because they contain hemoglobin, a powerful catalysts for initiation of oxidative reactions, RBCs are particularly susceptible to oxidative damage.\textsuperscript{67} Oxidative damage can change a number of RBC membrane properties, contributing to the pathophysiology of SCD.\textsuperscript{67, 86, 87} Reactions of ROS with proteins result in the modifications of various amino-acid side chains, leading to a partial or complete loss of protein functionality.\textsuperscript{84, 88} In addition, oxidative stress can cause the cross-linking of proteins, contributing to increased membrane rigidity and decreased deformability.\textsuperscript{67, 89} Lipids, the major components of the erythrocyte membrane, are vital for maintaining its structural and functional integrity.\textsuperscript{90} The excess production of ROS in SCD results in significantly enhanced lipid peroxidation compared to controls.\textsuperscript{69, 78, 79} Peroxidation of erythrocyte membrane lipids generates reactive peroxidation products, which, in turn, can react with nucleic acids and proteins causing further oxidative damage.

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Lipid peroxidation has been shown to have some effects on the activities of certain enzymes like the Na\(^+\)K\(^+\)−ATPase and (Ca\(^{2+}\)+Mg\(^{2+}\))−ATPase from sickle cell membranes.\(^{69, 93}\) MDA, a major lipid peroxidation product, induces decreased deformability of sickle erythrocytes, leading to increased rigidity, dehydration and decreased deformability of RBCs, and thus contributing to accelerated membrane senescence for these cells.\(^{69, 94}\) An asymmetric distribution of membrane phospholipids between the two lipid bilayers is characteristic of RBCs.\(^{76}\) Sphingomyelin and phosphatidylcholine (PC) are predominantly located in the outer bilayer of the membrane, and polar lipids such as phosphatidylethanolamine (PE) and phosphatidylserine (PS) are located in the inner bilayer of the membrane.\(^{76, 95}\) However, increased oxidative stress can cause membrane instability by inducing the movement of PS and PE from its inner bilayer to the outer bilayer, disrupting the normal lipid asymmetry in RBCs, and contributing to accelerated intravascular hemolysis that further drives increased endothelial ROS generation.\(^{74, 95, 96}\) Moreover, the abnormal exposure of phosphatidylserine on the outer surface of the erythrocyte triggers the removal of cells by macrophages with PS-specific receptors.\(^{97, 98}\) In addition, increased oxidative stress in SCD can also impair cation homeostasis, resulting in a rise in intracellular calcium levels, which activate the calcium-activated potassium channel (K−Ca), leading to dehydration and cell shrinkage with increased cytoplasmic viscosity and a less deformable cytoskeleton.\(^{70, 99}\) Furthermore, the high concentration of calcium may be a factor in the potentiation of peroxidation of erythrocyte membrane lipids.\(^{90}\)

The vascular endothelium is a major target of oxidative damage in SCD owing to its proximity to cell-free hemoglobin and the sites of ischemia-reperfusion injury, the
primary localization of an abundance of xanthine oxidase in endothelial cells, along with the lack of critical oxygen reactive scavenging enzymes such as catalase and glutathione peroxidase, and the production of ROS in endothelial cells.\textsuperscript{73, 92, 100} The endothelial damage that contributes to SCD-related chronic organ complications, e.g., cerebral vasculopathy, pulmonary hypertension, and retinopathy, can be fostered by oxidative stress-related mechanisms.\textsuperscript{73} Thus, high levels of ROS inflict direct damage to endothelial cells by causing lipid peroxidation, enzyme and mitochondrial dysfunction, and DNA damage, leading to endothelial cell swelling and detachment from the underlying basement membrane.\textsuperscript{73, 80} This results in the exposure of sub-endothelial structures and proteins including tissue factor (TF), which further contributes to a condition of hypercoagulability.\textsuperscript{73, 101} In addition to being directly toxic to the endothelial cells, ROS can also cause endothelial damage via the deactivation of NO.\textsuperscript{77, 92} In the presence of increased ROS, especially $O_2^-$, NO preferentially forms ONOO$^-$ that is a powerful and highly reactive oxidant.\textsuperscript{65, 102} Both $O_2^-$ and ONOO$^-$ can alter endothelial cell gene expression.\textsuperscript{92} Moreover, ONOO$^-$ exists in equilibrium with peroxynitrous acid (ONOOH), which can further form $\cdot$OH and NO$_2$$\cdot$ molecules, resulting in peroxidation of the lipid membrane, and may predispose the endothelial cell to apoptosis.\textsuperscript{82, 92} Furthermore, ROS can also contribute to endothelial damage through alteration in vascular tone by upregulating expression of endothelin-1, a potent vasoconstrictor and an important mediator of vascular tone, thereby increasing vaso-occlusion.\textsuperscript{80, 92} Additionally, ROS can act as second messengers to enhance gene expression and production of adhesion molecules and proinflammatory cytokines through activation of the redox-sensitive nuclear factor-κB (NF-κB).\textsuperscript{73, 102} NF-κB exposed to ROS is activated by
phosphorylation and translocated to the nucleus, where it alters gene expression of vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1), leading to their increase.\textsuperscript{65, 102-104} The adhesion molecules of sVCAM-1 and ICAM-1 can facilitate binding of sickle RBCs and WBCs to the endothelium, thereby contribute to the propagation of vaso-occlusion.\textsuperscript{92} Increased levels of inflammatory cytokines such as tumor necrosis factor-\( \alpha \) (TNF-\( \alpha \)) and interleukin-1\( \beta \) (IL-1\( \beta \)), which can regulate NF-\( \kappa \)B and influence the expression of adhesion molecules, are observed in SCD patients, further augmenting a proinflammatory state.\textsuperscript{65, 105} Oxidative stress-induced externalization of PS to the outer leaflet of sickle erythrocyte cell membrane is associated with hypercoagulability and increased endothelial activation.\textsuperscript{74, 106}
1.6 References.


sickle cell disease exhibit oxidative stress that can be ameliorated by antioxidants.


CHAPTER 2

HOHA-LACTONE INDUCES P53 EXPRESSION AND APOPTOSIS IN PRIMARY HUMAN RETINAL PIGMENT EPITHELIAL CELLS
2.1 Background

Age-related macular degeneration (AMD), a chronic, degenerative disorder in the maculae of the retina, has become the leading cause of irreversible blindness among the elderly.\textsuperscript{1-4} Although the pathogenesis of AMD is complex and remains poorly understood, the retinal pigment epithelium (RPE), the key supportive retinal epithelium, is considered to be a primary site of pathology of AMD.\textsuperscript{2, 5} The RPE, a monolayer of cells interposed between the photoreceptors and Bruch’s membrane-choroid complex, is critical for the maintenance and survival of the photoreceptors. RPE cell dysfunction and death play a vital role in the pathogenesis of AMD.\textsuperscript{2, 6, 7} It has been suggested that both inflammation and oxidative stress contribute to RPE dysfunction in AMD.\textsuperscript{8, 9} Due to its structural and functional features, the retina is an ideal environment for the generation of reactive oxygen species (ROS). It experiences high levels of oxygen consumption and cumulative irradiation, contains an abundance of photosensitizers and undergoes active phagocytosis of photoreceptor outer segments by the RPE. In addition, it contains high levels of lipids that are highly susceptible to oxidation, with docosahexaenoic acid (DHA) accounting for more than 80% of polyunsaturated fatty acids (PUFAs), manly esterified into phospholipids, in photoreceptor disk membranes.\textsuperscript{1, 3, 8, 10}

Under physiological conditions, ROS are rapidly neutralized by effective enzymatic and non-enzymatic defense mechanisms. However, excess ROS production results in an imbalance between prooxidant and antioxidant processes, leading to oxidative stress.\textsuperscript{3, 11} Under oxidative stress, the membrane lipids, mainly phospholipids containing PUFAs, are predominantly susceptible to oxidative stress-mediated lipid peroxidation (LPO), which has been implicated in the pathogenesis of many degenerative ocular diseases.
including AMD.\textsuperscript{1, 3, 12} Lipid peroxidation leads to the generation of a variety of reactive molecules including relatively stable and toxic electrophiles such as 4-hydroxynonenal (4-HNE).\textsuperscript{13, 14} LPO products have been widely implicated to contribute to RPE dysfunction, leading to AMD. In previous studies, modification of biomolecules by 4-HNE was suggested to contribute to the initiation of light-induced retinal degeneration.\textsuperscript{15, 16} In recent years, 4-HNE has been recognized as an important signaling molecule that can modulate various signaling pathways such as cell cycle procession, cell proliferation and apoptosis in many cell types of different origins.\textsuperscript{13, 14, 17}

DHA, an omega-3 fatty acid, comprises 40\%, 60\% and 80\% of the PUFAs in the brain, retina and photoreceptor disk membranes, respectively.\textsuperscript{10, 18} Owing to the presence of six double bonds between carbon atoms and five doubly allylic methylene groups in its polyene chain (C=C), DHA is exquisitely susceptible to lipid peroxidation.\textsuperscript{10} Free radical-induced oxidative cleavage of phospholipids containing DHA generates a large family of reactive aldehydes including the 4-hydroxy-7-oxohept-5-enoic acid ester of 2-lyso-phosphatidylcholine (HOHA-PC). HOHA–PC reacts with primary amino group of protein or ethanolamine phospholipids to generate biologically active 2-ω-carboxyethylpyrrole (CEP) derivatives.\textsuperscript{19–21} Recently, HOHA-PC was found to undergo rapid and spontaneous deacylation to generate 2-lyso-PC and a stable five-membered ring lactone aldehyde, 3-(5-oxotetrahydrofuran-2-yl)acrylaldehyde (HOHA-lactone).\textsuperscript{22} More recently, HOHA-lactone was shown to react with the primary amino groups of biomolecules to produce CEP derivatives (Scheme 2.1, see page 45).\textsuperscript{23} CEP derivatives were found to accumulate in the retinas and blood plasma of individuals with AMD and are considered to be a biomarker of AMD.\textsuperscript{19, 20, 24} Animal model studies also
demonstrated that CEP derivatives induce choroidal neovascularization, promote wound healing and tumor growth in a toll-like receptor 2 (TLR2) dependent manner.\textsuperscript{20, 25, 26} Furthermore, immunization of mice with CEP-modified mouse serum albumin induces AMD-like lesions in their retinas.\textsuperscript{27} Since DHA, the precursor of HOHA-lactone is present in abundance in the retina and HOHA-lactone is an important precursor of CEP, which is highly related with AMD, it is reasonable to presume that HOHA-lactone contributes directly to the development of AMD. Because damage to the RPE is an early and crucial event in the molecular pathways leading to AMD,\textsuperscript{3} it is important to delineate the role of HOHA-lactone in the degeneration of the RPE. Therefore, we examined the generation of CEP derivatives in a human RPE cell line (ARPE-19) and the primary human RPE cells (hRPE) upon exposure to HOHA-lactone. We also evaluated the generation of CEP derivatives in RPE cells caused by exposure to lipopolysaccharide (LPS) that exemplifies inflammatory stimulation and H\textsubscript{2}O\textsubscript{2} that exemplifies oxidative stress.

Because it incorporates the α,β-unsaturated aldehyde functional array characteristic of 4-HNE, HOHA-lactone was expected to act as a second messenger capable of inducing intracellular oxidative stress, causing cell signaling and cell death in analogy with the chemistry of 4-HNE. In vitro studies showed that oxidant-treated RPE cells undergo apoptosis, a possible mechanism by which RPEs are lost during the early phase of AMD.\textsuperscript{2} The present study investigated whether HOHA-lactone is cytotoxic, induces intracellular oxidative stress and causes RPE cell apoptosis. These studies established a molecular pathway involved in HOHA-lactone induced RPE cell apoptosis.
Scheme 2.1 Postulated CEP adduct generation \textit{in vivo}.
2.2 Results and Discussion

Synthesis of HOHA-lactone. Authentic HOHA-lactone was prepared using an efficient synthesis method as described previously with minor modifications (Scheme 2.2). Briefly, ethyl 3-(2-furyl)propanonate (2.1) was oxidatively ring opened with N-bromosuccinimide (NBS) to produce the keto aldehyde 2.2 that was further selectively protected by treatment with trimethyl orthoformate and Montmorillonite K10 to give 2.3. Then the ketone carbonyl group in 2.3 was reduced with sodium borohydride to provide the masked γ-hydroxyalkenal 2.4 in excellent yield followed by hydrolysis in the presence of strong acid catalysts, Amberlyst-15 and trifluoroacetic acid (TFA), to produce the target HOHA-lactone (2.5).

![Scheme 2.2 Synthesis of HOHA-lactone 2.5.](image)

Post-translational modification of RPE cells by HOHA-lactone. ARPE-19 cells, a cell line developed from the RPE of a 19-year-old adult male donor, retain many of the characteristics of RPE cells and have been widely used as an alternative to primary RPE cells to study the impact of oxidative stress due to its readily availability and feature stability in prolonged cultivation. However, ARPE-19 cells are transformed and therefore may respond differently to oxidative stress compared to primary RPE cells in
vitro and in vivo. Thus, it would be more appropriate to use primary RPE cells for AMD disease modeling. We recently reported that HOHA-lactone is an important precursor for the generation of CEP derivatives and CEP-positive immunostaining was observed in HOHA-lactone-treated ARPE-19 cells.\textsuperscript{23} The present study was conducted to evaluate whether primary human RPE cells (hRPE) possess a similar pattern of post-translational modification in response to HOHA-lactone as that previously reported for ARPE-19 cells. We therefore incubated both ARPE-19 and hRPE cells with 10 μM HOHA-lactone for two hours to investigate the formation of CEP derivatives. As shown in Figure 2.1 (see next page), incubation of ARPE-19 cells with HOHA-lactone led to the formation of CEP derivatives, which was consistent that previously reported for ARPE-19 cells.\textsuperscript{23} As expected, the generation of CEP adducts was observed when hRPE cells were treated with 10 μM HOHA-lactone although less intense CEP-positive immunostaining was observed in hRPE cells compared to ARPE-19 cells after treatment with HOHA-lactone (Figure 2.1, see next page).
**Figure 2.1** Post-translational modification of RPE cells by HOHA-lactone. (A) Images of the generation of CEP in RPE cells. (B) Quantification of the generation of CEP in ARPE-19 cells. (C) Quantification of the generation of CEP in hRPE cells. ARPE-19 and hRPE cells were treated with 10 μM HOHA-lactone for 2 h followed by incubation in basal medium overnight under 5% CO₂/95% air at 37 °C. Then cells were immunostained using mouse monoclonal anti-CEP antibodies /goat anti-mouse Alexa Fluor 488 antibodies and DAPI. CEP-modified biomolecules exhibit green fluorescence. The images were taken at 10x magnification. Data shown are representative of three independent experiments that show very similar results.
Generation of CEP derivatives in RPE cells consequent to inflammatory stimuli or oxidative stress. Because both local inflammation and oxidative stress are associated with RPE damage in AMD, and because CEP is a biomarker of AMD, we also examined the effect of inflammatory stimuli and oxidative stress on the generation of CEP derivatives in both ARPE-19 and hRPE cells. Inflammatory stimulation was accomplished by treatment with lipopolysaccharide (LPS) and oxidative stress was induced by treatment with H$_2$O$_2$. As shown in Figure 2.2 (see next page), treatment of RPE cells with 25 µM H$_2$O$_2$ generated CEP derivatives, demonstrating that H$_2$O$_2$ induced oxidative stress can cause CEP generation in RPE cells. Treatment with LPS also caused CEP generation (Figure 2.3, see page 51). These results indicate that CEP derivatives can arise in RPE cells that are exposed to an oxidative or inflammatory environment presumably through endogenous generation of HOHA-lactone.
Figure 2.2 Generation of CEP adducts in RPE cells under oxidative stress. (A) Images of the generation of CEP in RPE cells. (B) Quantification of the generation of CEP in ARPE-19 cells. (C) Quantification of the generation of CEP in hRPE cells. RPE cells were treated with 25 μM H₂O₂ for 2 hours followed by overnight recovery and then immunostained with mouse monoclonal anti-CEP antibody/goat anti-mouse Alexa Fluor 488 antibodies and DAPI. The figure is representative of two independent experiments that showed very similar results.
Figure 2.3 Generation of CEP adducts in RPE cells under inflammatory stimuli. (A) Images of the generation of CEP in RPE cells. (B) Quantification of the generation of CEP in ARPE-19 cells. (C) Quantification of the generation of CEP in hRPE cells. RPE cells were treated with 12 µg/ml LPS for overnight and then immunostained with mouse monoclonal anti-CEP antibody/goat anti-mouse Alexa Fluor 488 antibodies and DAPI. The figure shows a representative of two independent experiments, which showed very similar results.
**HOHA-lactone induces death of RPE cells.** In view of the high accumulation of CEP in human retina and blood plasma in individuals with AMD and the fact that HOHA-lactone is a major precursor of CEP, some HOHA-lactone could evade metabolic conversion by RPE cells into less toxic products. Thus, we assessed the cytotoxic effects of HOHA-lactone on RPE cells, including both the ARPE-19 cell line and primary human RPE cells, by determining the cell viability upon exposure of varying concentrations of HOHA-lactone overnight. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay is a rapid colorimetric method for determining mitochondrial activity. It is based on reduction of the yellow water-soluble tetrazolium dye MTT by NAD(P)H-dependent cellular oxidoreductase enzymes of living cells to produce crystals of water-insoluble purple E,Z-5-(4,5-dimethylthiazol-2-yl)-1,3-diphenylformazan that dissolves in dimethyl sulfoxide (DMSO) to produce a homogeneous solution suitable for measurement of optical density with a microplate reader. Because the tetrazolium ring of MTT is cleaved in active mitochondria, the reaction occurs only in living cells and thus the amount of formazan generated is directly proportional to the number of living cells over a wide range using a homogeneous cell population. Results of a MTT viability assay showed a progressive decrease in cell viability of ARPE-19 cells with increasing concentrations of HOHA-lactone (Figure 2.4A, see next page) with LC$_{50}$ = 42.2 ±5.4 μM. Compared to ARPE-19 cells, HOHA-lactone was less cytotoxic to the primary hRPE cells (Figure. 2.4B, see next page) and showed little or no effect, possibly weakly growth stimulating within the range of 0-50 μM. However, HOHA-lactone was cytotoxic to hRPE cells when its concentration in the cell
culture exceeded 50 µM, where cell viability declined precipitously with only 38.4% viable cells at 75 µM.

**Figure 2.4** Cytotoxicity of HOHA-lactone to (A) ARPE-19 cells and (B) primary hRPE cells. Eight replicate wells were used for each concentration of HOHA-lactone in these studies. Results presented are percent cell survival in HOHA-lactone treated groups with respect to control non-treated cells (mean ± SD; n = 8). Data are representative of three independent experiments that show very similar results.

**HOHA-lactone induces ROS accumulation in RPE cells.** As oxidative stress through the release of reactive oxygen species (ROS) is a crucial trigger for AMD pathogenesis,\(^2\) the generation of ROS in HOHA-lactone-treated RPE cells including both ARPE-19 and hRPE cells was studied with an oxidative stress probe, 2',7'-dichlorofluorescin diacetate (DCF-DA). The DCF-DA assay is based on the premise that the nonpolar, nonionic DCFH-DA diffuses passively through cellular membranes and is hydrolyzed enzymatically by intracellular esterases to nonfluorescent 2',7'-dichlorofluorescin (DCFH) that is further oxidized to highly fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of ROS.\(^{34-36}\) As shown in Figure 2.5 (see page 55), fluorescence levels increased in both ARPE-19 and hRPE cells exposed to HOHA-lactone compared to those of control RPE cells, indicating that HOHA-lactone treatment promoted intracellular ROS accumulation in a dose dependent manner. The cell morphology of ARPE-19 cells
changed significantly as the concentration of HOHA-lactone increased, and ARPE-19 cells treated with 30 μM HOHA-lactone exhibited characteristics typical of apoptosis including cell shrinkage, nuclear chromatin condensation, and segmentation of the nucleus (see phase-contrast panel corresponding to 30 μM HOHA-lactone in Figure 2.5). However, the effect of 30 μM of HOHA-lactone on primary hRPE cells was less detrimental. This is consistent with the results of the MTT assay. This suggests that hRPE cells are more effective at detoxifying HOHA-lactone, or protecting against HOHA-lactone induced oxidative injury, or both.
Figure 2.5 Detection of ROS in RPE cells challenged with various concentrations of HOHA-lactone. (A) Images of the generation of ROS in RPE cells. (B) Quantification of the generation of ROS in ARPE-19 cells. (C) Quantification of the generation of ROS in
hRPE cells. Cells were treated with DCFH-DA (15 µg/ml) in the basal medium under 5% CO₂/95% air at 37 °C for one hour and then were exposed to HOHA-lactone for two hours. Images were taken at 10X magnification. Data shown are representative of three independent experiments that showed very similar results.

**HOHA-lactone provokes apoptosis in RPE Cells.** Since HOHA-lactone can cause RPE cell death and induce ROS accumulation, and since one potential important consequence of ROS signaling is the induction of apoptosis, a key mechanism of RPE loss during AMD, we next examined whether HOHA-lactone could induce RPE apoptosis. As caspases are central to the execution of programmed cell death and their activation constitutes the biochemical hallmark of apoptosis, we firstly examined in situ caspase activity in HOHA-lactone-treated or untreated ARPE-19 cells using a NucView 488 caspase-3 assay kit. The top panel of Figure 2.6A shows fluorescence images of ARPE-19 cells challenged with or without 15 µM HOHA-lactone overnight followed by staining with the green fluorogenic DEVD-NucView488™ caspase-3 substrate, which consists of a green fluorogenic DNA dye coupled to the caspase-3/7 DEVD recognition sequence. The initial non-fluorescent substrate is cleaved by caspase-3/7 to release the high-affinity fluorogenic DNA dye in the cytoplasm of apoptotic cells. Then the released dye migrates to the cell nucleus and stains DNA with bright green fluorescence. We used this assay to test if HOHA-lactone treatment of ARPE-19 cells leads to the production of active caspase 3 owing to cleavage of pro-caspase 3. As shown in top panel of Figure 2.6A, 15 µM HOHA-lactone-treated ARPE-19 cells showed a marked increase in fluorescence compared to control cells treated with PBS. In addition, the specificity of the cytosolic enzyme capable of proteolysis of DEVD-NucView-488 in ARPE-19 cells
was verified by pre-incubating the cells with the plasma membrane permeable caspase 3/7 inhibitor Ac-DEVD-CHO before incubation with DEVD-NucView 488 substrate. As shown in the bottom panel of Figure 2.6A, the NucView488™ fluorescence signal generated in the presence of the inhibitor was suppressed and resembled that found for cells treated with PBS. Thus, pre-treatment with the inhibitor essentially abolished the proteolysis of the caspase-3/7 specific substrate in the cells challenged with HOHA-lactone.

**Figure 2.6** HOHA-lactone induces apoptosis in ARPE-19 cells. (A) The microscopy imaging of caspase activation in ARPE-19 cells using the NucView 488 caspase-3 assay kit. ARPE-19 cells were challenged with 15 µM HOHA-lactone in DMEM/F12 medium for 18 hours. Cells were then stained using 5 µM DEVD-NucView 488-substrate to detect the activation of Caspase 3/7. The inhibitor DEVD-CHO was used to ensure the fluorescence observed in the HOHA-lactone-treated cells is due to the active caspase 3/7. (B) The fluorescence images of ARPE-19 cells stained with CF™488A-Annexin V and Propidium iodide (PI). Cells were stained with Annexin V to study the presence of
phosphatidylserine on the outer membrane and PI was used to establish the integrity of the plasma membrane. Data are representative of three independent experiments that show very similar results.

To independently confirm that apoptosis is indeed induced in the ARPE-19 cells treated with HOHA-lactone, we carried out CF\textsuperscript{TM}488A-Annexin V and Propidium iodide (PI) staining by using fluorescence microscopy. Annexin V, which is a phospholipid binding protein and interacts strongly and specifically with phosphatidylserine (PS), is conjugated to a green fluorescent dye CF\textsuperscript{TM}488A to detect apoptosis by detecting the loss of plasma membrane asymmetry. In normal viable cells, PS is typically found on the inner leaflet of the cell membrane. However, when a cell undergoes apoptosis, plasma membrane asymmetry is lost, which is an early event in apoptosis independent of the cell type, resulting in the presence of PS on the outer leaflet of the plasma membrane.\textsuperscript{38, 39} Propidium iodide (PI), a red fluorescent membrane-impermeant DNA-binding dye, is widely used in conjunction with Annexin V to discriminate between viable, apoptotic or necrotic cells on the basis of differences in plasma membrane integrity and permeability. The ability of PI to enter a cell is dependent upon the integrity of the plasma membrane. Usually, PI does not stain live or early apoptotic cells, which apparently exclude this dye due to the presence of an intact plasma membrane. However, the integrity of the plasma and nuclear membranes becomes compromised in late apoptotic and necrotic cells, which allow PI to pass through the membrane to gain access to the nucleus where it complexes with DNA rendering red fluorescence.\textsuperscript{39-41} As shown in the left panel of Figure 2.6B (see page 57) above, most of the PBS-treated ARPE-19 cells were negative to both Annexin V and PI, indicating that they are viable with only a few early apoptotic cells. The right
panel of Figure 2.6 B (see page 57) shows that most of the ARPE-19 cells treated with 15 μM HOHA-lactone were annexin V positive and PI negative, indicating that most of these cells were in the early stages of apoptosis with few late apoptotic or necrotic cells.

To confirm DEVD-NucView 488 staining results, we further evaluated intracellular caspase-3 activity in HOHA-lactone-treated ARPE-19 cells by immunoblotting using a specific anti-caspase-3 antibody. In the effector stage of apoptosis, pro-caspase-3 is activated by proteolytic cleavage of this inactive 32-kDa caspase-3 precursor (CPP32) at Asp-28-Ser-29 to generate the mature 17 kDa fragment. Activation of caspase 3 was detected by the appearance of a band at 17kDa. Immunoblot analyses presented in Figure 2.7 (see next page) show that HOHA-lactone caused a dose dependent cleavage from the caspase-3 zymogen of a 17-kDa fragment with the highest level of the active enzyme being generated upon treatment with 40 μM HOHA-lactone. These immunoblotting results were in good correlation with the data obtained from in situ immunofluorescence studies described above which also showed enhanced activity of caspase-3 in HOHA-lactone-treated ARPE-19 cells compared to the untreated cells (Figure 2.6A, see page 57). Taken together, the present observations clearly demonstrate that HOHA-lactone can induce cell death and activate caspase-3 cascades in ARPE-19 cells.
Figure 2.7. The effect of HOHA-lactone on caspase-3 activation. (A) Western blot analysis of caspase-3 in ARPE-19 cells incubated overnight with various concentrations of HOHA-lactone. (B) Densitometric analysis of the Western blot shown in (A). Immunoblotting was conducted using a rabbit polyclonal anti-caspase-3 antibody. GAPDH was used as a loading control. Results are representative of three independent experiments that show very similar results.
**HOHA-lactone induces accumulation of p53 in ARPE-19 cells.** P53, the tumor suppressor and transcription factor, is activated in response to stimuli such as UV, DNA damaging agents, ionizing radiation, hypoxia or oxidative stress.\(^{43, 44}\) Current evidence suggests that activation of p53 triggers cell cycle arrest, cellular senescence or apoptosis.\(^{43, 45}\) P53 has been documented to be involved in the 4-HNE-induced apoptotic process in ARPE-19 cells.\(^{13}\) Therefore, to determine whether apoptosis induced by HOHA-lactone, a structural analog of 4-HNE, is correlated with p53 expression, we evaluated expression of the p53 protein in HOHA-lactone-treated ARPE-19 cells by Western blotting. As shown in Figure 2.8A (see next page), a dose dependent increase in the intracellular levels of p53 was observed in ARPE-19 cells upon HOHA-lactone treatment. The results presented in Figure 2.8B (see next page) also indicated that there was a time dependent increase in p53 expression, at least up to 2 h, in ARPE-19 cells after exposure to 20 \(\mu\)M HOHA-lactone. Taken together, the results of these experiments demonstrated that HOHA-lactone induces a time-and dose-dependent accumulation of p53 in ARPE-19 cells.
Figure 2.8 Effect of HOHA-lactone on induction and phosphorylation of p53 in ARPE-19 cells: Western blot analysis of cell extracts collected from HOHA-lactone-treated ARPE-19 cells. (A) Dose-dependent expression of total p53 protein in ARPE-19 cells treated with HOHA-lactone for 2h. (B) Time-dependent expression of p53 in ARPE-19 cells treated with 20 µM HOHA-lactone. (C) The expression of phosphorylated p53 in ARPE-19 cells treated with HOHA-lactone for 2h. Immunoblotting for A and B was conducted using total anti-p53 antibody and for C using anti-phosphoserine-15 antibody, respectively, as the primary antibodies. GAPDH was used as a loading control (30 µg/well of a protein from ARPE-19 lysate was loaded). Data shown are representative of three independent experiments that show very similar results.

HOHA-lactone induces phosphorylation of p53 in ARPE-19 cells. Post-translational modifications of p53, such as phosphorylation on Ser and Thr residues
within its N and C-terminal regions, has been shown to influence p53 activity under physiological and stress conditions.\textsuperscript{13, 44}\ Therefore, we assessed the phosphorylation levels of p53 at serine 15 in HOHA-lactone-treated ARPE-19 cells by Western blotting using an anti-phosphoserine-15 antibody. As shown in Figure 2.8C, a dose dependent increase in phosphorylated p53 compared to the loading control was observed in HOHA-lactone-treated cells, which correlated well with the dose dependent accumulation of p53 protein (Figure 2.8A, see page 62).

**HOHA-lactone causes enhanced nuclear accumulation of p53 in ARPE-19 cells.** It has been reported that p53, which is mainly localized in the cytoplasm under basal conditions, undergoes modification in response to cellular stress and eventually translocates to the nucleus.\textsuperscript{45} In order to investigate whether or not HOHA-lactone facilitates nuclear accumulation of p53, we measured p53 expression in the cytoplasmic and nuclear compartments of ARPE-19 cells after incubation with 20 µM HOHA-lactone for up to 2 h by Western blot analysis. As shown in Figure 2.9 (see next page), a time dependent translocation of p53 from the cytosol to the nucleus was observed in ARPE-19 cells exposure to 20 µM HOHA-lactone, demonstrated by decreasing p53 levels in the cytosol and increasing p53 levels in the nucleus with increasing time of exposure.
**Figure 2.9** The translocation of p53 in ARPE-19 cells treated with 20 µM HOHA-lactone. Two different lysis buffers were used to extract the cytosolic component and membrane/nucleus component. GAPDH was used as a loading control.

**HOHA-lactone causes degradation of MDM2.** The mouse double minute (MDM2), an ubiquitin ligase, acts a negative regulator of the p53 protein that binds directly with the transactivation domain of p53 and assists in the ubiquitin-proteasomal degradation of p53. Under basal conditions, the half-life of the p53 protein in many cells varies between 6 and 20 minutes. When under cellular stress, MDM2 polyubiquitylates itself, resulting in the degradation of MDM2 and an increase in the half-life of p53 from minutes to hours. Therefore, we investigated the effect of HOHA-lactone on MDM2 in ARPE-19 cells. As shown in Figure 2.10 (see next page), the intracellular levels of MDM2 in ARPE-19 cells decreased with increasing concentrations of HOHA-lactone. This is consistent with the accumulation of p53 in ARPE-19 cells after treatment with HOHA-lactone. The decrease in the MDM2 levels clearly evident at 40 µM HOHA-lactone is in a good agreement with a drastic decrease in cell viability at 40 µM concentration of HOHA-lactone (Figure 2.4A, see page 53). Though not investigated in detail, the present studies suggested that HOHA-lactone induces p53-mediated apoptosis in ARPE-19 cells.
Figure 2.10 HOHA-lactone induces degradation of MDM2 in ARPE-19 cells. Western blot analysis of SDS PAGE separation of cell extracts collected from ARPE-19 cells after treatment with various concentrations of HOHA-lactone and probed with anti-MDM2 antibodies. GAPDH was used as a loading control.
2.3 Conclusions

The RPE, a primary site of pathology in AMD, is at high risk for oxidative stress due to its structural and functional features.\textsuperscript{2, 5} Under oxidative stress, DHA, an omega-3 fatty acid that comprises 60\% of the PUFAs in the retina,\textsuperscript{10} is exquisitely susceptible to lipid peroxidation to generate a multitude of reactive aldehydes including HOHA-lactone owing to the presence of the six double bonds between carbon atoms in its polyene chain (C=C) and five doubly allylic methylene groups.\textsuperscript{10, 22} Recently, HOHA-lactone was shown to react with the primary amino groups of biomolecules to produce CEP derivatives, thus acting as an important precursor of CEP.\textsuperscript{23} Previous studies suggested a significant association between CEP derivatives and AMD.\textsuperscript{19, 20, 24} This suggested that HOHA-lactone might be involved in the pathogenesis of AMD.

In the present study, authentic HOHA-lactone was efficiently synthesized using the method as described previously with minor modifications.\textsuperscript{23, 28} Considering that CEP-positive immunostaining was observed in HOHA-lactone-treated ARPE-19 cells, a spontaneously formed cell line from human RPE cells,\textsuperscript{23} and that this transformed cell line may respond differently to oxidative challenge compared to primary RPE and in vivo RPE,\textsuperscript{29, 30} it is important to also examine the post-translational modification of primary RPE cells to exposure to HOHA-lactone. Therefore, the post-translational modification of hRPE cells by HOHA-lactone was compared to that of ARPE-19 cells. It was found that hRPE cells possessed a similar pattern of post-translational modification in response to HOHA-lactone as ARPE-19 cells. However, HOHA-lactone caused less intense CEP-positive immunostaining in hRPE cells than ARPE-19 cells. Because both oxidative stress and inflammation have been implicated in the pathogenesis of AMD and CEP is a
biomarker of AMD, the effect of LPS, an inflammatory stimulus, and of H$_2$O$_2$-induced oxidative stress on the generation of CEP derivatives was also studied in both ARPE-19 and hRPE cells. As expected, the generation of CEP derivatives was observed under conditions of both inflammation and oxidative stress. Taken together, these results indicated that CEP derivatives accumulated in RPE cells either by exogenous addition of HOHA-lactone or by HOHA-lactone generated endogenously in RPE cells exposed to oxidative or inflammatory insults.

Given that oxidative stress contributes to RPE dysfunction in AMD and 4-HNE-treated RPE cells undergo apoptosis, a possible mechanism by which RPE cells are lost in AMD, HOHA-lactone, a structural analog of 4-HNE, was expected to induce RPE apoptosis, contributing to AMD. Thus, we firstly examined the cytotoxic effects of HOHA-lactone on RPE cells both in the ARPE-19 cell line and in primary human RPE cells by MTT assay. The results indicated that HOHA-lactone causes cell death in a concentration dependent manner. HOHA-lactone was less cytotoxic to the primary hRPE cells than to ARPE-19 cells. In addition, the present study was also undertaken to investigate the generation of ROS in both ARPE-19 and hRPE cells after treatment with HOHA-lactone using the DCF-DA. The results showed that HOHA-lactone treatment promotes intracellular ROS accumulation in a HOHA-lactone dose dependent manner in both ARPE-19 and hRPE cells. To examine whether HOHA-lactone toxicity to RPE cells involves the onset of apoptosis, we next tested whether HOHA-lactone induces apoptosis in ARPE-19 cells by three independent methods: in situ detection of activated caspase 3/7 using the NucView 488 caspase-3 assay, in situ detection of phosphatidylserine exposure using the Annexin V/PI assay and detection of caspase-3 activity by immunoblotting.
using a specific anti-caspase-3 antibody. The experiments clearly demonstrated that HOHA-lactone treatment activates caspase-3 apoptotic signaling in ARPE-19 cells. Finally, we investigated the molecular mechanisms for apoptosis in ARPE-19 cells after HOHA-lactone treatment. That study demonstrated that addition of HOHA-lactone to the medium of ARPE-19 cells in culture led not only to the induction and nuclear accumulation of p53 but also to the generation of elevated levels of phosphorylated p53. Along with the induction and phosphorylation of p53, HOHA-lactone was also found to induce degradation of MDM2 in ARPE-19 cells. Though not investigated in detail, the present studies suggested that HOHA-lactone induces apoptosis in ARPE-19 cells through p53 activation.

In conclusion, it has been documented that CEP derivatives accumulate in RPE cells either upon exposure to exogenous HOHA-lactone or by the putative endogenous generation HOHA-lactone in RPE cells caused by exposure to oxidative or inflammatory insults. We found that HOHA-lactone treatment induces cell death and increases oxidative stress in RPE cells and could activate caspase-3 apoptotic signaling in ARPE-19 cells. Though the detailed molecular basis for HOHA-lactone-induced RPE apoptosis remains to be thoroughly elucidated, that p53 is involved in the HOHA-lactone induced apoptotic process in ARPE-19 cells is indicated by the induction and phosphorylation of p53, nuclear accumulation of p53, and the degradation of MDM2. Therefore, HOHA-lactone may be an important mediator of oxidative stress-induced apoptosis that is likely to contribute to the etiology of AMD.
2.4 Experimental Procedures

Materials. Dulbecco’s modified Eagle’s (DMEM)/F12 medium, Dulbecco’s phosphate buffered saline (DPBS), fetal bovine serum (FBS) and 2’,7’-dichlorofluorescein diacetate (DCFDA) were purchased from Fisher Scientific (Pittsburgh, PA). Retinal pigment epithelial cell basal medium (RtEBM), optimized mixture of growth factors and supplements for primary hRPE cells (SingleQuots™ Kit) were obtained from Lonza (Allendale, NJ). Goat anti rabbit FITC antibody and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Invitrogen (Carlsbad, CA). The in situ apoptosis detection kits, NucView 488 Caspase 3 assay (DEVD-NucView 488-substrate) for live cells and CF488A-Annexin V-propidium iodide apoptosis assay kits were purchased from Biotium (Hayward, CA). All other chemicals and reagents were obtained from Sigma–Aldrich unless specified.

Antibodies against monoclonal mouse anti-human caspase-3, mouse anti-human total p53 antibody were purchased from Genetex (Irvine, CA). Anti-human phosphorylated p53 (ser15) antibody was obtained from Cell Signaling Technology (Danvers, MA). Mouse monoclonal antibodies against MDM2 and GAPDH as well as horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-mouse and anti-rabbit) were procured from Santa Cruz Biotechnology (Santa Cruz, CA). A monoclonal mouse anti-CEP antibodies was raised and characterized in this laboratory.19

RIPA lysis buffer was obtained from Cell Signaling Technology (Danvers, MA). The Western blot stripping buffer as well as the enhanced chemiluminescence (ECL) Western blot detection system were obtained from Pierce Biotechnology (Rockford, IL) via Fisher
Scientific (Pittsburgh, PA). Reagents and pre-cast gels (4-20% gradient and 16%) for SDS–PAGE were purchased from Invitrogen Life Technologies (Carlsbad, CA).

**General Methods.** Proton magnetic resonance (\(^1\)H NMR) spectra and carbon magnetic resonance (\(^{13}\)C NMR) spectra were recorded on a Varian Inova AS400 spectrometer operating at 400 MHz and 100 MHz, respectively. Proton chemical shifts are reported as parts per million (ppm) on the δ scale relative to CDCl\(_3\) (δ 7.26) or CD\(_3\)OD (δ 3.31). \(^1\)H NMR spectral data are tabulated in terms of multiplicity of proton absorption (s, singlet; d, doublet; dd, doublet of doublet; t, triplet; q, quartet; m, multiplet; br, broad), coupling constants (Hz), number of protons. Carbon chemical shifts are reported relative to CDCl\(_3\) (δ 77.0) or CD\(_3\)OD (δ 49.0). Flash chromatography was performed with ACS grade solvents from Fisher Scientific (Hanover Park, IL). R\(_f\) values are quoted for TLC plates of thickness 0.25 mm from Whatman (Florham Park, NJ). The plates were visualized with iodine, dinitrophenylhydrazine or phosphomolybdic acid reagents. For all reactions performed in an inert atmosphere, argon was used unless otherwise specified.

**Preparation of (E)-ethyl 4,7-dioxohept-5-enoate (2.2).** N-bromosuccinimide (NBS) (4.8 g, 27 mmol) was dissolved in THF-acetone-H\(_2\)O (5:4:1, 35 mL) and slowly added dropwise to a solution of ethyl 3-(furan-2-yl)propanoate (2.1, 3.08 g, 18 mmol) and pyridine (3 mL, 36 mmol) in THF-acetone-H\(_2\)O (5:4:1, 15 mL) at -20 °C under argon. The solution was stirred for 1 h at -20 °C and then slowly warmed to room temperature with the exclusion of light. Once TLC showed the complete disappearance of 2.1, the solvent was removed under reduced pressure. Then more ddH\(_2\)O was added and the residue was extracted with ethyl acetate (3 x 20 mL) and 50 mL of brine, followed by
drying of the combined extracts over anhydrous sodium sulfate. The solvents were evaporated under reduced pressure and the residue was purified by flash chromatography on a silica gel column (30% ethyl acetate in hexanes, TLC: Rf = 0.3) to give 2.2 (1.83 g, 55%). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 9.80 (d, $J = 7.0$ Hz, 1H), 6.86 (dt, $J = 16.3$, 11.7 Hz, 2H), 4.15 (q, $J = 7.1$ Hz, 2H), 3.02 (t, $J = 6.4$ Hz, 2H), 2.70 (t, $J = 6.4$ Hz, 2H), 1.26 (t, $J = 7.1$ Hz, 3H).

**Preparation of (E)-ethyl 7,7-dimethoxy-4-oxohept-5-enoate (2.3).** Compound 2.2 (670 mg, 3.7 mmol) was dissolved in dry dichloromethane (6 mL) and then mixed with Montmorillonite K-10 (1.2 g) and trimethyl orthoformate (1.2 mL). The reaction mixture was stirred at room temperature. Once TLC showed complete disappearance of compound 2.2, the reaction mixture was filtered through a pad of Celite 545 (10 g), followed by washing the residue with 15 mL of dichloromethane. The combined filtrate was dried by evaporation under reduced pressure. The crude compound 2.3 was purified by flash chromatography on a silica gel column (hexanes/ethyl acetate/triethylamine 80:20:1, TLC: Rf = 0.25) to give compound 2.3 (596 mg, 70%). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 6.60 (dd, $J = 16.2$, 4.0 Hz, 1H), 6.36 (dd, $J = 16.2$, 1.3 Hz, 1H), 4.92 (dd, $J = 4.0$, 1.3 Hz, 1H), 4.17 – 3.97 (m, 3H), 3.31 (s, 6H), 2.88 (t, $J = 6.6$ Hz, 2H), 2.59 (t, $J = 6.6$ Hz, 2H), 1.23 – 1.19 (m, 3H).

**Preparation of (E)-ethyl 4-hydroxy-7,7-dimethoxyhept-5-enoate (2.4).** Dimethyl acetal 2.3 (429 mg, 1.87 mmol) was dissolved in ethanol (12 mL). Then 160 µL triethylamine (TEA) were added. Then a solution of sodium borohydride (84 mg, 2.2 mmol) in ethanol (4.0 mL) were slowly added to the ice-cold reaction mixture. The reaction was stirred at 4 °C under Argon. Once TLC showed complete disappearance of
compound 2.3, methanol (4.0 mL) was added to destroy excess borohydride. Then saturated sodium chloride solution was added and the resulting mixture was extracted with ethyl acetate (3 x 50 mL). The organic extracts were combined and then dried with anhydrous sodium sulfate. The solvent was removed by rotary evaporation under reduced pressure. The residue was purified by flash chromatography on a silica gel column (hexanes/ethyl acetate/triethylamine 70:30:1, TLC: \( R_f = 0.19 \)) to give compound 2.4 (390 mg).

Preparation of (E)-3-(5-oxotetrahydrofuran-2-yl)acrylaldehyde (HOHA-lactone, 2.5). An ice-cold mixture of trifluoroacetic acid-water (3:2, 5.00 mL) was slowly added dropwise to a solution of compound 2.4 (116 mg, 0.5 mmol) in ice-cold chloroform (2 mL). The solution was stirred on ice for 4 h until the complete disappearance of compound 2.4. The solvent was removed by rotary evaporation under reduced pressure. The residue was purified by flash chromatography on a silica gel column (ethyl acetate / hexanes = 1:1, \( R_f = 0.2 \)) to give pure HOHA-lactone 2.5. (32 mg, 45%).\(^1\)H NMR (400 MHz, CDCl\(_3\)) \( \delta \) 9.62 (d, \( J = 7.5 \) Hz, 1H), 6.79 (dd, \( J = 15.8, 4.7 \) Hz, 1H), 6.35 (ddd, \( J = 15.8, 7.5, 1.5 \) Hz, 1H), 5.26 – 5.16 (m, 1H), 2.66 – 2.52 (m, 3H), 2.18 – 2.02 (m, 1H). HRMS (EI): m/z calcd for C\(_7\)H\(_8\)O\(_3\) (M\(^+\)), 140.0473; found, 140.0479.

Cell culture. The cell line ARPE-19 (ATCC; CRL-2302) derived from spontaneously arising retinal pigment epithelia of a healthy person, as described by Dunn et al.\(^{29}\) was obtained from the American Type Culture Collection (Manassas, VA). The stock cells were grown on 100-mm dishes in a humidified CO\(_2\) incubator at 37 °C and 5% CO\(_2\) in Ham’s F12 medium and Dulbecco’s modified Eagle’s medium (DMEM) (50:50),
containing L-glutamine and 10% heat-inactivated FBS. Cells were trypsinized and passed every 2-3 days.

Clonetics™ human primary retinal pigmented epithelial cells (hRPE) (passage 2) were obtained from Lonza (Allendale, NJ) and maintained and sub-cultured in RtEBM- Retinal Pigment Epithelial Cell Basal Medium supplemented with an optimized mixture of growth factors and supplements (SingleQuots™ Kit) and 10% heat-inactivated FBS. hRPE cells were passaged every 4-5 days.

**Post-translational modification of hRPE and ARPE-19 cells by HOHA-lactone.**

Either ARPE-19 cells or hRPE cells (2.5 x 10^4 cells/ per well) were plated on an 8-chamber well (Lab-Tek II Chamber Slide System, Nunc, Rochester, NY) in the corresponding complete medium (with 10% FBS) at 37 °C and 5% CO₂ overnight. After starving the cells in the corresponding basal medium for 4-5 hours, the cells were washed three times with the corresponding basal medium. Then, the cells were exposed to 0 or 10 µM HOHA-lactone in PBS buffer for 2 h at 37 °C and 5% CO₂. After the wells were aspirated, the corresponding basal medium was added to each well and the cells were incubated overnight at 37 °C and 5% CO₂. After incubation, the plates were centrifuged at 500g for 5 min and the medium was aspirated from each well. Then, the cells were aspirated, fixed and permeabilized with acetone at -20 °C. The slides were blocked with 1:100 diluted normal goat serum for 1 h. The cells were probed with mouse anti-CEP-monoclonal antibody (1:100 in NGS) followed by incubation with goat anti-mouse Alexa Fluor 488 antibody (1:200; Invitrogen, Carlsbad, CA). The slides were mounted in VectaShield containing DAPI mounting medium (Vector Laboratories, Burlingame, CA).
Cell images were taken at 10x magnification using a Leica DMI 6000 B inverted microscope.

**Detection of CEP in RPE cells incubated with H$_2$O$_2$.** Either ARPE-19 cells or hRPE cells (2.5 x 10$^4$ cells/ per well) were plated on an 8-chamber well (Lab-Tek II Chamber Slide System, Nunc, Rochester, NY) in the corresponding complete medium (with 10% FBS) at 37 °C and 5% CO$_2$ overnight. After starving the cells in the corresponding basal medium overnight, the cells were washed three times with the corresponding basal medium. Then the cells were exposed to 0 or 25 µM H$_2$O$_2$ in PBS buffer for 2 h at 37 °C and 5% CO$_2$. The cells were aspirated and washed three times with the corresponding basal medium followed by overnight incubation at 37 °C and 5% CO$_2$. After incubation, the plates were centrifuged at 500g for 5 min and the medium was aspirated from each well. Cell fixation in cold acetone and immunostaining was performed as described above for HOHA-lactone treated RPE cells.

**Detection CEP in RPE cells incubated with LPS.** Either ARPE-19 cells or hRPE cells (2.5 x 10$^4$ cells/ per well) were plated on an 8-chamber well (Lab-Tek II Chamber Slide System, Nunc, Rochester, NY) in the corresponding complete medium (with 10% FBS) at 37 °C and 5% CO$_2$ overnight. After starving the cells in the corresponding basal medium overnight, the cells were washed three times with the corresponding basal medium. Then, the cells were exposed to 0 or 12 µg/mL LPS in PBS buffer overnight at 37 °C and 5% CO$_2$. After incubation, the plates were centrifuged at 500g for 5 min and the medium was aspirated from each well. Cell fixation in cold acetone and immunostaining was performed as described above for HOHA-lactone treated RPE cells.
Cell viability assay (MTT assay). The sensitivity of hRPE and ARPE-19 cells to HOHA-lactone was evaluated using MTT as described by van Meerloo et al.33 Briefly, either hRPE or ARPE-19 cells were seeded in 96-well flat bottom plates at a density of 4.5x10^4 cells per well in 200 µl of the corresponding cell culture medium supplemented with 10% FBS and allowed to attach to culture plates in a humidified CO₂ incubator at 37 °C and 5% CO₂. After starving the cells in the corresponding basal medium for 4 to 5 hours, the medium was changed to 200 µl of basal medium containing various concentrations of HOHA-lactone (0-100 μM). After incubation overnight (about 16 h) at 37 °C in 5% CO₂, the supernatants were removed and the cells were washed three times with the corresponding basal medium. Then 20 µL of MTT solution (5 mg/ml in basal medium) plus 180 µL of basal medium were added to each well, and the plates were incubated for an additional 4 h in a CO₂ incubator at 37 °C and 5% CO₂. The plates were then centrifuged at 1,000 g for 5 min and the medium was aspirated from each well. 200 µL of dimethylsulfoxide (DMSO) was added to each well and the water-insoluble intracellular formazan crystals were dissolved by carefully pipetting each well. The absorbance was then measured with a plate reader (Model M3, Molecular Device) at 570/670 nm. The concentration of HOHA-lactone resulting in a 50% decrease in formazan formation was calculated as the LC₅₀ value of HOHA-lactone.

Visualization of intracellular reactive oxygen species. Intracellular generation of ROS in hRPE and ARPE19 cells as the result of HOHA-lactone treatment was evaluated using DCFH-DA according to the method of Wang and Joseph47 with minor modifications. Briefly, either hRPE or ARPE-19 cells (4.5 x 10⁴ cells/ per well) were plated on an 8-chamber well (Lab-Tek II Chamber Slide System, Nunc, Rochester, NY)
in the corresponding complete medium (with 10% FBS). After starving the cells in the corresponding basal medium for 4-5 hours, the cells were pre-incubated with 13.3 μM DCFDA for 45 min. After removing the medium, the cells were further incubated with medium containing various concentrations of HOHA-lactone (0 - 30 μM) for another 30 min at 37 °C in 5% CO₂. Images were acquired on a Leica DMI 6000 B inverted microscope (Leica Microsystems Wetzlar, Germany) using a Retiga EXI camera (Q-imaging, Vancouver, British Columbia). Image analysis was performed using Metamorph Imaging Software (Molecular Devices, Downington, PA).

**In situ detection of caspase-3/7 activity using fluorescence microscopy.** Visualization of activation of caspase 3/7 activity in ARPE-19 cells after treatment with HOHA-lactone was done by using an in situ apoptosis detection kit, NucView 488 caspase 3 assay kit (DEVD-NucView 488-substrate) for live cells. Briefly, ARPE-19 cells (2 x 10⁴ cells/ per well) were plated in 96-well microtiter plates in complete DMEM/F12 medium overnight under 5% CO₂/95% air at 37 °C. The following day, the cells were starved in the basal medium for 4-5 hours. The cells were then incubated with 0 or 15 μM HOHA-lactone overnight under 5% CO₂/95% air at 37°C. Then the plates were centrifuged at 400g for 5 min and washed with basal DMEM /F12 medium followed by detection of apoptotic cells with the NucView 488 caspase 3 assay kit according to the manufacturer’s suggested protocol. Finally, images were acquired at 10x magnification with a Leica DMI 6000 B inverted microscope.

**Detection of apoptosis using an Annexin V assay.** Detection of PS exposure in ARPE-19 cells as the result of HOHA-lactone treatment was carried out by the CF488A-Annexin V - propidium iodide (PI) apoptosis assay kit using fluorescence microscopy.
ARPE-19 cells (2 x 10⁴ cells/ per well) were plated in 96-well microtiter plates in complete DMEM/ F12 medium overnight under 5% CO₂/95% air at 37°C. The following day, the cells were starved in basal DMEM /F12 medium for 4-5 hours. The cells were then incubated with 0 or 15 µM HOHA-lactone overnight in 5% CO₂/95% air at 37 °C. Then the plates were centrifuged at 400g for 5 min and washed with basal DMEM/F12 medium followed by staining with Annexin V and PI according to the instructions of the kit. Finally, images of the stained cells were acquired at 10x magnification with a Leica DMI 6000 B inverted microscope.

**Cell treatment of ARPE-19 cells with HOHA-lactone for signaling studies.**

Monolayers of ARPE-19 cells were grown in 100 mm culture dishes in complete DMEM/ F12 medium overnight under 5% CO₂/95% air at 37 °C. The following day, the cells were starved in the basal medium overnight and washed with the corresponding basal medium. For studies of dose-dependence, the starved ARPE-19 cells were challenged with HOHA-lactone (0 - 40 µM) for 2 h at 37 °C, 5% CO₂. For time course studies, the starved ARPE-19 cells were treated with 20 µM HOHA-lactone for either 0, 15, 30, 60, 90, or 120 minutes in 5% CO₂/95% air at 37 °C. The cells were then scraped with a rubber policeman, transferred to 15-ml conical tubes and centrifuged at 480g 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 by 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 1x HALT protease inhibitor cocktail for 15 min at 4 °C. The cell lysate was placed on ice and sonicated [Model CL-18, Fisher Scientific] 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.

**Western blot analysis.** Extracts from ARPE-19 cells (25-30 μg protein/lane) were separated by SDS-PAGE (4 - 20% gels) and the proteins were electrotransferred onto a nitrocellulose 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 with the indicated antibody overnight 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 1 h. The membrane was again washed with TBST and the immunoblot was developed with the SuperSignal West Pico Chemiluminescent Substrate from Pierce Biotechnology (Rockford, IL) according to the manufacturer's instructions. For the detection of caspase-3, 16% gels were used. Representative Western blots from three independent experiments are shown.

**Statistical Analysis.** Statistical analyses were performed by using Student’s t test. P value <0.05 is considered as statistically significant. Representatives of p-value in figures include “*” p<0.05, “**” p<0.002, “***” p<0.0001. Data are presented as mean ± SD.

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CHAPTER 3

HOHA-LACTONE INDUCES ANGIOGENESIS THROUGH SEVERAL MOLECULAR PATHWAYS
3.1 Background

The retina, a light-sensitive layer that lines the back of the eye, is particularly susceptible to oxidative damage due to its intensive oxygenation and high levels of polyunsaturated fatty acids (PUFAs).\textsuperscript{1-3} The retinal pigment epithelium (RPE) plays critical roles not only in the maintenance of the normal functions of the retina and but also in the homeostasis of different retinal structures like photoreceptors or choriocapillaries by secreting various growth factors including vascular endothelial growth factor (VEGF).\textsuperscript{2, 4} RPE dysfunction has been implicated as the cause of many retinal diseases, including retinitis pigmentosa and age-related macular degeneration (AMD).\textsuperscript{1, 2, 5}

Angiogenesis is the process of forming new blood vessels from pre-existing blood vessels. It plays a central role in physiological processes such as embryogenesis and wound healing and is an important pathway in the progression of diseases states such as cancer and inflammation.\textsuperscript{6} VEGF, a homo-dimeric glycoprotein, is known to be directly implicated in the angiogenesis of cancers and also in the retinal microenvironment.\textsuperscript{5, 7} In the retina, VEGF is essential for the development of retinal and choroidal vascularization as well as the neuroretina.\textsuperscript{5} However, VEGF is now emerging as a risk factor for AMD in which vascular hyperpermeability and neovascularization are observed.\textsuperscript{8} It has been shown that VEGF levels are significantly increased in retinas and plasma of AMD subjects.\textsuperscript{8, 9} There is evidence suggesting that human retinal pigmented epithelial (RPE) cells could be the source of VEGF in the choroidal neovascularization of advanced AMD.\textsuperscript{5} The successful clinical applications of the anti-VEGF compounds bevacizumab, ranibizumab, and pegaptanib in AMD strongly support the importance of VEGF
involvement in the progression of neovascular AMD.\textsuperscript{2,10,11} However, the stimuli leading to enhanced VEGF secretion from RPE cells and the subsequent neovascularization in AMD remain unclear.

Oxidative stress is considered to be one of the major detrimental factors in the etiology of AMD.\textsuperscript{11,12} Under oxidative stresses, PUFA phospholipids, especially those containing docosahexaenoic acid (DHA), an omega-3 fatty acid that comprises 60\% of the PUFAs in the retina, undergo oxidation and truncation to generate a multitude of reactive aldehydes including HOHA-PC. (Scheme 3.1).

\textbf{Scheme 3.1} Generation and reactions of HOHA-PC from DHA-PC.

HOHA-PC has an extraordinary proclivity for deacylation (\(t_{1/2} = 30\) minutes at 37 °C) producing 3-(5-oxotetrahydrofuran-2-yl)acrylaldehyde (HOHA-lactone), a notable degradation product.\textsuperscript{3,13} Recently, this \(\alpha,\beta\)-unsaturated lactone was demonstrated to react with the primary amino groups of biomolecules to produce carboxyethylpyrrole (CEP) derivatives. (Scheme 3.1)\textsuperscript{14} CEP, a biomarker of AMD, was found to be more abundant in AMD than in normal Bruch’s membrane/RPE/choroid tissues.\textsuperscript{15} The levels of CEP derivatives had also been shown to be elevated in human plasma from AMD donors in
comparison to the levels of CEP in normal healthy donors.\textsuperscript{16, 17} Recently, animal model studies also demonstrated that CEP adducts stimulate angiogenesis that leads to choroidal neovascularization, and promotes wound healing and tumor growth through toll-like receptor 2 (TLR2) signaling.\textsuperscript{18, 19} In addition, in vitro studies carried out with a human retinal pigmented epithelial cell line (ARPE-19), a spontaneously formed cell line from human RPE cells, revealed two main pathways of HOHA-lactone metabolism. These are: (i) oxidation to a carboxylic acid by aldehyde dehydrogenase (ALDH), and (ii) conjugation with glutathione (GSH) to form an aldehyde adduct HOHA-lactone-GSH(=O) (3.2) that is then reduced to an alcohol HOHA-lactone-GSH(-OH) (3.3)\textsuperscript{20} (Scheme 3.2, see next page). GSH-HOHA-lactone(=O), but not HOHA-lactone-GSH(-OH), was found to produce CEP derivatives when incubated with proteins or with Ac-Gly-Lys-OMe dipeptide.\textsuperscript{20} No data clearly identified the GSH conjugates of HOHA-lactone in human primary retinal pigmented epithelial cells (hRPE), and although glutathione Michael adducts of HOHA-lactone were identified as important metabolites of HOHA-lactone, the cytotoxicity of these GSH conjugates had not been investigated.
Scheme 3.2 Structures of GSH conjugates of HOHA-lactone in ARPE-19 cells.

Oxidative stress has been found not only to increase the production of VEGF but also to be involved in the upregulation of VEGF expression. Reactive oxygen species (ROS) and lipid peroxidation products, including oxidized phospholipids (OxPLs), malondialdehyde (MDA) and 4-hydroxynonenal (HNE), significantly induce the secretion of VEGF in RPE cells. Interestingly, ROS and HNE exhibit hormetic (biphasic effects) on cellular activity; low levels of ROS or HNE induce proliferation and cell growth of vascular cells, however high levels of these stimuli induce apoptosis. Similar to the reported hormetic effect of HNE, our recent studies showed for the first time that HOHA-lactone, a structural analog of HNE, promotes VEGF secretion at low concentrations, and inhibits VEGF secretion at high concentrations in ARPE-19 cells. These findings pose an intriguing question as to how HOHA-lactone is able to exert such effects on cellular processes.
Since elevated oxidative stress and VEGF levels as well as reduced plasma GSH were observed with AMD,\textsuperscript{26, 29} one objective of this study was to determine whether HOHA-lactone depletes GSH and thus induces oxidative stress and VEGF secretion in ARPE-19 cells. Therefore we investigated the dose-dependent effect of HOHA-lactone on cell viability, VEGF secretion by ARPE-19 cells, intracellular GSH levels, and oxidative stress. The second objective of this study was to determine whether the secretion of VEGF from RPE cells induced by HOHA-lactone has physiological consequences. To this end, the effects of HOHA-lactone induced VEGF secreted from RPE cells on migration and tube formation of human umbilical vein endothelial cells (HUVECs) were examined in vitro. Moreover, the formation of GSH conjugates of HOHA-lactone (GSH-HOHA-lactone (\(=\text{O}\)) and GSH-HOHA-lactone (-OH)) in hRPE cells were also investigated. More importantly, the cytotoxicity of the resulting GSH metabolites as well as their possible physiological consequences were also examined in vitro. Furthermore, the pro-angiogenic properties of GSH conjugates of HOHA-lactone toward HUVECs were investigated. The results of these studies show for the first time, that HOHA-lactone causes angiogenesis in HUVECs by more than one molecular pathway. Previous observations showed that HOHA-lactone serves as an important precursor of CEPs, which can promote angiogenesis at sub-micromolar concentrations. The present study shows that HOHA-lactone may contribute to choroidal neovascularization either through the induction of VEGF secretion by RPE cells, or by conjugation with GSH or with the primary amino groups of biomolecules to form the corresponding GSH conjugates or CEPs that, in turn, also promote angiogenesis in surrounding endothelial cells.
3.2 Results and Discussion

HOHA-lactone promotes biphasic concentration-dependent effects on the survival of ARPE-19 cells. To assess the effect of HOHA-lactone accumulation on RPE cells, we examined the cytotoxicity of HOHA-lactone to ARPE-19 cells by MTT and LDH assays. The MTT assay is based on the ability of metabolically active prokaryotic and eukaryotic cells to perform intracellular reduction of the yellowish water-soluble tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) into a purple water-insoluble formazan product (E,Z-5-(4,5-dimethylthiazol-2-yl)-1,3-diphenylformazan; with an absorbance maximum near 570 nm) by NAD(P)H-dependent cellular oxidoreductase enzymes of viable cells. Biochemical evidence indicates that MTT is taken up by the live cells and is mainly reduced in the cytoplasm by NADH (and in lesser amount by NADPH) and dehydrogenases associated with the endoplasmic reticulum (ER) endosome/lysosome vesicles, plasma membrane and mitochondria (~25%). Given that during glucose metabolism, two molecules of NADH are produced during glycolysis (in the cytoplasm), two more during synthesis of acetyl CoA (connecting reaction) and six NADH are produced per one glucose molecule during the Krebs cycle which takes place in mitochondria, the MTT assay can be considered as a “genuine” indicator of live cell “redox activity” or cell viability. Because the reduction of MTT mostly occurs by NADH and NADPH in cytosol along with dehydrogenases associated with cell mitochondria, measurement of the intracellular levels of accumulated formazan is an indirect measure of the function of mitochondria, i.e. when the cell dies mitochondria cease to function and produce NADH needed to reduce MTT. Thus, in the MTT assay, when the same number of cells, i.e., ARPE-19 cells, are treated with the
cytotoxic agent HOHA-lactone, which interferes with the function of mitochondria to produce NADH leading to cell death (e.g. apoptosis), the decrease in the levels of intracellular insoluble formazan compared to the levels of intracellular insoluble formazan in the control (untreated) cells is a measure of the decrease in the production of NADH by mitochondria and is also a measure of the number of cells capable of reducing MTT. The opposite effect, i.e., increased NADH production and concomitant reduction of MTT, of a substance on live cells indicates cell growth and proliferation. When the same number of cells, i.e. ARPE-19 cells, is treated with HOHA-lactone and shows an increase in the levels of intracellular insoluble formazan compared to the levels of intracellular insoluble formazan in the control (untreated) cells, a corresponding increase in the number of mitochondria capable of producing NADH corresponding to an increase in cell numbers owing to proliferation is indicated. Because the reduction of MTT occurs intracellularly in the cytosol and/or on the mitochondrial membrane, it indicates that the cell plasma membrane is intact. It also suggests that the cell death occurs through apoptosis. We used the following formula to evaluate the cell viability using the MTT assay: Cell survival (%) = 100x(A_{sample} - A_b)/(A_c - A_b) where A_{sample} is the absorbance of sample, A_b is the absorbance of blank well and A_c is the absorbance of the control untreated cell well.

Our data show that HOHA-lactone treatment of ARPE-19 elicited a biphasic dose-response, where higher levels of HOHA-lactone (>10 μM) decreased the cell number and low levels of HOHA-lactone (0.1-10 μM) increased the cell number (Figure 3.1A, see next page). Treatment with higher concentrations of HOHA-lactone (>10 μM) significantly decreased cell viability, with LC_{50} = 38.6 ± 6.4 μM. However, the toxicity
of HOHA-lactone was not significant at relatively lower (< 10 µM) concentrations, with more than 90% cell viability after overnight incubation (Figure 3.1A). More interestingly, HOHA-lactone at concentrations less than 1 µM considerably stimulated cell proliferation. Cell viability above 100% was observed when ARPE-19 cells were incubated with 1-500 nM HOHA-lactone. The maximum of 155.2 ± 15.0% cell viability, which is significantly higher than control cells (p-value < 0.0001), was observed with 10 nM HOHA-lactone, a much lower physiologically relevant concentration. These results indicated that HOHA-lactone induces ARPE-19 cell growth at low concentrations.

**Figure 3.1** Effect of HOHA-lactone on the survival of RPE cells. (A) ARPE-19 cells were treated with 0 – 100 µM of HOHA-lactone overnight (approximately 16 hours) and assayed for cytotoxicity by MTT (Panel A) and LDH assay (Panel B). The plots show the percent cell survival (mean ± SD, n = 8) at various concentrations of HOHA-lactone.

There are two major forms of cell death observed in the normal and disease states, apoptosis and necrosis. Unlike apoptotic cells, necrotic cells are characterized by swelling and rupture of intracellular organelles, that eventually lead to the breakdown of
the plasma membrane and concomitantly to the release of intracellular contents into the extracellular medium.\textsuperscript{34} The lactate dehydrogenase (LDH) assay is used to measure the degree of cell necrosis, i.e., plasma membrane damage. It is based on the measurement of LDH enzymatic activity, which is released from necrotic cells into the cell culture medium immediately after cell damage by a cytotoxic substance, e.g., HOHA-lactone. In this assay, LDH promotes conversion of lactate to pyruvate using NAD\textsuperscript{+} as a cofactor through a coupled enzymatic reaction with diaphorase that, using NADH as a cofactor, converts MTT a yellow tetrazolium salt into a pink formazan product that can be measured quantitatively using a microplate reader.\textsuperscript{35} We used the following formula to evaluate the cell viability using this assay: Cell survival (\%)=100\times\frac{1-(A_{\text{sample}}-A_{c})}{(A_{\text{max}}-A_{c})} where A_{\text{sample}} is the absorbance of sample well, A_{c} is the absorbance of the control untreated cell well and A_{\text{max}} is the absorbance of the maximum LDH activity controls.

The results of LDH leakage (Figure 3.1B, see page 95) from the cells were consistent to those of MTT assay, indicating that HOHA-lactone starts to damage the cell plasma membrane of ARPE-19 cells only at a concentration above 40 \(\mu\text{M}\) (Figure 3.1B, see page 95). Apoptosis is detectable at lower concentrations of HOHA-lactone, i.e., between 10 and 20 \(\mu\text{M}\) (Figure 3.1A, see page 95).

**HOHA-lactone promotes VEGF secretion from ARPE-19 cells.** Recent studies showed that 4-HNE exerts concentration dependent opposite effects on the secretion of VEGF from RPE cells. At low levels, 4-HNE causes increased secretion of VEGF from RPE cells, but at higher concentration it inhibits VEGF secretion.\textsuperscript{28} Given that HOHA-lactone incorporates the \(\alpha,\beta\)-unsaturated aldehyde functional array of 4-HNE, we hypothesized that it would elicit a response similar to that caused by 4-HNE, i.e., to
stimulate the secretion of VEGF by RPE cells at sub-micromolar concentrations. To test this proposition, we treated ARPE-19 cells with various concentrations of HOHA-lactone, and determined VEGF levels in the extracellular medium using a human VEGF-A ELISA kit (Pierce, Rockford, IL). The data (Figure 3.2, see next page) shows that while untreated control ARPE-19 cells had 1295±50 pg/ml levels of VEGF-A in extracellular medium, ARPE-19 cells treated with 0.1 to 10 μM HOHA-lactone exhibited significantly increased VEGF secretion, which reached a maximal level at 0.1 μM with 3168 ± 125 pg/ml (2.45-fold). Elevated secretion of VEGF was still evident when ARPE-19 cells were maintained in the presence of 10 μM HOHA-lactone, which elicited 1780±205 pg/ml levels (37% increase over the normal levels). On the other hand, the production of VEGF-A by ARPE-19 cells at higher levels of HOHA-lactone, e.g., 25 μM, significantly diminished VEGF secretion.
Figure 3.2 Effect of HOHA-lactone on VEGF secretion by ARPE-19 cells. ARPE-19 cells grown in a 96-well plate (25,000 cells per well) were treated with serum-free culture media containing varying concentrations (0-25 μM) of HOHA-lactone. After 16 h of incubation, supernatants were collected and secreted VEGF in the supernatants was measured using a human VEGF-A ELISA kit. The data represent the mean SD (n = 8) (*** p<0.0001).

HOHA-lactone induced VEGF secretion is dependent of oxidative stress in ARPE-19 cells. Given that HOHA-lactone is a structural analog of 4-HNE, which is known to induce VEGF secretion via formation of intracellular ROS,26 we hypothesized that HOHA-lactone induces the secretion of VEGF through a similar mechanism. To strengthen the hypothesis that oxidative stress played a role in HOHA-lactone-induced production of VEGF, the oxidative stress was measured in HOHA-lactone-treated ARPE-19 cells by 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) assay. The theory behind

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using the DCFDA assay is that cell-permeant DCFDA is hydrolyzed enzymatically by intracellular esterases to nonfluorescent 2',7'-dichlorofluorescin (DCFH) that cannot leave the cell due to its charged nature, which is further oxidized to highly fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of ROS, which are generated in HOHA-lactone-treated ARPE-19 cells.\textsuperscript{36} As shown in Figure 3.3 (see next page), HOHA-lactone exposure at 0.1-1.0 µM triggered a significant increase of ROS in ARPE-19 cells compared to the control untreated cells, as measured by the increase of DCF fluorescence intensity. Compared to untreated ARPE-19 cells, the fluorescence intensity from DCF was increased 2.5-fold (p<0.0001) and 4-fold upon treatment ARPE-19 cells with 0.1 µM and 0.5 µM HOHA-lactone, respectively. These data indicated that exposure to HOHA-lactone caused oxidative stress, which coincided with HOHA-lactone induced VEGF production in a concentration-dependent manner.
Figure 3.3 HOHA-lactone at sub-micromolar concentrations induces generation of intracellular reactive oxygen species in ARPE-19 cells. A: representative micrographs of cells with various treatment as indicted. B: quantification of the fluorescence intensities. After DCFDA pretreatment, ARPE-19 cells (4.5 x 10^4 cells) were further incubated with 0-1 μM HOHA-lactone before analysis. The images are representative of four independent experiments showing very similar results. The data in the bar graph represents the mean ± SD (n = 4).

The involvement of intracellular GSH in HOHA-lactone induced production of VEGF in ARPE-19 cells. GSH is the major intracellular cytosolic antioxidant that is directly involved in detoxification of ROS. To confirm that the induction of oxidative
stress by HOHA-lactone in ARPE-19 cells is related with GSH levels in particular. GSH levels were measured in both ARPE-19 control cells and in ARPE-19 cells challenged with HOHA-lactone. Intracellular GSH levels were tested after treating ARPE-19 cells with low concentrations of HOHA-lactone using a spectrofluorometric microplate method described earlier.\textsuperscript{37} This assay involves the reaction of GSH with the sulfhydryl reagent 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) to produce a yellow derivative 5′-thio-2-nitrobenzoic acid (TNB) and oxidized glutathione–TNB adduct (GS–TNB). Then, GS-TNB and glutathione disulfide (GSSG) is reduced to GSH by glutathione reductase (GR) in the presence of β-NADPH, recycling the GSH. The TNB formed can be quantitated at 412 nm.\textsuperscript{37} Treatment of ARPE-19 cells with various amounts of HOHA-lactone for two hours resulted in a concentration-dependent progressive decrease of intracellular levels of GSH from 52.0±2.5 nmol/mg protein in untreated cells to 21.5±0.5 nmol/mg protein in cells exposed to 10 μM HOHA-lactone (Figure 3.4, see next page). Furthermore, as shown in Figure 3.4 (see next page), even exposure to 1.0 μM HOHA-lactone decreases the GSH level by 23%. These data suggested that the induction of VEGF secretion by HOHA-lactone may result from depletion of intracellular GSH levels and resulting increase in oxidative stress in ARPE-19 cells.
Figure 3.4 Concentration-dependent effect of HOHA-lactone on intracellular GSH levels in ARPE-19 cells. 2×10⁶ ARPE-19 cells were incubated with various concentrations of HOHA-lactone. After 2 h incubation, cells were harvested and intracellular GSH was determined by a colorimetric assay. Data are expressed as a mean ± S.D for n = 3. Data shown are representative of three independent experiments that show very similar results.

HOHA-lactone induced VEGF secretion from ARPE-19 cells increases the migration and wound healing capability of HUVECs in vitro. Because VEGF is a primary inducer of pathological neovascularization, and because cell migration is necessary for the involvement of endothelial cells in neovascularization,²⁸ the ability of ARPE-19 cells to show pro-angiogenic tendencies after HOHA-lactone treatment was tested using an in vitro “wound healing” migration assay with HUVECs.³⁸ As shown in Figure 3.5 (see next page), as control, some degree of cell migration was observed when the conditioned medium (CM) from PBS-treated ARPE-19 cells was used on HUVECs, which is reasonable considering that ARPE-19 is a cancer cell line and secrets basal
levels of angiogenic cytokines. The conditioned medium (CM) derived from HOHA-lactone treated ARPE-19 cells produced a significantly higher level of HUVEC migration than the CM derived from PBS-treated ARPE-19 cells.

Figure 3.5 Effect of ARPE-19 conditioned medium on HUVECs in the wound healing assay. A: representative micrographs of cells with various treatment as indicted. B: quantification of wound healing assay. HUVECs (1x10^5 cells in 300 µl of a cell culture medium) were injured by a scratch with a 200 µL pipette tip. Wounded cells were allowed to heal for 24 h in the presence of conditioned medium from control and HOHA-lactone-treated (0-2.0 µM) ARPE-19 cells. The images are representative of four independent experiments showing very similar results. The data in bar graph represents the mean ± SD (n = 4).

HUVECs maintained in the CM from ARPE-19 cells exposed to 0.1-2 µM HOHA-lactone for 24 h showed robust dose-dependent progressive reoccupation of the wounded region and reached the maximal level at 2 µM HOHA-lactone. These data provide, for
the first time, clear in vitro evidence for HOHA-lactone-induced wound healing. The secretion of VEGF from ARPE-19 cells likely contributes to this response of HUVECs.

**HOHA-lactone induced secretion of VEGF from ARPE-19 cells increases tube formation by HUVECs in an in vitro angiogenesis assay.** To further investigate the pro-angiogenic effects of VEGF secreted from HOHA-lactone-treated ARPE-19 cells, the matrigel tube formation assay, which is a convenient and quantifiable method to test the vascular formation properties on HUVECs in vitro, was performed. As shown in Figure 3.6A, treatment of HUVECs with CM from HOHA-lactone treated ARPE-19 cells increased the total length of tube-like structures in comparison with the control PBS treatment. The photographs were image-analyzed and the total tube lengths were measured (Figure 3.6B, see next page). When the HUVECs were incubated with the CM from ARPE-19 cells exposed to 0.1-1 µM HOHA-lactone, the tube lengths increased in a dose-dependent manner and reached the greatest level at 0.25 µM HOHA-lactone. These results further confirm that the release of VEGF by HOHA-lactone treated ARPE-19 cells can lead to angiogenesis in vitro.
Figure 3.6 Effect of RPE conditioned medium on HUVEC tube formation. A: representative micrographs of cells with various treatments as indicted. B: quantification of tube formation assay. HUVECs (2.5x10^4 cells/well) in 24-flat bottom tissue culture plate were allowed to grow on matrigel (175µL) for 4 h in presence of conditioned medium from ARPE-19 cells treated with 0 – 1 µM of HOHA-lactone for 16 hours. The cells were stained with Calcein AM for 1 hour at 37 °C in a CO₂ incubator. The images are representative of four experiments showing very similar results. The data in the bar graph represents the mean ± SD (n = 4).

Formation and export of the glutathione conjugate of HOHA-lactone in RPE cells. As described above, HOHA-lactone is toxic and significantly decreases cell viability at high concentrations. Similar to 4-HNE, a structural analog of HOHA-lactone, HOHA-lactone was expected to be detoxified in living cells by oxidative, reductive and
conjugative pathways.\(^9\) In addition, the previous cellular metabolism studies of HOHA-lactone from our laboratory have characterized the degree to which oxidative, reductive, and conjugative metabolic pathways function simultaneously to rapidly eliminate free HOHA-lactone in cultured ARPE-19 cells.\(^\text{20}\) In the current studies, we evaluated the capacity of RPE cells, including both ARPE-19 cells developed from the retina of adult young male and human primary retinal pigmented epithelial cells (hRPE), to eliminate HOHA-lactone on the basis of the activity per \(10^6\) of cells, as well as the fate of the resulting metabolites. Relatively low concentrations of HOHA-lactone (10 \(\mu\)M) were used to simulate pathological conditions of mammalian retina under chronic oxidative stress.

To characterize the metabolites of HOHA-lactone in RPE cells, authentic samples of HOHA-lactone 3.1, the glutathione adduct HOHA-lactone-GSH(=O) 3.2 and the reduced glutathione adduct HOHA-lactone-GSH(-OH) 3.3 were prepared by unambiguous chemical syntheses\(^\text{20}\) (Scheme 3.3), and a quantitative LC-/MS/MS method was developed comprising a reverse-phase chromatographic separation of analytes and electrospray mass spectrometry.

![Scheme 3.3 Chemical synthesis of authentic aldehyde 3.2 and alcohol 3.3.](image)

After treating RPE cells (both ARPE-19 and hRPE cells) with 10 \(\mu\)M HOHA-lactone, 20.0 \(\mu\)L of both intra-cellular (cell lysate, CL) and extra-cellular medium (ECM),
presumably containing GSH metabolites of HOHA-lactone, was directly injected for LC-MS/MS analysis. A MS/MS scan of the collision-induced dissociation (CID) generated ions at m/z 448.4 and 450.4 for compound 3.2 and 3.3, respectively, was set in positive ion mode from m/z 200 to 400, and MS/MS collision energy was set at 35%. Representative daughter ions, m/z 301 and 321, were chosen as MRM transitions for fragmentations of the corresponding parent ions m/z 448.5 and 450.5 for 3.2 and 3.3 analysis, respectively. The proposed structures of the major fragments in the MS2 spectrum of 3.2 and 3.3 are shown in Scheme 3.4.

![Scheme 3.4 Proposed structures of m/z 448.4 and 450.4 CID mass spectrometric fragment ions.](image)

As shown in Figure 3.7A (see next page), the mass chromatograms relative to compound-specific MRM transitions in the positive-ion mode of the corresponding [M+H]+ ions m/z 448.4 to the fragment ions at m/z 301 showed that treatment of ARPE-19 cells with 10 µM HOHA-lactone resulted in a significant peak that elutes at the same
retention time as the authentic 3.2 in both intra- and extracellular fractions, while the 10 μM HOHA-lactone treated hRPE cells only had a peak at this retention time in ECM. Similarly, a significant peak in LC-MS/MS spectrum was observed in the transition 450.4→321 with the same retention as authentic 3.3 in both ECM and CL from 10 μM HOHA-lactone treated RPE cells (Figure 3.7B). These results confirmed that both compound 3.2 and 3.3 were major metabolites of HOHA-lactone in RPE cells.

Figure 3.7 LC-MS/MS analysis (positive ion mode) of metabolites (A) 3.2 and (B) 3.3 in HOHA-lactone treated ARPE-19 and hRPE cells. A: LC chromatograms showing the MS2 transition m/z 448.4→301.0; B: LC chromatograms showing the MS2 transition m/z 450.4→321.0. For compound 3.2 analysis, ARPE-19 and hRPE cells were incubated with 10 μM HOHA-lactone for 15 min and 30 min, respectively. For compound 3.3 analysis, ARPE-19 and hRPE cells were incubated with 10 μM HOHA-lactone for 30 min and 45 min, respectively.
The GSH metabolites 3.2 and 3.3 of HOHA-lactone in both CL and ECM from RPE cells were quantitated by LC-MS/MS analysis over the time course of treatment and expressed in pmol/10⁶ cells. MS/MS analysis was performed in the positive ion mode monitoring the transition 448.4→301 for 3.2 and 450.4→321 for 3.3, respectively. As shown in Figure 3.8, treatment of RPE cells with HOHA-lactone engendered a time-dependent increase of HOHA-lactone-GSH levels. Intracellular levels of metabolite 3.2 increased exponentially and rapidly reached maximal concentrations of 845±225 pmol per 10⁶ ARPE-19 cells during the first 5 min of incubation and then were eliminated immediately (Figure 3.8A).

**Figure 3.8** Time course and quantification of HOHA-lactone-derived GSH conjugates in (A) ARPE-19 cells and (B) hRPE cells after treatment with 10 μM HOHA-lactone. Quantitation of 3.3 (■), 3.2 (●) in extra-cellular media (ECM) and 3.3 (▲), 3.2(▼) in cell lysates (CL). Values represent means ± S.D. of three independent experiments.

Paralleling elimination of metabolite 3.2 from the CL, extracellular levels of metabolite 3.2 in ARPE-19 cells treated with 10 μM HOHA-lactone increased, establishing that there is a unidirectional export of 3.2 from the cytosol to ECM. 10 μM
of HOHA-lactone exogenously added to RPE cells resulted in maximal extracellular concentrations for metabolite 3.2 of 1620±48 pmol per 10⁶ ARPE-19 cells⁻¹ at 15 min of incubation, which then rapidly decreased to a level with no difference from that of 0 min. Over the time course studied during ARPE-19 exposure to 10 μM of HOHA-lactone, LC-MS/MS data showed that formation of metabolite 3.3 occurred at a much slower rate than metabolite 3.2 in both intra- and extra-cellular fractions. The metabolite 3.3 is the reduction product of HOHA-lactone derived aldehyde glutathione adduct 3.2. The reduction was presumably catalyzed by alcohol dehydrogenase (ADH). Similar to the formation time course of metabolite 3.2 in the CL, the metabolite 3.3 accumulated with the maximal concentrations of 284±75 pmol per 10⁶ ARPE-19 cells⁻¹ at 15 min and then was rapidly removed from the intracellular fraction (Figure 3.8A, see page 109). The rapid formation of metabolites 3.2 and 3.3 in the cell lysate demonstrated that HOHA-lactone can easily diffuse through the cell membrane into ARPE-19 cells and be metabolized in those cells by conjugation with GSH. The metabolite 3.3 accumulated rapidly in ECM during the first 45 min of incubation after addition of HOHA-lactone to ARPE-19 cells and then attained these apparent steady-state levels up to 120 min (Figure 3.8A, see page 109).

Having shown the formation and export of the glutathione conjugates of HOHA-lactone in ARPE-19 cells, which are derived from a human retinal pigmented epithelial cell line, we next tested primary human RPE (hRPE) cells. Upon treatment with HOHA-lactone, they showed similar behavior. As shown in Figure 3.8B (see page 109), the reduced GSH metabolite 3.3 of HOHA-lactone appeared rapidly in the intracellular fraction achieving concentrations of 13.4 ± 3.8 pmol per 10⁶ hRPE cells⁻¹ at 5 min and
then was efficiently eliminated, coinciding with a rise in the levels of 3.3 in the extracellular medium. The aldehyde-GSH metabolite of HOHA-lactone, 3.2, rapidly reached maximal extracellular concentrations of $1230 \pm 160$ pmol $10^6$ hRPE cells$^{-1}$ after incubation for 45 min and was eliminated thereafter. In contrast to cell lysates from ARPE-19 cells, compound 3.2, which may be below detectable limits by LC-MS/MS analysis, was not detected in the cytosol from hRPE cells treated with 10 μM HOHA-lactone during the time-course experiment.

Addition of 10 μM HOHA-lactone to RPE cells resulted in a notable drop in intracellular GSH (Figure 3.9) coincident with rapid production of HOHA-lactone-GSH adducts. Presumably, conjugation with GSH through Michael addition of its nucleophilic sulfur to the electrophilic C-3 of HOHA-lactone in RPE cells is catalyzed by glutathione S-transferase (GST). Following rapid depletion of cellular GSH, its level remained constant, only increasing 120 min after exposure to HOHA-lactone, especially in hRPE cells.

**Figure 3.9** Time course of intracellular glutathione levels in (A) ARPE-19 cells and (B) hRPE cells after treatment with 10 μM HOHA-lactone. Values represent means ± S.D. of three independent experiments.
Next we examined concentration-dependent formation of HOHA-lactone-GSH adducts in ARPE-19 cells treated with HOHA-lactone over the concentration range from 0 to 100 µM for 2 h by LC-MS/MS described above. As shown in Figure 3.10, levels of HOHA-lactone-GSH adducts in ARPE-19 cells increased in a concentration dependent manner in the HOHA-lactone concentration range examined. The extracellular levels of both metabolites, aldehyde 3.2 and alcohol 3.3, increased significantly and then plateaued with the increase in amount of HOHA-lactone added. Incubation of HOHA-lactone with ARPE-19 cells resulted in a concentration-dependent formation of alcohol 3.3 in the cell lysate whereas no aldehyde 3.2 was observed over the range from 0 to 100 µM concentrations of added HOHA-lactone. Apparently, both metabolites are efficiently transported out of the cells.

![Figure 3.10](image_url)

**Figure 3.10** Concentration-dependent formation of HOHA-lactone-derived GSH conjugates in ARPE-19 cells over the concentration range from 0 to 100 µM HOHA-lactone. Quantitation of 3.3 (■), 3.2 (●) in extra-cellular media (ECM) and 3.3 (▲) in cell lysates (CL). Values represent means ± S.D. of three independent experiments.
Cytotoxicity of HOHA-lactone-GSH conjugates in HUVECs. Although it is well-known that conjugation with GSH is a major detoxification pathway, there is evidence indicating that several classes of compounds are converted to cytotoxic, genotoxic, or mutagenic metabolites after conjugation with GSH. Therefore, to test whether this conjugative metabolism may result in toxicity of HOHA-lactone, the cytotoxicity of HOHA-lactone-GSH (=O) 3.2 and HOHA-lactone-GSH (-OH) 3.3 in HUVECs was evaluated based on its effects on cell viability. Incubation of HUVECs with various concentrations of 3.2 or 3.3 overnight, caused an increase compared to the untreated control, in cell viability for all concentrations of HOHA-lactone-GSH conjugates tested (Figure 3.11, see next page). The dose-response to treatment of HUVECs with HOHA-lactone-GSH conjugates was biphasic. Although HOHA-lactone-GSH conjugates can stimulate HUVEC cell proliferation, the effect was not significant at concentrations less than 10 µM and 1 µM for 3.2 and 3.3, respectively. During these low effect range, the maximum increase of 105.7± 5.0% and 133.6± 6.8% in cell viability was achieved at the corresponding concentration of 0.005µM and 0.075µM for 3.2 and 3.3, respectively. However, treatment of higher concentrations of HOHA-lactone-GSH conjugates (>10 µM for 3.2 and 1 µM for 3.3) significantly increased cell viability, with the maximum of 137.5± 7.0% and 181.0± 2.5% cell viability at 75µM and 2.5µM for 3.2 and 3.3, respectively. Interestingly, a significant decrease in the cell viability (58.1± 8.9%) was observed in cells treated with 100 µM of 3.2 while no drop was observed in cells with 100 µM of 3.3 treatment. Taken together, these data show that HOHA-lactone-GSH conjugates are not toxic metabolites. Instead, they can stimulate HUVEC cell proliferation in a dose-dependent manner.
Figure 3.11 Concentration-dependent effect of (A) HOHA-lactone-GSH (=O) and (B) HOHA-lactone-GSH (-OH) on HUVEC cell viability. HUVECs were incubated with 0 – 100 μM of HOHA-lactone-GSH conjugates overnight (approximately 16 hours) and assayed for cytotoxicity by MTT. Results expressed as % of untreated control are means ± SD of three independent experiments (n = 8).

**HOHA-lactone-GSH conjugates induce angiogenesis in vitro on HUVECs.** As described above, HOHA-lactone-GSH conjugates can increase cell viability at the concentrations tested. Therefore, the possible pro-angiogenic effects of HOHA-lactone-GSH conjugates on angiogenesis were also investigated using cell migration and tube formation assays.

In a wound healing assay, HOHA-lactone-GSH (=O) accelerated wound “closure” in cultured HUVECs (Figure 3.12A, see next page) in comparison with the control HUVECs treated with PBS. As shown in Figure 3.12B (see next page), HUVECs treated with HOHA-lactone-GSH (=O) at concentrations of 0.1 to 2 μM showed significant increases of “wound healing” (scratch closure) in a dose-dependent manner compared with HUVECs treated with PBS, with the maximal increase at 1 μM HOHA-lactone-
GSH (=O). Similarly, HOHA-lactone-GSH (-OH) also caused a stimulation of cell migration in a dose-dependent manner (Figure 3.13, see page 116) and reached the maximal level at 0.25 µM (Figure 3.13B, see page 116).

**Figure 3.12** The pro-angiogenic effect of HOHA-lactone-GSH (=O) on HUVECs in a wound healing assay. A: representative micrographs of HUVECs incubated in the presence of various concentrations of HOHA-lactone-GSH (=O). B: quantification of wound healing assay. HUVECs (1x10\(^5\) cells in 300 µl of a cell culture medium) were injured by a scratch with a 200 µL pipette tip. Wounded cells were allowed to heal for 24 h in the presence of various concentrations of HOHA-lactone-GSH (=O) (0-2.0 µM). The images are representative of four independent experiments showing very similar results. The data in the bar graph represents the mean ± SD (n = 4).
Figure 3.13 The pro-angiogenic effect of HOHA-lactone-GSH (-OH) on HUVECs in a wound healing assay. A: representative micrographs of HUVECs incubated in the presence of various concentrations of HOHA-lactone-GSH (-OH). B: quantification of wound healing assay. HUVECs (1x10^5 cells in 300 µl of a cell culture medium) were injured by a scratch with a 200 µL pipette tip. Wounded cells were allowed to heal for 24 h in the presence of various concentrations of HOHA-lactone-GSH (-OH) (0-2.0 µM). The images are representative of four independent experiments showing very similar results. The data in bar graph represents the mean ± SD (n = 4).

We also investigated the effects of HOHA-lactone-GSH conjugates on tube formation, a marker of angiogenesis, in human endothelial cells. As shown in Figure 3.14 (see page 118), the effect of HOHA-lactone-GSH (=O) on the tube-forming activity of HUVECs
was not similar to that on migration activity. When the HUVECs were pretreated with HOHA-lactone-GSH (=O) at concentrations between 0.1 and 2 µM, the quantification of tube formation (Figure 3.14B, see page 118) indicated that the maximal increase in the total tube length observed at 0.1 µM decreased with further increases in HOHA-lactone-GSH (=O) concentrations to a level with no difference from that of control PBS-treated cells at 1 µM, and it dropped further at 2 µM. Upon treatment with HOHA-lactone-GSH (-OH) at concentrations between 0.1 and 2 µM (Figure 3.15, see page 119), tube-forming activity first increased significantly and reached a maximum level at 0.5 µM compared with control. Higher concentrations (2 µM) of HOHA-lactone-GSH (-OH) reduced the total tube length back to that found for the control PBS-treated HUVECs.
Figure 3.14 The pro-angiogenic effect of HOHA-lactone-GSH (=O) on HUVECs in a tube formation assay. A: representative micrographs of HUVECs incubated in the presence of various concentrations of HOHA-lactone-GSH (=O). B: quantification of tube formation. HUVECs (2.5x10^4 cells/well) in 24-flat bottom tissue culture plate were allowed to grow on matrigel (175µL) for 4 h in the presence of various concentrations of HOHA-lactone-GSH (=O) (0 - 2.0 µM) of HOHA-lactone for 16 hours. The cells were stained with Calcein AM for 1 hour in 37 °C in a CO₂ incubator. The images are representative of four experiments showing very similar results. The data in the bar graph represents the mean ± SD (n = 4).
Figure 3.15 The pro-angiogenic effect of HOHA-lactone-GSH (-OH) on HUVECs in a tube formation assay. A: representative micrographs of HUVECs incubated in the presence of various concentrations of HOHA-lactone-GSH-(OH). B: quantification of tube formation. HUVECs (2.5x10^4 cells/well) in 24-flat bottom tissue culture plate were allowed to grow on matrigel (175µL) for 4 h in presence of various concentrations of HOHA-lactone-GSH (-OH) (0-2.0 µM) of HOHA-lactone for 16 hours. The cells were stained with Calcein AM for 1 hour in 37 °C in a CO₂ incubator. The images are representative of four experiments showing very similar results. The data in the bar graph represents the mean ± SD (n = 4).
3.3 Conclusions

Although AMD, a slow, progressive, multifactorial, polygenic disease, is the most common cause of legal blindness in the elderly population in developed countries, the cellular, biochemical, and molecular events contributing to the etiology of AMD remain poorly understood.\textsuperscript{16, 18} Oxidative stress has long been suspected of contributing to the pathogenesis of AMD.\textsuperscript{11, 12} Direct evidence of oxidative damage in AMD includes elevated levels of ROS\textsuperscript{21-23} and retinal lipid peroxidation products such as HNE\textsuperscript{26-28} and CEP adducts.\textsuperscript{15-17} The retina is particularly susceptible to oxidative damage due to its intensive oxygenation and high levels of polyunsaturated fatty acids (PUFAs).\textsuperscript{1-3} Considering that DHA, the precursor of HOHA-lactone is present in abundance in the retina,\textsuperscript{3, 13} it is likely that retinal degeneration associated with aging may be mediated via HOHA-lactone. Because RPE damage is an early event in AMD, it is important to delineate the role of HOHA-lactone in the degeneration of RPE.

Angiogenesis can either promote host defense and tissue repair or exacerbate organ dysfunction resulting in disease.\textsuperscript{19} In the retina VEGF, the main factor in angiogenesis, is constitutively secreted by the RPE to exert its physiological functions. For example, VEGF is an important protective factor for retinal cells and the choroid and is essential for the development of retinal and choroidal vascularization.\textsuperscript{5} However, VEGF is now emerging as a pathogenic factor in several retinal conditions, most notably in edema, retinopathy of prematurity and choroidal neovascularization.\textsuperscript{8} Overexpression of VEGF in RPE cells of the retina was suggested to be a responsible factor in the development of choroidal neovascularization in vivo. Recently, oxidative stress causing agents and
electrophiles like 4-HNE were found to significantly induce VEGF secretion from RPE cells.\textsuperscript{26, 28}

Given that 4-HNE, a structural analog of HOHA-lactone, stimulates angiogenesis by induction of VEGF secretion in vitro,\textsuperscript{26, 28} the present study was undertaken to investigate the potential roles of HOHA-lactone treatment in producing VEGF secretion in ARPE-19 cells, which in turn induce angiogenesis in HUVECs. The results of this study indicated that HOHA-lactone induces the secretion of VEGF in the human retina cell line in a concentration dependent manner. Similar to the reported hormetic effect of 4-HNE on ARPE-19, HOHA-lactone at low levels promotes VEGF secretion in cells and inhibits this secretion at high levels. In addition, we observed that HOHA-lactone induces oxidative stress in ARPE-19 cells, indicated by increased fluorescence intensity generated from 2,7-DCFDA, an oxidative stress probe. Moreover, following HOHA-lactone treatment, ARPE-19 cells exhibited decreased levels of GSH that is an indication of oxidative stress in these cells. The HOHA-lactone induced secretion of increased levels of VEGF by ARPE-19 cells correlated well with an increase in intracellular ROS and a decrease in intracellular GSH. During this study, we have investigated the physiological consequences of the secretion of VEGF induced by low levels of HOHA-lactone. Using wound healing and tube formation assays, we provided the first in vitro evidence for the release of VEGF by HOHA-lactone treated ARPE-19 cells that leads to increased cell migration and tube formation by HUVECs treated with conditioned medium from HOHA-lactone treated ARPE-19 cells. These data indicated that HOHA-lactone can stimulate vascular growth in HUVECs through a VEGF-dependent pathway.
In a previous study from our laboratory, it had been confirmed that HOHA-lactone can be detoxified by oxidative, reductive and conjugative pathways in cultured ARPE-19 cells, a cell line that spontaneously emerged from human RPE cells. In this chapter, we have extended the investigation of HOHA-lactone metabolism to evaluate the ability of both, ARPE-19 cells and human primary retinal pigmented epithelial (hRPE) cells to detoxify HOHA-lactone, as well as to establish the trafficking and some biological activities of the resulting metabolites.

LC-MS/MS analyses of both intra-cellular components and extra-cellular medium from RPE cells treated with 10 μM HOHA-lactone demonstrated that HOHA-lactone readily diffuses into cells where it becomes conjugated with GSH in the cytosol, forming two major metabolites 3.2 and 3.3, that are transported from cytosol to extracellular medium. The Michael adduct of HOHA-lactone 3.2, was detected in both the cell lysates and the extracellular medium from ARPE-19 cells. However, it was only detected in extracellular medium from hRPE cells demonstrating more efficient transport out of these cells than from ARPE-19 cells. The reduced conjugated metabolite 3.3 is more prominent compared to the aldehyde 3.2, demonstrated by the detection in both intra- and extra-cellular fractions from ARPE-19 and hRPE cells. The addition of HOHA-lactone to RPE cells resulted in a time-dependent depletion of intracellular reduced GSH with the concomitant production of HOHA-lactone-GSH adducts, consistent with GST-catalyzed conjugation of HOHA-lactone with GSH. The generation of alcohol 3.3 in RPE cells presumably involves the co-operative action of both GST and ADH. Previously, Hua et al. demonstrated that HOHA-lactone-GSH adduct 3.2 can react with proteins and Ac-
Gly-Lys-OMe dipeptide to produce CEP derivatives. The generation and conjugative metabolic pathways for HOHA-lactone are summarized in Scheme 3.5.

![Scheme 3.5 Pathways for HOHA-lactone generation and its conjugative metabolism with GSH and adduction with primary amino groups of biomolecules. GSH, glutathione; GST, glutathione S-transferase; ADH, alcohol dehydrogenases.](image)

Results from the MTT assay revealed that low concentrations of HOHA-lactone-GSH conjugates had little effect on HUVEC cell proliferation, but levels above 10 μM of the aldehyde 3.2 or 1.0 μM alcohol 3.3 dose dependently increased the cell number. Since HOHA-lactone-GSH conjugates can stimulate HUVEC cell proliferation, the possible pro-angiogenic effects of HOHA-lactone-GSH conjugates were also investigated by cell migration and tube formation assays. These experiments demonstrated that exposure of HUVECs to HOHA-lactone-GSH conjugates increased both wound healing and tube formation activities in vitro. These results indicated that besides VEGF, HOHA-lactone...
stimulates the formation of other pro-angiogenic factors, such as CEP and HOHA-lactone-GSH conjugates.

These results, coupled with the previous study that HOHA-lactone is a major precursor of CEP adducts\textsuperscript{14} and the demonstration that CEP adducts promote angiogenesis in a TLR2 dependent manner, a novel mechanism that is independent of hypoxia-triggered VEGF expression,\textsuperscript{18, 19} indicate that HOHA-lactone can stimulate angiogenesis by four different molecular mechanisms. One indirect mechanism for in vitro HOHA-lactone stimulation of angiogenesis was that HOHA-lactone induces the secretion of VEGF by retinal pigmented epithelial cells that can contribute to the induction of angiogenesis. Another two molecular mechanisms by which HOHA-lactone induces angiogenesis are related to either the formation of HOHA-lactone-GSH conjugates or CEP adducts, which we now showed can promote angiogenesis in vitro. The fourth angiogenesis pathway induced by HOHA-lactone may involve the formation of HOHA-lactone-GSH 3.2, which then produces CEP derivatives by reaction with primary amino groups of biomolecules such as protein lysyl residues or ethanolamine phospholipids. The proposed angiogenesis pathways mediated by HOHA-lactone are summarized in Scheme 3.6 (see next page).
Scheme 3.6 Angiogenesis pathways mediated by HOHA-lactone.

The contribution of HOHA-lactone to angiogenesis may vary in different physiological/pathological settings, depending on the extent of oxidative stress. Low levels of HOHA-lactone may be generated as a consequence of a low level of oxidative stress and promote protective mechanisms in four different pro-angiogenic signaling pathways, thereby contributing to accelerated wound healing and tissue recovery under physiological conditions. If high levels of HOHA-lactone accumulate due to persistent oxidative stress in tissues, it may lead to excessive vascularization, for example, in tumors. Currently, anti-VEGF therapies are the standard of care for treating AMD and other neovascular retinal diseases. Recently, anti-CEP therapy was suggested to be a promising therapeutic intervention to staunch the growth of tumors, especially in
diseases resistant to anti-VEGF therapy. The present study suggests that HOHA-lactone-driven angiogenesis may also be a therapeutic target and anti-HOHA-lactone therapy may be effective independently or as a complement to anti-VEGF or anti-CEP therapies for the inhibition of neovascular AMD. Owing to molecular similarities, it also seems possible that anti-CEP antibodies will cross react with GSH-HOHA-lactone adducts, and that the efficacy of those antibodies for inhibiting angiogenesis and tumor growth may include blocking the angiogenic activities of those adducts. Further studies should test this hypothesis.
3.4 Experimental Procedures

Materials. Dulbecco’s modified Eagle’s (DMEM)/F12 medium, Dulbecco’s phosphate buffered saline (DPBS), fetal bovine serum (FBS) and 2’,7’-dichlorofluorescein diacetate (DCFDA) were purchased from Fisher Scientific (Pittsburgh, PA). Human VEGF-A ELISA kit was purchased from Piercenet (Thermo Fisher Scientific, Rockford, IL). Retinal pigmented epithelial cell basal medium (RtEBM), optimized mixture of growth factors and supplements for primary hRPE cells (SingleQuots™ Kit ) were obtained from Lonza (Allendale, NJ). RIPA lysis buffer was obtained from Cell Signaling Technology (Danvers, MA). Goat anti rabbit FITC antibody and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Invitrogen (Carlsbad, CA). All other chemicals and reagents including L-glutathione reduced, sodium borohydride, glutathione reductase (GR) (250 units• ml⁻¹), 5,5’-dithio-bis(2-nitrobenzoic acid) (DTNB), and β-NADPH were purchased from Sigma–Aldrich (St. Louis, MO). HOHA-lactone 3.1 was prepared as described in Chapter 2.

Cell culture and treatment. 2 x 10⁶ of ARPE-19 cells or 1.5 x 10⁶ of hRPE cells were grown in 60 mm culture dishes and washed with the corresponding basal medium. For studies of dose-dependence, 3 mL of basal medium containing various concentrations of HOHA-lactone (0-100 μM) were added and the mixture was incubated for 2 h at 37 °C, 5% CO₂. For time course studies, 3 mL of basal medium containing 10 μM of HOHA-lactone were added and the mixture was incubated for up to 2 h at 37 °C, 5% CO₂. At the end of incubation, the supernatants were collected and designated the “extracellular medium” (ECM). Then the cell dishes were washed three times with the
corresponding basal medium, followed by the addition of 400 μL of basal medium. The cells were scraped and collected, and then subjected to sonication at 4 °C and designated the “cell lysate” (CL). The ECM and CL were kept at -20 °C until analysis.

**Cell viability assays.** ARPE-19 or HUVEC cells (4.5x10⁴ cells per well) were seeded in 96-well plates in DMEM/F12 or HUVEC cell culture medium supplemented with 10% heat-inactivated FBS at 37 °C, 5% CO₂ overnight. After starving the cells in a basal medium for 4 to 5 hours, nothing or 200 μl of basal cell culture medium containing various concentrations of HOHA-lactone or HOHA-lactone-GSH conjugates (0-100 μM) were added. After incubation at 37 °C, 5% CO₂ overnight (about 16 h), cell viability was estimated by MTT assay for both HOHA-lactone and HOHA-lactone-GSH conjugates and lactate dehydrogenase (LDH) assay only for HOHA-lactone according to the manufacturer’s instructions.

For MTT assay, in brief, at the end of the overnight incubation, the supernatants were removed and the cells were washed with basal cell culture medium three times and then incubated with 20 μL of MTT solution (5 mg/ml in basal medium) plus 180 μL of basal medium at 37 °C and 5% CO₂ for 4 h. The plates were then centrifuged at 1,000g for 5 min and the medium was aspirated from each well. Dimethylsulfoxide (DMSO, 200 μL) was added to each well and the absorbance of this solution was measured with a plate reader (Model M3, Molecular Device) at 570/670 nm.

For LDH assay, 10μL of lysis buffer (10X) was added to the wells serving as the maximum LDH activity controls followed by incubation at 37 °C, 5% CO₂ for 45min. Then, the plates were centrifuged at 1,000g for 5 min and the supernatants were collected. 100 μL of each sample was transferred to a 96-well plate and mixed with 100 μL of LDH
reagent mixture. After incubation for another 30 min at room temperature, 50 μL of stop solution was added and the absorbances of the samples were measured at 490/680 nm.

**Measurement of secreted VEGF-A from ARPE-19 cells.** ARPE-19 cells grown in 96-well plates (2.5x10⁴ cells per well) were incubated with 200 μL of basal DMEM/ F12 media containing various concentrations (0 - 25 μM) of HOHA-lactone. After incubation for 16 h, supernatants were collected to estimate secreted VEGF using an enzyme-linked immunosorbent assay (ELISA) according to manufacturer’s protocol (Human VEGF-A ELISA kit, Piercenet). Absorbance was measured using a plate reader (Molecular Devices) with a test wavelength of λ = 450 nm and a reference wavelength set at λ = 550 nm.

**Measurement of oxidative stress.** 4.5 x 10⁴ ARPE-19 cells were plated in 8-chamber slides (LabTek II) in complete DMEM/F12 culture medium overnight at 37 ºC and 5% CO₂. ARPE-19 cells were starved the following day by incubation in basal DMEM/F12 medium for 4 to 5 hours. Then cells were pre-incubated with 13.3 μM DCFDA for 45 min, washed and further incubated with 0 - 30 μM HOHA-lactone for another 30 min at 37 ºC, 5% CO₂. Images were acquired on a Leica DMI 6000 B inverted microscope (Leica Microsystems Wetzlar, Germany) using a Retiga EXI camera (Q-imaging, Vancouver, British Columbia). Image analysis was performed using Metamorph Imaging Software (Molecular Devices, Downington, PA).

**Quantification of intracellular GSH in hRPE and ARPE-19 cells.** Aliquots (10 μL) of hRPE or ARPE cell lysates from time-course and dose-dependence studies were assayed to determine intracellular GSH using a spectrofluorometric microplate method described earlier.³⁷ In this experiment, all the reagents were prepared in 0.1M potassium
phosphate buffer with 5 mM EDTA disodium salt, pH 7.5 (KPE buffer). Briefly, 10 μL of KPE buffer, GSH standards or samples were added to the corresponding microplate wells, followed by the addition of 120 μL of a freshly prepared mixture of DTNB (1mg/3ml) and GR (5U/3ml). Then, 60.0 μL of β-NADPH (2mg/3ml) was added and mixed well. The plate was immediately placed in a microplate reader (Molecular Devices) and absorbance was measured at a wavelength of 412 nm after mixing for 2 min or 10 min for ARPE-19 or hRPE cells, respectively.

**Wound healing assay.** An in vitro wound-healing assay was performed using a previously described method. In brief, HUVECs (1x10^5 cells /well) were grown to confluence on a 24-well tissue culture plate at 37 °C and 5% CO2 overnight. The cell monolayer was scraped in a straight line to create a “scratch” with a sterile 200 μL micropipette tip. Then the plate was washed once and 450 μL of basal HUVEC medium was added, followed by the addition of 50 μL of HOHA-lactone-GSH conjugate or the conditioned media obtained from ARPE-19 cell cultures with or without HOHA-lactone treatment to the corresponding wells to make final concentrations from 0.1 μM to 2.0 μM. Cells were photographed immediately and 24 h after wounding. The images were acquired on a Leica DMI 6000 B inverted microscope (Leica Microsystems Wetzlar, Germany) using a Retiga EXI camera (Q-imaging, Vancouver, British Columbia). Image analysis was performed using Metamorph Imaging Software (Molecular Devices, Downington, PA).

**Tube Formation Assay.** HUVECs were starved in basal medium at 37 °C and 5% CO2 overnight and then seeded onto the 48-well plates (2.5x10^4 cells/well), which were pretreated with growth factor-reduced (GFR) matrigel (Trevigen Inc., Gaithersburg, MD)
(175 µl/well) for 1 h at 37 °C to allow the gel to solidify. Then HOHA-lactone-GSH conjugates or the conditioned medium obtained from ARPE-19 cell cultures with or without HOHA-lactone treatment was added to the corresponding cells to make final concentrations from 0.1 µM to 2.0 µM. Cells are incubated at 37 °C and 5% CO₂ overnight. The following day, cells were stained with Calcein AM (BD Biosciences) solution by adding 50 µL of working solution (0.01 mg/ml) to each well. After incubating for 45 min at 37 °C, images were acquired using a Leica DMI 6000 B inverted microscope (FITC filter, Leica Microsystems Wetzlar, Germany) equipped with a Retiga EXI camera (Q-imaging, Vancouver, British Columbia). Image analysis was performed using Metamorph Imaging Software (Molecular Devices, Downingtown, PA).

**Synthesis of HOHA-lactone-GSH adduct (3.2).** HOHA-lactone-GSH adduct 3.2 was synthesized as described previously with small modifications. In brief, 100 µmol of HOHA-lactone (3.1) was reacted with 200 µmol of reduced glutathione in 3.0 mL water at room temperature for 3 h. The excess of GSH was removed by solid-phase extraction (SPE) through a strata-X 33U polymeric reversed phase cartridge (Phenomenex, 500 mg/6ml). The cartridge was prewetted with 6 mL of methanol containing 0.1% formic acid and equilibrated with 6 mL of water containing 0.1% formic acid. After loading the sample, the cartridge was rinsed with 12 mL of water containing 0.1% formic acid to remove excess GSH and the adduct 3.2 was then eluted with 18 mL of 10% acetonitrile containing 0.1% formic acid. Structural identity of compound 3.2 was confirmed by mass spectroscopic analysis, which was compared with data obtained previously. ESI-MS: m/z calcd for C₁₇H₂₆N₃O₅S (M+H)⁺, 448.47, found 448.26.
Synthesis of reduced HOHA-lactone-GSH adduct (3.3). HOHA-lactone-GSH adduct 3.3 was prepared as described previously. Briefly, 10 μmol of HOHA-lactone-GSH adduct 3.2 was reduced by 12 μmol of sodium borohydride in 1.5 mL PBS buffer (pH = 7.4) at 4 °C for 5 h. Then the excess NaBH$_4$ was destroyed by adding 2 μL of formic acid. Its purification was performed by SPE as described above. ESI-MS: m/z calcd for C$_{17}$H$_{30}$N$_3$O$_9$S (M+H)$^+$, 450.48, found 450.76.

High performance liquid chromatography/mass spectrometry. LC-ESI-MS/MS analysis was performed on a Thermo Finnigan LCQ™ Deca system in the positive ion mode using nitrogen as the sheath and auxiliary gas. The heated capillary temperature was 300 °C, the source voltage was 4.5 kV, and the capillary voltage was 31.00 V. Chromatographic separation was carried out with a Surveyor LC system equipped with a Luna C18 column (150× 2.0 mm i.d. 5 μm, Phenomenex). The mobile phase consisted of solvent A (HPLC grade water containing 0.1% formic acid) and solvent B (HPLC graded methanol containing 0.1% formic acid). HPLC gradient steps were as follows: 0 – 10 min, linear gradient from 2 to 100% solvent B; 10 – 15 min, isocratic at 100% solvent B; 15 – 16 min, linear gradient from 100 to 2% solvent B; 16 – 25 min, isocratic at 2% solvent B. A flow rate of 200 μL/min and a run time of 25 min were used for all analyses. Before analysis, the collected cell lysates and extracellular media were centrifuged at 10,000 g for 5 min and an aliquot (20 μL) of the sample solution was employed for each LC–MS/MS analysis. All data were processed with Qual browser in Xcalibur software.

Statistical Analysis. Statistical analyses were performed by using Student’s t test. P value <0.05 is considered as statistically significant. Representatives of p-values in figures include “*” p<0.05, “**” p<0.002, “***” p<0.0001.
(ANOVA) was used for the analysis of VEGF secretion on exposure to HOHA-lactone. Data are presented as mean ± SD.

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CHAPTER 4

TOXICITY AND DETOXIFICATION OF HOOA-LACTONE, A PRECURSOR OF CARBOXYPROPYLPYRROLES, IN CULTURED RETINAL PIGMENTED EPITHELIAL CELLS
4.1 Background

Oxidative damage has been implicated in the development of many human diseases such as heart disease, cancer, cardiovascular diseases, neurodegenerative disorders and age-related macular degeneration (AMD). Lipids are essential cell membrane components and contain abundant polyunsaturated fatty acids (PUFAs), which are particularly susceptible to oxidative damage. Arachidonic acid (AA) is an omega-6 long-chain PUFA with 4 double bonds that is abundant in neural and vascular tissue of the retina and brain. Highest concentrations of AA in human retina are found in phosphatidylcholine (PC) and the next highest in phosphatidylethanolamine (PEA). The peroxidation of AA leads to the formation of aldehydes such as malondialdehyde (MDA), 4-hydroxynonenal (4-HNE) and 4-oxo-2-nonenal (4-ONE) (Scheme 4.1), which are highly reactive with cellular nucleophiles such as amino and thio functional groups in proteins and nucleic acids, causing lipid-derived oxidative damage in the form of covalent adducts and crosslinks.

Scheme 4.1 Structures of major aldehydes generated from AA lipid peroxidation.

Oxidative cleavage of phospholipids containing docosahexaenoate (C22) and arachidonate (C20) generates oxidatively truncated derivatives such as the 4-hydroxy-7-oxohept-5-enoic acid ester of 2-lyso-phosphatidylcholine (HOHA-PC) and the 5-hydroxy-8-oxo-6-octenoic acid ester of 2-lyso-PC (HOOA-PC). These γ-hydroxyalkenal phospholipids can react with primary amino groups in biomolecules leading to the generation of biologically active carboxyalkylpyrrole derivatives, i.e., 2-(ω-
carboxyethyl)pyrroles (CEPs), and 2-(ω-carboxypropyl)pyrroles (CPPs), respectively (Scheme 4.2). For example, CEPs and CPPs can be produced through adduction with the primary amino groups of protein lysyl residues followed by PLA2-catalyzed hydrolysis to deliver carboxyalkylpyrrole protein derivatives.

Alternatively, free fatty acids are released from membrane phospholipids under catalysis of phospholipase A2 (PLA2) during oxidative stress. These free fatty acids are also subject to oxidative truncation to generate γ-hydroxyalkenals that react with the primary amino groups of protein lysyl residues to produce carboxyalkylpyrrole protein derivatives (Scheme 4.2). Recently, HOHA-PC and HOOA-PC were found to undergo spontaneous deacylation to generate 2-lyso-PC and the corresponding HOHA-lactone and

**Scheme 4.2** Postulated CPP adduct generation *in vivo.*

Alternatively, free fatty acids are released from membrane phospholipids under catalysis of phospholipase A2 (PLA2) during oxidative stress. These free fatty acids are also subject to oxidative truncation to generate γ-hydroxyalkenals that react with the primary amino groups of protein lysyl residues to produce carboxyalkylpyrrole protein derivatives (Scheme 4.2). Recently, HOHA-PC and HOOA-PC were found to undergo spontaneous deacylation to generate 2-lyso-PC and the corresponding HOHA-lactone and
HOOA-lactone under physiological conditions, that is, 37 °C and pH 7.4. More recently, it was reported that HOHA-lactone serves as an intermediate for the generation of CEP derivatives. CEP derivatives are produced upon reaction of HOHA-lactone with biomolecules such as Ac-Gly-Lys-OMe, proteins and ethanolamine phospholipids. It seemed reasonable to anticipate that the reaction of HOOA-lactone with protein primary amino groups would generate CPP derivatives in analogy with the chemistry of HOHA-lactone (Scheme 4.2, see page 143). As one of end products of AA peroxidation, CPPs were found not only to promote physiological activity, i.e., angiogenesis required for wound healing, but also to accumulate in melanoma where they promote pathological effects, i.e., angiogenesis and consequent tumor growth. Thus, it is likely that CPPs contribute to the etiology of human diseases associated with oxidative stress.

Oxidative stress-induced lipid peroxidation (LPO) has been implicated in the pathogenesis of many degenerative ocular diseases, especially AMD, the leading cause of blindness in the developed world. In view of high consumption of oxygen, high levels of cumulative irradiation, abundance of photosensitizers and high proportion of PUFAs, the retina is particularly susceptible to oxidative stress. Retinal pigment epithelium (RPE) cells, which play a central role in retinal physiology by forming the outer blood-retinal barrier and supporting the function of the photoreceptors, are known to be rich in PUFAs. Under oxidative stresses, PUFAs, especially AA, undergo peroxidation and truncation to generate a multitude of LPO products including γ-hydroxy-α, β-unsaturated aldehydes such as 4-HNE. These γ-hydroxy-α, β-unsaturated aldehydes are reactive electrophiles that contain three functional groups: (i) a C=C double bond that can not only form a “Michael adduct” with nucleophiles like the thiol group of cysteine, the imidazole group of histidine
residues and the primary amino groups in lysyl residues by Michael additions, but also undergo reduction or epoxidation, (ii) a C=O carbonyl group which can not only be target to Schiff-base formation, oxidation, or reduction, but also can form acetal/thio acetals, (iii) a hydroxyl group than can undergo oxidation to form a ketone. 4,8 4-HNE, one of the most abundant α,β-unsaturated aldehydes, has been most intensively studied in relation not only to its signaling role to modulate various signaling pathways but also to its cytotoxic role causing long-lasting biological consequences, in particular by covalent modification of macromolecules. 4,15 13 4-HNE has been detected at elevated levels in several different models of retinal damage. 16,17 It has also been suggested that 4-HNE, acting as a secondary messenger, may induce signaling in RPE that leads to apoptosis. 18 Possessing the same α,β-unsaturated aldehyde functional array as 4-HNE, it is conceivable that HOOA-lactone would be anticipated to act similarly as a second messenger capable of inducing intracellular oxidative stress, causing cell signaling and cell death at high levels in RPE cells. We therefore herein investigated whether HOOA-lactone is capable of inducing intracellular oxidative stress and causing cell apoptosis using a human retinal pigmented epithelial cell line (ARPE-19), a spontaneously formed cell line from human RPE cells.

The metabolism of toxic aldehydes to less reactive molecules is required to ensure cell survival and normal functioning. 4-HNE can be detoxified either by oxidation or reduction of the aldehyde carbonyl group or conjugation with glutathione (GSH) that involve multiple enzymatic pathways. 14 In chapter 3, HOHA-lactone was demonstrated to conjugate with GSH to form HOHA-lactone-GSH (\(=O\)) aldehyde, and then to be reduced to HOHA-lactone-GSH (-OH) alcohol in RPE cells. In analogy with 4-HNE and HOHA-
lactone, HOOA-lactone was also expected to be detoxified by conjugation with GSH in ARPE-19 cells.

In this present study, we efficiently synthesized HOOA-lactone and accessed whether HOOA-lactone mimics HOHA-lactone to covalently modify the primary amino groups of lysyl residues to produce CPP derivatives. In addition, with ARPE-19 cells, we also investigated whether HOOA-lactone plays a role in cell signaling, focusing on apoptosis. Finally, we also examined whether HOOA-lactone can be detoxified by conjugation with GSH in ARPE-19 cells.
4.2 Results and Discussion

Synthesis of HOOA-lactone. HOOA-lactone was prepared using an efficient unambiguous chemical synthesis as described previously with minor modifications (Scheme 4.3). In brief, the regio- and stereoselective tributylstannylcupration of 3,3-diethoxyprop-1-yn (4.1) with the Lipshutz reagent, Bu3SnCu(Bu)CNLi2, readily produced vinyltin 4.2. The resulting 4.2 underwent acylation with glutaric acid monomethyl ester chloride catalyzed by PdCl2(CH3CN)2 to generate ketone 4.3, which was further reduced by sodium borohydride to give the stable masked γ-hydroxyalkenal 4.4. The target HOOA-lactone (4.5) was produced from 4.4 in the presence of strong acid catalysts: Amberlyst-15 and trifluoroacetic acid (TFA).

Scheme 4.3 Synthesis of HOOA-lactone.

HOOA-lactone reacts with Ac-Gly-Lys-OMe dipeptide to form CPP-dipeptide. To investigate whether HOOA-lactone can covalently modify the primary amino groups of lysyl residues to produce the corresponding CPP derivatives in analogy with the chemistry of HOHA-lactone, HOOA-lactone (4.5) was incubated with Ac-Gly-Lys-OMe dipeptide (4.6) in pH 7.4 phosphate buffered saline (PBS) at 37 °C for up to 7 days (Scheme 4.4, see next page). The resulting reaction mixture was analyzed by reverse phase HPLC-ESI/MS.
in the positive ion mode using authentic CPP-dipeptide (4.7) prepared by unambiguous chemical synthesis as a standard of comparison (Scheme 4.5).

Scheme 4.4 HOO\(_2\)-lactone (4.5) reacts with Ac-Gly-Lys-OMe (4.6) to form CPP-dipeptide (4.7).

Scheme 4.5 Chemical synthesis of authentic CPP-dipeptide.

The authentic CPP-dipeptide was prepared as described in Dr. Li Hong’s thesis. In brief, the propylene acetal group of 7-(1,3-dioxan-2-yl)-5-oxoheptanoic acid (4.8) from Dr. Li Hong was removed by incubation with aqueous acetic acid to give the γ-keto aldehyde carboxylic acid 4.9. Then the Paal-Knorr synthesis between free acid 4.9 and dipeptide 4.6 was successfully applied to generate CPP-dipeptide (4.7, Scheme 4.5).

As shown in Figure 4.1 (see next page), the mass spectrum of authentic CPP-dipeptide (4.7) in the positive ion mode shows three peaks at m/z 396.5, 418.5 and 434.5, which correspond to the [M+H]\(^+\), [M+Na]\(^+\) and [M+K]\(^+\) ions from CPP-dipeptide (4.7), respectively. Thus, a peak in the selected ion chromatogram (SIC) with a selected ion m/z
396.5 and a retention time comparable to the authentic standard that was available from unambiguous total syntheses was used for quantitative determination of CPP-dipeptide (4.7) from the incubation mixture of HOOA-lactone with dipeptide 4.6. Figure 4.2 (see next page) shows the SICs of m/z 396.5 from authentic CPP-dipeptide and reaction mixture of HOOA-lactone and Ac-Gly-Lys-OMe dipeptide at 37 °C after 4 days incubation. The results indicated that HOOA-lactone can modify dipeptide to produce CPP-dipeptide, evident by the significant peak at 5.40 min from the reaction mixture after 4-day incubation with a retention time that is identical to the standard 4.7.

**Figure 4.1** ESI-MS scan of authentic CPP-dipeptide 4.7 in the positive ion mode.
Figure 4.2 Selected ion chromatograms (SICs) in the positive ion mode of m/z 396.5 from (A) authentic CPP-dipeptide standard and (B) the reaction mixture of HOOA-lactone and Ac-Gly-Lys-OMe dipeptide at 37 °C after four-day incubation.

The generation of CPP-dipeptide during the incubation of HOOA-lactone with Ac-Gly-Lys-OMe dipeptide as a function of incubation time was then quantified from the peak area of SIC m/z 396.5 by comparison with a calibration curve established with authentic CPP-dipeptide standard (Figure 4.3A). As shown in Figure 4.3B, the levels of CPP-dipeptide increased nearly linearly with time.

Figure 4.3 (A) Calibration curve for CPP-dipeptide and (B) yield of CPP-dipeptide in the reaction of HOOA-lactone with Ac-Gly-Lys-OMe dipeptide as a function of time at SIC of m/z 396.5. The data was obtained from three replicates.
HOOA-PC reacts with Ac-Gly-Lys-OMe dipeptide to form CPP-PC-dipeptide. In a previous study, the reaction of HOHA-PC with Ac-Gly-Lys-OMe led to the formation of CEP-PC-dipeptide. More recently, CEP-dipeptide was also found to be the major CEP derivatives formed in the reaction of HOHA-PC with Ac-Gly-Lys-OMe. Since HOOA-PC (4.10) was found to produce HOOA-lactone in analogy with the chemistry of HOHA-PC, it seemed likely that CPP-dipeptide (4.7) would be generated in addition to CPP-PC-dipeptide (4.11) in the reaction of HOOA-PC (4.10) with Ac-Gly-Lys-OMe (4.6) (Scheme 4.6). HOOA-PC (4.10) was incubated with Ac-Gly-Lys-OMe (4.6) in a binary solution of PBS and methanol (9:1 v/v) at 37 °C for up to six days (Scheme 4.6). The generation of the CPP derivatives 4.6 and 4.11 was analyzed by LC-MS comparisons with the authentic samples prepared by unambiguous chemical syntheses.

![Scheme 4.6](image)

**Scheme 4.6** HOOA-PC (4.10) modifies dipeptide 4.6 to give CPP-PC-dipeptide (4.11).

Authentic CPP-PC-dipeptide (4.11) was efficiently prepared in a manner similar to that described for the synthesis of CEP-PC-dipeptide. Briefly, esterification of acid 4.8 with 1-palmityl-sn-glycero-3-phosphatidylcholine (2-lyso-PC) provided the six-member-ring dioxane protected ester 4.13, from which DOOA-PC ester 4.14 was generated. Then, the Paar-Knorr reaction between DOHA-PC ester 4.14 and Ac-Gly-Lys-OMe (2.6) was successfully applied to generate the target CPP-PC-dipeptide (4.11, Scheme 4.7, see next page).
Scheme 4.7 Synthesis of authentic CPP-PC-dipeptide 4.11.

Analysis of CPP-dipeptide (4.7) and CPP-PC-dipeptide (4.11) in the product mixture after incubation of HOOA-PC with Ac-Gly-Lys-OMe for 3 days by LC-MS in the positive ion mode using the corresponding authentic adducts prepared by unambiguous chemical synthesis for comparison. No peak was observed in the SIC at m/z 396.5 in Figure 4.4C (see next page) corresponding to the peak at 5.42 min for the authentic CPP-dipeptide in Figure 4.4A (see next page), indicating that detectable amounts of CPP-dipeptide were not generated. Only one major peak was observed in the SIC at m/z 873.5 at 11.86 min in Figure 4.4D (see next page), that might correspond to CPP-PC-dipeptide (4.11). However, authentic 4.11 exhibits an LC retention time of 12.68 min (Figure 4.4B, see next page) that is readily distinguishable from the peak at 11.86 min in Figure 4.4D (see next page). Furthermore, Figures 4.4 E and F (see next page) show the ESI full mass spectra of the LC/MS peaks at 12.68 min from authentic CPP-PC-dipeptide (Figure 4.4B, see next page) and of the peak at 11.86 min in the product mixture from reaction of HOOA-PC with Ac-Gly-Lys-OMe (Figure 4.4D, see next page), respectively. That the spectra are completely different, further confirmed that the major product from the reaction of HOOA-PC with Ac-Gly-Lys-OMe is not 4.11. In conclusion, CPP-dipeptide (4.7) and CPP-PC-dipeptide
(4.11) were not detectable in the product mixture after 3 days reaction of HOOA-PC with Ac-Gly-Lys-OMe.

Figure 4.4 Selected ion chromatograms (SICs) in the positive ion mode for CPP-dipeptide (4.7, m/z 396.5) and for CPP-PC-dipeptide (4.11, m/z 873.5). (A) Authentic CPP-dipeptide. (B) Authentic CPP-PC-dipeptide. (C) SIM for CPP-dipeptide in the product mixture from incubation of HOOA-PC with Ac-Gly-Lys-OMe dipeptide after 3 days. (D) SIM for CPP-PC-dipeptide in the reaction product mixture from incubation of HOOA-PC with Ac-Gly-Lys-OMe dipeptide after 3 days. (E) ESI full mass spectrum of authentic CPP-PC-dipeptide
peak that appears at 12.68 min in B. (F) ESI full mass spectrum of the reaction product that appears at 11.86 min in D.

**Post-translational modification of ARPE-19 cells by HOOA-lactone to generate CPP adducts.** As described above, HOOA-lactone reacts with Ac-Gly-Lys-OMe dipeptide to form CPP derivatives. We next investigated whether exposure of ARPE-19 cells to HOOA-lactone causes post-translational modification resulting in the formation of CPP derivatives. ARPE-19 cells were exposed to various concentrations of HOOA-lactone for 2 h followed by incubation in basal medium overnight in 5% CO₂/95% air at 37 °C. Immunostaining of HOOA-lactone treated ARPE-19 cells showed that CPP adducts were produced in ARPE-19 cells in a dose-dependent manner (Figure 4.5, see next page).
Figure 4.5 HOOA-lactone induces CPP generation in ARPE-19 cells. (A) Images of ARPE-19 cells treated with various concentrations of HOOA-lactone. (B) Quantification
of the generation of CPP in ARPE-19 cells. Cells were stained using rabbit anti CPP antibodies/ mouse anti rabbit TXRED antibodies and DAPI. The red fluorescence represents the presence of CPP-modified biomolecules. The blue fluorescence represents the presence of ARPE-19 cells. The images were taken at 10x.

Cytotoxicity of HOOA-lactone to ARPE-19 cells. In order to test whether HOOA-lactone can result in toxicity to ARPE-19 cells in analogy with the chemistry of HOHA-lactone, the cytotoxicity of HOOA-lactone to ARPE-19 cells was evaluated based on its effects on cell viability by MTT assay. As shown in Figure 4.6 (see next page), HOOA-lactone treatment of ARPE-19 also elicited a biphasic dose-response, where higher levels of HOOA-lactone (>0.075 μM) decreased the cell number and low levels of HOHA-lactone (0-0.01 μM) increased the cell number. Treatment of ARPE-19 cells with high concentrations of HOOA-lactone significantly decreased cell viability with increasing concentrations of HOOA-lactone. The LC$_{50}$ of HOOA-lactone for ARPE-19 cells is 43 ± 6.4 μM (n = 8). In view of their structural and functional similarity it is not surprising to find that there is no significant difference between the toxicity of HOHA-lactone (LC$_{50}$ = 42.2 ± 5.4 μM) and HOOA-lactone.
**Figure 4.6** Concentration-dependent effect of HOOA-lactone on ARPE-19 cell viability. ARPE-19 cells were incubated with 0–100 µM of HOOA-lactone for 2 h and then assayed for cytotoxicity by MTT. Results expressed as % of untreated control are means ± SD of independent experiments (n = 8).

**Effect of HOOA-lactone on the formation reactive oxygen species and oxidative stress.** To establish whether the cell death induced by HOOA-lactone in ARPE-19 cells was related to increased oxidative stress, we examined the formation of reactive oxygen species (ROS) in HOOA-lactone-treated ARPE-19 cells by the 2’,7’–dichlorofluorescin diacetate (DCF-DA) assay. As shown in Figure 4.7 (see next page), intracellular ROS in HOOA-lactone-treated ARPE-19 cells increased in a dose-dependent manner, demonstrated by the increased green fluorescence with the increase of the added HOOA-lactone.
Figure 4.7 HOOA-lactone induces generation of intracellular reactive oxygen species in ARPE-19 cells in a dose-dependent manner. (A) Images of intracellular ROS in ARPE-19 cells treated with different concentrations of HOOA-lactone. (B) Quantification of the generation of ROS in ARPE-19 cells. After DCF-DA pretreatment, ARPE-19 cells (4.5 x 10^4 cells) were further incubated with 0-30 μM HOOA-lactone before analysis. PHASE images were taken to observe cell morphology. The images are representative of four independent experiments showing very similar results. Images were taken at 20X magnification.
**HOOA-lactone induces apoptosis in ARPE-19 cells.** There are two major types of cell death: apoptosis and necrosis. Since HOOA-lactone was able to induce cell death (Figure 4.6, see page 157), in order to further determine if the cell death is due to apoptosis or necrosis, we carried out CF-TM488A-Annexin V and Propidium iodide (PI) staining by using fluorescent microscopy. In normal viable cells, phosphatidylserine (PS) is found at the cytoplasmic face of the plasma membrane. However, in apoptotic cells, this plasma membrane asymmetry is lost, resulting in the exposure of PS residues on the outer plasma membrane. Annexin V, which interacts strongly and specifically with PS, is conjugated to a green fluorescent dye CFTM488A to detect apoptosis by binding cell surface PS that is a measure of the loss of plasma membrane asymmetry. PI is a red fluorescent membrane-impermeant DNA-binding dye that stains DNA of both necrotic and late apoptotic cells that have compromised membrane integrity.19, 20 As shown in Figure 4.8 (see next page), most of PBS-treated ARPE-19 cells were viable and negative to both Annexin V and PI with only a few early apoptotic cells and a few necrotic cells. Most of the ARPE-19 cells after treatment with 15 µM HOOA-lactone were positive to Annexin V and negative to PI, indicating that most of these cells were early apoptotic cells.
Figure 4.8 HOOA-lactone induced apoptosis in ARPE-19 cells after incubation for 2 h. Viable cells are colorless (Annexin V−/PI−), early apoptotic cells (Annexin V+/PI−) are green, and necrotic cells (Annexin V−/PI+) are red. The images are representative of three independent experiments showing very similar results.

In situ analysis of activated caspase-3/7 in ARPE-19 cells. Apoptosis is central to a number of pathological proliferative disorders, including cancer. Activation of caspases is the biochemical hallmark of apoptosis, in view of the central role of this class of enzymes as death effector mediators. Therefore, in order to investigate the possible mechanism of HOOA-lactone induced apoptosis, we examined in situ caspase activity in HOOA-lactone-treated or untreated ARPE-19 cells using the NucView 488 caspase-3 assay kit. The green fluorogenic DEVD-NucView488™ caspase-3 substrate consists of a green fluorogenic DNA dye coupled to the caspase-3/7 DEVD recognition sequence. The initial non-fluorescent substrate is cleaved by caspase-3/7 to release the high-affinity fluorogenic DNA dye into the cytoplasm of apoptotic cells. The released dye migrates to the cell nucleus to stain DNA with bright green fluorescence. As shown in Figure 4.9 (see next page), compared to the cells treated with PBS, those treated with 15 μM HOOA-lactone showed a marked increase in fluorescence. In contrast, the NucView488™ fluorescence...
signal was similar to that resulting from PBS treatment for cells treated with 15 µM HOOA-lactone after pretreatment with the caspase-3/7 inhibitor Ac-DEVD-CHO. This confirmed that the NucView488™ signal is caspase-3/7 dependent.

Figure 4.9 Microscopy imaging of caspase activation in ARPE-19 cells using the NucView 488 caspase-3 assay kit. Cells were stained using 5 µM DEVD-NucView 488-substrate to study the activation of caspase 3/7. The caspase-3/7 inhibitor Ac-DEVD-CHO was used to ensure the fluorescence observed is due to caspase 3/7 activity. The images are representative of three independent experiments showing very similar results.

Formation and export of the glutathione conjugate of HOOA-lactone in ARPE-19 cells. HOOA-lactone reacts with primary amino groups of biomolecules to form CPP derivatives in vitro and vivo. In addition, as described above, at µM levels HOOA-lactone is toxic and promotes oxidative stress, causing cell apoptosis in ARPE-19 cells. The extent and severity of damage produced by HOOA-lactone will depend, in part, upon how the particular cell type detoxifies this aldehyde. Given its structural and functional similarity to HOHA-lactone, HOOA-lactone, in analogy with the metabolism of HOHA-lactone and HNE, was expected to be detoxified in living cells by conjugation with glutathione (GSH)
to form HOOA-lactone-GSH (=O) (4.15), catalyzed by glutathione-S-transferase (GST), and then be reduced to HOOA-lactone-GSH (-OH) (4.16) catalyzed by alcohol dehydrogenases (ADH) in ARPE-19 cells (Scheme 4.8). To confirm this presumption, the capacity of ARPE-19 cells to eliminate HOOA-lactone and to produce GSH conjugated metabolites was investigated by LC-MS analysis.

Scheme 4.8 Structures of GSH conjugates of HOOA-lactone in ARPE-19 cells.

ARPE-19 cells were incubated with either 0 µM or 10 µM HOOA-lactone for 2 h. Then 20 µL of each extracellular medium (ECM) was directly injected for LC-MS analysis. Figure 4.10 (left, see next page) shows the selected ion monitoring (SIM) chromatograms at m/z 464.4 in the positive ion mode of the ECM after incubation for 2 h. A peak with retention time (R.T.) 4.60 min was detected only in the ECM from cells treated with 10 µM HOOA-lactone and not in the ECM from untreated cells. That the peak at a R.T. of 4.60 min was 4.16, was confirmed by its mass spectrum in the positive ion mode. As shown in Figure 4.10 C, ions with m/z values of 464.07, 486.13 and 502.08, corresponded to the
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[M+H]+, [M+Na]+ and [M+K]+ ions from HOOA-lactone-GSH (-OH) (4.16), respectively. An additional prominent ion with m/z 335.18 is ascribed to a daughter ion [MH + H – COCH₂CH₂CH(NH₂)COOH]+ from HOOA-lactone-GSH (-OH) (4.16). Structure identity of the putative metabolite HOOA-lactone-GSH (-OH) (4.16) was further established by MS/MS analysis in the positive mode of the [M+H]+ ion at 464.4. Figure 4.11A (see next page) shows the fragmentation pattern of the metabolite presumed to be 4.16. The collision-induced dissociation (CID) produced ions with m/z 300, 318, 389 and 446. These are
consistent with \([\text{M}+\text{H} - \text{H}_2\text{O} - \text{NH}_2\text{COCH}_2\text{CH}_2\text{CH(}\text{NH}_2\text{)}\text{COOH}]^+\), \([\text{M}+\text{H} - \text{NH}_2\text{COCH}_2\text{CH}_2\text{CH(}\text{NH}_2\text{)}\text{COOH}]^+\), \([\text{M}+\text{H} - \text{NH}_2\text{CH}_2\text{COOH}]^+\) and \([\text{M}+\text{H} - \text{H}_2\text{O}]^+\), respectively (Figure 4.11B).

**Figure 4.11** MS/MS analysis of metabolite 4.16 in the ECM of HOOA-lactone-treated ARPE-19 cells. (A) CID spectrum of m/z 464.4 from ECM of ARPE-19 cells incubated with 10 μM HOOA-lactone for 2 hours. (B) Proposed structures of CID fragments of m/z 464.4 in mass spectrometry.

To further confirm that this metabolite is identical to compound 4.16, authentic samples of the predicted glutathione adduct HOOA-lactone-GSH (=O) (4.15) and the reduced glutathione adduct HOOA-lactone-GSH (-OH) (4.16) were synthetized by unambiguous chemical syntheses from HOOA-lactone (4.5) (Scheme 4.9, see next page).
Scheme 4.9 Chemical synthesis of authentic 4.15 and 4.16.

The generation of metabolite 4.16 in both ECM and cell lysate (CL) after treating ARPE-19 cells with 10 µM HOOA-lactone for 30 min was monitored by reverse phase HPLC-ESI/MS/MS by monitoring the MRM transition m/z 464.4 to 335 in the positive ion mode and comparison with an authentic sample prepared by unambiguous chemical synthesis. As shown in Figure 4.12 (see next page), LC–MS/MS analysis of ECM (Figure 4.12B, see next page) or CL (Figure 4.12C, see next page) produced MRM chromatographs essentially identical to that produced from authentic 4.16 (Figure 4.12A, see next page). These results confirmed that compound 4.16 is a major metabolite of HOOA-lactone in ARPE-19 cells.
Figure 4.12 MRM chromatographs monitoring the transition m/z 464.4→335.0 in the positive ion mode of (A) authentic standard 4.16, (B) ECM or (C) cell lysate (CL) from ARPE-19 cells incubated with 10 µM HOOA-lactone for 30 min.

Having demonstrated that metabolite 4.16 is generated in both intra- and extra-cellular medium from ARPE-19 cells upon treatment with HOOA-lactone, we next studied the kinetics of formation of compound 4.16 in ARPE-19 cells by LC-MS/MS analysis in the positive ion mode monitoring the transition m/z 464.4→335.0 over the time course of treatment. As shown in Figure 4.13 (see next page), the level of the metabolite 4.16 in the cell lysate rises very rapidly. It reaches a maximum in the CL after 5 min of incubation followed by a rapid decline and a concomitant accumulation in ECM, demonstrating that HOOA-lactone can easily diffuse through the cell membrane into ARPE-19 cells to conjugate with GSH to form GSH adduct 4.16 in cells, which is then transported
unidirectionally from the cytosol to the ECM. The metabolite 4.16 accumulated rapidly in ECM during the first 30 min of incubation after addition of HOOA-lactone to ARPE-19 cells and then maintained a steady state level for 120 min.

**Figure 4.13** Time course of formation of 4.16 in the ECM (■) and CL (○) from ARPE-19 cells exposed to 10 μM HOOA-lactone. Values, expressed as arbitrary units (a.u), were calculated as mass chromatogram peak areas per 10^6 of cells. Values represent means ± S.D. of three independent experiments.

Similarly, an LC-/MS/MS method was developed to quantitate 4.15. Figure 4.14A (see next page) shows that CID fragmentation of authentic 4.15 in the positive ion mode produced ions with m/z 308, 315, 333, 387 and 444, that are consistent with [GSH+H]^+, [MH+H – H2O – COCH2CH2CH(NH2)COOH]^+, [MH+H – COCH2CH2CH(NH2)COOH]^+, [M+H – NH2CH2COOH]^+ and [M+H – H2O]^+, respectively (Figure 4.14B, see next page). The transition of m/z 462.4→315.0 was chosen to study the generation of metabolite 4.15 in ARPE-19 cells treated with HOOA-lactone. Unlike the detection of the reduced HOOA-lactone-GSH (-OH) 4.16 in both ECM and CL from ARPE-19 cells treated with 10 μM HOOA-lactone, the metabolite 4.15 was only
detectable in the ECM evidenced by a significant peak from the ECM that eluted at the same retention time as the authentic 4.15 (Figure 4.15A, see next page). As shown in Figure 4.15B (see next page), extracellular levels of metabolite 4.15 increased rapidly reaching a maximum in the first 15 min of incubation and then decreased to zero over by 120 min of incubation. This presumably resulted from the reduction of 4.15 to 4.16 under the catalysis of ADH.

Figure 4.14 MS/MS analysis of metabolite 4.15. (A) CID spectrum of m/z 462.4 from authentic HOOA-lactone-GSH (=O) 4.15. (B) Proposed structures of CID fragments of m/z 462.4 in mass spectrometry.
Figure 4.15 LC-MS/MS analysis of metabolite 4.15 production in ARPE-19 cells treated with HOOA-lactone. (A) MRM chromatographs of authentic standard 4.15 and ECM of ARPE-19 cells incubated with 10 μM HOOA-lactone for 5 min in the transition m/z 462.4 → 315.0 in positive ion mode. (B) Time course of 4.15 formation in ECM from ARPE-19 cells treated with 10 μM HOOA-lactone. Values, expressed as arbitrary units (a.u), were calculated as mass chromatogram peak areas per 10^6 of cells. Values represent means ± S.D. of three independent experiments.

The concentration dependence of the formation of HOOA-lactone-GSH adducts 4.15 and 4.16 in ARPE-19 cells treated with concentrations of HOOA-lactone from 0 to 100 μM for 2 h was also examined by LC-MS/MS as described above. As shown in Figure 4.16A (see next page), levels of HOOA-lactone-GSH (-OH) (4.16) in both ECM and CL of ARPE-19 cells rose and plateaued with increasing amounts of added HOOA-lactone. HOOA-lactone treatment also resulted in a concentration dependent appearance of
metabolite 4.15 in the ECM of ARPE-19 cells that plateaued. No accumulation of 4.15 was detected in the CL over concentrations of added HOOA-lactone from 0 to 100 µM (Figure 4.16B, see next page).

**Figure 4.16** Concentration-dependent formation of (A) HOOA-lactone-GSH (-OH) (4.16) and (B) HOOA-lactone-GSH (=O) (4.15) in ARPE-19 cells treated with 0 to 100 µM HOOA-lactone. Insert: the concentration dependent formation of the metabolite 4.16 in the CL with amplified vertical axis to highlight the trend. (■) and (●) represent ECM and CL, respectively. Values, expressed as arbitrary units (a.u), were calculated as mass chromatogram peak areas per 10^6 of cells. Values represent means ± S.D. of three independent experiments.
4.3 Conclusions

In the present study, chemical synthesis of HOOA-lactone was achieved with minor modifications of an efficient method described previously. In an in vitro model study monitoring the reaction by reverse phase HPLC-ESI/MS in the positive ion mode and comparison with authentic CPP-dipeptide prepared by unambiguous chemical synthesis, HOOA-lactone (free acid) was shown to react with Ac-Gly-Lys-OMe dipeptide to form CPP-dipeptide. Surprisingly, neither CPP-dipeptide nor CPP-PC-dipeptide were detected in the product mixture from reaction after 3 days incubation of HOOA-PC (phospholipid ester) with the dipeptide. Furthermore, using immunostaining analysis, we found that HOOA-lactone post-translationally modifies ARPE-19 cells, leading to the formation of CPP derivatives in a dose-dependent manner. Taken together, these observations indicated that HOOA-lactone can serve as a precursor of CPP modifications in vivo.

In analogy with the biological activity of HOHA-lactone, the homologous HOOA-lactone was found by the MTT assay to be toxic to ARPE-19 cells in a dose-dependent manner. Using the DCF-DA assay, cell death induced by HOOA-lactone in ARPE-19 cells was demonstrated to be related to increased oxidative stress and, using CFTM488A-Annexin V and Propidium iodide (PI) staining and fluorescent microscopy, was further shown to be due to apoptosis. Considering that caspase-3 and caspase-7 are central to the execution of programmed cell death, and that their activation reflects progression into apoptosis, the effect of HOOA-lactone treatment of ARPE-19 cells on caspase3/7 activity was also investigated using the NucView 488 caspase-3 assay kit. This immunofluorescence study showed a HOOA-lactone-induced increase in the activation of caspase3/7 in ARPE-19 cells.
The demonstration that HOOA-lactone is toxic and promotes oxidative stress, causing cell apoptosis in ARPE-19 cells, suggests that the RPE would become more vulnerable to damage by HOOA-lactone with increasing production of HOOA-lactone and a decrease in its detoxification that might occur with aging. Thus, in view of the toxic potential of HOOA-lactone to ARPE-19 cells, it is important to investigate how HOOA-lactone is detoxified in this cell type. Our results suggested that HOOA-lactone can easily diffuse through the cell membrane into ARPE-19 cells to be detoxified by conjugation with GSH to form HOOA-lactone-GSH (=O) facilitated by GST. This adduct can be efficiently reduced to HOOA-lactone-GSH (-OH) under catalysis by ADH. Both of the GSH conjugates of HOOA-lactone are transported unidirectionally from the cytosol to the ECM in ARPE-19 cells. Among the glutathione conjugates of HOOA-lactone in ARPE-19 cells, the reduced form HOOA-lactone-GSH (-OH) is predominant.
4.4 Experimental Procedures

**Materials.** Dulbecco’s modified Eagle’s (DMEM)/F12 medium, Dulbecco’s phosphate buffered saline (DPBS), fetal bovine serum (FBS) and 2’,7’-dichlorofluorescein diacetate (DCFDA) were purchased from Fisher Scientific (Pittsburgh, PA). RIPA lysis buffer was obtained from Cell Signaling Technology (Danvers, MA). Goat anti rabbit FITC antibody and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Invitrogen (Carlsbad, CA). The in situ apoptosis detection kits, NucView 488 Caspase 3 assay (DEVD-NucView 488-substrate) for live cells and CF488A-Annexin V -propidium iodide apoptosis assay kits were purchased from Biotium (Hayward, CA). The Ac-Gly-Lys-OMe dipeptide was purchased from Bachem (Torrance, CA). LysoPC (13:0) (1-tridecanoyl-2-hydroxy-sn-glycero-3-phosphocholine) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). All other chemicals and reagents including L-glutathione reduced, and sodium borohydride were purchased from Sigma–Aldrich (St. Louis, MO). 7-(1.3-Dioxan-2-yl)-5-oxoheptanoic acid (4.8) and HOOA-PC (4.10) were prepared by Dr. Li Hong and Dr. Jaewoo Choi according to the methods described in their theses, respectively. Rabbit anti-CPP polyclonal antibody was raised and characterized as described previously.5

**General Methods.** Proton magnetic resonance (1H NMR) spectra and carbon magnetic resonance (13C NMR) spectra were recorded on a Varian Inova AS400 spectrometer operating at 400 MHz and 100 MHz, respectively. Proton chemical shifts are reported as parts per million (ppm) on the δ scale relative to CDCl3 (δ 7.26) or CD3OD (δ 3.31). 1H NMR spectral data are tabulated in terms of multiplicity of proton absorption (s, singlet; d, doublet; dd, doublet of doublet; t, triplet; q, quartet; m, multiplet; br, broad), coupling
constants (Hz), number of protons. Carbon chemical shifts are reported relative to CDCl₃ (δ 77.0) or CD₃OD (δ 49.0). Flash chromatography was performed with ACS grade solvents from Fisher Scientific (Hanover Park, IL). Rₚ values are quoted for TLC plates of thickness 0.25 mm from Whatman (Florham Park, NJ). The plates were visualized with iodine, dinitrophenylhydrazine or phosphomolybdic acid reagents. For all reactions performed in an inert atmosphere, argon was used unless otherwise specified.

**High Performance liquid chromatography/mass spectrometry.** Chromatographic separation was carried out with a Surveyor LC system equipped with a Luna C18 column (150× 2.0 mm i.d. 5 µm, Phenomenex). The mobile phase consisted of solvent A (HPLC grade water containing 0.1% formic acid) and solvent B (HPLC graded methanol containing 0.1% formic acid). For HOOA-lactone and HOOA-PC modified dipeptide analysis, the total run time was 26 min. HPLC gradient steps were as follows: 0-2 min, isocratic at 40% solvent B; 2-8 min, linear gradient from 40 to 100% solvent B; 8-15 min, isocratic at 100% solvent B; 16-26 min, isocratic at 40% solvent B. The flow rate employed was 200 µL/min. For analysis of HOOA-lactone-GSH adducts, the total run time was 25 min. The HPLC gradient steps were set as follows: 0-10 min, linear gradient from 2 to 100% solvent B; 10-15 min, isocratic at 100% solvent B; 16-25 min, isocratic at 2% solvent B. The flow rate employed was 200 µL/min.

ESI mass spectrometry was performed on a Thermo Finnigan LCQ™ Deca system in the positive ion mode using nitrogen as the sheath and auxiliary gas. The heated capillary temperature was 300 °C, the source voltage was 4.5 kV, and the capillary voltage was 31.00 V. MS scan at 50-1200 were obtained for standard compounds. All data were processed with Qual browser in Xcalibur software.
Modification of Ac-Gly-Lys-OMe dipeptide with HOOA-lactone or HOOA-PC.

Ac-Gly-Lys-OMe dipeptide (2 mM) was incubated with either HOOA-lactone (1 mM) or HOOA-PC (0.2 mM in a binary solution mixture of phosphate buffered saline (PBS) and methanol (9:1, v/v) under the protection of argon at 37 °C. An aliquot of 50 μL of the reaction mixture was withdrawn after various reaction times and mixed with 100 μL of methanol. 20 μL of this diluted solution was injected for HPLC-ESI/MS analysis.

Preparation of (E)-tributyl(3,3-diethoxyprop-1-en-1-yl)stannane (4.2). Compound 4.2 was prepared following the procedure described previously with minor modifications.22, 23 In brief, dry copper (I) cyanide (269 mg, 3.0 mmol) was mixed with dry THF (6.0 mL) and cooled to -78 °C and then n-butyllithium in hexanes (2.52 mL, 6.0 mmol) was added dropwise via a syringe. The resulting mixture was warmed slightly to yield a pale yellow homogenerous solution and then was recooled to -78 °C followed by the addition of n-tributyltin hydride (1.6 mL, 6.0 mmol). The reaction mixture was stirred for 25 min to give the Lipshutz reagent, Bu3SnCu(Bu)CNLi2. Then, 3,3-diethoxy-1-propyne 4.1 (0.388 mL, 2.7 mmol) was added dropwise via a syringe to this Bu3SnCu(Bu)CNLi2 solution and the reaction mixture was stirred for 2.5 h at -78 °C before quenching with water. The dark solution was extracted with ether (3 x 20 mL), followed by drying of the combined extracts over anhydrous sodium sulfate. The solvents were evaporated under reduced pressure and the residue was purified by flash chromatography on a silica gel column (hexanes/ethyl acetate/triethylamine 98:2:1, TLC: Rf = 0.24) to give 4.2 (0.9622 g, 85%). 1H NMR (400 MHz, CDCl3) δ 6.46 – 6.20 (m, 1H), 6.08 – 5.82 (m, 1H), 4.82 – 4.76 (m, 1H), 3.63 (dq, J = 9.5, 7.1 Hz, 2H), 3.48 (dq, J = 9.5, 7.1 Hz, 2H), 1.52 – 1.43 (m, 6H), 1.28 (dd, J = 15.1,
7.2 Hz, 6H), 1.24 – 1.19 (m, 6H), 0.94 – 0.80 (15H). The $^1$H NMR spectral data for stannane 4.2 is in agreement with that reported previously.

**Preparation of methyl (E)-8,8-diethoxy-5-oxooct-6-enoate (4.3).** The procedure was modified from that reported.\textsuperscript{23} Vinylstanane 4.2 (0.84 g, 2.0 mmol) and PdCl\textsubscript{2}(MeCN)\textsubscript{2} (5.2 mg, 20 μmol) were dissolved in dry DMF (3 mL). Then glutaric acid monomethyl ester chloride (279 μL, 2.0 mmol) was added dropwise via a syringe to the reaction mixture in an ice bath. The resulting red brown mixture was allowed to stir at 0 °C for 1.0 hour and then incubated for another 4 h at room temperature. Finally, saturated aqueous sodium fluoride (3.0 mL) and acetone (3.0 mL) were added to the reaction mixture to remove tributyltin chloride. The mixture was then extracted with ethyl acetate (3 x 20 mL), followed by combining the extracts and drying over anhydrous sodium sulfate. The solvent was removed under reduced pressure and the crude product was purified by flash chromatography on a silica gel column (hexanes/ethyl acetate/triethylamine 80:20:1) to give 4.3 (284 mg, 55%). TLC: (hexanes/ethyl acetate/triethylamine 80:20:1, R\textsubscript{f} = 0.32). $^1$H NMR (400 MHz, CDCl\textsubscript{3}) δ 6.63 (dd, $J = 16.2, 4.3$ Hz, 1H), 6.36 – 6.28 (m, 1H), 5.03 (dd, $J = 4.3, 1.3$ Hz, 1H), 3.68 – 3.59 (m, 6H), 3.51 (dq, $J = 9.4, 7.0$ Hz, 2H), 2.65 (t, $J = 7.2$ Hz, 2H), 2.34 (d, $J = 6.9$ Hz, 2H), 1.93 (td, $J = 7.2, 2.3$ Hz, 2H), 1.21 (dd, $J = 8.4, 5.7$ Hz, 6H).

**Preparation of methyl (E)-8,8-diethoxy-5-hydroxyoct-6-enoate (4.4).** Sodium borohydride (3.4 mg, 0.09 mmol) in ethanol (0.5 mL) was added dropwise via a syringe to a stirred solution of ketone 4.3 (23.3 mg, 0.09 mmol) in ethanol (3.0 mL) with 1% triethylamine at 0 °C. The resulting mixture was allowed to react for 4 h at 0 °C and then quenched by addition of methanol (1.0 mL) to destroy excess sodium borohydride. Then
saturated sodium chloride solution was added and the mixture was extracted with ethyl acetate (3 x 15 mL). The aqueous phase was collected and solvents were removed under reduced pressure and the crude product was purified by flash chromatography on a silica gel column (hexanes/ethyl acetate/triethylamine 70:30:1) to give 4.4 (20 mg, 87%). TLC (hexanes/ethyl acetate/triethylamine 70:30:1, R_f = 0.24): 1H NMR (400 MHz, CDCl_3) δ 5.84 (ddd, J = 15.7, 6.0, 1.0 Hz, 1H), 5.68 (ddd, J = 15.7, 5.0, 1.2 Hz, 1H), 4.87 (d, J = 5.0 Hz, 1H), 4.17 – 4.06 (m, 3H), 3.66 – 3.58 (m, 2H), 3.51 – 3.43 (m, 2H), 2.35 – 2.28 (m, 2H), 1.78 – 1.62 (m, 2H), 1.55 (dd, J = 15.0, 7.4 Hz, 2H), 1.25 – 1.18 (m, 8H).

**Preparation of (E)-3-(6-oxotetrahydro-2H-pyran-2-yl)acrylaldehyde (HOOA-lactone, 4.5).** Acetal 4.4 (16 mg, 0.06 mmol) was dissolved in mixture of TFA and water (95:5, v/v, 1.5 mL). Then Amberlyst-15 (10 mg) was added and the resulting mixture was stirred at room temperature for 1.5 hour. TLC (hexanes/EA=55:44, v/v) was used to monitor the reaction. Then solvent was evaporated under reduced pressure and the residue purified by flash chromatography on a silica gel column (hexanes/EA=55:44) to give lactone 4.5 (3.6 mg, 40%). TLC (hexanes/EA=55:44, R_f = 0.13): 1H NMR (400 MHz, CDCl_3) δ 9.61 (d, J = 7.6 Hz, 2H), 6.76 (dd, J = 15.8, 4.3 Hz, 2H), 6.37 (ddd, J = 15.8, 7.6, 1.7 Hz, 2H), 5.11 (dtd, J = 9.9, 4.0, 1.7 Hz, 2H), 2.72 – 2.62 (m, 2H), 2.55 (ddd, J = 18.0, 8.3, 7.1 Hz, 2H), 2.18 – 2.09 (m, 2H), 2.04 – 2.00 (m, 1H), 2.00 – 1.93 (m, 3H), 1.79 – 1.71 (m, 3H).

**Preparation of 5,8-dioxooctanoic acid (4.9).** The acetal 4.8 (42.8 mg, 0.186 mmol) was dissolved in 12 mL of AcOH/H2O (3:1, v/v) solution and the mixture was stirred at 50 °C for 6 h. Once TLC analysis showed the complete removal of the acetal, the solvent
was removed by rotary evaporation to give the crude $\gamma$-ketoaldehyde 4.9, which was directly used for the next step.

**Preparation of 4-(1-[5-(2-Acetamidoacetamido)-6-methoxy-6oxohexyl]-1H-pyrrol-2-yl)butanoic acid (CPP-dipeptide, 4.7).** Crude $\gamma$-ketoaldehyde 4.9 was mixed with methyl 6-amino-2-((2-acetylamino)acetyl)amino) hexanoate (4.6, Ac-Gly-Lys-OMe, 50 mg, 0.193 mmol) in methanol (7 mL). The resulting mixture was stirred for 24 h at room temperature under argon. The solvent was then removed by rotary evaporation and the residue was purified by flash chromatography on a silica gel column (chloroform/methanol 10:1) to give pure 4.7 (35 mg, 47%). TLC (chloroform/methanol 10:1, $R_f = 0.24$): $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.20 (d, $J = 7.9$ Hz, 1H), 7.05 (s, 1H), 6.54 (s, 1H), 6.05 (d, $J = 2.6$ Hz, 1H), 5.86 (d, $J = 3.0$ Hz, 1H), 4.54 (dd, $J = 12.1, 7.3$ Hz, 1H), 4.02 (ddd, $J = 21.5, 13.7, 5.1$ Hz, 2H), 3.86 – 3.78 (m, 2H), 3.71 (s, 3H), 2.63 – 2.54 (m, 2H), 2.44 (t, $J = 6.3$ Hz, 2H), 2.07 (s, 3H), 1.94 – 1.84 (m, 3H), 1.77 – 1.63 (m, 3H), 1.33 (ddd, $J = 25.5, 15.7, 10.6$ Hz, 2H). $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 176.81, 172.55, 172.18, 169.59, 132.18, 119.81, 107.17, 105.77, 52.73, 52.40, 46.13, 43.41, 33.35, 31.75, 31.55, 25.61, 24.95, 22.84, 22.77. ESI-MS: m/z calcd for C$_{19}$H$_{30}$N$_3$O$_6$ (M+H)$^+$, 396.21, found 396.07; m/z calcd for C$_{19}$H$_{29}$N$_3$O$_6$Na (MNa$^+$), 418.19; found, 418.13; m/z calcd for C$_{19}$H$_{29}$N$_3$O$_6$K (MK$^+$), 434.19; found, 434.13.

**1-Palmityl-2-(4,7-dioxoheptanoyl)-sn-glycero-3-phosphatidylcholine (4.13).** 1-palmityl-sn-glycero-3-phosphatidylcholine 4.12 (2-lyso-PC, 148.5 mg, 0.3 mmol) was mixed with carboxylic acid 4.8 (46 mg, 0.2 mmol) in a flame-dried 25 mL round-bottom flask, which was further thoroughly dehydrated by azeotropic evaporation of molecular
sieve dried toluene (15 mL). Then anhydrous chloroform (9 mL), dicyclohexylcarbodiimide (DCC, 61.8 mg, 0.3 mmol) and N,N-dimethylamino-pyridine (DMAP, 36.6 mg, 0.3 mmol) were added. The reaction mixture was stirred overnight under argon. The solvent was then removed by rotary evaporation and the residue was purified by flash chromatography on a silica gel column (chloroform/methanol/H2O 16:9:1, Rf = 0.20) to give pure 4.13 (85 mg, 60%). 1H NMR (400 MHz, CD3OD) δ 5.28 – 5.19 (m, 1H), 4.57 (t, J = 5.0 Hz, 1H), 4.43 (dt, J = 12.0, 3.5 Hz, 1H), 4.28 (d, J = 2.3 Hz, 2H), 4.18 (ddd, J = 11.5, 6.6, 4.7 Hz, 1H), 4.06 – 3.97 (m, 4H), 3.76 (td, J = 12.4, 2.4 Hz, 2H), 3.68 – 3.62 (m, 2H), 3.24 (s, 9H), 2.53 (ddd, J = 9.7, 7.1, 2.8 Hz, 4H), 2.38 – 2.30 (m, 4H), 1.98 (qt, J = 12.5, 5.0 Hz, 1H), 1.87 – 1.77 (m, 4H), 1.64 – 1.56 (m, 2H), 1.35 – 1.26 (25H), 0.90 (t, J = 6.8 Hz, 3H). 13C NMR (101 MHz, CD3OD) δ 210.34, 174.76, 172.80, 147.92, 101.10, 66.64, 66.24, 63.7, 62.5, 62.29, 59.26, 53.52, 53.48, 53.44, 40.98, 37.97, 36.39, 36.37, 33.84, 33.64, 32.92, 32.70, 31.87, 29.6, 29.57, 29.44, 29.28, 29.25, 29.24, 28.99, 25.71, 24.78, 22.54, 18.82, 13.27.

1-Palmityl-2-(Ac-Gly-Lys-OMe-CPP)-sn-glycerophosphatidylcholine (CPP-PC-dipeptide, 4.11) Propylene acetal 4.13 (40 mg, 0.056 mmol) was dissolved in 8 mL of AcOH-water (3:1) solution, and the mixture was stirred at 50 °C overnight. TLC analysis showed complete reaction. Then the solvent was removed by rotary evaporation to give the crude DOOA-PC ester 4.14, which was directly used for the next step.

Crude γ-ketoaldehyde 4.14 was mixed with Ac-Gly-Lys-OMe 4.6 (20 mg, 0.08 mmol) in methanol (10 mL). The mixture was stirred at room temperature overnight under argon. The solvent was then removed by rotary evaporation and the residue was purified by flash chromatography on a silica gel column (chloroform/methanol/H2O = 16:9:2, Rf = 0.50) to
give pure 4.11 (32 mg, 65%). $^1$H NMR (400 MHz, CD$_3$OD) δ 6.58 (dd, $J = 4.5, 2.1$ Hz, 1H), 5.94 (dd, $J = 5.7, 2.5$ Hz, 1H), 5.79 (d, $J = 1.3$ Hz, 1H), 5.28 – 5.22 (m, 1H), 4.42 (ddd, $J = 9.2, 6.4, 3.8$ Hz, 2H), 4.27 (d, $J = 2.6$ Hz, 2H), 4.19 (dt, $J = 12.4, 6.3$ Hz, 1H), 4.04 – 3.99 (m, 2H), 3.90 – 3.80 (m, 4H), 3.69 (s, 3H), 3.65 – 3.59 (m, 2H), 3.24 – 3.18 (m, 11H), 2.59 (dd, $J = 14.4, 6.8$ Hz, 2H), 2.45 – 2.38 (m, 2H), 2.32 (dt, $J = 15.0, 7.4$ Hz, 2H), 2.01 (s, 3H), 1.95 – 1.79 (m, 4H), 1.77 – 1.65 (m, 3H), 1.63 – 1.54 (m, 2H), 1.29 (25H), 0.90 (t, $J = 6.8$ Hz, 3H). $^{13}$C NMR (101 MHz, CD$_3$OD) δ 173.73, 173.08, 172.43, 170.77, 170.47, 131.20, 119.89, 106.14, 105.55, 63.56, 62.50, 62.27, 60.50, 59.29, 53.48, 53.44, 53.41, 52.29, 51.52, 45.72, 42.06, 33.84, 33.62, 33.20, 32.97, 31.88, 30.88, 29.6, 29.57, 29.56, 29.44, 29.29, 29.25, 29.23, 29.19, 28.99, 28.97, 25.11, 24.78, 22.71, 22.55, 21.27, 13.27. ESI-MS: m/z calcd for C$_{43}$H$_{78}$N$_4$O$_{12}$P (M+H)$^+$, 872.53, found 873.27; m/z calcd for C$_{43}$H$_{77}$N$_4$O$_{12}$PNa (MNa$^+$), 895.53; found, 895.20.

**Cell culture and treatment.** 2 x $10^6$ ARPE-19 cells were grown in 60 mm culture dishes and washed with basal DMEM/F12 medium. For studies of dose-dependence, ARPE-19 cells were exposed to 3 mL of basal medium containing various concentrations of HOOA-lactone (0-100 μM) for 2 h at 37 °C, 5% CO$_2$. For time course studies, ARPE-19 cells were exposed to 3 mL of basal medium containing 10 μM of HOOA-lactone for different time periods at 37 °C under 5% CO$_2$. At the completion of incubations, the supernatants were collected and designated the “extracellular medium” (ECM). After washing the cell monolayer three times with basal DMEM/F12 medium, the cells were re-suspended in 400 μL of basal medium. The cells were scraped using a rubber scraper and collected, and then subjected to sonication at 4 °C and designated the “cell lysate” (CL). The ECM and CL were kept at -20 °C until analysis.
Post-translational modification of ARPE-19 cells by HOOA-lactone. Cells (2.5 x 10^4 cells per well) were plated on a 8-chamber well (Lab-Tek II Chamber Slide System, Nunc, Rochester, NY) in DMEM/F12 complete medium (medium with 10% FBS supplementatio) in at 37 °C, 5% CO2 overnight. After starving the cells in basal DMEM/F12 medium for 4 - 5 hours, the cells were washed three times with basal DMEM/F12 medium. Then, the cells were exposed to various concentrations of HOOA-lactone (0-15 μM) in PBS buffer for 2 h at 37 °C under 5% CO2. After the wells were aspirated, basal medium was added to each well and the cells were incubated overnight at 37 °C and 5% CO2. After incubation, the plates were centrifuged at 500g for 5 min and the medium was aspirated from each well. Then, the cells were aspirated, fixed and permeabilized with acetone (-20 °C). The slides were blocked with 1:100 diluted normal goat serum (NGS) for 1 h. The cells were then probed with rabbit anti CPP-polyclonal antibody5 (1:100 in NGS) followed by incubation with Texas Red goat anti-rabbit IgG (H&L) antibody (1:200; Invitrogen, Carlsbad, CA). The slides were mounted in VectaShield containing DAPI mounting medium (Vector Laboratories, Burlingame, CA). The cell images were taken at 10x magnification using a Leica DMI 6000 B inverted microscope.

Cell viability assay (MTT assay). ARPE-19 cells were seeded in 96-well plates at a density of 4.5x10^4 cells per well in DMEM/F12 culture medium supplemented with 10% heat-inactivated FBS at 37 °C, 5% CO2 overnight. After starving the cells in a basal DMEM/F12 medium for 4 to 5 hours, the medium was changed to 200 µl of basal medium containing various concentrations of HOOA-lactone (0-100 μM). After incubation overnight (about 16 h) at 37 °C, 5% CO2 overnight, the supernatants were removed and the
cells were washed three times with basal DMEM/F12 medium. Then 20 μL of MTT solution (5 mg/ml in basal medium) plus 180 μL of basal medium were added to each well. After an additional 4 h of incubation at 37 °C under 5% CO₂, the plates were centrifuged at 1,000g for 5 min and the medium was aspirated from each well. 200 μL of dimethylsulfoxide (DMSO) was added to each well to dissolve the formazan crystals, and the absorbance was then measured with a plate reader (Model M3, Molecular Device) at 570/670 nm.

**Measurement of oxidative stress.** 4.5 x 10⁴ ARPE-19 cells were plated in 8-chamber slides (LabTek II) in complete DMEM/F12 culture medium overnight at 37 °C and 5% CO₂. After starving the cells in basal DMEM/F12 medium for 4 to 5 hours, the cells were pre-incubated with 13.3 μM DCFDA for 45 min. After removing the medium, the cells were further incubated with medium containing various concentrations of HOOA-lactone (0 - 30 μM) for another 30 min at 37 °C under 5% CO₂. Images were acquired on a Leica DMI 6000 B inverted microscope (Leica Microsystems Wetzlar, Germany) using a Retiga EXI camera (Q-imaging, Vancouver, British Columbia). Image analysis was performed using Metamorph Imaging Software (Molecular Devices, Downington, PA).

**Determination of apoptosis and necrosis using fluorescence microscopy.** The two major types of cell death, necrosis and apoptosis, produced by HOOA-lactone on ARPE-19 cells were detected using a CF488A-Annexin V-propidium iodide (PI) apoptosis assay kit and fluorescence microscopy. Briefly, ARPE-19 cells (2 x 10⁴ cells/ per well) were plated in 96-well microtiter plates in complete DMEM/ F12 medium overnight under 5% CO₂/95% air at 37 °C. The following day, the cells were starved in basal DMEM/F12 medium for 4 - 5 hours. Then, the cells were incubated with 0 or 15 μM HOOA-lactone
overnight under 5% CO\textsubscript{2}/95% air at 37 °C. After overnight incubation, the plates were centrifuged at 400 g for 5 min and washed with basal DMEM/F12 medium followed by staining with Annexin V and PI according to the instructions of the kit. Finally, the images of the stained cells were taken at 10X magnification with a Leica DMI 6000 B inverted microscope.

**Measurement of caspase-3/7 activity using fluorescence microscopy.** Visualization of activation of caspase 3/7 activity in ARPE19 cells after treatment with HOOA-lactone was accomplished with a NucView 488 caspase 3 assay (DEVD-NucView 488-substrate) *in situ* apoptosis detection kit for live cells. Briefly, ARPE-19 cells (2 x 10\textsuperscript{4} cells/ per well) were plated in 96-well microtiter plates in complete DMEM/F12 medium overnight under 5% CO\textsubscript{2}/95% air at 37 °C. The following day, the cells were starved in the basal medium for 4-5 hours. The cells were then incubated with 0 and 15 µM HOOA-lactone overnight under 5% CO\textsubscript{2}/95% air at 37 °C. Then the plates were centrifuged at 400g for 5 min and washed with basal DMEM /F12 medium followed by detection of apoptotic cells with the NucView 488 caspase 3 assay kit according to the manufacturer’s suggested protocol. Finally, the images were taken at 10X magnification with a Leica DMI 6000 B inverted microscope.

**Synthesis of HOOA-lactone-GSH adduct (=O) (4.15).** HOOA-lactone (4.5, 15.4mg, 100 µmol) was incubated with reduced glutathione (61.5 mg, 200 µmol) in water (3.0 mL) at room temperature for 4 h. The excess of GSH was removed by solid-phase extraction (SPE) through a strata-X 33U polymeric reversed phase cartridge (Phenomenex, 500 mg/6 mL). The cartridge was prewetted with 6 mL of methanol containing 0.1% formic acid and equilibrated with 6 mL of water containing 0.1% formic acid. After loading the sample,
the cartridge was rinsed with 12 mL of water containing 0.1% formic acid to remove excess GSH and the adduct **4.15** was then eluted with 18 mL of 10% acetonitrile containing 0.1% formic acid. The structural identity of compound **4.15** was confirmed by mass spectroscopic analysis. ESI-MS: m/z calcd for $\text{C}_{18}\text{H}_{28}\text{N}_3\text{O}_9\text{S}$ (M+H)$^+$, 462.15, found 462.00.

**Synthesis of reduced HOOA-lactone-GSH adduct (-OH) 4.16.** 10 μmol of HOOA-lactone-GSH adduct (=O) **4.15** was reduced by 12 μmol of sodium borohydride in pH 7.4 PBS buffer (1.5 mL) at 4 °C for 5 h. Then excess NaBH$_4$ was destroyed by adding 2 μL of formic acid. Purification was performed by SPE as described above for ketone **4.15**. ESI-MS: m/z calcd for $\text{C}_{18}\text{H}_{30}\text{N}_3\text{O}_9\text{S}$ (M+H)$^+$, 463.16, found 464.73.

**Statistical Analysis.** Statistical analyses were performed by using Student’s t test. P value <0.05 is considered as statistically significant. Representatives of p-value in figures include “*” p<0.05, “**” p<0.002, “***” p<0.0001. Data are presented as mean ± SD.

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2.5 References


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CHAPTER 5

IDENTIFICATION OF NOVEL BIOACTIVE 4-OXO-HEPTANEDIOIC AMIDE (OHDIA) DERIVATIVES IN VITRO AND VIVO BY LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY
5.1 Background

Free radicals such as reactive oxygen species (ROS), which are molecules or molecular fragments containing one or more unpaired electrons, are produced from both endogenous sources such as mitochondria and exogenous substances such as xenobiotics.\(^1\) The steady-state formation of ROS is normally balanced by antioxidant defense mechanism including antioxidant enzymes such as superoxide dismutases in addition to nonenzymatic antioxidants such as glutathione (GSH).\(^2\) The imbalance between prooxidant and antioxidant processes, which results from a lack of antioxidant capacity or by an over-abundance of ROS, leads to oxidative stress.\(^3\) Oxidative stress causes overproduction of ROS, which are able to directly modify biomolecules, such as nucleic acids, lipids, and proteins, causing oxidative damage. Oxidative stress has been implicated in various and numerous pathological states including inflammation, atherosclerosis, neurodegenerative diseases, and cancer.\(^2,4\) Although ROS can react with all biomacromolecules in the cell, among these targets, the membrane lipids, mainly phospholipids containing polyunsaturated fatty acids (PUFAs) are predominantly susceptible to free radical-initiated oxidation.\(^5\) Lipid peroxidation proceeds by a free radical chain reaction mechanism and yields a variety of unsaturated lipid hydroperoxides as major initial reaction products. Subsequently, the decomposition of lipid hydroperoxides generates numerous mono- and bi-functional reactive carbonyl-containing products called lipoxidation (LPO) products.\(^3,6,7\) On the basis of their structural features, these reactive aldehydes, produced during peroxidation of polyunsaturated fatty acids, can be classified as: (1) long-chain aldehydes that contain the carboxyl terminus of the acyl chain such as 9-hydroxy-12-oxododec-10-enoic acid (HODA),
9-keto-12-oxododec-10-enoic acid (KODA), 4-hydroxy-7-oxohept-5-enoic acid (HOHA) and 4-keto-7-oxohept-5-enoic acid (KOHA); or (2) short-chain aldehydes that contain the methyl terminus, such as 4-hydroxy-2-nonenal (4-HNE), 4-oxo-2-nonenal (4-ONE) and 4-hydroxy-2-hexenal (4-HHE) (Scheme 5.1).\textsuperscript{7-10} Compared to free radicals, the short-chain aldehydes have prolonged half-lives that allow them to travel over long distances to attack targets far from the site of their origin\textsuperscript{2} while the long-chain aldehydes are anchored to membranes. The toxicity of these compounds has been mostly related to their ability to readily modify biological macromolecules such as proteins, DNA, and ethanolamine phospholipids to form a variety of biologically relevant intra and intermolecular covalent adducts that have been demonstrated to be involved in several pathological processes.\textsuperscript{7,11}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{scheme51.png}
\caption{Structures of some $\alpha$, $\beta$-unsaturated aldehyde lipid peroxidation products.}
\end{figure}
During the past few decades, characterization of the structures of protein and DNA modifications by LPO-derived aldehydes, especially α,β-unsaturated aldehydes, has attracted tremendous attention. Among these aldehydes, 4-HNE, a highly reactive bifunctional aldehyde derived from the peroxidation ω-6 PUFAs (linoleic and arachidonic acids), has been the best recognized and most studied cytotoxic product of lipid peroxidation.\textsuperscript{5,7,11} The reactivity of 4-HNE arises from three functional groups: an aldehyde group, a C=C double bond, and a hydroxyl group. 4-HNE can form Michael adducts with cysteine (Cys), histidine (His), and lysine (Lys) residues of proteins. The Michael adducts formed with Cys and His are relatively stable while the lysine Michael adducts are readily reversible. 4-HNE can also form Schiff base adducts with a protein Lys residue, leading to a 2-pentylpyrrole (PP) that is very stable and represents the most stable end product of 4-HNE protein modification. In addition, HNE also has protein crosslinking ability.\textsuperscript{2,4,7,12} The 4-keto cousin of 4-HNE, 4-ONE, arising independently and not from oxidation of 4-HNE, was shown to be even more reactive and toxic than 4-HNE.\textsuperscript{11,13} It was demonstrated that 4-ONE can modify Lys residues of proteins to form another stable advanced lipoxidation end product: the Lys-derived keto-amide.\textsuperscript{8,14} Similarly, KODA, the carboxy-terminating 4-ONE-like product of oxidation of linoleic acid, was also found to form the same 4-ketoamide construct with the Lys residues of proteins.\textsuperscript{8,15} The proposed mechanism for the formation of such amides involves a facile tautomerization of a carbinolamine precursor to the 4-oxo-2-enal-derived Schiff base (Scheme 5.2, see next page).\textsuperscript{8}
Docosahexaenoic acid (DHA; 22:6; ω-3) has been a focus of interest owing to the fact that it is highly enriched in important neural structures including the brain and retina, where it comprises 40% and 60% of total fatty acids, respectively.\textsuperscript{16, 17} Because DHA has six C=C bonds and 5 doubly allylic methylenes, and because the susceptibility of PUFAs to peroxidation is linearly proportional to the degree of unsaturation, DHA is a prime target for oxidative stress, resulting in more toxic oxidation products than linoleic, linolenic and arachidonic acids.\textsuperscript{16, 18} In analogy with the chemistry of arachidonate and linoleate phospholipids, oxidative cleavage of phospholipids containing DHA produces reactive aldehydic acid esters of 2-lyso-phosphatidylcholine (PC) including the HOHA ester (HOHA-PC) and KOHA ester (KOHA-PC).\textsuperscript{19} HOHA-PC reacts with lysyl ε-amino residues of proteins or the amino group of ethanolamine phospholipids to generate biologically active 2-ω-carboxyethylpyrrole (CEP) derivatives.\textsuperscript{19, 20} CEP derivatives are biomarkers of AMD. Elevated levels are found in the retinas and blood plasma of individuals with AMD compared to healthy individuals.\textsuperscript{19, 20} CEP adducts were shown to induce choroidal
neovascularization, and to promote wound healing and tumor growth through toll-like receptor 2 (TLR2) signaling.\textsuperscript{21, 22}

\begin{scheme}
\textbf{Scheme 5.3} Proposed mechanism for the formation of CEP and OHdiA from DHA-PC.

By analogy with the chemistry of 4-ONE and KODA, we postulated that KOHA-PC can modify biomolecules to give similar stable 4-ketoamide adducts: 4-oxo-heptanedioic amide
\end{scheme}
(OHdiA) derivatives. The proposed mechanism by which CEP and OHdiA could be formed from free radical-initiated lipid peroxidation of DHA-PC is outlined in Scheme 5.3 (see page 193). In brief, oxidative cleavage of DHA-PC generates KOHA-PC. The reactive aldehyde group of KOHA-PC can react with the free lysine ε-amine group of proteins or the amino group of phosphatidylethanolamine to form a hemiaminal intermediate, which undergoes tautomerization to generate a bis-enol intermediate that can be further converted into OHdiA adducts. Given that DHA is highly concentrated in neural structures, one of our motivations for investigating whether OHdiA could be formed from KOHA involves the possibility that OHdiA may prove to be a unique biomarker of neurological diseases and further be used for understanding of the pathological involvements of lipid oxidation in these diseases. Furthermore, OHdiA could potentially exert biological activity.

In the present study, we first designed a LC-MS/MS method to identify the OHdiA adducts *in vitro* and *in vivo*. After achieving an efficient synthesis of authentic standards, we investigated whether KOHA-PC was able to modify protein lysine residue to form OHdiA adducts. Then we examined the generation of OHdiA adducts during free-radical-induced oxidation of DHA-PC in the presence of human serum albumin (HSA) or 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE) and determined the product evolution profiles. In addition, as a prelude to exploring *in vivo* formation of OHdiA adducts as well as their relevance in diseases, we performed a preliminary investigation of the generation of OHdiA adducts in normal human plasma and plasma from patients with sickle cell disease (SCD) and compared the levels of OHdiA-protein adducts and OHdiA-PE adducts in human plasma. In addition, collaborating with researchers at the Cleveland Clinic
and Dr. Xiaoxia Z. West from Dr. Salomon’s group, the angiogenic properties of OHdiA adducts were examined using adhesion and tube formation assays with human umbilical vein endothelial cells (HUVECs). Furthermore, the mechanism of OHdiA-driven angiogenesis was studied by Dr. Xiaoxia Z. West with respect to toll-like receptors (TLRs). Moreover, anti-OHdiA polyclonal antibody was purified and its structural selectivity and specificity were also examined compared to similar haptens.
5.2 Results and Discussion

Strategy for identification of OHdiA derivatives of biological amines. As shown in Scheme 5.4, our strategy is to heat the OHdiA adducts with 6 N hydrochloric acid (HCl) for about 5 hours at 100 °C to give the corresponding carboxylic acid, which was characterized by LC-tandem mass spectrometry (LC-MS/MS). To further verify the structures of OHdiA modification in complex systems, the mixtures were treated with pentafluorobenzyl bromide (PFB-Br) to derivatize the carboxylic acid functionality (360 Da mass addition) and compared with authentic samples and deuterium labeled derivatives that are available through the unambiguous total syntheses described below. The overall workflow for analysis of OHdiA adduct formation is shown in Scheme 5.5 (see next page).

Scheme 5.4 Strategy for the identification of OHdiA modifications.
Scheme 5.5 Schematic of workflow for analysis of OHdiA adducts by LC–MS/MS.
Syntheses of OHdiA-PFB and OHdiA-PFB-D4. To facilitate the detection and characterization of OHdiA modifications by LC-ESI-MS/MS, authentic OHdiA-PFB (5.3) and d4-labeled OHdiA-PFB (OHdiA-PFB-D4, 5.5) were synthesized (Scheme 5.6). The two carboxylic acid groups of 4-oxoheptanedioic acid (5.1) were derivatized with PFB-Br (5.2) in anhydrous acetonitrile containing N,N-diisopropylethylamine (DIPEA) to give compound 5.3. Based on a protocol kindly provided by Dr. Guofang Zhang, four deuterium atoms, which are stable and nonexchangeable, were introduced into 5.1 by a base (NaOD/D2O) catalyzed deuterium exchange of 5.1 to generate the tetradeutero derivative 3,3,5,5-d4-4-oxoheptanedioic acid (5.4). The carboxyl groups in 5.4 were then derivatized with PFB-Br (5.2) to give deuterium labeled 3,3,5,5-d4-OHdiA-PFB (d4-OHdiA-PFB, 5.5).

\[
\text{Scheme 5.6 Syntheses of OHdiA-PFB and d4-OHdiA-PFB.}
\]

Validation of acid-based hydrolysis for OHdiA adducts. The efficacy of HCl-mediated deacylation was tested using synthetic ethanolamine amide 5.6 of OHdiA, which was expected to produce 4-oxoheptanedioic acid (5.1) (Figure 5.1A, see next page). Thus, a sample of pure OHdiA-ethanolamine amide 5.6 (prepared as detailed in the Experimental
Procedures) was hydrolyzed with 6 N HCl at 100 °C for 5 hours. Then the pH of reaction mixture was adjusted to 3-4 by addition of 10 N KOH, and then extracted with ethyl acetate three times. Solvent was removed from the combined organic layers, and the residue was dissolved in CD$_3$OD for $^1$H NMR analysis. As shown in Figure 5.1C (see next page), the hydrolysis of OHdiA-ethanolamine amide 5.6 went to completion delivering 4-oxoheptanedioic acid, as confirmed by $^1$H NMR identity with authentic 4-oxoheptanedioic acid (Figure 5.1B, see next page). Therefore, HCl-mediated amide hydrolysis could be used for measurement of OHdiA derivatives in biological samples.

Figure 5.1 Hydrolysis of OHdiA-ethanolamine amide 5.6 to 4-oxoheptanedioic acid by 6 N HCl. (A) The expected product after 6 N HCl-mediated hydrolysis of OHdiA-ethanolamine amide 5.6 with 6 N HCl for 5 hours at 100 °C. (B) $^1$H NMR of authentic 4-oxoheptanedioic acid. (C) $^1$H NMR of hydrolysis product from OHdiA-ethanolamine amide 5.6.
KOHA-PC reacts with Ac-Gly-Lys-OMe to form OHdiA. Keto aldehyde KOHA-PC (5.10) was prepared by an efficient synthesis from 3-furan-2-ylpropionic acid (5.8) as described previously (Scheme 5.7). Briefly, esterification of 2-lyso-PC (5.7) with 5.8 produced the carboxyethylfuryl PC (CEF-PC, 5.9). Oxidative ring opening of 5.9 gave KOHA-PC (5.10) in good yield.

Scheme 5.7 Syntheses of KOHA-PC (5.10).

To test the hypothesis that KOHA-PC reacts with the ε-amino group of a peptide lysyl residue to form an OHdiA adduct, a 2.5-fold excess of the KOHA-PC (100 ng/µL) was incubated with the dipeptide, Ac-Gly-Lys-OMe (5.11, 40 ng/µL) in methanol at room temperature for 48 hours. To detect the formation of OHdiA-PC-dipeptide (5.12), the strategy described above was used (Scheme 5.8, see next page). Thus, after the removal of solvents, the crude reaction product was hydrolyzed with 6 N HCl at 100 °C for 5.0 hours.
and then extracted with ethyl acetate three times. The combined organic extracts were dried under a flow of dry N₂ and the solvent free sample was stored at -80 °C until analysis. Reverse phase HPLC-ESI/MS in the negative ion mode was used to detect the formation of 5.1 from KOHA-PC incubated with dipeptide 5.11 followed by 6 N HCl hydrolysis by comparison with a sample of commercially available authentic 5.1. As a control experiment,

![Scheme 5.8](image)

**Scheme 5.8** KOHA-PC reacts with Ac-Gly-Lys-OMe to form OHdiA-PC-dipeptide (5.12).

similar treatment of KOHA-PC without dipeptide 5.11 followed by acid-catalyzed hydrolysis resulted in a minor if any peak in the SIC at m/z 173.2 (Figure 5.2B, see next page) corresponding to the peak at 2.42 min for the authentic sample of 5.1 (Figure 5.2A, see next page). Thus, 5.1 is not generated from KOHA-PC under these conditions. In contrast, a significant peak at 2.40 min corresponding to 5.1 was detected in the product mixture from reaction of KOHA-PC with dipeptide 5.11 followed by acid hydrolysis of the putative amide intermediate 5.12 (Figure 5.2C, see next page). This observation provides presumptive evidence that KOHA-PC can modify the dipeptide to produce OHdiA-PC-dipeptide (5.12) which is hydrolyzed to form 5.1.
Figure 5.2 Selected ion chromatograms (SICs) in the negative ion mode of m/z 173.2 from (A) pure authentic 5.1 standard, (B) the reaction product mixture from incubation of KOHA-PC for 48 h without dipeptide 5.11 followed by hydrolysis with 6 N HCl at 100 °C for 5 h, and (C) incubation of KOHA-PC with dipeptide 5.11 for 48 h followed by hydrolysis with 6 N HCl at 100 °C for 5 h.

To confirm the identity of the putative product 5.1 from incubation of KOHA-PC with dipeptide followed by hydrolysis with 6 N HCl at 100 °C for 5 h, it was further analyzed by reverse phase HPLC/ESI/MS/MS using multiple reaction monitoring (MRM) in the negative ion mode. A MRM method was developed based on the collision induced
disassociation (CID) spectrum of [M−H]− of the parent ion for authentic 4-oxoheptanedioic acid (5.1) (Figure 5.3). Four representative daughter ions, m/z 85, 111, 129.2, and 137.1, were chosen as MRM transitions for fragmentations of the parent ion m/z 173.2 for analysis of 4-oxoheptanedioic acid. The proposed structure of each daughter ion is shown in the inset.

![CID spectrum](image)

**Figure 5.3** CID spectrum of the m/z 173.2 ion from authentic 4-oxoheptanedioic acid (5.1). Inset: interpretation of major product ions.

As shown in Figure 5.4 (see next page), treatment of KOHA-PC with dipeptide Ac-Gly-Lys-OMe for 48 hours followed by acid-catalyzed hydrolysis generates a significant peak in the negative-ion LC-MS/MS chromatogram at 2.11 min. An identical peak appears in the authentic 4-oxoheptanedioic acid. This further confirms the formation of OHdiA adduct from KOHA-PC modification.
Figure 5.4 HPLC/ESI/MS/MS analysis of OHdiA adduct production in the reaction of KOHA-PC with Ac-Gly-Lys-OMe dipeptide. (A) Authentic 4-oxoheptanedioic acid standard. (B) Reaction product mixture after 48 h incubation at room temperature.

To further confirm the identity of the peak detected, the 4-oxoheptanedioic acid produced from the acid-catalyzed hydrolysis of the product mixture of KOHA-PC with dipeptide was treated with pentafluorobenzyl bromide (PFB-Br) to derivatize the two carboxylic acid functional groups for analysis by reverse phase HPLC/ESI/MS/MS using multiple reaction monitoring (MRM) in the positive ion mode. Pentafluorobenzyl esterification of the two carboxylic acid functional groups results in a net increase of 360 Da. The pentafluorobenzyl ester of pure 4-oxoheptanedioic acid, OHdiA-PFB (5.3), was prepared and purified as described in the previous paragraph to provide an authentic sample of 5.3. Similarly, a MRM method was developed based on the CID spectrum of [M+H]+ of the parent ion for authentic
OHdiA-PFB \((5.3)\) (Figure 5.5). Three representative daughter ions, \(m/z\) 111.1, 181.2, and 337.0, were chosen as MRM transitions for fragmentations of the parent ion \(m/z\) 535 for OHdiA-PFB analysis. The proposed structure of each daughter ion is shown in the inset.

**Figure 5.5** CID spectrum of \(m/z\) 535.0 of authentic OHdiA-PFB \((5.3)\). Inset: Interpretation of major product ions.

Analyses following treatment with PFB-Br are shown in Figure 5.6. After reaction with PFB-Br, a major peak with a retention time at 11.2 min that is identical to the derivatized standard (Figure 5.6A, see next page) is present in the positive-ion MRM chromatogram of the product mixture from KOHA-PC treated dipeptide (Figure 5.6B, see next page), demonstrating the generation of OHdiA derivative.
Figure 5.6 HPLC/ESI/MS/MS analysis after pentafluorobenzyl esterification of OHdiA derivative production in the reaction of KOHA-PC with Ac-Gly-Lys-OMe dipeptide. (A) Authentic OHdiA-PFB standard. (B) Reaction product mixture from incubation of KOHA-PC with dipeptide for 48 h at room temperature followed by hydrolysis 6 N HCl and pentafluorobenzyl esterification.

Generation of OHdiA by in vitro oxidation of DHA-PC in the presence of human serum albumin (HSA). Previously, it was demonstrated that free radical-induced oxidative cleavage of DHA-PC promoted by myeloperoxidase or copper ions generates a large number of reactive electrophiles such as HOHA-PC and KOHA-PC.\textsuperscript{10} It was established that condensation of HOHA-PC with the ε-amino group of a lysine containing dipeptide or protein could form CEP derivatives.\textsuperscript{10, 19} In addition, in vitro experiments also demonstrated that CEP derivatives could be generated during autoxidation of DHA-PC in the presence of
protein. Since the studies described above showed that KOHA-PC could modify Ac-Gly-Lys-OMe dipeptide to generate OHdiA derivatives, it seemed likely that OHdiA derivatives would be also generated during autoxidation of DHA-PC in the presence of protein. Therefore, we next investigated the formation of OHdiA derivatives during in vitro free radical initiated oxidation of DHA-PC in the presence of HSA by the HPLC-ESI/MS/MS analysis using MRM developed as described above. As shown in Figure 5.7A (see next page), little or no OHdiA was detected in the reaction mixture before (0 h) oxidation of DHA-PC in presence of HSA followed by acid-catalyzed hydrolysis. After 48 h oxidation, a significant peak was observed in the negative-ion LC-MS/MS chromatogram at 2.12 min as also observed for an authentic 4-oxoheptanedioic acid standard (Figure 5.7B, see next page). These results demonstrated that OHdiA derivative is produced during autoxidation of DHA-PC in the presence of protein.
Figure 5.7 HPLC/ESI/MS/MS analysis of OHdiA derived from reaction of DHA-PC with HSA after (A) 0 h incubation and (B) 48 h incubation followed by hydrolysis in the presence of 6 N HCl at 100 °C.

To further characterize the putative OHdiA formed in the oxidation product mixture from DHA-PC in the presence of HSA after acid-catalyzed hydrolysis, it was subjected to pentafluorobenzyl esterification followed by analysis by the reverse phase HPLC-ESI/MS/MS using MRM in the positive ion mode as described above. Analyses following treatment with PFB-Br are shown in Figure 5.8 (see next page). DHA-PC in the presence of HSA prior to oxidation (0 h) produced detectable but low levels of OHdiA while oxidation for 48 h resulted in a significantly greater level of OHdiA. These results further confirmed
the generation of OHdiA derivatives during autoxidation of DHA-PC in the presence of protein.

Figure 5.8 HPLC/ESI/MS/MS analysis of OHdiA in the reaction mixture of DHA-PC with HSA (A) prior to oxidation (0 h incubation) and (B) after 48 h oxidation followed by hydrolysis at 100 °C with 6 N HCl and esterification with pentafluorobenzyl bromide.

I next examined the time course for the formation of OHdiA derivatives during autoxidation of DHA-PC in the presence of HSA. The copper-promoted oxidation of DHA-PC vesicles with HSA in PBS was performed up to 48 h at 37 °C under air and aliquots were withdrawn after various reaction times and quenched by the addition of butylated hydroxytoluene (BHT) and ethylenediaminetetraacetic acid (EDTA). The reaction mixture was then dialyzed against PBS containing EDTA for 48 h. The reaction mixture was then combined with 6 N HCl in a vial and heated for 5 h at 100 °C. The concentration of
endogenous OHdiA was quantified by LC-MS/MS using a calibration curve established with the commercially available authentic standard 4-oxoheptanedioic acid (Figure 5.9A). The product evolution profile showed that the formed OHdiA derivative increased steadily in the first 30 h and more slowly afterward during autoxidation of DHA-PC in the presence of HSA (Figure 5.9B).

**Figure 5.9** (A) Calibration curve for OHdiA derivative in MRM transition 173.2>111 and (B) evolution profile of OHdiA generation in the reaction of DHA-PC with HSA. The data was obtained from three replicated experiments.

Generation of OHdiA by in vitro oxidation of DHA-PC in the presence of DPPE followed by hydrolysis with 6 N HCl at 100 °C. Although Lys ε-amino groups in proteins are a major target of LPO derived aldehydes, such as α,β-unsaturated aldehydes, and various adducts have been identified, the primary amino groups of phosphatidylethanolamines (PEs) are also targets for covalent modification by lipid peroxidation products such as 4-HNE, acrolein and isolevuglandins.23-25 On the basis of these findings, it was expected that PEs would be modified by KOHA-PC generated from oxidation of DHA-PC to produce OHdiA.
derivatives in analogy with the reaction of ε-amino groups of protein lysyl residues with oxidatively generated KOHA-PC as described above. To test the hypothesis that KOHA-PC generated from oxidation of DHA-PC covalently binds with the primary amino groups of PEs in vitro to generate OHdiA derivatives, the copper-catalyzed oxidation of DHA-PC was performed in the presence of 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE) followed by hydrolysis with HCl at 100 °C and analysis of the reaction product mixture by the LC-MS/MS method described above for detecting the final product OHdiA. As shown in Figure 5.10 (see next page), the copper-promoted oxidation of DHA-PC in the presence of DPPE for 48 h followed by acid-catalyzed hydrolysis, produced high levels of OHdiA as demonstrated by the detection of significant peak with the same retention time as the authentic standard 4-oxoheptanedioic acid in the corresponding MRM transitions (Figure 5.10C, see next page) while a barely detectable level of OHdiA was observed prior to oxidation of DHA-PC in the presence of DPPE (Figure 5.10B, see next page). In addition, the copper-promoted oxidation of DHA-PC in the absence of DPPE for 48 h followed by acid hydrolysis produced very low levels of OHdiA (Figure 5.10A, see next page). Collectively, these results indicated that covalent binding of KOHA-PC, generated from free radical-induced oxidation of DHA-PC in vitro, with the primary amino group of PEs produces OHdiA derivatives.
Figure 5.10 HPLC/ESI/MS/MS analysis of OHdiA produced by copper-catalyzed oxidation of DHA-PC for 48 h (A) without DPPE or (B) with DPPE after 0 h incubation or (C) with DPPE after 48 h incubation followed by hydrolysis with 6 N HCl at 100 °C for 5 h.

To further probe for the formation of OHdiA derivatives by copper-promoted oxidation of DHA-PC in the presence of DPPE, the product mixture of oxidation of DHA-PC in the presence or absence of DPPE followed by acid-catalyzed hydrolysis were also treated with PFB-Br to derivatize the two carboxylic acid functional groups, and then analyzed by LC-MS/MS. As shown in Figure 5.11 (see next page), strong peaks with the same retention time as the synthetic authentic standard OHdiA-PFB appeared in the MRM chromatograms of the product mixture generated from DHA-PC oxidation in the presence of DPPE (C) for 48 h, but not (B) 0 h or (A) for 48 h in the absence of DPPE as detected in peaks for three MRM transitions with a retention time that is identical to that of an authentic OHdiA-PFB standard.
Figure 5.11 HPLC/ESI/MS/MS analysis of OHdiA-PFB generated after acid hydrolysis followed by pentafluorobenzyl esterification of the reaction product mixtures from copper-catalyzed oxidation of (A) DHA-PC for 48 h or (B) DHA-PC with DPPE for 0 h or (C) DHA-PC with DPPE for 48 h.

The time course for the formation of OHdiA derivatives during oxidation of DHA-PC in the presence of DPPE was also investigated and the concentration of endogenous OHdiA was quantified by LC-MS/MS using a calibration curve established with the commercially available authentic standard 4-oxoheptanedioic acid (Figure 5.12A, see next page). The product evolution profile for oxidation of DHA-PC in the presence of DPPE was shown in Figure 5.12B (see next page). Initially, OHdiA derivative was formed quickly in the first 10 h and then more slowly afterward.
Figure 5.12 (A) Calibration curve for OHdiA detected by the MRM transition 173.2>111 and (B) evolution profile of OHdiA generated by the copper-catalyzed oxidation of DHA-PC in the presence (■) or absence of DPPE (●) followed by hydrolysis with 6 N HCl at 100 °C for 5 h. The data represents three replicated experiments.

Detection of OHdiA derivatives in human plasma. As described above, KOHA-PC generated from free radical-induced oxidation of DHA-PC was shown to modify the primary amino groups from both proteins and phosphatidylethanolamines in vitro to form OHdiA derivatives. OHdiA derivatives were also expected to be formed in vivo. For detecting their presence in vivo, we initially examined whether they could be detected in human plasma samples from healthy individuals. Analysis of normal human plasma treated with acid hydrolysis followed by PFB esterification provided evidence for the presence of OHdiA derivatives in vivo. Representative LC-MS/MS chromatograms of OHdiA detected in normal human plasma are shown in Figure 5.13 B (see next page). The MRM transitions corresponding to OHdiA-PFB coeluted at a single retention time identical with that of the pure synthetic OHdiA-PFB standard (Figure 5.13A, see next page). Heptanedioic acid
(pimelic acid), an endogenous compound in human, results from the β-oxidation of nonanedioic acid (azelaic acid).\textsuperscript{26, 27} It is convincible that the 4-oxoheptanedioic acid after acid hydrolysis may come from heptanedioic acid in vivo. However, to our knowledge, there is no evidence showing that heptanedioic acid can undergo oxidation to give 4-oxoheptanedioic acid.

**Figure 5.13** LC/ESI/MS/MS evidence for OHdiA derivatives in human plasma. OHdiA-PFB was detected after acid hydrolysis followed by PFB esterification. (A) MRM for synthetic authentic standard OHdiA-PFB. (B) MRM for PFB derivative of 4-oxoheptanedioic acid from normal human plasma after acid hydrolysis and PFB derivatization. (C) MRM for PFB derivative of 4-oxoheptanedioic acid obtained from human plasma samples from sickle cell patients after acid hydrolysis and PFB derivatization.

Sickle cell disease (SCD) is a genetic blood disorder of hemoglobin resulting in harmful pathophysical effects, including sickling, vaso-occlusion, inflammation, and ischemia-reperfusion injury.\textsuperscript{28, 29} These phenomena can produce more ROS, resulting in the formation of oxidative stress. The oxidative stress further intensifies the symptoms of SCD and
exacerbates the disease, generating a vicious circle. Studies have shown that there is elevated oxidative stress in SCD and antioxidant therapeutic strategies are effective to reduce pathological consequences of the disease, strongly supporting the importance of oxidative stress in progression of SCD. Since PUFA phospholipids, especially those containing DHA, undergo oxidation and truncation to generate a multitude of reactive aldehydes including KOHA-PC and lipid peroxidation levels are increased in sickle cell patients, OHdiA adducts were expected to be present in increased levels in sickle cell patients, and OHdiA may be useful as a marker of SCD severity. Therefore, we next investigated the presence of OHdiA derivatives in human plasma from SCD patients using the procedure involving LC-MS/MS developed above. As shown in Figure 5.13C (see page 215), the MRM transitions corresponding to OHdiA-PFB detected in human plasma from SCD patients treated with acid hydrolysis followed by PFB esterification also have the single retention time as that of the pure synthetic OHdiA-PFB standard (Figure 5.13C, see page 215), demonstrating the presence of OHdiA adducts in blood from individuals with SCD. It was found that OHdiA adducts were present in similar levels in blood from SCD patients and from healthy people (Figure 5.13B and C, see page 215). It is possible, but remains to be explored, that elevated levels of OHdiA may be found in blood collected from SCD patients during hospitalization for an episode of cell sickeling.

**Measurement of OHdiA modified PE and protein in human plasma.** Evidence described above supports the conclusion that KOHA-PC generated from oxidation of DHA-PC can generate OHdiA derivatives of the primary amino groups of lysine residues of proteins and the ethanolamine headgroup of PEs in vitro, and supports the conclusion that
OHdiA derivatives are present in human plasma samples. The levels of the putative OHdiA-PE and OHdiA-protein adducts in human plasma samples were next compared by LC-MS/MS. Before acid hydrolysis, biological samples were extracted with chloroform/methanol by the Bligh and Dyer method to separate proteins and phospholipids.\textsuperscript{31} To increase the sensitivity and minimize any matrix effect for the biological samples, the isotope-labeled internal standard OHdiA-PFB-d\textsubscript{4} was introduced prior to LC-MS analysis. The integrated peak area of OHdiA-PFB in the MRM transition m/z 535>181.2 from different runs was normalized by comparing with that of the internal standard OHdiA-PFB-d\textsubscript{4} in the MRM transition m/z 539>181. As shown in Figure 5.14 (see next page), both OHdiA-PE adducts and protein adducts were detected in normal human plasma samples (n = 3) and the ratio of OHdiA-PE to protein adducts was 0.35, indicating that OHdiA-protein adducts approximately twice as abundant as PE adducts in normal human plasma. Previously, it was shown that modification of proteins by LPO-derived aldehydes such as 4-HNE and isolevuglandin (referred to by the misnomer “isoketal”) inhibits their degradation by the proteasome.\textsuperscript{32, 33} Therefore, it is likely that OHdiA modification of proteins would similarly impair the clearance of these proteins, potentially leading to their accumulation, consistent with our results.
Figure 5.14 Levels of OHdiA-PE and OHdiA-protein derivatives in normal human plasma (n = 3, P = 0.027) measured by LC-MS/MS. Results are presented as mean ± SD. The peak area ratio of OHdiA-PFB in the MRM transition m/z 535>181.2 to the internal standard OHdiA-PFB-d$_4$ in the MRM transition m/z 539>181 from the LC-MS/MS analysis was used for analysis.

Similarly, the presence of both OHdiA-PE adducts and protein adducts were also examined in blood plasma samples from a hospitalized (n=1) and a clinical (n=1) patient with SCD. As shown in Table 5.1 (see next page), both OHdiA modified PE and protein adducts were detected in these SCD samples. This confirmed that OHdiA derivatives of the primary amino groups of lysine residues of proteins and the ethanolamine headgroup of PEs are formed in vivo and are present in SCD plasma.
Table 5.1 Analysis of OHdiA-PE and OHdiA-protein adducts in SCD plasma samples.

<table>
<thead>
<tr>
<th></th>
<th>OHdiA-PE (Ratio of OHdiA-PFB/OHdiA-PFB-D4)</th>
<th>OHdiA-Protein (Ratio of OHdiA-PFB/OHdiA-PFB-D4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC-H (N=1)</td>
<td>0.067</td>
<td>0.107</td>
</tr>
<tr>
<td>SC-C (N=1)</td>
<td>0.080</td>
<td>0.064</td>
</tr>
</tbody>
</table>

**Recovery of OHdiA and OHdiA-PFB in the sample pretreatment step.** To assess the recovery of 4-oxoheptanedioic acid in extraction step, the ratios of pure authentic 4-oxoheptanedioic acid concentration after ethyl acetate extraction to that without extraction at the concentration of 1 µg/mL were determined by LC-MS/MS. As is shown in Figure 5.15 A (see next page), the recovery of 4-oxoheptanedioic acid in extraction step is 56.2±3.1%, 58.9±5.7%, 58.3±4.3% and 52.9±7.7% in the MRM channel of 173.2>85, 173.2>111, 173.2>129.2 and 173.2>137.1, respectively. In addition, the recovery of OHdiA-PFB in extraction step was also determined by the ratios of pure authentic OHdiA-PFB concentration with ethyl acetate extraction to that without extraction at the concentration of 1 µg/mL by LC-MS/MS. As is shown in Figure 5.15 B (see next page), the recovery of OHdiA-PFB in extraction step is 91.6±2.2%, 91.1±1.3% and 92.9±2.0% in the MRM channel of 535>111.1, 535>181.2 and 535>337, respectively.
Figure 5.15 Recovery ratio of (A) 4-oxoheptanedioic acid and (B) OHdiA-PFB at the concentration of 1 µg/mL in the sample pretreatment steps. Data points represent the means of triplicate determinations from an experiment performed on three separate occasions.

**OHdiA-modified proteins induce toll-like receptor 2 (TLR2) receptor-dependent angiogenesis in vitro.** The biological activity of OHdiA derivatives such as OHdiA-bovine serum albumin (OHdiA-BSA) was investigated using two different *in vitro* models of angiogenesis with human umbilical vein endothelial cells (HUVECs) by Dr. Xiaoxia Z. West from Dr. Salomon’s group. As shown in Figure 5.16 (see page 222), low concentrations of OHdiA-BSA provided by Dr. Hong Li from Dr. Salomon’s lab had a proangiogenic effect comparable to that of vascular endothelial growth factor (VEGF) in adhesion and tube formation assays. In both adhesion and tube formation experiments, 1 µg/mL of OHdiA had no significant effect on angiogenesis compared to control BSA without OHdiA modification. However, in both experiments, 10 µg/mL of OHdiA greatly promoted angiogenesis in HUVECs. Presumably, OHdiA also promotes angiogenesis by HUVEC cells *in vivo*. Considering that CEP adducts are also derived from the peroxidation
of DHA and CEP adducts have a proangiogenic effect comparable to that of VEGF,\textsuperscript{21} the proangiogenic effects of OHdiA adducts and CEP adducts were compared using tube formation assay by Dr. West. As shown in Figure 5.17 (see page 223), the tube length increased firstly with the increase of concentration of both CEP-dipeptide and OHdiA-dipeptide and then reached the plateau. In the tested concentration range, OHdiA-dipeptide showed a lower proangiogenic effect than CEP-dipeptide at each concentration.
Figure 5.16 OHdiA derivatives induce angiogenesis in vitro in HUVECs in both (A) adhesion and (B) tube formation assays. Left: representative micrographs of HUVEC cells treated with OHdiA-BSA (1 or 10 µg/mL), BSA (control), or VEGF (60 ng/mL). Right: quantification of adhesion and tube formation experiments reported as means S.D. (n = 3). NS represent no significant difference, *** represents p<0.001. These experiments were performed by Dr. Xiaoxia Z. West.
Figure 5.17 The comparison of the proangiogenic effect of CEP and OHdiA derivatives in HUVECs in a tube formation assay. Data reported as means S.D. (n = 3)

Having established that OHdiA-BSA has proangiogenic effects on HUVECs, Dr. West explored the mechanism of OHdiA-BSA-induced angiogenesis. To identify receptors mediating OHdiA-BSA-induced angiogenesis, she tested the role of toll-like receptors (TLRs) such as TLR2 and TLR4, as they are not only expressed on the endothelium and implicated in angiogenesis, but also recognize a broad range of protein and lipid ligands like CEPs. The results of tube formation assays with HUVECs treated with OHdiA-BSA, VEGF or control BSA in the presence of anti-TLR2, anti-TLR4 and anti-isotype control (IgG2a) antibodies are shown in Figure 5.18 (see next page). Anti-TLR2, but not antibodies against TLR4, inhibited OHdiA-BSA-induced, but not VEGF-induced, tube formation. Thus, OHdiA-driven angiogenesis is similar to that promoted by CEP which is also TLR2 dependent and VEGF-independent. Therefore, OHdiA-BSA-promoted
angiogenesis may be an attractive new therapeutic target, especially in cancers resistant to anti-VEGF therapy as an adjunct to anti-VEGF therapy.

**Figure 5.18** HUVEC tube formation stimulated by OHdiA-BSA is TLR2 dependent. (A) Representative micrographs of HUVECs treated with OHdiA-BSA (10 µg/mL), VEGF (60 ng/mL) or control BSA in the presence of anti-TLR2, anti-TLR4 or isotype control (IgG2a) antibodies as indicated. (B) Quantification of tube formation reported as means and showing S.D. (n = 4). NS represents no significant difference, *** represents p<0.001. These experiments were performed by Dr. Xiaoxia Z. West.

**Purification of the anti-OHdiA polyclonal antibody with a protein-A/G column.** Multiple reactive aldehydes are generated from peroxidation of different PUFAs. Each unique aldehyde can produce unique derivatives of biomolecules. Because the level of specific derivatives is very low, their detection requires sensitive and specific techniques. A significant effort has been made to develop immunochemical tools that are highly sensitive and specific. To develop an immunoassay for OHdiA derivatives, a polyclonal rabbit antibody (anti-OHdiA) was raised against OHdiA-keyhole limpet hemocyanin (KLH) by
Dr. Li Hong from Dr. Salomon’s lab. Following immunization of a New Zealand white rabbit, the anti-OHdiA antibody titer increased and then plateaued over a 4 month period. I purified the anti-OHdiA polyclonal antibody on a protein A/G column. Briefly, rabbit serum was diluted with binding buffer (PBS, 10 mM, pH 7.4) and then loaded onto an equilibrated protein A/G column packed with immunopure protein A/G plus-agarose. To remove non-immunoglobulin G (non-IgG) proteins, pH 7.4 binding buffer was first passed through the column. Then the bound IgG was eluted with elution buffer (100 mM glycine-HCl containing 150 mM NaCl, pH 2.5). The purified polyclonal antibody solution was neutralized immediately and then dialyzed against 10 mM PBS (pH 7.4) at 4 ºC for 38 h. The resulting solutions of anti-OHdiA polyclonal antibody contained 0.528 mg/mL purified IgGs, as determined by Pierce bicinchoninic acid (BCA) protein assay.

**Cross-reactivity of the anti-OHdiA polyclonal antibody.** The utility of immunoassays largely depends on their structural specificity, in another words, its ability to distinguish between epitopes with structural similarities. To assess the structural specificity of the anti-OHdiA polyclonal antibody, competitive inhibition of antibody binding to OHdiA-BSA by various haptens was examined through competitive enzyme-linked immunosorbent assay (ELISA) studies. For these cross-reactivity studies, OHdiA-BSA was used as the coating agent and OHdiA-human serum albumin (HSA) was used as a standard. The concentration at 50% inhibition (IC₅₀) for OHdiA-HSA was defined as 100% cross-reactivity. The ELISAs were done as described previously for similar studies with CEP-KLH antibodies. Duplicates of serial dilutions of all inhibitors were used and final curves were constructed using mean absorbance values. As shown in Figure 5.19 (see next page), CEP-HSA, which
contains a carboxyethyl group appended to the 2-position of a pyrrole (Scheme 5.9), showed a significant inhibition of OHdiA antibody binding to OHdiA-BSA. The IC$_{50}$ values for OHdiA-HSA and CEP-HSA using OHdiA-BSA as coating agent were 0.084 µM and 0.291 µM, respectively. Thus, the OHdiA antibody exhibits 29% cross reactivity with CEP-HSA. Perhaps the antibodies that crossly react with CEP specifically recognize the pentacyclic OHdiA-BSA pyrrolidone tautomer that has a carboxyethyl group appended to the pyrrolidone 2 position (Scheme 5.10, see next page). Under acid catalyzed conditions, γ-keto amides tautomerize completely to the cyclic carbinol amide, i.e., pyrrolidone, form in aqueous solution. Thus, tautomerization of OHdiA-BSA to a pyrrolidone, which contains a carboxyethyl chain and a ring similar to those of CEP, is strongly favored thermodynamically. Consequently, the antigen-binding site of OHdiA antibody may readily accommodate the CEP owing to their similar structures. In contrast, carboxypropylpyrrole (CPP)-HSA and carboxyheptylpyrrole (CHP)-HSA, which have larger carboxylic acid side chains — three and seven CH$_2$ groups, respectively — than CEP-HSA (Scheme 5.9), and would not be expected to fit in the OHdiA binding site, clearly showed no cross-reactivity.

Scheme 5.9 The structure of carboxyalkylpyrrole compounds.
**Figure 5.19** Inhibition curves for binding of the anti-OHdiA-KLH polyclonal antibody to OHdiA-BSA by OHdiA-HSA (●), CEP-HSA (▼), CPP-HSA (▼), and CHP-HSA (♦).

**Scheme 5.10** Tautomeric amide and pyrrolidone forms of OHdiA.

**Cross-reactivity of the anti-CEP polyclonal antibody.** In a previous study by Dr. Gu,\textsuperscript{19} the anti-CEP polyclonal antibody showed high structural selectivity in competitive binding inhibition assays with CPP-HSA and CHP-HSA even though the CEP epitope differs from CPP epitope by only a single CH\textsubscript{2} group. Since CEP-HSA showed significant cross-reactivity with the anti-OHdiA polyclonal antibody as described above, it was important to address the cross-reactivity of the anti-CEP polyclonal antibody with OHdiA-protein. For this purpose, CEP-BSA was used as coating agent and CEP-HSA as a standard for binding.
anti-CEP polyclonal antibody. The concentration at 50% inhibition (IC\textsubscript{50}) for CEP-HSA was defined as 100% cross-reactivity. The IC\textsubscript{50} value for CEP-HSA using CEP-BSA as coating agent was 0.1555 µM. As shown in Figure 5.20, the anti-CEP polyclonal antibody showed remarkable selectivity for not binding with CPP-HSA or CHP-HSA, which is in accordance with previous report. Especially noteworthy is the fact that OHdiA-HSA did not show significant inhibition of anti-CEP compared to CEP-HSA. This may be a consequence of the nonplanarity of the carboxyethylpyrrolidone tautomer of OHdiA-HSA that requires a larger binding pocket than the planer pyrrole ring of the the carboxyethylpyrrole ring of CEP-HSA, which may thus not fit the antigen binding site of anti-CEP antibody. Therefore, anti-CEP polyclonal antibody exhibits high structural sensitivity and specificity for detecting CEP epitopes, and minimal cross-reactivity with other analogous compounds including OHdiA epitopes.

![Inhibition curve for binding of the anti-CEP polyclonal antibody to CEP-BSA](image)

**Figure 5.20** Inhibition curve for binding of the anti-CEP polyclonal antibody to CEP-BSA by OHdiA-HSA (●), CEP-HSA (▼), CPP-HSA (▲) and CHP-HSA (♦).
5.3 Conclusions

Previously, 4-oxo-2-nonenal (4-ONE), a bifunctional oxidative cleavage product generated from peroxidation of polyunsaturated fatty acids, was shown to modify proteins to form 4-ketoamides of 4-oxononanoic acid with the epsilon amino group of protein lysyl residues. By analogy with the chemistry of ONE, we postulated that KOHA-PC, an oxidatively truncated docosahexaenoate phospholipid that also incorporates 4-oxo-2-alkenal functionality, can modify biomolecules to give similar 4-ketoamides, i.e., OHdiA. OHdiA was detected by LC-MS/MS in the reaction mixture produced by incubation of KOHA-PC in the presence of the dipeptide Ac-Gly-Lys-OMe followed by HCl-catalyzed hydrolysis. This provided presumptive evidence for the formation and acid catalyzed hydrolysis of OHdiA derivatives. In contrast, similar treatment of KOHA-PC in the absence of the dipeptide did not produce OHdiA. In addition, the formation of OHdiA was detected by LC-MS/MS of the reaction product mixture from autoxidation of DHA-PC in the presence of HSA or DPPE followed by HCl catalyzed hydrolysis, indicating that KOHA-PC not only modifies the primary ε-amino group of protein lysyl residues but also those of the ethanolamine headgroup of phosphatidylethanolamines.

OHdiA derivatives were detected in similar levels in blood from SCD patients and from healthy individuals, supporting the conclusion that OHdiA derivatives are present in vivo. Interestingly, OHdiA-protein derivatives were present at twice the levels of OHdiA-PE derivatives in normal human plasma samples while the levels of OHdiA-protein and OHdiA-PE level were almost the same in SCD blood.

Both adhesion and tube formation experiments done by Dr. West demonstrated that
OHdiA-BSA induces angiogenesis in HUVECs, providing additional evidence for the importance of lipid oxidation in promoting angiogenesis in a VEGF-independent manner. Moreover, Dr. West also found that OHdiA-driven angiogenesis is TLR2 dependent similar to that promoted by CEP but different from VEGF promoted angiogenesis. It is tempting to speculate that OHdiAs and CEPs may have similar angiogenicity due to their structural similarity (Scheme 5 and 5.10). In addition, anti-OHdiA antibody was found to exhibit significant cross-reactivity with CEP-HSA while anti-CEP antibody showed high structural specificity and did not show cross-reactivity with OHdiA-HSA. These results suggested that anti-OHdiA therapy may be effective independently or as a complement to anti-VEGF or anti-CEP therapies to inhibit angiogenesis in growth of tumors, especially in cancers resistant to anti-VEGF therapy or after failure of anti-VEGF therapy.
5.4 Experimental Procedures

Materials. 1,2-Dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE), 1-tridecanoyl-2-hydroxy-sn-glycero-3-phosphocholine (Lyso–PC) and 1-palmityl-2-docosahexaenoyl-sn-glycero-3-phosphocholine (DHA-PC) were purchased from Avanti Polar Lipids (Alabaster, AL). The methyl 6-amino-2-((2-acetylamino)acetyl)amino) hexanoate (Ac-Gly-Lys-OMe) was purchased from Bachem (Torrance, CA). Human serum albumin (HSA, Cat. A3782) was purchased from Sigma (Milwaukee, WI). 2,2’-Azino-di-[3-ethylbenzthiazoline sulfonate (ABTS) solution, sodium deuteroxide (30 % in D$_2$O, 99 %) and deuterium oxide (99.9 %) were obtained from Sigma-Aldrich (St. Louis, MO). Goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody was procured from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal rabbit anti-CEP-KLH antibody was prepared by Dr. Xiaorong Gu, in Dr. Salomon’s group. CEP-HSA, CPP-HSA, CHP-HSA, CEP-dipeptide, OHdiA-dipeptide, OHdiA-BSA and OHdiA-HSA were prepared by Dr. Li Hong from Dr. Salomon’s group. All other chemicals and reagents were purchased from Sigma–Aldrich (St. Louis, MO).

General Methods. Proton magnetic resonance ($^1$H NMR) spectra and carbon magnetic resonance ($^{13}$C NMR) spectra were recorded on a Varian Inova AS400 spectrometer operating at 400 MHz and 100 MHz, respectively. Proton chemical shifts are reported as parts per million (ppm) on the δ scale relative to CDCl$_3$ (δ 7.26), CD$_3$OD (δ 3.31) or D$_2$O (δ 4.79). $^1$H NMR spectral data are tabulated in terms of multiplicity of proton absorption (s, singlet; d, doublet; dd, doublet of doublet; t, triplet; q, quartet; m, multiplet; br, broad),
coupling constants (Hz), number of protons. Carbon chemical shifts are reported relative to CDCl$_3$ (δ 77.0) or CD$_3$OD (δ 49.0). Flash chromatography was performed with ACS grade solvents from Fisher Scientific (Hanover Park, IL). R$_f$ values are quoted for TLC plates of thickness 0.25 mm from Whatman (Florham Park, NJ). The plates were visualized with iodine, dinitrophenylhydrazine or phosphomolybdic acid reagents. For all reactions performed in an inert atmosphere, argon was used unless otherwise specified.

**High Performance liquid chromatography/mass spectrometry.** LC-ESI/MS/MS analysis of OHdiA adducts was performed on a Quattro Ultima triple-quadrupole mass spectrometer (Micromass, Wythenshawe, UK) equipped with a Waters Alliance 2690 HPLC system with an auto-injector (Waters, Milford, MA). The chromatographic separation was achieved using on a Luna C18 column (150x2.0 mm i.d. 5 µm, Phenomenex). The source and desolvation temperature was maintained at 120 °C and 250 °C, respectively. The drying gas (N$_2$) and cone flow gas was kept at ca. 650 L/h and 65 L/h, respectively. The multiplier was set at an absolute value of 600.

For 4-oxoheptanedioic acid analysis, LC-MS/MS analysis was performed in the negative ion mode and the total run time was 30 min. The mobile phase consisted of solvent A (HPLC grade water containing 2 mM ammonium acetate) and solvent B (HPLC graded methanol containing 2 mM ammonium acetate). HPLC gradient steps were as follows: 0-8 min, isocratic at 5 % solvent B; 8-10 min, linear gradient from 5 to 100 % solvent B; 10-20 min, isocratic at 100 % solvent B; 21-30 min, isocratic at 5 % solvent B. The flow rate employed was 200 µL/min.
For analysis of OHdiA-PFB, LC-MS/MS analysis was performed in the positive ion mode and the total run time was 35 min. The mobile phase consisted of solvent A (HPLC grade water containing 0.1% formic acid) and solvent B (HPLC graded methanol containing 0.1% formic acid). The HPLC gradient steps were set as follows: 0-2 min, isocratic at 75% solvent B; 2-15 min, linear gradient from 75 to 100% solvent B; 15-24 min, isocratic at 100% solvent B; 25-35 min, isocratic at 75% solvent B. The flow rate employed was 200 µL/min.

Optimized parameters for 4-oxoheptanedioic acid and OHdiA-PFB were determined with authentic samples. MS scans at m/z 50-900 were obtained for standard compounds. For multiple reaction monitoring experiments, argon was used as collision gas at a pressure of 5 psi. The optimum collision energy and other parameters were determined for each individual analyte. (Table 5.2 and Table 5.3)

<table>
<thead>
<tr>
<th>Ion mode</th>
<th>4-oxoheptanedioic acid</th>
<th>OHdiA-PFB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capillary(kV)</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Cone (V)</td>
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<tr>
<td>Hex 1 (V)</td>
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<td>40</td>
</tr>
<tr>
<td>Aperture (V)</td>
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<td>0</td>
</tr>
<tr>
<td>Hex 2 (V)</td>
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<td>1.0</td>
</tr>
<tr>
<td>LM 1 Resolution</td>
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<tr>
<td>HM 1 Resolution</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Ion Energy 1</td>
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<td>0.5</td>
</tr>
<tr>
<td>LM 2 Resolution</td>
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<td>15</td>
</tr>
<tr>
<td>HM 2 Resolution</td>
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</tr>
<tr>
<td>Ion Energy 2</td>
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<td>1.5</td>
</tr>
</tbody>
</table>

Table 5.2 Optimized parameters for the mass spectrometer
Table 5.3 MRM transition ion pairs and optimized collision energy for the analytes

<table>
<thead>
<tr>
<th>Analytes</th>
<th>MRM transition ion pair (m/z)</th>
<th>Collision energy (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-oxoheptanedioic acid</td>
<td>173.2 &gt; 85.0</td>
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</tr>
<tr>
<td></td>
<td>173.2 &gt; 111.0</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>173.2 &gt; 129.2</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>173.2 &gt; 137.1</td>
<td>5</td>
</tr>
<tr>
<td>OHdiA-PFB</td>
<td>535.0 &gt; 111.1</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>535.0 &gt; 181.2</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>535.0 &gt; 337.0</td>
<td>5</td>
</tr>
<tr>
<td>OHdiA-PFB-D4</td>
<td>539.0 &gt; 114.0</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>539.0 &gt; 181.0</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>539.0 &gt; 341.0</td>
<td>5</td>
</tr>
</tbody>
</table>

Preparation of bis(perfluorophenyl) 4-oxoheptanedioate (OHdiA-PFB, 5.3).

Authentic 4-oxoheptanedioic acid (5.1, 18.4 mg, 0.1 mmol) was suspended in the 1.5 mL of anhydrous acetonitrile containing pentafluorobenzyle bromide (5.2, 39.2 mg, 0.15 mmol) and N,N-diidopropylethylamine (DIPEA, 100 µL, 0.57 mmol). The reaction mixture was incubated at room temperature under Ar protection for 5.5 hours. Then solvent was evaporated under a stream of dry N₂. Water (1 mL) was added and the resulting mixture was extracted with ethyl acetate (3 x 2 mL). The combined organic extracts were dried with NaSO₄, filtered and the solvents were evaporated under reduced pressure to give the crude compound bis(perfluorophenyl) 4-oxoheptanedioate (OHdiA-PFB, 5.3). The crude OHdiA-PFB (5.3) was purified by flash chromatography on a silica gel column (hexane:ethyl acetate 4:1, TLC: Rₗ = 0.27) to give pure OHdiA-PFB (5.3, 48.2 mg, 0.09 mmol, 88 %). ¹H NMR (400 MHz, CDCl₃) δ 5.18 (d, J = 1.4 Hz, 4H), AA’XX’(δ 2.79-2.74, 4H; δ 2.63-2.58, 4H). ESI-MS: m/z calcd for C₂₁H₁₃F₁₀O₅ [M+H]⁺, 535.06, found 535.00; m/z calcd for C₂₁H₁₂F₁₀O₅Na [M+Na]⁺, 557.04, found 556.87.

Preparation of 4-oxoheptanedioic-3,3,5,5-d₄ acid (5.4). 4-Oxoheptanedioic acid (5.1,
522.2 mg, 3 mmol) was incubated overnight in 5 mL of mixture of D₂O and NaOD (763.8 mg of 40 % NaOD in D₂O, 7.6 mmol) at room temperature under Ar. The solvents were then evaporated under reduced pressure to remove the HOD product. Then another 5 mL of D₂O were added and 360.2 mg of 40 % NaOD in D₂O was used to adjust the pH to 12. The mixture was allowed to react for another 6.5 hours. The NMR spectrum showed that the wash out of α-hydrogens was complete. Then the pH of reaction mixture was adjusted 2 by addition of concentrated deuterium chloride. The resulting mixture was extracted with ethyl acetate (3 x 10 mL). The combined organic extracts were dried with MgSO₄, filtered and the solvents were evaporated under reduced pressure to give pure 4-oxoheptanedioic-3,3,5,5-d₄ acid (5.4, 499 mg, 2.8 mmol, 93 %). ¹H NMR (400 MHz, D₂O) δ 2.26 (s, 4H). ESI-MS: m/z calcd for C₇H₅D₄O₅ [M-H]⁻, 177.07, found 177.13.

**Preparation of bis((perfluorophenyl)methyl) 4-oxoheptanedioate-3,3,5,5-d₄ (OHdiA-PFB-D₄, 5.5).** Pure 5.4 (38.3 mg, 0.2 mmol) was suspended in 2.0 mL of anhydrous acetonitrile containing pentafluorobenzyl bromide (5.2, 78.4 mg, 0.3 mmol) and N,N-diisopropylethylamine (DIPEA, 200 µL, 1.14 mmol). The reaction mixture was incubated for 8 h at room temperature under Ar. Then the solvents were evaporated under reduced pressure. Water (2 mL) was added and the resulting mixture was extracted with ethyl acetate (3 x 4 mL). The combined organic extracts were dried with anhydrous NaSO₄, filtered and the solvents were evaporated under reduced pressure to give the crude compound bis((perfluorophenyl)methyl) 4-oxoheptanedioate-3,3,5,5-d₄ (OHdiA-PFB-D₄, 5.5). The crude OHdiA-PFB-D₄ (5.5) was purified by flash chromatography on a silica gel column (hexane:ethyl acetate 4:1, TLC: Rf = 0.27) to give pure OHdiA-PFB-D₄ (5.5, 97.8
mg, 0.18 mmol, 91\%). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 5.18 (s, 4H), 2.59 (s, 4H). ESI-MS: m/z calcd for C$_{21}$H$_9$D$_4$F$_{10}$O$_5$ [M+H]$^+$, 539.09, found 539.24.

**Synthesis of 7-((2-hydroxyethyl)amino)-4,7-dioxoheptanoic acid (OHdiA-ethanolamine, 5.6).** A synthesis of 5.6 was achieved as outlined in Scheme 5.11. 4-Oxoheptanedioic acid (5.1, 0.8814 g, 5 mmol) was heated in acetyl chloride (10 mL) overnight at 60 °C under Ar. The solvents were then evaporated under reduced pressure to give the anhydride, 1,5-dioxocane-2,8-dione (5.11, 750 mg, 4.8 mmol, 96\%) that was used without further purification for the synthesis of 5.6 (*vide infra*). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 2.87 (ddd, J = 17.9, 11.3, 9.1 Hz, 2H), 2.68 – 2.53 (m, 4H), 2.40 (ddd, J = 13.8, 11.3, 9.5 Hz, 2H)

A solution of tert-butyldimethylchlorosilane (TBDMSCl, 3.15 g, 20.9 mmol) in dichloromethane (10 mL) was added dropwise to a stirred solution of ethanolamine (1.2 mL, 20.7 mmol) and imidazole (2.778 g, 40.8 mmol) in dichloromethane (20 mL) at room temperature, and the resulting mixture was stirred overnight at room temperature. Water (20 mL) was then added, and the phases were separated. The aqueous phase was extracted with
dichloromethane \( (3 \times 20 \text{ mL}) \), and the combined organic phases were dried with MgSO\(_4\), filtered and the solvents were evaporated under reduced pressure to give the crude compound 2-(tert-butyldimethylsiloxy)ethanamine (5.12, 2.5381 g, 14.5 mmol, 70%) that was used without further purification for the synthesis of 5.6. \(^1\)H NMR (400 MHz, CDCl\(_3\)) \( \delta 3.67 - 3.49 \text{ (m, 2H)}, 2.75 \text{ (t, } J = 5.2 \text{ Hz, 2H)}, 0.82 - 0.80 \text{ (m, 9H)}, -0.02 \text{ (dd, } J = 4.4, 2.6 \text{ Hz, 6H)} \)

Crude 5.11 (234.2 mg, 1.5 mmol) was allowed to react with crude 5.12 (178.7 mg, 1 mmol) in 15.0 mL of dichloromethane at room temperature under Ar for 24 h. The solvents were then evaporated under reduced pressure and the residue was purified by flash chromatography on a silica gel column (CH\(_2\)Cl\(_2\) : MeOH =25:1, TLC: \( R_f = 0.18 \)) to give pure 7-(2-((tert-butyldimethylsilyl)oxy)ethyl)amino)-4,7-dioxoheptanoic acid (5.13, 165 mg, 0.5 mmol, 50%). \(^1\)H NMR (400 MHz, CDCl\(_3\)) \( \delta 6.36 - 6.13 \text{ (m, 1H)}, 3.71 - 3.57 \text{ (m, 2H)}, 3.30 \text{ (p, } J = 5.0 \text{ Hz, 1H)}, 2.85 - 2.65 \text{ (m, 1H)}, 2.63 - 2.53 \text{ (m, 1H)}, 2.44 \text{ (dd, } J = 9.7, 6.5 \text{ Hz, 1H)}, 0.97 - 0.77 \text{ (m, 2H)}, 0.19 - 0.15 \text{ (m, 1H)}. \) ESI-MS: m/z calcd for C\(_{15}\)H\(_{28}\)NO\(_5\)Si [M-H]\(^-\), 330.17, found 330.33.

Pure 5.13 (32.7 mg, 0.1 mmol) was incubated in 10 mL of mixture of AcOH, H\(_2\)O and THF (3:1:1, V:V:V) for 3.5 h at 50 °C under Ar. The solvents were then evaporated under reduced pressure and the residue was purified by flash chromatography on a silica gel column (CH\(_2\)Cl\(_2\):MeOH 5:1, TLC: \( R_f = 0.2 \)) to give pure 7-((2-hydroxyethyl)amino)-4,7-dioxoheptanoic acid (OHdiA-ethanolamine, 5.6, 10 mg, 46%). \(^1\)H NMR (400 MHz, CD\(_3\)OD) \( \delta 3.58 \text{ (dd, } J = 7.1, 4.6 \text{ Hz, 2H)}, 3.27 \text{ (dd, } J = 8.0, 3.6 \text{ Hz, 2H)}, 2.83 - 2.72 \text{ (m, 4H)}, 2.54 \text{ (t, } J = 6.5 \text{ Hz, 2H)}, 2.47 \text{ (t, } J = 6.8 \text{ Hz, 2H)}. \) ESI-MS: m/z calcd for C\(_9\)H\(_{14}\)NO\(_5\)-
[M-H], 216.09, found 216.67; m/z calcd for C_{9}H_{15}NO_{5}Na [M+Na]^+, 240.08, found 240.80.

**Preparation of 1-Palmitoyl-2-(3-(2-furyl)propanoyl)-sn-glycero-3-phosphatidylcholine (CEF-PC, 5.9).** A mixture of 3-furanyl-2-propionic acid (5.8, 170.0 mg, 1.2 mmol) and lyso-PC (5.7, 300.0 mg, 0.6 mmol) was dried by evacuation through a Dry Ice-acetone cooled trap with a vacuum pump for 4.0 h at room temperature. Then the mixture was dissolved in 10 mL of dry chloroform. Then N,N-dimethylaminopyridine (DMAP, 72.0 mg, 0.6 mmol) and 3600 µL of 1M dicyclohexylcarbodiimide (DCC) solution (3.6 mmol) were added into the mixture sequentially. The resulting mixture was allowed to react for 2 days at room temperature under Ar. The solvents were then removed under reduced pressure and the residue was purified by flash chromatography on a silica gel column (CHCl₃:MeOH:H₂O 15:9:1, TLC: Rₙ = 0.28) to give pure 2-(3-(2-furyl)propanoyl)-1-palmitoyl-sn-glycero-3-phosphatidylcholine (CEF-PC, 5.9, 296.5 mg, 0.48 mmol, 80 %).

$^1$H NMR (400 MHz, CDCl₃) δ 7.26 (td, $J = 2.2$, 0.8 Hz, 1H), 6.23 (dd, $J = 3.2$, 1.9 Hz, 1H), 5.98 (ddd, $J = 6.6$, 3.2, 0.8 Hz, 1H), 5.22 – 5.12 (m, 1H), 4.33 (dd, $J = 12.1$, 2.9 Hz, 1H), 4.22 (d, $J = 13.9$ Hz, 2H), 4.09 (dd, $J = 12.1$, 7.2 Hz, 1H), 3.95 – 3.83 (m, 2H), 3.73 (s, 2H), 3.30 (s, 9H), 2.93 – 2.86 (m, 2H), 2.69 – 2.57 (m, 2H), 2.27 – 2.18 (m, 2H), 1.57 – 1.47 (m, 2H), 1.21 (s, 24H), 0.84 (t, $J = 6.9$ Hz, 3H). ESI-MS: m/z calcd for C₃₁H₅₇NO₉P [M+H]^+, 618.38, found 618.58.

**Preparation of 1-Palmitoyl-2-(4,7-dioxohept-6-enoyl)-sn-glycero-3-phosphatidylcholine (KOHA-PC, 5.10).** N-Bromosuccinimide (NBS, 16 mg, 0.09 mmol) was dissolved in 6.0 mL of THF-acetone-water (V/V/V, 5:4:2) and then slowly added into a solution of 5.9 (38.2 mg, 0.06 mmol) in 4.0 mL of THF-acetone-water (V/V/V, 5:4:2) that was kept at
-20 °C. Then pyridine (10.1 µL, 0.12 mmol) was added. The resulting mixture was stirred for 1.0 h at -20 °C and then kept at room temperature for 6.0 h. The solvent was then removed under reduced pressure and the residue was purified on a silica gel column (CHCl₃:MeOH:H₂O 15:9:1, TLC: Rₚ = 0.15) to give pure 1-palmitoyl-2-(4,7-dioxohept-6-enoyl)-sn-glycero-3-phosphatidylcholine (KOHA-PC, 5.10, 28.5 mg, 0.045 mmol, 75 %).

1H NMR (400 MHz, CDCl₃) δ 9.80 (d, J = 7.3 Hz, 1H), 6.97 (dd, J = 16.3, 4.7 Hz, 1H), 6.81 (dd, J = 16.3, 7.3 Hz, 1H), 5.18 (s, 1H), 4.32 (d, J = 12.1 Hz, 3H), 4.14 (dd, J = 11.9, 7.0 Hz, 1H), 3.95 (s, 2H), 3.78 (s, 2H), 3.34 (s, 9H), 3.12 – 2.99 (m, 2H), 2.68 (ddd, J = 18.5, 11.9, 6.0 Hz, 2H), 2.27 (d, J = 7.9 Hz, 3H), 1.56 (s, 2H), 1.24 (s, 24H), 0.86 (d, J = 6.4 Hz, 3H). ESI-MS: m/z calcd for C₃₁H₅₇NO₁₀P [M+H]⁺, 634.37, found 634.60.

Free radical-induced oxidation of DHA-PC in the presence of HSA. Small unilamellar vesicles (SUVs) comprised of DHA-PC (2 mg/mL) were prepared by extrusion (11 times) through a 0.1 µm polycarbonate filter using an Avanti Mini-Extruder (Avanti Polar Lipids, Inc., Alabaster, AL) in argon sparged phosphate buffered saline (PBS, 10 mM, pH 7.4). Liposome solution (1 mL) containing DHA-PC (2 mg/mL) was mixed with HSA (16.5 mg) in PBS (4 mL) to give final concentrations of DHA-PC (0.4 mg/mL) and HSA (3.3 mg/mL). Autoxidation was initiated by the addition of 1 M CuSO₄ (5.0 µL) and 5 M H₂O₂ (10 µL). The autoxidation proceeded at 37 ºC. Aliquots (1200 µL/each) were withdrawn and autoxidation was terminated by the addition of 200 mM BHT (6.0 µL) and 0.1 mM EDTA (2.4 µL) after various reaction times (0 h, 20 h, 30 h and 48 h). Then, all aliquots were dialyzed against 10 mM PBS, pH 7.4 (3 x 2 L) containing Na₂EDTA (1 mg/mL) for 48 h at room temperature. After dialysis, each aliquot was divided into six
portions (200 μL/each). Each aliquot (200 μL) at various reaction times was hydrolyzed in 2000 μL of 6 N HCl at 100 °C using a Fisher Scientific™ isotemp™ block heater (Model 2050FS) under Ar protection for 6 h. After adjusting the pH to 1-2 with 10 N NaOH, the mixture was extracted with ethyl acetate (3 x 4 mL). Volatiles were removed from the combined organic extracts under reduced pressure. The dried sample was redissolved in 800 μL of methanol and was divided equally into two vials. The solvent was then removed under a stream of dry N₂. Then half of the dried sample was kept at -80 °C under argon until LC-MS/MS analysis. The other half of the dried sample was used for PFB derivatization followed by extraction with ethyl acetate and ddH₂O as described above. The PFB derivatized product was dried and kept at -80 °C under argon until LC-MS/MS analysis. For LC-MS/MS analysis, the dried samples were redissolved in MeOH (60 μL). After vortexing and centrifuging for 10 min, 20.0 μL of this solution was used for LC-MS/MS.

**Free radical-induced oxidation of DHA-PC in the presence of DPPE.** Small unilamellar vesicles (SUVs) comprised of DHA-PC (1 mg/mL) and DPPE (0.09 mg/mL) were prepared by extrusion (11 times) through a 0.1 μm polycarbonate filter using an Avanti Mini-Extruder (Avanti Polar Lipids, Inc., Alabaster, AL) in argon sparged pH 7.4 10 mM PBS. Autoxidation was initiated by exposure to 1 mM CuSO₄ and 10 mM H₂O₂. The autoxidation proceeded at 37 °C. Aliquots (600 μL/each) were withdrawn and the autoxidation was terminated by the addition of 100 mM BHT (6.0 μL) and 0.2 mM EDTA (0.6 μL) after various reaction times (0 h, 2 h, 5 h, 10 h, 22 h, 34 h and 48 h). Each aliquot was divided into six portions (100 μL/each). Then, 100 μL of each aliquot was hydrolyzed using 6 N HCl at 100 °C using a Fisher Scientific™ isotemp™ block heater (Model 2050FS).
under Ar protection for 6 h. After adjusting the pH to 2-3 with 10 N NaOH, the mixture was extracted with ethyl acetate (3 x 3 mL). The combined organic phases were dried under reduced pressure. Then half of the dried sample was kept at -80°C under argon before LC-MS/MS analysis. The other half of the dried sample was used for PFB derivatization followed by extraction with ethyl acetate and ddH$_2$O as described above. The PFB derivatization product was dried and kept at -80 °C under argon until LC-MS/MS analysis. For LC-MS/MS analysis, the dried samples were redissoved in 50 μL of MeOH. After vortexing and centrifuging for 10 min, 15.0 μL of this solution was used for LC-MS/MS.

**Extraction of human plasma lipids.** To separate protein from phospholipids, plasma samples were extracted by a modified Bligh & Dyer method adapted for small-scale extraction with precautions to prevent *in vitro* oxidation. In brief, 200 μL of human plasma from SCD patients or normal controls was mixed with 750 μL of chloroform/methanol (1:2, v/v) containing 1 mM BHT and the resulting mixture was vortexed vigorously for 1 min. Then 250 μL of chloroform was added and the resulting mixture was vortexed vigorously for 1 min. Then 250 μL of 1.5% aqueous sodium chloride solution was added and the resulting mixture was vortexed vigorously for 1 min. The resulting mixture was then centrifuged for 10 min at 14,000 rpm to give a three-phase system (aqueous top, protein disk and organic bottom). The lower organic layer containing phospholipids was carefully transferred to a new tube, making sure to avoid the interface or upper layer. After transferring the upper aqueous layer to another separate tube, the protein disk at the interphase between the aqueous and organic phase was left. The solvents were then evaporated from the fractions under a stream of dry nitrogen at room temperature. The dry
protein and lipid fractions were hydrolyzed using 6 N HCl at 100 °C as described above. After adjusting the pH of reaction mixture to 2-3 with 10 N NaOH, the resulting mixture was extracted with ethyl acetate three times. The organic phases were combined and dried under a stream of dry nitrogen. Half of the dried sample was stored under argon at -80 °C until being analyzed using LC-MS/MS and half was used for PFB derivatization. After PFB derivatization, ethyl acetate and ddH2O was used to extract the PFB derivative from the reaction product mixture. The organic phase was combined and dried under a stream of nitrogen. The dried sample was stored under argon at -80 °C until being analyzed using LC-MS/MS. For LC-MS/MS analysis, the dried sample was redissolved in 50 µL of MeOH containing OHdiA-PFB-D4 (5.0 µg/mL) as quantitation standard. After vortexing and centrifuging for 10 min, 15.0 µL of the solution was injected for LC-MS/MS analysis.

**Cell adhesion assay.** A 96-well plate was coated with fibronectin (1 µg/mL) for 1 h at 37 °C followed by blocking with 1 % BSA for 1 h. Then human umbilical vein endothelial cells (HUVECs) were added to the fibronectin coated 96-well plate (10⁴ cells per well) and incubated for 1 h in the presence of various concentrations of OHdiA-BSA, BSA or VEGF as indicated. The cells were then washed with Dulbecco’s modified Eagle’s (DMEM)/F12 medium and fixed with 4 % paraformaldehyde (PFA) in PBS. Then hematoxylin was added to stain the cells for 30 min. Then the cells were washed with PBS and photographs were taken. These experiments were performed by Dr. Xiaoxia Z. West.

**Tube formation assay.** HUVECs were seeded onto matrigel-coated 48-well plates (BD Bioscience). Then cells were incubated with various concentrations of OHdiA-BSA, BSA or VEGF as indicated at 37 °C for 8 h. Tube formation was observed using a phase contrast
inverted microscope, and photographs were taken of each well. The data were quantified by measuring the length of tubes with ImagePro software. These experiments were performed by Dr. Xiaoxia Z. West.

**OHdiA Antibody purification by protein A/G column.** The protein A/G column was packed with Pierce protein A/G plus agarose (cat# B1811, 5.0 mL) and was then equilibrated with 10 column volumes (about 25 mL) of binding buffer (10 mM PBS, pH 7.4). 1.0 mL of crude OHdiA-KLH antibody serum from the bleeding of the rabbit was mixed with 1.0 mL of binding buffer. The resulting mixture was vortexed for 60 seconds and then centrifuged for 10 min at 5,000 rpm. The supernatant was filtered through 0.22 µm cellulose ester filters and loaded onto the equilibrated protein A/G column. The sample was allowed to flow through the column by gravity. The non-protein-bound material was eluted with the binding buffer (about 26 mL) when the color of the mixture of 100 µL of quick start bradford dye reagent and 100 µL of effluent became the same as the color of the mixture of 100 µL of quick start bradford dye reagent and 100 µL of binding buffer. The bound IgG protein was then eluted with about 40 mL of elution buffer (100 mM glycine-HCl containing 150 mM NaCl, pH 2.5). The eluates were collected into 1 mL of fractions containing 100 µL of 1 M Tris buffer (pH 8.7). The pH was finally adjusted to 10 by 1 N NaOH. According to the color of the mixture of 100 µL of quick start bradford dye reagent and 100 µL of eluate (the more blue, the more protein), different fractions containing IgG proteins were combined together and dialyzed against 10 mM PBS (pH 7.4) (3 x 2 L) at 4 °C for 38 h. The resulting solutions of anti-OHdiA polyclonal antibody contained 528 µg/mL purified IgGs, as determined by Pierce BCA protein assay.
Enzyme-linked immunosorbent assay (ELISA) for competitive antibody binding inhibition studies. For antibody binding inhibition studies to measure cross-reactivities, OHdiA-BSA and CEP-BSA were used as coating agents and OHdiA-HSA and CEP-HSA were used as standards for purified anti-OHdiA-KLH and anti-CEP-KLH, respectively. For each inhibitor or standard, up to ten serial dilutions of an initial concentration, a blank, and a positive control containing no inhibitor and standard, were run. Each well of the 96-well plate was pre-coated with 100 µL of OHdiA-BSA or CEP-BSA (25 pmol/mL). The plate was covered with a plastic lid and placed in an incubator at 37 °C for 1 h and then allowed to come to room temperature. After discarding the supernatant, each well was washed with 10 mM pH 7.4 PBS (3 x 300 µL) and then blocked by incubating 1 h at 37 °C with 300 µL of 3 % BSA in 10 mM pH 7.4 PBS. After coming to room temperature, the supernatant was discarded and the wells were washed with 0.3% BSA with 0.05 % Tween 20 in PBS (3 x 300 µL). For each inhibitor or standard, up to ten serial dilutions were prepared in PBS buffer from the initial concentration of 25 pmol/mL using a dilution factor of 0.2. 120 µL of each of diluted inhibitor or standard solution was preincubated with another 120 µL of antibody solution at 37 °C for 1 h. The antibody solutions were prepared by adding the required amount of protein A/G column-purified antibody in PBS to 0.3 % BSA in PBS (1:500 dilution for anti-OHdiA-KLH and 1:2000 dilution for anti-CEP-KLH). Blank wells were filled with 100 µL of 0.3 % BSA in pH 7.4 PBS. Positive control wells were filled with the antibody solution (50 µL) and PBS (50 µL). To the rest of the sample wells, 100 µL of antibody-antigen complex solution were added in duplicate. The plate was then incubated at room temperature on a shaker for 1 h. After discarding the supernatant, the
wells were washed with 0.3 % BSA with 0.05 % Tween 20 in PBS (3 x 300 µL). Then, 100 µL of goat anti-rabbit IgG- horseradish peroxidase conjugate solution, which was prepared by a 1:2000 dilution of commercially available enzyme-linked secondary antibody with 0.3 % BSA in PBS, was added to each well. The plate was then incubated at room temperature for 1 h while gently agitating on a shaker. After discarding the supernatant, the wells were washed with 0.3 % BSA with 0.05 % Tween 20 in PBS (3 x 300 µL). Then, 100 µL of ready-to-use ABTS solution was added to each well. The absorbance in each well was measured with a plate reader (Model M3, Molecular Device) at 405 nm relative to 655 nm. Absorbance values for duplicate assays were averaged and then scaled so that the maximum curve fit value is close to 100 percent. The averaged and scaled percent absorbance values were plotted against the log of concentration. Theoretical curves for each plot were fit to the absorbance data with a four parameter logistic function using SigmaPlot® 12.5 from Jandel Scientific Software, San Rafael, CA.

**Statistical analysis.** Statistical analyses were performed by using Student’s t test. P value <0.05 is considered as statistically significant. All data are presented as mean ± SD for all studies. Probability values were based on the paired t test: NS – not significant, * P< 0.05, ** P< 0.01, *** P< 0.001.

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5.5 References


CHAPTER 6

QUANTITATIVE ANALYSIS OF CARBOXYALKYLPYRROLE
AND PENTYLPYRROLE DERIVATIVES OF ETHANOLAMINE
PHOSPHOLIPIDS
6.1 Background

It is well-established that free radicals are continuously produced in vivo and play a dual role as both deleterious and beneficial species in biological systems. At low concentrations, free radicals in cells act as secondary messengers in intracellular signaling cascades, showing beneficial effects. However, at high concentrations, free radicals can be important mediators of damage to cell structures, including lipids and membranes, proteins and nucleic acids. Even though organisms have evolved not only antioxidant defense systems but also repair systems to balance the harmful effects of free radicals, oxidative damage accumulates during the life cycle, and radical-related cell damage to biomolecules has been proposed to play a crucial and causative role in the pathogenesis of a wide variety of human diseases, including cancer, arteriosclerosis, arthritis, neurodegenerative disorders and other conditions.

Although free radicals can cause damage to all biomacromolecules in the cell, among these targets, the most sensitive cellular target of free radical reactions is the membrane lipids, mainly phospholipids, containing polyunsaturated fatty acids (PUFAs). PUFAs such as linoleic acid (LA), arachidonic acid (AA) and docosahexaenoic acid (DHA) are susceptible to free radical-initiated oxidation to yield a variety of corresponding unsaturated lipid hydroperoxides. The breakdown of these lipid hydroperoxides generates a large number of mono- and bi-functional reactive carbonyl-containing products called lipoxidation (LPO) products. On the basis of their structural features, two types of these reactive aldehydes are: (1) aldehydes that contain the carboxyl terminus of the acyl chain such as 9-hydroxy-12-oxododec-10-enoic acid (HODA), 5-hydroxy-8-oxooct-6-enoic acid (HOOA) and 4-hydroxy-7-oxhept-5-enoic acid (HOHA); or (2) aldehydes that contain the
methyl terminus, such as 4-hydroxy-2-nonenal (4-HNE) and 4-hydroxy-2-hexenal (4-HHE) (Scheme 6.1).\textsuperscript{7, 9-11}

\textbf{Scheme 6.1} Two classes of aldehydes generated by free radical-initiated oxidation of PUFA.

Compared to free radicals, these aldehydes are more stable, and thus are likely to diffuse through cellular compartments to attack targets far from the site of their generation.\textsuperscript{12} Some of these oxidized lipids are restricted to diffuse laterally if they are esterified to phospholipids within membranes. Some of these aldehydes are highly reactive and can easily modify biological macromolecules such as proteins, DNA and ethanolamine phospholipids to form biologically relevant adducts that have been demonstrated to be involved in several pathological processes.\textsuperscript{13} Therefore, they are not only end products and remnants of lipid peroxidation processes but also may be considered as second cytotoxic
messengers, which disseminate and increase initial free radical events.\textsuperscript{6, 12} Protein modification by LPO-derived aldehydes, especially γ-hydroxy-α,β-unsaturated aldehydes, has been extensively studied (Scheme 6.2). 4-HNE, one of the best recognized and most studied cytotoxic lipid peroxidation products produced by oxidative fragmentation of either LA or AA, forms Schiff base adducts with lysine residue of proteins, leading to a 2-pentylpyrrole (PP) derivatives, which are the most stable end products of 4-HNE protein modification.\textsuperscript{7, 14, 15} In analogy with the chemistry of 4-HNE, the carboxy-terminating γ-hydroxy-α, β-unsaturated aldehydes like HODA, HOOA and HOHA, which are formed upon free radical-induced oxidation of LA, AA, or DHA, respectively, generate the

\textbf{Scheme 6.2} The formation from γ-hydroxy-α, β-unsaturated aldehydes of alkyl- and carboxyalkyl pyrrole derivatives of proteins.
corresponding carboxyheptylpyrrole (CHP), carboxypropylpyrrole (CPP), and carboxyethylpyrrole (CEP) derivatives of proteins.\textsuperscript{9, 16, 17}

Protein modification by these active LPO-derived aldehydes has been demonstrated in a wide range of physiological and pathological conditions. In conditions of oxidative stress, protein modification can contribute to protein dysfunction or tissue damage and thus may play important roles in disease progression. PP modified proteins accumulate in the blood of individuals with atherosclerosis and in brain neurons of patients with Alzheimer’s disease.\textsuperscript{14, 15} Immunoassay-based studies also revealed the presence of CHPs and CPPs in oxidized low-density lipoprotein (oxLDL).\textsuperscript{9} In addition, there is immunological evidence that mean levels of CHP derivatives of plasma proteins are significantly elevated in renal failure and atherosclerosis patients compared with healthy volunteers.\textsuperscript{9} CEPs were found to be more abundant in age-related macular degeneration (AMD) than in normal Bruch’s membrane/RPE/choroid tissues.\textsuperscript{18} The levels of CEP derivatives are elevated in human plasma from AMD donors relative to normal healthy donors.\textsuperscript{17} Moreover, CEP derivatives can stimulate choroidal neovascularization and promote wound healing and tumor growth through toll-like receptor 2 (TLR2) signaling.\textsuperscript{19, 20}

While the formation of protein adducts by γ-hydroxy-α, β-unsaturated aldehydes such as 4-HNE, HODA, HOOA and HOHA has been well established vivo and presumed to mediate some biological effects of these active aldehydes due to the increased levels of alkyl- and carboxyalkyl pyrrole-modified proteins in several pathological conditions including eye diseases, the extent of their adduction to ethanolamine phospholipids (EPs) in vivo and the biological activities of those modified EPs remain poorly characterized. EPs account for 25% of mammalian phospholipids and are particularly enriched in the
brain where the EPs comprise 45% of total phospholipids. EPs play an important role in stabilizing membrane proteins and cell function. Additionally, PEs act as important precursors, substrates, or donors in several biological pathways. Thus, excessive modification of EPs by LPO-derived aldehydes may also have biological relevance since the modification may alter the phospholipid membrane distribution and change its fluidity, resulting in changes in membrane structure and function. Therefore, it is possible that EP modification may play an important role in the pathobiology of oxidative injury.

Analogously to proteins, EPs rapidly react with γ-hydroxy-α, β-unsaturated aldehydes, and thus are expected to compete with proteins to be modified by these aldehydes. The carbonyl-amine reaction between these reactive aldehydes and EPs may be facilitated by the location of the EPs in cell membranes and lipoprotein outer shells where the LPO-derived aldehydes are generated and the amphiphatic nature of phospholipids may also trap the small aldehydes in the lipophilic cores of membranes and lipoprotein particles. EPs modified by LPO-derived aldehydes generated in vivo, such as 4-HNE, isolevuglandin (isoLG), acrolein and HOHA, have been reported in recent years. Analogous to the well-known reaction of lysyl ε-amino groups of proteins with active aldehydes, the primary amino group in EPs was found to react with these aldehydes to produce Schiff bases and, ultimately, pyrroles. These aldehyde-PE adducts have important biological activities and may be involved in the pathogenesis of diseases associated with oxidative injury. Levels of isolevuglandin-modified EPs are elevated in plasma of patients with AMD as well as in the liver of ethanol-fed mice. CEP-EPs derived from HOHA are not only present in human blood at 4.6-fold higher levels in AMD plasma than in normal plasma but also exhibit proangiogenic activity that is dependent on TLR 2 signaling similar to
CEP-protein derivatives. On the basis of these findings, other carboxy-terminating γ-hydroxy-α, β-unsaturated aldehydes like HODA and HOOA were also expected to covalently modify the primary amino group of EPs to give the corresponding carboxyalkyl pyrrole (CAP) adducts in analogy with HOHA. In the current studies, we intended to explore the possibility and develop analytical methods that allow quantification of global CAP- and PP-modified EPs present in vivo. This is of considerable interest because these adducts could potentially be involved in the pathogenesis of diseases associated with oxidative injury besides having potential utility as disease biomarkers that may be useful for early diagnosis and for assessing the efficacy of therapeutic interventions.

Simultaneous measurement of both CAP- and PP-modified EPs may facilitate studies to understand the relative rates of formation under various conditions in vivo and how these various pyrrole-modified EPs influence the processes of diseases. To our knowledge, there have not been studies attempting a comprehensive systematic study of EP modifications by γ-hydroxy-α, β-unsaturated aldehydes resulting from peroxidation of a variety of PUFAs. For the simultaneous measurement of both CAP- and PP-modified EPs in biological samples, there are many challenging problems. Firstly, multiple reactive aldehydes are generated from peroxidation of different FUFAs in vivo, which requires careful analysis with mass spectrometry (MS). MS, particularly liquid chromatography–tandem MS (LC–MS/MS), not only allows the use of internal standards to correct for different sources of analytical variability but also the use of chromatography to separate different analytes to identify specific modifications of EPs, but at the same time allows the simultaneous measurement of various pyrrole-modified EPs. Secondly, isotope-labeled internal standard can be used to minimize any matrix effects for the biological samples and
to increase the sensitivity of characterization of EP modifications by LPO aldehydes. Furthermore, considering that EPs tend to exist in 1, 2-diacyl, 1-alkyl-2-acyl, and 1-vinyl-2-acyl forms, each of which can potentially differ in acyl chain length and unsaturation, a complex mixture of pyrrole-modified EPs with different molecular weights are expected to be generated, in which the amount of each species could be lower than the lowest limit of mass spectrometric detection. To overcome this problem, phospholipase A2 (PLA2) was employed previously to convert the putative complex mixture of isoLG-modified EPs into a much simpler mixture by hydrolyzing the sn-2 acyl chain releasing 2-lysophospholipid. However, this strategy only removes the differences in mass related to sn-2 position. To provide an even more sensitive index of oxidative stress, phospholipase D (PLD) from *Streptomyces chromofuscus* can be used to remove all of the differences in mass related to the glycerophospholipid moieties by hydrolyzing various EP adducts to their corresponding modified ethanolamine (ETN) derivatives. This maneuver greatly simplifies the number of total masses that must be quantified. Analogously, PLD (*Streptomyces chromofuscus*) was anticipated to hydrolyze various CAP- and PP-modified EPs to release a single CAP- or PP-ETN from each, respectively (Scheme 6.3).

![Scheme 6.3](image)

**Scheme 6.3** Hydrolysis of CAP- or PP-EPs catalyzed by PLD delivers CAP- or PP-ETN.
In this chapter, an analytical assay using LC-MS/MS to simultaneously analyze both CAP- and PP- modified EPs in biological samples, e.g. human blood plasma, was developed. In order to establish the method and minimize the variability in the analytical procedure, a series of authentic samples and deuterium labeled internal standards were prepared. The proposition that pyrrole derivatives of EPs are substrates of PLD was tested \textit{in vitro}. The presence of CAP and PP derivatives of EPs in human blood plasma was investigated, and their levels were also quantified by the LC-MS/MS methodology.
6.2 Results and Discussion.

Strategy for analysis of CAP- and PP- modified EPs in biological samples. As shown in Scheme 6.4, our strategy is to isolate total phospholipids from biological samples by a modified Bligh & Dyer method, and then make these lipid extracts into small unilamellar vesicles (SUV) by extrusion followed by the addition of internal standards CAP-and PP-ETN-d₄. Then phospholipase D from *Streptomyces chromofuscus* is used to catalyze hydrolysis of phospholipids to release CAP- and PP-ETN, which are analyzed by the LC-MS/MS.

![Scheme 6.4 Strategy for analysis of CAP- and PP- modified EPs in biological samples.](image)
Syntheses of CAP and PP derivatives of ETN and deuterated ETN as well as of DPPEs. All CAP derivatives of ETN and deuterated ETN as well as of DPPEs were prepared by unambiguous chemical syntheses. The non-deuterated authentic CEP-ETN (6.3a) and deuterated authentic internal standard CEP-ETN-d₄ (6.3b) were synthesized as described previously.²⁷ Briefly, the propylene acetal group of 6-[1,3]dioxolan-2-yl-4-oxo-hexanoic acid-9H-fluoren-9-yl-methyl ester (6.1) prepared by Dr. Li Hong in Dr. Salomon’s lab was removed by treatment with aqueous acetic acid to give the 9H-fluoren-9-ylmethyl ester of 4,7-dioxo-heptanoic acid (DOHA-Fm, 6.2). Finally, CEP-ETN (6.3a) and CEP-ETN-d₄ (6.3b) were generated by the reaction of 6.2 with ETN or ethanol-1,1,2,2-d₄-amine (ETN-D₄) through a Paal-Knorr reaction followed by the removal of the Fm ester by 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU)(Scheme 6.5).

Scheme 6.5 Synthesis of CEP derivatives of ETN and deuterated ETN.

For other non-deuterated authentic CAP-ETN derivatives (CPP-ETN and CHP-ETN) and deuterated CAP-ETN-d₄ derivatives (CPP-ETN-d₄ and CHP-ETN-d₄) as well as authentic CAP-DPPE derivatives (CEP-DPPE, CPP-DPPE and CPP-DPPE), there is no need to protect the carboxyl group to obtain a high level of modification. In brief, the
propylene acetal group of the dioxolane-protected carboxylic acid provided by Dr. Li Hong in Dr. Salomon’s lab was removed by treatment with aqueous acetic acid to give the corresponding γ-keto aldehyde carboxylic acid that generated carboxyalkylpyrrole derivatives by Paal-Knorr synthesis (Scheme 6.6).

Scheme 6.6 Syntheses of CPP, CHP derivatives of ETN, deuterated ETN and CAP-DPPE.

For the synthesis of non-deuterated authentic PP-ETN and deuterated authentic internal standard PP-ETN-D₄, the Paal-Knorr synthesis was successfully applied to the generation of PP derivatives from 4-oxononanal. 4-oxononanal was prepared by a method published
elsewhere with minor modification. Briefly, the commercially available starting material γ-nonalactone was firstly reduced to nonane-1,4-diol by LiAlH₄, which was then converted to 4-oxononanal through Swern oxidation. (Scheme 6.7).

![Chemical structure](image)

**Scheme 6.7** Synthesis of PP modified ETN and deuterated ETN as well as DPPE.

Test the hypothesis that pyrrole modified EPs are substrates for PLD from *Streptomyces chromofuscus*. Although PLD is an efficient lipase of levuglandin and isolevuglandin derivatives of EPs (also referred to as γ-KA-EPs) and CEP-EPs, whether PLD can hydrolyze other pyrrole modified EPs was not known. Therefore, the ability of PLD (*Streptomyces chromofuscus*) to catalyze the hydrolysis of pyrrole derivatives of EP was evaluated by treatment authentic CAP-DPPE with PLD from *Streptomyces chromofuscus* followed by analysis by LC-MS in the negative ion mode. As shown in Figure 6.1A-C (see next page), significant peaks were observed in the selected ion chromatograms (SICs) corresponding to CAP-ETNs after hydrolysis of CAP-DPPEs catalyzed by PLD from *Streptomyces chromofuscus*, confirming that CAP-DPPEs are substrates for PLD (*Streptomyces chromofuscus*). To verify that the CAP-ETN was produced by PLD-catalyzed hydrolysis of CAP-DPPE, the hydrolysis product from CHP-
DPPE without PLD (Streptomyces chromofuscus) treatment was also studied by LC-MS as a representative. As shown in Figure 6.1D, no peak corresponding to CHP-ETN was observed in the SIC of the reaction product mixture from hydrolysis of CHP-DPPE in the absence of PLD.

Figure 6.1 LC-MS data supports the conclusion that PLD from Streptomyces chromofuscus catalyzes the hydrolysis of CAP-DPPE to deliver the corresponding CAP-ETN. CAP-DPPE standards were treated either with or without PLD overnight. Then CAP-ETN was analyzed by LC-MS in the negative ion model. (A) CEP-DPPE plus PLD. (B) CPP-DPPE plus PLD. (C) CHP-DPPE plus PLD. (D) CHP-DPPE without PLD. Top traces: selected ion chromatograms (SICS) of CEP-ETN (m/z 182.5), CPP-ETN (m/z 196.6) and CHP-ETN (m/z 252.5) detected in the reaction mixture in the negative ion mode. Bottom traces: total ion chromatograms (TICS) of the reaction mixtures.

LC-MS/MS analysis of authentic CAP and PP derivatives of ETN and deuterated ETN. For the analysis of CAP and PP derivatives of EPs in biological samples by reverse phase HPLC/ESI/MS/MS using multiple reaction monitoring (MRM), a MRM method was developed based on the collision induced dissociation (CID) spectrum of [M+H]+ of the parent ion for authentic CAP and PP derivatives of ETN and deuterated ETN in the positive ion mode. Figure 6.2-6.5 (see page 266-269) shows representative CID spectra of authentic
CAP and PP derivatives of ETN and deuterated ETN and interpretation of major product ions.

**Figure 6.2** (A) CID spectrum of m/z 184.3 of authentic CEP-ETN (6.3a) and interpretation of major product ions. (B) CID spectrum of m/z 188.4 of authentic CEP-ETN-d$_4$ (6.3b) and interpretation of major product ions.
Figure 6.3 (A) CID spectrum of m/z 198.4 of authentic CPP-ETN (6.10a) and interpretation of major product ions. (B) CID spectrum of m/z 202.4 of authentic CPP-ETN-d₄ (6.10b) and interpretation of major product ions.
Figure 6.4  (A) CID spectrum of m/z 254.3 of authentic CHP-ETN (6.11a) and interpretation of major product ions. (B) CID spectrum of m/z 258.4 of authentic CHP-ETN-d₄ (6.11b) and interpretation of major product ions.
Figure 6.5 (A) CID spectrum of m/z 182.4 of authentic PP-ETN (6.18a) and interpretation of major product ions. (B) CID spectrum of m/z 186.4 of authentic PP-ETN-d4 (6.18b) and interpretation of major product ions.

MRM of the transitions 184.3 → 124.2, 188.4 → 128.2, 198.4 → 162.5, 202.4 → 166.4, 254.3 → 124.1, 258.4 → 128.1, 182.4 → 94.1 and 186.4 → 98.2 were used to analyze CEP-ETN (6.3a), CEP-ETN-d4 (6.3b), CPP-ETN (6.10a), CPP-ETN-d4 (6.10b), CHP-ETN (6.11a), CHP-ETN-d4 (6.11b), PP-ETN (6.18a) and PP-ETN-d4 (6.18b), respectively. Therefore, these eight MRM transitions were set to monitor the LC-MS/MS of a mixture of all eight authentic standards. As shown in Figure 6.6 (see next page), there are strong
signals for each channel in the MRM. Signals for the CEP, CPP, CHP and PP derivatives are well separated by LC. Consequently, this LC-MS/MS method can be used for the analysis of CAP-and PP-ETN derivatives in biological samples.

Figure 6.6 MRM chromatographs from LC/MS/MS analysis of a mixture of all eight authentic standards (CEP-ETN, CEP-ETN-d₄, CPP-ETN, CPP-ETN-d₄, CHP-ETN, CHP-ETN-d₄, PP-ETN and PP-ETN-d₄) in the positive ion mode using 0.1% formic acid as additive.
LC-MS/MS demonstration that CAP and PP derivatives of EPs are present in human plasma. Sickle cell disease (SCD) is a class of hemoglobinopathy in humans clinically characterized by chronic hemolysis, inflammation and vaso-occlusion, causing a reduced quality of life and life expectancy. Chronically elevated oxidative stress is an important feature of SCD and might play a significant role in the pathophysiology of a cascade of SCD-related debilitating conditions. Despite the mounting evidence of oxidative stress in SCD, there is still no practical biomarker for determining the degree of oxidative stress and disease severity in SCD due to either the high reactive instability of reactive oxygen species (ROS) or the lack of a specific and sensitive assay for measuring the more stable “end” products of oxidative reactions. Since CAP and PP derivatives have been measured immunologically in human blood plasma, we anticipated that CAP and PP derivatives of EPs exist in human plasma and might serve as markers of disease severity in SCD.

Based on the workflow developed for detection of CAP and PP derivatives of EPs in biological samples (Scheme 6.4, see page 261), a small pilot study was launched to examine their presence in blood plasma samples from hospitalized and clinical patients with SCD. In brief, phospholipids were first extracted from 200 μL of human plasma samples from hospitalized and clinical patients with SCD by a modified Bligh & Dyer method. Then lipid extracts were prepared as SUV followed by the addition of a fixed amount of each of the internal standards, CAP-ETN-d₄ and PP-ETN-d₄ (0.01 nmol) and the mixtures were then hydrolyzed by 280 units of PLD (Streptomyces chromofuscus). Then the amounts of CAP-ETN and PP-ETN released from blood lipids in the hydrolysis reaction product mixtures were determined by the LC-MS/MS methods described above.
To determine the linearity and sensitivity of the assay for measurement of CAP-ETN and PP-ETN, calibration curves were constructed with authentic CAP-ETN and PP-ETN in 200 µL of HBSS buffer in the concentration range from 4 to 80 nM spiked with a fixed amount of each of internal standards, CAP-ETN-d₄ and PP-ETN-d₄ (0.01 nmol). The peak area ratio of CAP-ETN or PP-ETN to CAP-ETN-d₄ or PP-ETN-d₄ from the LC-MS/MS analysis was plotted against calibrator concentrations to generate the corresponding curves and equations as shown in Figures 6.7-6.10A (see page 272-274). These equations were then used to calculate the measured amount of CAP-ETN and PP-ETN in the hydrolysis reaction product mixtures from human blood plasma samples. For human plasma samples, the CAP or PP-EP concentration was calculated as follows:

\[ M(\text{CAP-or PP-EP}) = \frac{[\text{Area (CAP-ETN or PP-ETN)/Area (CAP-ETN-d₄ or PP-ETN-d₄)} - \text{intercept}] \times 1000}{2 \times \text{slope} (\text{nM})}. \]

**Figure 6.7** (A) Calibration curve for PP-ETN. Data points represent mean ± SD of three independent experiments; (B) Levels of PP-ETN in human plasma samples from clinical SCD patients (SC-C, n = 5) and from hospitalized SCD patients (SC-H, n = 6) determined by LC-MS/MS. * = p < 0.05.
Figure 6.8 (A) Calibration curve for CEP-ETN. Data points represent mean ± SD of three independent experiments; (B) Levels of CEP-ETN in human plasma samples from clinical SCD patients (SC-C, n = 5) and from hospitalized SCD patients (SC-H, n = 6) determined by LC-MS/MS. ** = p < 0.002.

Figure 6.9 (A) Calibration curve of CPP-ETN. Data points represent mean ± SD of three independent experiments; (B) Levels of CPP-ETN in human plasma samples from clinical SCD patients (SC-C, n = 5) and from hospitalized SCD patients (SC-H, n = 6) determined by LC-MS/MS. ** = p < 0.002.
Figure 6.10 (A) Calibration curve of CHP-ETN. Data points represent mean ± SD of three independent experiments; (B) Levels of CHP-ETN in human plasma samples from clinical SCD patients (SC-C, n = 5) and from hospitalized SCD patients (SC-H, n = 6) determined by LC-MS/MS. “NS”-not significant.

The LC-MS/MS analysis of lipid extracts after treatment with PLD revealed the presence of all CAP-EP and PP-EP derivatives in human blood plasma. Representative LC-MS/MS chromatograms of CAP-ETN and PP-ETN detected in PLD-hydrolyzed lipid extracts from human blood plasma samples from hospitalized and clinical patients with SCD are shown in Figures 6.11 and 6.12 (see page 275-276).
Figure 6.11 Representative LC-MS/MS chromatograms of CAP-ETN and PP-ETN generated by PLD hydrolysis of phospholipids extracted from human blood plasma samples from hospitalized patients with SCD.
Figure 6.12 Representative LC-MS/MS chromatograms of CAP-ETN and PP-ETN generated by PLD hydrolysis of phospholipids extracted from human blood plasma samples from clinical patients with SCD.

In the present small pilot clinical study, we compared CAP-EP and PP-EP levels in human blood plasma from hospitalized SCD patients (SC-H, n = 6) with those from clinical
SCD patients (SC-C, n = 5) by LC-MS/MS. The results are summarized in Table 6.1. As shown in Figure 6.7B (see page 272) and Table 6.1, the PP-EP derivative concentration in plasma samples from clinical SCD patients is nearly 4.8-fold higher than its level in plasma samples from hospitalized SCD patients (7.06 ± 4.05 vs 1.48 ± 0.92 nM; p < 0.05). As shown in Figure 6.8B (see page 273) and Table 6.1, two fold higher levels of CEP-EPs (P<0.002) were detected in plasma samples from clinical SCD patients (63.9 ± 9.7 nM, n = 6 patients) compared to those of hospitalized SCD patients (27.6 ± 3.6 nM, n = 5). Plasma samples from clinical SCD patients demonstrated a four-fold higher concentration of CPP-EPs compared with those of hospitalized SCD patients (45.1 ± 10.9 vs 10.9 ± 3.4 nM; p < 0.002) (Figure 6.9B, see page 273 and Table 6.1). As shown in Figure 6.10 (see page 274) and Table 6.1, the mean level of CHP-EPs detected in plasma from clinical SCD patients (29.1 ± 15.1 nM, n = 6 patients) was not significantly different (NS) compared with plasma from hospitalized SCD patients (22.9 ± 7.5 nM, n = 5). Our preliminary results demonstrated that elevated levels of CAP-EPs and PP-EPs in vivo in SCD patients are generally found in blood from clinical samples compared to hospitalized individuals during episodes of platelet sickling.

Table 6.1 Analysis of SCD plasma samples with PLD treatment.

<table>
<thead>
<tr>
<th></th>
<th>PP-EP (nM) (mean ± SD)</th>
<th>CEP-EP (nM) (mean ± SD)</th>
<th>CPP-EP (nM) (mean ± SD)</th>
<th>CHP-EP (nM) (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC-H (N = 6)</td>
<td>1.48 ± 0.92</td>
<td>27.6 ± 3.6</td>
<td>10.9 ± 3.4</td>
<td>22.9 ± 7.5</td>
</tr>
<tr>
<td>SC-C (N = 5)</td>
<td>7.06 ± 4.05</td>
<td>63.9 ± 9.7</td>
<td>45.1 ± 10.9</td>
<td>29.1 ± 15.1</td>
</tr>
</tbody>
</table>

**Syntheses of deuterium labeled CAP- and PP-EP derivatives.** As shown in Scheme 6.4 (see page 261), the analytical workflow for quantification of EP modifications in
biological samples is complicated. Ideally, in order to correct for all experimental variability, an internal standard should be added to the sample at the beginning of the analytical procedure.\textsuperscript{33} Considering that the first step in the analytical procedure for quantification of CAP- and PP modified EPs in biological samples is the extraction of total phospholipids from the biological matrix, it is better to add the internal standard to the sample prior to the initial extraction step. Therefore, to optimize the method, a phospholipid internal standard instead of an ETN standard is desirable. In addition, hydrolysis of lipid extracts from biological samples by phospholipase D from \textit{Streptomyces chromofuscus} is an enzymatic process, the efficiency of which depends on a multitude of factors. To correct for variability resulting from variations in phospholipolysis efficiency and interference from complex biological matrices that may vary among different biological samples, the ideal internal standard has to be added to the sample before hydrolysis and should be a mixture containing phospholipids with various fatty acyl side chains, strongly mimicking the natural multiplicity of phospholipid fatty acyl side chains in biological matrices. Therefore, deuterium labeled CAP-EP and PP-EP standards (CAP-EP-d\textsubscript{4} and PP-EP-d\textsubscript{4}) were prepared from L-\textit{α}-phosphatidylcholine (egg PC) by an enzyme-mediated transphosphatidylation as described previously.\textsuperscript{27} In brief, L-\textit{α}-phosphatidylcholine (egg PC, Chicken) was allowed to react with deuterium labeled CAP-ETN-d\textsubscript{4} or PP-ETN-d\textsubscript{4} via a transphosphatidylation reaction mediated by PLD from \textit{Streptomyces sp.}, a type of PLD enzyme that has a higher transphosphatidylation than hydrolysis activity, to give the corresponding deuterium labeled CAP-EP-d\textsubscript{4} or PP-EP-d\textsubscript{4} derivatives (CEP-EP-d\textsubscript{4}, \textbf{6.19}; CPP-EP-d\textsubscript{4}, \textbf{6.20}; CHP-EP-d\textsubscript{4}, \textbf{6.21}; and PP-EP-d\textsubscript{4}, \textbf{6.22}) (Scheme 6.8, see next page), which are now available to be used as internal standards.
instead of CAP-ETN-d₄ and PP-ETN-d₄ in future studies.

**Scheme 6.8** Chemical syntheses of CAP-EP and PP-EP internal standards from egg PC and CAP-ETN-d₄ or PP-ETN-d₄ through transphosphatidylidation mediated by PLD (*Streptomyces sp.*).
6.3 Conclusion

Oxidative stress, the imbalance between prooxidant and antioxidant processes in living cells, causes overproduction of ROS. PUFAs, the most sensitive cellular target of free radical reactions, are susceptible to a series of free radical initiated chain reactions that can generate a large number of reactive aldehyde species. Protein modification by those reactive aldehydes, especially γ-hydroxy-α,β-unsaturated aldehydes, has been extensively studied. For example, the γ-hydroxy-α,β-unsaturated aldehydes 4-HNE, HODA, HOOA and HOHA, generate the corresponding PP, CHP, CPP, and CEP derivatives upon reaction with proteins. These oxidative lipid-derived proteins mediate various biological activities, and there can be increased levels of these modifications in several pathological conditions, including eye diseases, where the biological activities contribute to disease pathology. While the formation of protein adducts by these γ-hydroxy-α,β-unsaturated aldehydes has been well established in vivo, the extent of the formation of analogous phospholipid adducts to the primary amino groups of EPs in vivo and the biological activities of those modified EPs remain poorly characterized. In analogy with proteins, EPs exhibit a high proclivity to rapidly react with these γ-hydroxy-α,β-unsaturated aldehydes and thus are expected to compete with proteins. Recently, modification of EPs by reactive aldehydes, such as 4-HNE, isoLGs, acrolein and HOHA, have been reported. By analogy, γ-hydroxy-α,β-unsaturated aldehydes like HODA and HOOA were also expected to covalently modify the primary amino group of EPs to give the corresponding CAP derivatives. Such EP derivatives may play an important role in the pathobiology of oxidative injury.

In the present study, a series of authentic samples and deuterium labeled internal standards were prepared and an LC-MS/MS assay was developed by measuring levels of
CAP-and PP-ETN released through hydrolysis of lipid extracts under catalysis by PLD from *Strdptomycyes chromofuscus*. In vitro experiments demonstrated that pyrrole derivatives of EPs are substrates of this PLD. The new analytical method was successfully applied for simultaneous measurement of both CAP and PP modified EPs derivatives in human blood samples from SCD patients. The presence of CAP-EPs and PP-EPs in vivo was established. A small pilot study revealed that levels of CAP-EPs and PP-EPs, except CHP-EPs, are significantly elevated in plasma samples from clinical SCD patients compared to those of hospitalized SCD patients. The elevations are greater than those observed previously for CEP-PEs in blood from individuals with age-related macular degeneration. Levels in blood drawn from hospitalized SCD patients were low and similar to levels detected in blood from the healthy controls from the AMD study. The levels of CEP-EPs and CPP-EPs are strongly associated with SCD and may act as biomarkers for detecting SCD severity. Furthermore, since CEP and CPPs are biologically active, the elevated levels found in SCD blood may have biological significance for the pathology of SCD.

In the previous study, CEP and CPP derivatives have been found to represent a novel non-canonical ligand for toll like receptor 9 (TLR9) and can promote platelet activation in vitro and accelerate thrombosis in vivo in TLR9/MyD88 dependent manner. Thus, the increased CEP and CPP derivatives in SCD may potentially result in the permanent condition of hypercoagulability causing thrombosis and thereby play an essential role in vaso-occlusive events (VOEs), an important pathophysiologic feature of SCD. Additionally, the accumulation of CEP and CPP adducts in SCD may contribute to anemia by destabilizing the normal membrane lipid asymmetry similar to MDA, leading to the
excess exposure of phosphatidylserine on the outer surface of the erythrocyte, which triggers the removal of cells by macrophages.\textsuperscript{35-37} Although the fundamental processes of apoptosis and angiogenesis in mammalian biology are distinct in effect and apparent biologic intent, they become intricately intertwined in the biology of endothelial cells.\textsuperscript{38} This linkage is provided by the control role of endothelial apoptosis in the overall process of angiogenesis, with some endothelial proangiogenic factors such as VEGF exerting powerful anti-apoptotic effects.\textsuperscript{38} Angiogenesis is under tight regulation in most healthy tissues and is most probably controlled by the balance between proangiogenic and anti-angiogenic factors.\textsuperscript{39} The disruption of such a balance has been thought to potentially impact on sickle disease pathobiology.\textsuperscript{38, 39} Previous studies showed that the overproduction of growth factors such as VEGF in patients with SCD compared to production in healthy patients induced compensatory increase in neovascularization to overcome the vaso-occlusive crisis.\textsuperscript{40} Since both CEP and CPP adducts are shown to have proangiogenic effects and promote angiogenesis through MyD88-dependent TLR2 signaling that is independent of VEGF/VEGFR2 signaling,\textsuperscript{20} the increase of CEP and CPP adducts in SCD may also promote neovascularization to overcome the vaso-occlusive crisis in complementary to other growth factors such as VEGF. In addition, it has been previously demonstrated that the vascular pathobiology of SCD comprises a state of abnormally enhanced anti-apoptotic tone for endothelial cells and elevated VEGF was suggested to be a reflection of an anti-apoptotic state in patients with SCD.\textsuperscript{38, 41} Similarly, CEP and CPP-adducts may be also responsible for the enhanced anti-apoptotic tone for endothelial cells. Moreover, the abnormal angiogenesis induced by enhanced basal VEGF level in SCD has been demonstrated to contribute to the development of pulmonary hypertension,
proliferative retinopathy, moya–moya vasculopathy and leg ulcers in SCD. In analogy of VEGF, elevated levels of CEP and CPP adducts in SCD may also cause abnormal angiogenesis and contribute to these conditions. Therefore, it is anticipated that excessive CEP and CPP adduct generation plays a role in various aspects of the endothelial pathobiology of SCD. Since both CEP and CPP adducts promote angiogenesis that is independent of VEGF expression and VEGF-driven angiogenesis has been suggested to be an attractive therapeutic target for SCD treatment in previous studies, here the levels of CEP and CPP adducts in SCD may present another potential opportunity for novel therapeutic approaches that target CEP/CPP-driven angiogenesis for SCD especially in SCD resistant to anti-VEGF therapy.

To potentially optimize the new analytical method, deuterium labeled CAP-EP-d₄ or PP-EP-d₄ derivatives were synthesized through the transphosphatidylation mediated by PLD from *Streptomyces sp.* These derivatives are likely to be superior internal standards compared to CAP-EP-d₄ and PP-EP-d₄.
6.4 Experimental Procedures

**Materials.** 1,2-Dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE) and egg PC (L-α-phosphatidyl-choline) were purchased from Avanti Polar Lipids (Alabaster, AL). Phospholipase D (PLD) from *Streptomyces sp.* and *Streptomyces chromofuscus* were obtained from Enzo Life Sciences (Farmingdale, NY). Hank's balanced salt solution (HBSS) buffer was purchased from Thermo Scientific (Waltham, MA). Ethanol-1,1,2,2-d$_4$-amine (ETN-d$_4$, 99 atom % D) was purchased from C/D/N Isotopes (Quebec, Canada). 6-[1,3]Dioxolan-2-yl-4-oxo-hexanoic acid-9H-fluoren-9-yl methyl ester (6.1), and 6-(1,3-dioxan-2-yl)-4-oxohexanoic acid (6.4) were prepared by Dr. Li Hong in Dr. Salomon’s lab. 7-(1.3-Dioxan-2-yl)-5-oxoheptanoic acid (6.5) and 11-(1,3-dioxan-2-yl)-9-oxoundecanoic acid (6.6) were prepared by Dr. Liang Xin in Dr. Salomon’s lab. All other chemicals and reagents were obtained from Sigma–Aldrich unless specified.

**General Methods.** Proton magnetic resonance (1H NMR) spectra and carbon magnetic resonance (13C NMR) spectra were recorded on a Varian Inova AS400 spectrometer operating at 400 MHz and 100 MHz, respectively. Proton chemical shifts are reported as parts per million (ppm) on the δ scale relative to CDCl$_3$ (δ 7.26) or CD$_3$OD (δ 3.31). 1H NMR spectral data are tabulated in terms of multiplicity of proton absorption (s, singlet; d, doublet; dd, doublet of doublet; t, triplet; q, quartet; m, multiplet; br, broad), coupling constants (Hz), number of protons. Carbon chemical shifts are reported relative to CDCl$_3$ (δ 77.0) or CD$_3$OD (δ 49.0). Flash chromatography was performed with ACS grade solvents from Fisher Scientific (Hanover Park, IL). R$_f$ values are quoted for TLC plates of thickness 0.25 mm from Whatman (Florham Park, NJ). The plates were visualized with
iodine, dinitrophenylhydrazine or phosphomolybdic acid reagents. For all reactions performed in an inert atmosphere, argon was used unless otherwise specified.

4,7-Dioxo-heptanoic acid 9H-fluoren-9-ylmethyl ester (DOHA-Fm, 6.2). The procedure was used from that reported. In brief, ester acetal 6.1 (300 mg, 0.76 mmol) in 40 mL of AcOH/H$_2$O (3:1, v/v) was stirred at 50 °C for 5 h under Ar protection. The reaction was monitored by TLC (hexane/ethyl acetate/diethyl ether 2:1:1). After TLC analysis showed completion of the reaction, the solvents were evaporated under reduced pressure and the residue was purified by flash chromatography on a silica gel column (CH$_2$Cl$_2$, TLC: R$_f$ = 0.17) to give pure DOHA-Fm (6.2, 223.3 mg, 0.66 mmol, 88%). $^1$H NMR (400 MHz, CDCl$_3$) δ 9.79 (s, 1H), 7.77 (d, J = 7.5 Hz, 2H), 7.59 (dd, J = 7.5, 0.8 Hz, 2H), 7.41 (t, J = 7.4 Hz, 2H), 7.32 (td, J = 7.5, 1.2 Hz, 2H), 4.39 (d, J = 7.1 Hz, 2H), 4.22 (t, J = 7.1 Hz, 1H), 2.79 – 2.69 (m, 8H).

3-(1-(2-Hydroxyethyl)-1H-pyrrol-2-yl)propanoic acid (CEP-ETN, 6.3a). DOHA-Fm (6.2, 92.5 mg, 0.27 mmol) reacted with ethanolamine (0.33 mmol) in 5.0 mL of mixture of MeOH/CH$_2$Cl$_2$ (1:1, v/v) for 7 h at room temperature under nitrogen protection to afford CEP-ETN-Fm ester. Then, 75 µL of DBU was added and the mixture was incubated overnight at room temperature to remove the Fm group. TLC was used to monitor reaction progress (CH$_2$Cl$_2$/CH$_3$OH 5:1). After TLC analysis showed completion of the reaction, the solvents were evaporated under reduced pressure and the residue was purified by flash chromatography on a silica gel column (CH$_2$Cl$_2$/CH$_3$OH 5:1, TLC: R$_f$ = 0.4) to give pure CEP-ETN (6.3a, 33.0 mg, 0.18 mmol, 66%). $^1$H NMR (400 MHz, CD$_3$OD) δ 6.65 – 6.61 (m, 1H), 5.98 – 5.94 (m, 1H), 5.82 (dd, J = 3.0, 1.3 Hz, 1H), 3.95 (t, J = 5.9 Hz, 2H), 3.74 (t, J = 5.9 Hz, 2H), 2.87 (t, J = 7.6 Hz, 2H), 2.62 (dd, J = 8.4, 6.8 Hz, 2H). ESI-MS: m/z
calcd for C₉H₁₄NO₃ [M+H]⁺, 184.10, found 184.20; m/z calcd for C₉H₁₂NO₃ [M-H]⁻, 182.08, found 182.07.

3-(1-(2-Hydroxy-1,1,2,2-D,D,D,D-ethyl)-1H-pyrrol-2-yl)propanoic acid (CEP-ETN-d₄, 6.3b). DOHA-Fm (6.2, 48 mg, 0.14 mmol) was incubated with ethanol-1,1,2,2-d₄-amine (0.17 mmol) in 3.0 mL of a mixture of MeOH/CH₂Cl₂ (1:1, v/v) for 6 h at room temperature under nitrogen protection to afford CEP-ETN-d₄-Fm ester. Then, 32 μL of DBU was added and the mixture was incubated overnight at room temperature to remove the Fm group. TLC was used to monitor progress of the reaction (CH₂Cl₂/CH₃OH 5:1). After TLC analysis showed completion of the reaction, the solvents were evaporated under reduced pressure and the residue was purified by flash chromatography on a silica gel column (CH₂Cl₂/CH₃OH 5:1, TLC: Rf = 0.4) to give pure CEP-ETN-d₄ (6.3b, 16.8 mg, 0.09 mmol, 68%). ¹H NMR (400 MHz, CD₃OD) δ 6.63 (dd, J = 2.8, 1.8 Hz, 1H), 5.97 – 5.93 (m, 1H), 5.84 – 5.80 (m, 1H), 2.91 – 2.84 (m, 2H), 2.62 (dd, J = 8.4, 6.9 Hz, 2H). ESI-MS: m/z calcd for C₉H₁₀D₄NO₃ [M+H]⁺, 188.12, found 188.40; m/z calcd for C₉H₈D₄NO₃ [M-H]⁻, 186.11, found 186.08.

4,6-Dioxoheptanoic acid (DOHA, 6.7). Acetal 6.4 (240 mg, 1.2 mmol) in a solution of AcOH/H₂O (20 mL, 3:1, v/v) was stirred at 50 °C for 4 h under Ar protection. TLC (CH₂Cl₂/CH₃OH 10:1) was used to monitor progress of the reaction. Once the reaction was complete, the solvent was removed by rotary evaporation to give crude DOHA (6.7, 172.7 mg, 1.09 mmol, 91%), which was used without purification for pyrrole syntheses.

5,8-Dioxooctanoic acid (DOOA, 6.8). Acetal 6.5 (115 mg, 0.5 mmol) in 20 mL of AcOH/H₂O (3:1, v/v) was stirred at 50 °C for 5 h under Ar protection. TLC (CH₂Cl₂/CH₃OH 10:1) was used to monitor progress of the reaction. Once the removal of the acetal
was complete, the solvent was removed by rotary evaporation to give crude DOOA (6.8, 80 mg, 0.46 mmol, 93%), which was used without purification for pyrrole syntheses.

**9,12-Dioxododecanoic acid (DODA, 6.9).** Acetal 6.6 (114 mg, 0.40 mmol) in 20 mL of AcOH/H₂O (3:1, v/v) was stirred at 50 °C for 5 h under Ar protection. TLC (CH₂Cl₂/CH₃OH 10:1) was used to monitor progress of the reaction. Once the reaction was complete, the solvent was removed by rotary evaporation to give crude DODA (6.9, 86 mg, 0.38 mmol, 95%), which was used without purification for pyrrole syntheses.

**4-(1-(2-Hydroxyethyl)-1H-pyrrol-2-yl)butanoic acid (CPP-ETN, 6.10a).** The crude keto aldehyde 6.8 (17.2 mg, 0.1 mmol) was allowed to react with ethanolamine (0.15 mmol) in 8 mL of MeOH for 6.5 h at room temperature under Ar protection. TLC was used to monitor the reaction progress (CH₂Cl₂/CH₃OH 5:1). After TLC analysis showed completion of the reaction, the solvents were evaporated under reduced pressure and the residue was purified by flash chromatography on a silica gel column (CH₂Cl₂/CH₃OH 5:1, TLC: Rf = 0.45) to give pure CPP-ETN (6.10a, 14.2 mg, 0.072 mmol, 72%). ¹H NMR (400 MHz, CD₃OD) δ 6.65 – 6.60 (m, 1H), 5.96 (t, J = 3.1 Hz, 1H), 5.82 (s, 1H), 3.94 (t, J = 6.0 Hz, 2H), 3.73 (t, J = 5.9 Hz, 2H), 2.62 (t, J = 7.6 Hz, 2H), 2.36 (t, J = 7.3 Hz, 2H), 1.96 – 1.84 (m, 2H). ESI-MS: m/z calcd for C₁₀H₁₆N₂O₃ [M+H]⁺, 198.11, found 198.20.

**4-(1-(2-Hydroxyethyl-1,1,2,2-d₄)-1H-pyrrol-2-yl)butanoic acid (CPP-ETN-d₄, 6.10b).** The crude product 6.8 (25.8 mg, 0.15 mmol) was allowed to react with ethanol-1,1,2,2-d₄-amine (0.225 mmol) in 15 mL of MeOH for 6 h at room temperature under Ar protection. TLC was used to monitor the process of the reaction (CH₂Cl₂/CH₃OH 5:1). After TLC analysis showed the completion of the reaction, the solvents were evaporated under reduced pressure and the residue was purified by flash chromatography on a silica
gel column (CH$_2$Cl$_2$/CH$_3$OH 5:1, TLC: $R_f = 0.45$) to give pure CPP-ETN-d$_4$ (6.10b, 21.2 mg, 0.105 mmol, 70%). $^1$H NMR (400 MHz, CD$_3$OD) $\delta$ 6.62 (dd, $J = 2.8$, 1.8 Hz, 1H), 5.97 – 5.94 (m, 1H), 5.83 – 5.79 (m, 1H), 2.65 – 2.59 (m, 2H), 2.35 (t, $J = 7.3$ Hz, 2H), 1.93 – 1.85 (m, 2H). ESI-MS: m/z calcd for C$_{10}$H$_1$$_2$D$_4$NO$_3$ [M+H]$^+$, 202.14, found 202.20; m/z calcd for C$_{10}$H$_{10}$D$_4$NO$_3$ [M-H]$^-$, 200.12, found 200.20.

8-(1-(2-Hydroxyethyl)-1H-pyrrol-2-yl)octanoic acid (CHP-ETN, 6.11a). The crude product 6.9 (22.8 mg, 0.1 mmol) was allowed to react with ethanolamine (0.15 mmol) in 8 mL of MeOH for 8 h at room temperature under Ar protection. TLC was used to monitor progress of the reaction (CH$_2$Cl$_2$/CH$_3$OH 10:1). After TLC analysis showed the completion of the reaction, the solvents were evaporated under reduced pressure and the residue was purified by flash chromatography on a silica gel column (CH$_2$Cl$_2$/CH$_3$OH/ AcOH 10:0.3:0.1, TLC: $R_f = 0.2$) to give pure CHP-ETN (6.11a, 19.3 mg, 0.076 mmol, 76%). $^1$H NMR (400 MHz, CD$_3$OD) $\delta$ 6.63 – 6.59 (m, 1H), 5.94 (t, $J = 3.1$ Hz, 1H), 5.78 (s, 1H), 3.92 (t, $J = 6.0$ Hz, 2H), 3.72 (t, $J = 6.0$ Hz, 2H), 2.61 – 2.52 (m, 2H), 2.28 (t, $J = 7.4$ Hz, 3H), 1.61 (d, $J = 4.6$ Hz, 4H), 1.38 (s, 6H). ESI-MS: m/z calcd for C$_{14}$H$_{24}$NO$_3$ [M+H]$^+$, 254.18, found 254.50.

4-(1-(2-Hydroxyethyl-1,1,2,2-d$_4$)-1H-pyrrol-2-yl)butanoic acid (CHP-ETN-d$_4$, 6.11b). The crude product 6.9 (27.4 mg, 0.12 mmol) was allowed to react with ethanol-1,1,2,2-d$_4$-amine (0.18 mmol) in 15 mL of MeOH for 7 h at room temperature under Ar protection. TLC was used to monitor the process of the reaction (CH$_2$Cl$_2$/CH$_3$OH 10:1). After TLC analysis showed the completion of the reaction, the solvents were evaporated under reduced pressure and the residue was purified by flash chromatography on a silica gel column (CH$_2$Cl$_2$/CH$_3$OH/AcOH 10:0.3:0.1, TLC: $R_f = 0.2$) to give pure CHP-ETN-d$_4$
(6.11b, 24.1 mg, 0.094 mmol, 78%). $^1$H NMR (400 MHz, CD$_3$OD) δ 6.62 – 6.58 (m, 1H), 5.94 (t, $J = 3.1$ Hz, 1H), 5.77 (s, 1H), 2.59 – 2.51 (m, 2H), 2.15 (d, $J = 7.5$ Hz, 3H), 1.60 (s, 4H), 1.36 (d, $J = 12.6$ Hz, 6H). ESI-MS: m/z calcd for C$_{14}$H$_{20}$D$_4$NO$_3$ [M+H]$^+$, 258.20, found 258.30; m/z calcd for C$_{14}$H$_{18}$D$_4$NO$_3$ [M-H], 256.19, found 256.27.

**Preparation of the CEP derivative of 1,2-dipalmitoyl-glycero-3-phospho-ethanolamine (CEP-DPPE, 6.12).** A solution of CHCl$_3$/CH$_3$OH (6 mL, 2:1, v/v) was added into a vial containing crude DOHA 6.7 (23.7 mg, 0.15 mmol) and DPPE (69.2 mg, 0.1 mmol). Then triethylamine (TEA, 40 µL, 0.4 mmol) was added to the reaction mixture. Then the mixture was allowed to react overnight at room temperature under Ar protection. TLC (CH$_2$Cl$_2$/CH$_3$OH 10:1) was used to monitor progress of the reaction. After TLC analysis showed completion of the reaction, the solvents were evaporated under reduced pressure and the residue was purified by flash chromatography on a silica gel column (CH$_2$Cl$_2$/MeOH 10:1, $R_f$ = 0.28) to give pure CEP-DPPE (6.12, 56.9 mg, 0.07 mmol, 70%).

$^1$H NMR (400 MHz, CDCl$_3$/CD$_3$OD, 1/1): δ 6.61 (s, 1H), 6.00 – 5.95 (m, 1H), 5.82 (d, $J = 3.4$ Hz, 1H), 5.13 (s, 1H), 4.30 (dd, $J = 12.0, 2.9$ Hz, 1H), 4.05 (dt, $J = 11.5, 5.9$ Hz, 5H), 3.71 (d, $J = 4.0$ Hz, 2H), 2.88 (t, $J = 7.5$ Hz, 2H), 2.68 – 2.61 (m, 2H), 2.30 – 2.24 (m, 6H), 1.57 (s, 4H), 1.24 (s, 47H), 0.85 (d, $J = 6.8$ Hz, 6H). ESI-MS: m/z calcd for C$_{44}$H$_{79}$NO$_{10}$P$^+$ [M-H], 812.54, found 812.40; calcd for C$_{44}$H$_{81}$NO$_{10}$P$^+$ [M+H]$^+$ 814.56, found 814.13.

**Preparation of the CPP derivative of 1,2-dipalmitoyl-sn-glycero-3-phospho-ethanolamine (CPP-DPPE, 6.13).** Crude DOOA (6.8, 13.1 mg, 0.076 mmol) was allowed to react with DPPE (34.0 mg, 0.049 mmol) in CHCl$_3$/MeOH (6 mL, 2:1, v/v) containing TEA (30 µL, 0.296 mmol) for 7 hours at r.t under Ar protection to give the CPP-DPPE. The product was purified by silica gel flash chromatography (CHCl$_3$/MeOH 3:1, $R_f$ = 0.2)
to give pure CPP-DPPE (26.4 mg, 0.032 mmol, 65%). $^1$H NMR (400 MHz, CDCl$_3$/CD$_3$OD, 1:1): $\delta$ = 6.53 (s, 1 H), 5.93 (t, $J$ = 3.2 Hz, 1 H), 5.75 (s, 1 H), 5.07 (m, 1 H), 4.25 (dd, $J$ = 2.1, 12.0 Hz, 1 H), 4.07 (m, 1 H), 3.98 (m, 4 H), 3.63 (m, 2 H), 2.57 (t, $J$ = 8.0 Hz, 2 H), 2.29 (t, $J$ = 6.4 Hz, 2 H), 2.22 (m, 4 H), 1.85 (m, 2 H), 1.51 (m, 2 H), 1.18 (54 H), 0.80 (t, $J$ = 6.8 Hz, 6 H). ESI-MS: m/z calcd for C$_{45}$H$_{81}$NO$_{10}$P$^-$ [M-H]$^-$, 826.56, found 826.40; calcd for C$_{45}$H$_{83}$NO$_{10}$P$^+$ [M+H]$^+$ 828.56, found 828.40.

**Preparation of the CHP derivative of 1,2-dipalmitoylsn-glycero-3-phosphoethanolamine (CHP-DPPE, 6.14).** Crude DODA (6.9, 32.2 mg, 0.141 mmol) was allowed to react with DPPE (95.4 mg, 0.137 mmol) in CHCl$_3$/MeOH (12 mL, 2:1, v/v) containing TEA (60 μL, 0.592 mmol) for 7 hours at room temperature under Ar protection. The product was purified by silica gel flash chromatography (CHCl$_3$/MeOH 5:1, $R_f$ = 0.19) to give pure CHP-DPPE (82.3 mg, 0.093 mmol, 67%). $^1$H NMR (400 MHz, CDCl$_3$/CD$_3$OD 2:1): $\delta$ = 6.54 (s, 1 H), 5.92 (t, $J$ = 3.2 Hz, 1 H), 5.74 (s, 1 H), 5.07 (m, 1 H), 4.23 (dd, $J$ = 2.8, 12.0 Hz, 1 H), 4.00 (dd, $J$ = 7.2, 12.0 Hz, 1 H), 3.94 (m, 4 H), 3.65 (m, 2 H), 2.46 (t, $J$ = 7.6 Hz, 2 H), 2.22 (m, 6 H), 1.5 (m, 8 H), 1.18 (54 H), 0.80 (t, $J$ = 6.8 Hz, 6 H). ESI-MS: m/z calcd for C$_{49}$H$_{89}$NO$_{10}$P$^-$ [M-H]$^-$, 882.62, found 882.47; calcd for C$_{49}$H$_{91}$NO$_{10}$P$^+$ [M+H]$^+$ 884.63, found 884.33.

**Nonane-1,4-diol (6.16).** γ-Nonalactone (1 mL, 976 mg, 6.25 mmol) was added to 20 mL of tetrahydrofuran (THF) in a flask and the solution was cooled with a Dry Ice-acetone bath. Then 13.0 mL of 1 M of lithium aluminum hydride in THF (13 mmol) was added dropwise over 10 min while cooling continued. After the flask was allowed to warm to room temperature, the reaction mixture was stirred for an additional 3 h. After cooling in an ice bath, ice-cold saturated ammonium chloride (5.0 mL) was added until the evolution
of hydrogen stopped. Then the ice bath was removed and 40 mL of potassium sodium tartate tetrahydrate was added. The resulting mixture was allowed to stir slowly for 15 min. Then the mixture was transferred to separatory funnel and extracted with ethyl acetate and water. The upper organic phase was collected and dried over magnesium sulfate. The solvents were evaporated under reduced pressure to give crude nonane-1,4-diol (6.16, 702 mg, 4.38 mmol, 70%), which was used for the next step without purification. \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta 3.72 – 3.55 (m, 3H), 1.74 – 1.58 (m, 3H), 1.49 – 1.22 (m, 9H), 0.88 (q, J = 7.0 Hz, 3H).

**4-Oxononanal (6.17).** Oxalyl dichloride (2.5 mL, 3.69 g, 29 mmol) in a three-necked flask was cooled with a dry ice acetone bath. Then dimethyl sulfoxide (DMSO, 5 mL, 5.5 g, 70.4 mmol) was added and the resulting mixture was allowed to stir for 45 min while cooling continued. Crude 6.16 (1.16 g, 7.2 mmol) was added slowly to the mixture, which was then allowed to stir for another 70 min with continued cooling in a dry ice acetone bath. TEA (8 mL, 57 mmol) was then added slowly and the mixture was stirred for another 2 min. Then the mixture was allowed to warm to room temperature. The reaction was quenched by the addition of a mixture of water and dichloromethane. The mixture was extracted three times with dichloromethane and the lower organic phase was collected and dried over anhydrous sodium sulfate. The solvents were evaporated under reduced pressure and the residue was purified by flash chromatography on a silica gel column (15% ethyl acetate in hexanes, \(R_f = 0.2\)) to give pure 4-oxononanal (6.17, 968 mg, 6.2 mmol, 86%). \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta 9.77 (d, J = 0.6 Hz, 1H), 2.74 – 2.68 (m, 4H), 2.46 – 2.40 (m, 2H), 1.56 (ddd, J = 14.8, 10.8, 7.5 Hz, 2H), 1.31 – 1.21 (m, 4H), 0.86 (dd, J = 8.9, 5.2 Hz, 3H).
2-(2-Pentyl-1H-pyrrol-1-yl)ethan-1-ol (PP-ETN, 6.18a). 4-Oxononanal (6.17, 50 mg, 0.32 mmol) and ethanolamine (0.48 mmol) were incubated in 50.0 mL of MeOH/CH₂Cl₂ (1:1, v/v) overnight at room temperature under Ar protection. TLC was used to monitor progress of the reaction (30% ethyl acetate in hexanes). After TLC analysis showed completion of the reaction, the solvents were evaporated under reduced pressure and the residue was purified by flash chromatography on a silica gel column (15% ethyl acetate in hexanes, TLC: Rf = 0.15) to give pure PP-ETN (6.18a, 41.7 mg, 0.23 mmol, 73%). ¹H NMR (400 MHz, CD₃OD) δ 6.61 (dd, J = 2.8, 1.8 Hz, 1H), 5.96 – 5.93 (m, 1H), 5.78 (ddt, J = 3.4, 1.7, 0.8 Hz, 1H), 3.92 (t, J = 6.1 Hz, 2H), 3.72 (t, J = 6.0 Hz, 2H), 2.59 – 2.52 (m, 2H), 1.66 – 1.57 (m, 2H), 1.40 – 1.35 (m, 4H), 0.95 – 0.91 (m, 3H). ESI-MS: m/z calcd for C₁₁H₂₀NO [M+H]+, 182.15, found 182.40.

2-(2-Pentyl-1H-pyrrol-1-yl)ethan-1,1,2,2-d₄-1-ol (PP-ETN-d₄, 6.18b). 4-Oxononanal (6.17, 70 mg, 0.45 mmol) and ethanol-1,1,2,2-d₄-amine (0.68 mmol) in 50.0 mL of mixture of MeOH/CH₂Cl₂ (1:1, v/v) were incubated overnight at room temperature under Ar protection. TLC was used to monitor progress of the reaction (15% ethyl acetate in hexanes). After TLC analysis showed the completion of the reaction, the solvents were evaporated under reduced pressure and the residue was purified by flash chromatography on a silica gel column (15% ethyl acetate in hexanes, TLC: Rf = 0.15) to give pure PP-ETN-d₄ (6.18b, 59 mg, 0.32 mmol, 72%). ¹H NMR (400 MHz, CD₃OD) δ 6.60 (dd, J = 2.8, 1.8 Hz, 1H), 5.95 (dd, J = 3.4, 2.8 Hz, 1H), 5.78 (ddt, J = 3.4, 1.7, 0.8 Hz, 1H), 2.58 – 2.53 (m, 2H), 1.61 (tdd, J = 7.1, 5.7, 3.2 Hz, 2H), 1.38 (ddd, J = 4.4, 2.1, 0.9 Hz, 4H), 0.95 – 0.91 (m, 3H). ESI-MS: m/z calcd for C₁₁H₁₆D₄NO [M+H]+, 186.18, found 186.40.
**CAP-EP-d₄** and **PP-EP-d₄** from egg PC. The procedure for preparation of CAP-and PP-EP-d₄ was the same as that for synthesis of CEP-EP-d₄ describe previously. ²⁷ Briefly, 200 µL of 0.2 M sodium acetate (pH 5.6) containing 80 mM CaCl₂ were added to a vial containing CAP-ETN-d₄ or PP-ETN-d₄ (0.02 mmol). After vortexing, 80 units of phospholipase D (*Streptomyces sp.*) were added into the vial. Then the mixture was transferred to another vial containing egg PC (9.1 mg, 0.012 mmol) in 300 µL of ethyl acetate. The resulting mixture was incubated at 37 °C under continuous stirring. After 1.5 h, the reaction was terminated by extraction of the aqueous mixture three times with 500 µL of ethyl acetate. The upper organic phase was collected and dried under a stream of dry nitrogen. The crude product was purified by silica-gel column. For CEP-EP-d₄, CH₂Cl₂/MeOH (10:1, TLC: Rₐ = 0.2) was used. For CPP-EP-d₄, CH₂Cl₂/MeOH (5:1, TLC: Rₐ = 0.08) was used. For CHP-EP-d₄, CH₂Cl₂/MeOH (5:1, TLC: Rₐ = 0.23) was used. For PP-EP-d₄, CH₂Cl₂/MeOH (17:3, TLC: Rₐ = 0.21) was used.

**Extraction of lipids from blood plasma.** Human blood plasma samples from SCD patients were collected by Dr. Borys Hrinchenko at MetroHealth Medical Center in 7 ml vacutainer™ tubes (lavender top) containing EDTA (10.5 mg). After incubating the tubes at room temperature for 30 min, the upper yellow plasma layer was transferred into a 15 mL Falcon tube (Fisher Cat. # 14-959-70C). BHT (5 µL/mL) and 10 µL/mL of protease inhibitor cocktail (Sigma, Cat.# P8340) were added and mixed gently 5 - 6 times by inversion of the tube. Remaining blood cells were removed by centrifugation at 2500 rpm (1300 x g) for 20-30 min at 4 °C. Two mL of the supernatant was aliquoted into 8 vials (250 µL/vial) (Fisher Scientific Cat. No 02-681-343) with screw caps (Fisher Scientific Cat. No 02-681-358). The vials were flushed with argon, sealed with screw caps, and then
quench-frozen in liquid nitrogen for 1 min and then stored at -80 °C. Phospholipids were extracted from the plasma by a modified Bligh and Dyer method. In brief, 750 µL of chloroform/methanol (1:2, v/v) containing 1 mM BHT was added to the vial containing 200 µL of plasma. The mixture was vortexed vigorously. Then 250 µL of chloroform was added to the mixture, which was vortexed vigorously again. Aqueous sodium chloride solution (250 µL of 1.5%) was added and the resulting mixture was vortexed vigorously followed by centrifuging for 10 min at 3,000 RPM to give a three-phase system (aqueous top, protein disk, organic bottom). The organic phase was withdrawn very carefully with a Pasteur pipette. The lipids were dried under a stream of dry nitrogen. The residue was stored under argon at -80 °C until analysis.

**Phospholipase D (Streptomyces chromofuscus) mediated hydrolysis of lipids.** The isolated phospholipids were hydrolyzed with PLD (Streptomyces chromofuscus) by the method described previously for hydrolysis of CEP-modified EPs.²⁷ Briefly, lipids were resuspended in 50 µL of methanol followed by the addition of 450 µL of HBSS buffer supplemented with 5 mM CaCl₂ and 0.1 mM EDTA. Then the mixture was incubated at 37 °C for 30 minutes and then sonicated for 10 minutes. Finally, small unilammelar vesicles (SUV) were generated by passing the cloudy lipid mixture through a 0.1 µm polycarbonate filter (17 times) for extrusion using an Avanti Mini-Extruder Set (Avanti Polar Lipids, Inc., Alabaster, AL). Then, 280 units of phospholipase D (Streptomyces chromofuscus) were added into the LUV solution and the resulting mixture was shaken under argon protection at 37 °C overnight. Then the solvent was evaporated under a stream of dry nitrogen. The residue was stored at -80 °C under Ar and dissolved in 100 µL of methanol and 20 µL of this solution was injected when doing LC-MS/MS analysis.
High performance liquid chromatography/mass spectrometry. LC-ESI/MS/MS analysis of CAP-ETN and PP-ETN derivatives was performed on a Quattro Ultima triple-quadrupole mass spectrometer (Micromass, Wythenshawe, UK) equipped with a Waters Alliance 2690 HPLC system with an auto-injector (Waters, Milford, MA). The chromatographic separation was achieved using a Luna C18 column (150×2.0 mm i.d. 5 µm, Phenomenex). The source and desolvation temperature was maintained at 120 °C and 250 °C, respectively. The drying gas (N₂) and cone flow gas were kept at ca. 650 L/h and 65 L/h, respectively. The multiplier was set at an absolute value of 600.

LC-MS/MS analysis was performed in the positive ion mode and the total run time was 45 min. The mobile phase consisted of solvent A (HPLC grade water containing 0.1% formic acid) and solvent B (HPLC graded methanol containing 0.1% formic acid). The HPLC gradient steps were set as follows: 0-5 min, isocratic at 5% solvent B; 5-25 min, linear gradient from 5 to 100% solvent B; 25-35 min, isocratic at 100% solvent B; 35-45 min, isocratic at 5% solvent B. The flow rate employed was 200 µL/min.

Optimized parameters for detection of CAP-ETN and PP-ETN derivatives were determined with authentic samples. MS scans at m/z 30-330 were obtained for standard compounds. For multiple reaction monitoring experiments, argon was used as collision gas at a pressure of 5 psi. The optimum collision energy and other parameters were determined for each individual analyte as summarized in tables 6.2 and 6.3.
### Table 6.2 Optimized parameters for the mass spectrometer.

<table>
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<th>Ion mode</th>
<th>CAP and PP-ETN</th>
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<td>Ion mode</td>
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<td>Ion Energy 2</td>
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### Table 6.3 MRM transition ion pairs and optimized collision energy for the analytes.

<table>
<thead>
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<th>Analytes</th>
<th>MRM transition ion pair (m/z)</th>
<th>Collision energy (eV)</th>
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<tr>
<td>PP-ETN</td>
<td>182.4 =&gt; 94.1</td>
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<tr>
<td>PP-ETN-d4</td>
<td>186.4 =&gt; 98.2</td>
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<tr>
<td>CEP-ETN</td>
<td>184.3 =&gt; 124.2</td>
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<tr>
<td>CEP-ETN-d4</td>
<td>188.4 =&gt; 128.3</td>
<td>10</td>
</tr>
<tr>
<td>CPP-ETN</td>
<td>198.4 =&gt; 162.1</td>
<td>10</td>
</tr>
<tr>
<td>CPP-ETN-d4</td>
<td>202.4 =&gt; 166.2</td>
<td>10</td>
</tr>
<tr>
<td>CHP-ETN</td>
<td>254.3 =&gt; 124.2</td>
<td>15</td>
</tr>
<tr>
<td>CHP-ETN-d4</td>
<td>258.4 =&gt; 128.3</td>
<td>15</td>
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Statistical Analysis. Statistical analyses were performed by using Student’s t test. A P value <0.05 is considered as statistically significant. Representative p-values in figures include “NS”-not significant, “*” p<0.05, “**” p<0.002, “***” p<0.0001. Data are presented as mean ± SD.
6.5 References


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Appendix
Figure S1. The 400 MHz $^1$H NMR (CDCl$_3$) spectrum of $(E)$-ethyl 4,7-diohept-5-enoate (2.2).

Figure S2. The 400 MHz $^1$H NMR (CDCl$_3$) spectrum of $(E)$-ethyl 7,7-dimethoxy-4-oxohept-5-enoate (2.3).
**Figure S3.** The 400 MHz $^1$H NMR (CDCl$_3$) spectrum of (E)-3-(5-oxotetrahydrofuran-2-yl)acrylaldehyde (HOHA-lactone, 2.5).

**Figure S4.** The 400 MHz $^1$H NMR (CDCl$_3$) spectrum of (E)-tributyl(3,3-diethoxyprop-1-en-1-yl)stannane (4.2)
Figure S5. The 400 MHz $^1$H NMR (CDCl$_3$) spectrum of methyl (E)-8,8-diethoxy-5-oxooct-6-enoate (4.3).

Figure S6. The 400 MHz $^1$H NMR (CDCl$_3$) spectrum of methyl (E)-8,8-diethoxy-5-hydroxyoct-6-enoate (4.4).
Figure S7. The 400 MHz $^1$H NMR (CDCl$_3$) spectrum (E)-3-(6-oxotetrahydro-2H-pyran-2-yl)acrylaldehyde (HOOA-lactone, 4.5).

Figure S8. The 400 MHz $^1$H NMR (CDCl$_3$) spectrum of 4-(1-[5-(2-Acetamidoacetamido)-6-methoxy-6oxohexyl]-1H-pyrrol-2-yl)butanoic acid (CPP-dipeptide, 4.7).
Figure S9. The 100 MHz $^{13}$C NMR (CDCl$_3$) spectrum of 4-(1-[5-(2-Acetamidoacetamido)-6-methoxy-6oxohexyl]-1H-pyrrol-2-yl)butanoic acid (CPP-dipeptide, 4.7).

Figure S10. The 400 MHz $^1$H NMR (CD$_3$OD) spectrum of 1-Palmityl-2-(Ac-Gly-Lys-OMe-CPP)-sn-glycerophosphatidylcholine (CPP-PC-dipeptide, 4.11).
Figure S11. The 100 MHz $^{13}$C NMR (CD$_3$OD) spectrum of 1-Palmityl-2-(Ac-Gly-Lys-OMe-CPP)-sn-glycerophosphatidylcholine (CPP-PC-dipeptide, 4.11).

Figure S12. The 400 MHz $^1$H NMR (CD$_3$OD) spectrum of 1-Palmityl-2-(4,7-dioxoheptanoyl)-sn-glycero-3-phosphatidylcholine (4.13).
Figure S13. The 100 MHz $^{13}$C NMR (CD$_3$OD) spectrum of 1-Palmityl-2-(4,7-dioxoheptanoyl)-sn-glycero-3-phosphatidylcholine (4.13).

Figure S14. The 400 MHz $^1$H NMR (CD$_3$OD) spectrum of 4-oxoheptanedioic acid (5.1)
Figure S15. The 400 MHz 1H NMR (CDCl$_3$) spectrum of bis(perfluorophenyl) 4-oxoheptanedioate (OHdiA-PFB, 5.3).

Figure S16. The 400 MHz 1H NMR (D$_2$O) spectrum of 4-oxoheptanedioic-3,3,5,5-d$_4$ acid (5.4).
Figure S17. The 400 MHz 1H NMR (CDCl₃) spectrum of bis((perfluorophenyl)methyl) 4-oxoheptanedioate-3,3,5,5-d₄ (OHdiA-PFB-D₄, 5.5)

Figure S18. The 400 MHz 1H NMR (CDCl₃) spectrum of 7-((2-hydroxyethyl)amino)-4,7-dioxoheptanoic acid (OHdiA-ethanolamine, 5.6)
**Figure S19.** The 400 MHz $^1$H NMR (CDCl$_3$) spectrum of 1-Palmitoyl-2-(3-(2-furyl)propanoyl)-sn-glycero-3-phosphatidylcholine (CEF-PC, 5.9).

**Figure S20.** The 400 MHz $^1$H NMR (CDCl$_3$) spectrum of 1-Palmitoyl-2-(4,7-dioxohept-6-enoyl)-sn-glycero-3-phosphatidylcholine (KOHA-PC, 5.10).
**Figure S21.** The 400 MHz $^1$H NMR (CDCl$_3$) spectrum of 7-((2-((tert-butyldimethylsilyl)oxy)ethyl)amino)-4,7-dioxoheptanoic acid (5.13).

**Figure S22.** The 400 MHz $^1$H NMR (CDCl$_3$) spectrum of 4, 7-Dioxo-heptanoic acid 9H-fluoren-9-ylmethyl ester (DOHA-Fm, 6.2).
Figure S23. The 400 MHz $^1$H NMR (CD$_3$OD) spectrum of 3-(1-(2-hydroxyethyl)-1H-pyrrol-2-yl)propanoic acid (CEP-ETN, 6.3a).

Figure S24. The 400 MHz $^1$H NMR (CD$_3$OD) spectrum of 3-(1-(2-hydroxy-1,1,2,2-D,D,D,D-ethyl)-1H-pyrrol-2-yl)propanoic acid (CEP-ETN-d4, 6.3b).
Figure S25. The 400 MHz $^1$H NMR (CD$_3$OD) spectrum of 4-(1-(2-hydroxyethyl)-1H-pyrrol-2-yl)butanoic acid (CPP-ETN, 6.10a).

Figure S26. The 400 MHz $^1$H NMR (CD$_3$OD) spectrum of 4-(1-(2-hydroxyethyl-1,1,2,2-d4)-1H-pyrrol-2-yl)butanoic acid (CPP-ETN-d4, 6.10b).
**Figure S27.** The 400 MHz $^1$H NMR (CD$_3$OD) spectrum of 8-(1-(2-hydroxyethyl)-1H-pyrrol-2-yl)octanoic acid (CHP-ETN, 6.11a).

**Figure S28.** The 400 MHz $^1$H NMR (CD$_3$OD) spectrum of 4-(1-(2-hydroxyethyl-1,1,2,2-d4)-1H-pyrrol-2-yl)butanoic acid (CHP-ETN-d4, 6.11b).
Figure S29. The 400 MHz $^1$H NMR (CDCl$_3$/CD$_2$OD, 1/1) spectrum of 1,2-dipalmitoylsn-glycero-3-phosphoethanolamine (CEP-DPPE, 6.12).

Figure S30. The 400 MHz $^1$H NMR (CDCl$_3$/CD$_2$OD, 1/1) spectrum of 1,2-dipalmitoylsn-glycero-3-phosphoethanolamine (CPP-DPPE, 6.13).
Figure S31. The 400 MHz $^1$H NMR (CDCl$_3$/CD$_3$OD, 2/1) spectrum of 1,2-dipalmitoylsn-glycero-3-phosphoethanolamine (CHP-DPPE, 6.14).

Figure S32. The 400 MHz $^1$H NMR (CDCl$_3$) spectrum of nonane-1,4-diol (6.16).
Figure S33. The 400 MHz $^1$H NMR (CDCl$_3$) spectrum of 4-oxononanal (6.17).

Figure S34. The 400 MHz $^1$H NMR (CD$_3$OD) spectrum of 2-(2-pentyl-1H-pyrrol-1-yl)ethan-1-ol (PP-ETN, 6.18a)
Figure S35. The 400 MHz $^1$H NMR (CD$_3$OD) spectrum of 2-(2-pentyl-1H-pyrrol-1-yl)ethan-1,1,2,2-d$_4$-1-ol (PP-ETN-d$_4$, 6.18b).
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