Bioactive Oxidized Phosphatidylcholines cause Apoptotic Cell Death in Cardiomyocytes during Ischemia Reperfusion

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Abstract

Ischemic heart disease is one of the leading causes of death worldwide. The heart muscle is damaged during an ischemic period where coronary blood flow is reduced. The most common situation where this ischemia occurs is during a myocardial infarction. The most effective therapy for a myocardial infarction is to re-establish coronary blood flow to the heart muscle by means of percutaneous coronary intervention or thrombolytic therapies. However, upon this reperfusion of the myocardium, there is significant loss of previously functional cardiomyocytes contributing to loss of heart function. This subsequent reduction in heart function is termed as ischemia reperfusion injury, and the extent of damage is unpredictable. However, there has yet to be a therapy that adequately prevents ischemia reperfusion injury. During the reperfusion event, it has been established that there is significant reactive oxygen species production. These species are able to oxidize membrane phospholipids. The resulting oxidized phospholipids are potent bioactive signaling molecules in macrophages, vascular smooth muscle cells, and dendritic cells and are known to cause apoptotic cell death. The most abundant oxidized phospholipids are the oxidized phosphatidylcholines which are the primary focus of current research. These compounds are immunogenic and natural IgM antibodies, the EO antibodies, are produced *in vivo* that preferentially bind these compounds. The EO6 antibody is the best described EO antibody and could prevent the negative effects of oxidized phospholipids on cells. These findings have never been investigated in the cardiomyocytes within the heart. Using high performance liquid chromatography linked to tandem mass spectrometry, we were able to detect the production of oxidized phosphatidylcholines within cells that were exposed to simulated ischemia reperfusion.

Cell signaling effects were investigated by the exogenous administration of short-chain fragmented oxidized phosphatidylcholines, POVPC, PONPC, PGPC, and PAzPC to cells for cell viability and mitochondrial permeability assays. EO6 antibody was co-administered to cardiomyocytes to determine if any of the apoptotic signaling endpoints could be reversed. There was significant induction of cardiomyocyte cell death in a dose-dependent manner compared to a non-oxidized control PSPC. Mitochondrial permeability was increased after exposure to oxidized phosphatidylcholine molecules indicating an intrinsic mechanism of apoptotic cell death. EO6 antibody administration significantly blocked the apoptotic signaling that was initiated by fragmented oxidized phosphatidylcholines. For the first time, we have conclusively shown that during simulated ischemia reperfusion, cardiomyocytes endogenously produce oxidized phosphatidylcholines and that these compounds contribute to apoptotic cell death within this cell type. Additionally and most importantly, we show that the EO6 antibody could be effective in minimizing the injury sustained after reperfusion by rescuing cardiomyocytes that would die upon reperfusion. This research provides insight into the activity of the EO6 antibody and warrants further study into clinical application as a therapy for ischemia reperfusion injury.

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To my community of fellow followers of Jesus, thank you for encouragement and focus on the true purpose of life, God's love for all people shown on the Cross.

To my parents, Ian and Shawna, and family, Raisa, Elaine, and Joan, I love you and thank you for all you do in my life.

Most importantly, I thank God for creating the world and humans in His likeness, that once we sinned, He chose to save us from death by sending Yeshua ha'Meshiach. He will return victorious and I pray that you would find Him as your Saviour from sin and death.

Dedication

This thesis is dedicated to the beloved memory of my grandfather, Daniel Antoine Hoorne (July 23rd 1932 – May 21st 2013). I thank God for the years that we had with you and realize now that much more time could have been spent with you. Your perseverance to be a learning experience to the medical residents during the last few weeks of your life was inspiring as well as your decision to leave your heart behind as a sample for medical research was exceptional. You will be remembered with a wry grin on your face and camera in hand. Perhaps if we had investigated these compounds earlier the knowledge may have helped us to extend your life by a few more years. This work is closely tied to the disease that took your life and I hope that this knowledge may help another person live a little longer when stuck in your situation.

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List of abbreviations

AAR – Area-at-risk
A_3AR – A3 Adenosine Receptor
AIF – Apoptosis-inducing Factor
Akt – Protein Kinase B
Apaf-1 – Apoptotic Pepsidase-activating Factor 1
ApoE – Apolipoprotein E
ATF-6 – Activating Transcription Factor 6
ATP – Adenosine Tri-Phosphate
BMI – Body Mass Index
CAD – Caspase-activated Deoxyribonuclease
CCL7 – C-C Chemokine Ligand 7
CCR2 – C-C Chemokine Receptor 2
CRP – C-reactive Protein
CsA – Cyclosporin A
$\Delta\Psi_m$ – Mitochondrial Membrane Potential
DAMPS – Damage-associated Molecular Patterns
DD – Death Domain
DIABLO – Direct Inhibitor of Apoptosis-binding Protein with Low pI
DISC – Death-inducing Signaling Complex
DNPC – 1,2-Dinonanoyl-*sn*-glycero-3-phosphocholine
DP – Prostaglandin D2 Receptor
EC – Endothelial Cells
EP2 – Prostaglandin E2 Receptor
ERK1/2 – Extracellular Signal-regulated Kinase 1/2
ETC – Electron Transport Chain
ESI – Electrospray Ionization
$FADH_2$ – Reduced Flavin Adenine Dinucleotide
FADD – Fas-associated Death Domain
GPCR – G Protein-coupled Receptor
GROα – Growth-regulated Alpha Protein
HAEC – Human Aortic Endothelial Cells
HIF-1 – Hypoxia-inducible Factor 1
HPLC – High Performance Liquid Chromatography
HUVEC – Human Umbilical Vein Endothelial Cells
IAP – Inhibitor of Apoptosis
ICAD – Inhibitor of Caspase-activated Deoxyribonuclease
IgM – Immunoglobulin M
IL – Interleukin
iPSC – Induced Pluripotent Stem Cells
IR – Ischemia Reperfusion
IRE-1 – Inositol Requiring 1
JAK – Janus Kinase
JNK – *Jun* N-terminal kinase
KDdiA- PPC – 1-palmitoyl-2-(4-keto-dodec-3-enadioyl)-*sn*-glycero-3-phosphocholine

KOdiA-PPC – 1-palmitoyl-2-(5-keto-6-octene-dioyl)-*sn*-glycero-3-phosphocholine
LDH – Lactate Dehydrogenase
LDL – Low-Density Lipoprotein
LPS – Lipopolysaccharide
MACE – Major Adverse Cardiovascular Events
MALDI – Matrix-assisted Laser Desorption Ionization
MAPK – Mitogen-activated Protein Kinase
MCP – Monocyte Chemoattractant Protein
MIP-1α – Macrophage Inflammatory Protein-1 Alpha
MI – Myocardial Infarction
mPTP – Mitochondrial Permeability Transition Pore
MRM – Multiple Reaction Monitoring
N-Ab – Natural Antibody
NADPH – Nicotinamide Adenine Dincleotide Phosphate
NCMC – Post-natal Cardiomyocytes
NF-κB – Nuclear Factor Kappa-light-chain-enhancer of Activated B Cells
OxLDL – Oxidized Low-Density Lipoprotein
OxPAPC – Oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine
OxPC – Oxidized Phosphatidylcholine
OxPS – Oxidized Phosphatidylserine
OxPL – Oxidized Phospholipids
PAF – Platelet-activating Factor
PARP – Poly-ADP-ribose-polymerase
PAzPC – 1-palmitoyl-2-azelaoyl-*sn*-glycero-3-phosphocholine
PCI – Percutaneous Coronary Intervention
PC – Phosphatidylcholine
PE – Phosphatidylethanolamine
PEIPC – 1-palmitoyl-2-(5,6-epoxyisoprostane E2)-*sn*-glycero-3-phosphocholine
PERK – PKR-like ER Kinase
PGPC – 1-palmitoyl-2-glutaryl-*sn*-glycero-3-phosphocholine
PI – Phosphatidylinositol
PI3K – Phosphoinoside 3-Kinase
PKR - Double-stranded RNA-dependent Protein Kinase
PL – Phospholipid
PPARγ – Peroxisome Proliferator-activator Receptor Gamma
PONPC – 1-palmitoyl-2-(5'-oxo-valeroyl)-*sn*-glycero-3-phosphocholine
POVPC – 1-palmitoyl-2-(9'-oxo-nonanoyl)-*sn*-glycero-3-phosphocholine
PS – Phosphatidylserine
PSPC – 1-palmitoyl-2-stearoyl-*sn*-glycero-3-phosphocholine
PUFA – Poly-unsaturated Fatty Acid
RIP-1 – Receptor-interacting Protein 1
ROS – Reative Oxygen Species
SMAC – Second Mitochondria-derived Activator of Caspases
SOD – Superoxide Dismutase
SR-B1 – Scavenger Receptor Class B Type 1
STAT – Signal Transducer and Activator of Transcription

STEMI – ST-Elevated Myocardial Infarction
TF – Tissue Factor
TFPI – Tissue Factor Pathway Inhibitor
TLR – Toll-like Receptor
TNFα – Tumour Necrotic Factor Alpha
TNFR – TNF Receptor
TRADD – TNFR-associated Death Domain
TRAIL – TNF-related Apoptosis-inducing Ligand
VEGFR2 – Vascular Endothelial Growth Factor Receptor 2
VSMC – Vascular Smooth Muscle Cells
XBP-1 – X-box Binding Protein 1

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Chapter I: Literature Review

Introduction

Heart disease is one of the leading causes of death in developed countries.[2] In 2009, ischemic heart disease contributed to 15% of all deaths within Canada, and represents one of the greatest burdens to the health care system.[3] Coronary artery disease is a result of atherosclerosis, where deposits of cholesterol, calcium and cells begin to adhere to the coronary arteries causing them to narrow and resulting in an increase in the risk of cardiac and vascular problems like myocardial infarction (MI) and stroke, categorized together as major adverse cardiovascular events (MACE).[4] As coronary artery disease progresses, the plaque morphology changes and is correlated with increased risk of MACE. Small deposits of modified low-density lipoproteins (LDL) in the intima of the vessels are taken up by macrophages to form 'foam cells' making up the fatty streak and beginning the cascade of inflammatory signaling via TNFα, IL-6 and MCP-1.[5] The plaque progresses to a stage where the inner necrotic core can become unstable. The plaque can then rupture releasing the necrotic core consisting of the aforementioned cell types as well as pro-thrombotic, inflammatory, and apoptotic signaling molecules. This rupture can cause a thrombosis and blockage of a coronary artery resulting in the myocardium supplied by the blocked artery becoming ischemic. During a MI, the myocardium is ischemic, unable to contract effectively and unable to circulate adequate blood to the rest of the body. Removing the thrombus or plaque material blocking the coronary arteries is the best therapy to reduce mortality and allows for reperfusion of the ischemic myocardium and recovery of heart function. This is usually achieved by administration of thrombolytic enzymes or

percutaneous coronary intervention (PCI), which are the most effective strategies currently available for minimizing the effects of an infarct and improving the clinical outcome.[6] After a successful intervention, oxygen supply by the blood is re-established to the ischemic myocardium, called an infarct. However, despite restoration of blood flow to cardiomyocytes after ischemia, loss of cardiomyocytes continues. The cells in the area-at-risk (AAR) of an infarct have a balance of cell signaling that could lead to apoptosis, necrosis, or recovery. This phenomenon has been attributed directly to the cardiomyocytes that were functional just before reperfusion, but after reperfusion the cells are induced to undergo cell death.[7] Loss of cardiomyocytes, the functional cell type of the myocardium, causes akinesia, inability to contract, in the area of infarct.

Despite life-saving therapy, the recovery of cardiomyocytes in the AAR is not achieved, and there is reduced electrophysical and contractile responses in the myocardium as a result. [8-12] This reduction of cardiac function post-reperfusion is called ischemia reperfusion (IR) injury because it is due to initial ischemia and subsequent, but required, reperfusion of the myocardium. Many strategies have been proposed as therapy for IR injury, however, none have been successful. [13] The current inability to prevent or adequately treat IR injury is due to the complex nature of IR injury. A novel therapeutic approach is required to address the multifactorial process at a more proximal stage.

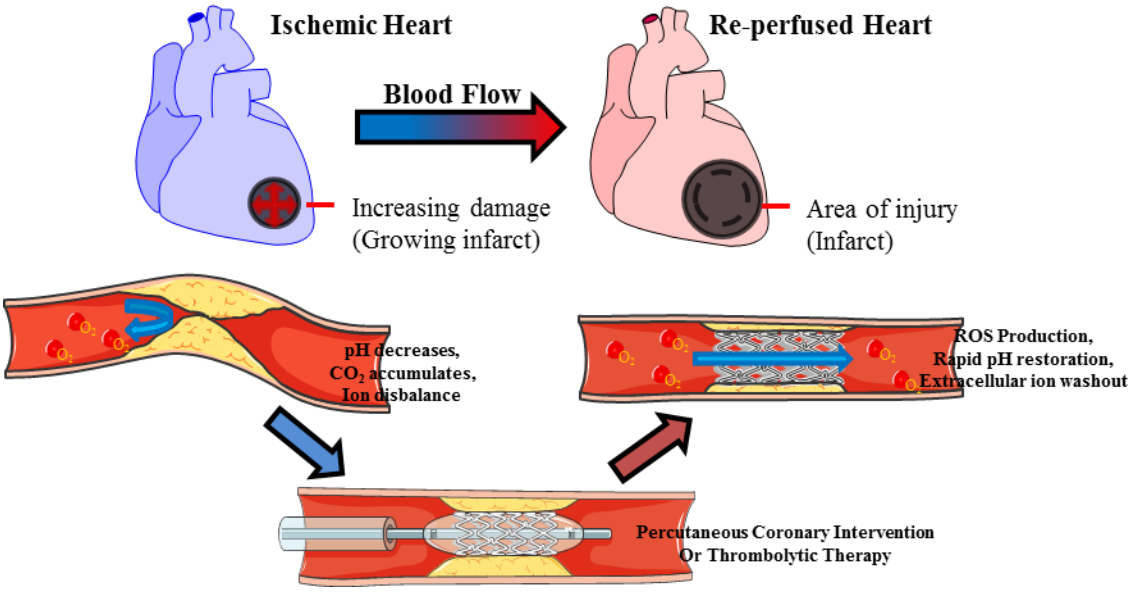

Figure 1.1: Coronary occlusion by either plaque or thrombus causes ischemia in the myocardium characterized by acidosis, carbon dioxide accumulation and sodium, potassium, calcium, and hydrogen ion dysbalance within the ischemic myocardium. These cause cardiomyocytes to be unable to contract, production of cytokines and cell signals that cause an influx of resident immune cells and upregulation of fibroblast activity. Restoration of blood flow either by PCI or thrombolytic therapy restores the pH, removes CO_2, and provides oxygen to ischemic cardiomyocytes. Adverse effects of reperfusion include increase in inflammatory cells, abrupt restoration of ion gradients causes ion exchangers on the cell surface and the mitochondria to become activated and cause hyper contracture of myocytes. Importantly ROS production occurs in activated neutrophils responding to injury and within re-activated mitochondria through electron shuttling proteins of the electron transport chain. The reperfused myocardium suffers from an infarcted area of injury which is often greater than the ischemic area as cellular signaling plays a key role in the injury.

Cardiac Ischemia Reperfusion Injury

Therapeutic early reperfusion of the myocardium after an MI is a double-edged sword as blood flow rapidly restores the environment to homeostasis, however, it causes a strong cellular response leading to inflammation and cell death, which reduces functional recovery. Ischemia reperfusion injury is the result of this deficiency in cardiac function and pathologically, IR injury can present as arrhythmias, myocardial stunning, microvascular obstructions, and/or, most importantly, cardiomyocyte cell death prompted by the reperfusion event.[14-17] During myocardial ischemia there are changes that occurs in oxygen utilization, ionic gradients, and metabolic processes. Lack of oxygen causes oxidative phosphorylation to stop and the cells begins to use anaerobic respiration. Pyruvate molecules are biochemically reduced by lactate dehydrogenase to lactate causing an increase in the intracellular hydrogen ion concentration. The sudden increase of hydrogen ions acts on ion exchangers for sodium and calcium to reverse their normal function and work in reverse, accumulating both sodium and calcium ions within the cardiomyocytes. This causes two important effects, first the sodium potassium ATPase stops functioning reducing the electrochemical gradient across the cell membrane and secondly, the contraction of the cardiomyocyte is inhibited. From the cellular level pathological changes, including cellular metabolic acidosis, ionic gradient changes, pro-inflammatory signals, ROS production, mitochondrial permeability transition pore (mPTP) opening, and release of pro-apoptotic signals, cause significant damage through loss of cardiac function and prevent functional recovery. These areas have been and continue to be investigated as important targets to find a therapy that allows for better functional

recovery of the myocardium and reduction in the morbidity and mortality associated with IR injury.

After the cardiomyocytes within the muscle become ischemic, oxidative phosphorylation, the cell's most efficient form of energy production, is interrupted. This causes the cell to switch to anaerobic respiration which causes increased lactate levels due to lactate dehydrogenase (LDH) activity on pyruvate. The decreased level of ATP production prevents cardiomyocytes from contracting when stimulated and increases the intracellular H^+ concentration.[18] Increased H^+ within the cytoplasm signals the cell to either remove hydrogen ions by the Na^+/H^+ exchanger in the plasma membrane or neutralize them using bicarbonate by the Na^+/HCO_3^- co-transporter. These transport mechanism causes the increased uptake of Na^+ into the cell and causes a depolarization of the cell which can contribute to fibrillation of cells, and results in the Na^+/Ca^{2+} exchanger removing sodium ions from the cell, but loading the cytoplasm with calcium ions which are further sequestered within the SR or the mitochondria, collapsing the negative electrochemical gradient. The ischemic environment causes the cells within the tissue begin to secrete pro-inflammatory cytokine, like tumor necrosis factor alpha (TNFα) and IL-8, in order to remove necrotic or apoptotic debris that may be accumulating in the myocardium. Damage associate molecular patterns (DAMPS) are released from cells and are recognized by TLR receptor on neutrophils, antibodies attracting immune mediators, or are taken up by antigen presenting cells that activate innate immunity and stimulate adaptive immune cells. The inflammatory processes attract neutrophils and macrophages which are capable of producing ROS by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase which are released into the area of damage.[19] The ROS are characteristic of oxidative stress and

can also be produced in the mitochondria by a particular superoxide dismutase (SOD).[20-22] Superoxide radicals, $OONO^{\cdot}$ and $O_2^{\bullet}$, produced in the inner mitochondrial matrix are converted into hydrogen peroxide which normally becomes detoxified to water. Under ischemic conditions, the hypoxia-inducible factor-1 (HIF-1) transcription factor induces the transcription of genes that are considered to be hypoxia-sensitive. HIF-1 regulation is an example of how low oxygen levels reduces the degradation of the HIF-1 protein by the ubiquitin-proteasome system by preventing it's hydroxylation at proline residues.[23, 24]

These cellular processes altered by ischemia begin to be reversed after reperfusion, however changes that occur abruptly are not well tolerated by cardiomyocytes in the AAR around the ischemic area and can cause increased loss of previously functional cardiomyocytes unaffected directly by ischemia. Reperfusion allows blood to offload oxygen to the cardiomyocytes and remove carbon dioxide to allow for the cells to use aerobic respiration. Cells convert pyruvate to acetyl-CoA within the mitochondria and produce $NADH^{+}$ and $FADH_2$ which starts the electron transport chain again which drives the reformation of the mitochondrial membrane potential ($\Delta\Psi_m$) used to drive ATP synthesis. The oxidative stress within the mitochondria is due to the sudden increase in the ETC function and generates increased amounts of free radicals. The rapid re-establishment of energy metabolism causes increased activity of the electron transport chain which causes complex I to produce radicals by a flavin mononucleotide mechanism and complex III to transport electrons from coenzyme Q to cytochrome C inadvertently passing them to oxygen. ROS production increases as the mitochondria becomes re-energized and causes significant redox signalling that causes non-enzymatic oxidation of cellular proteins,

carbohydrates, nucleic acids, and phospholipids that result in the generation of potent bioactive molecules influencing inflammation, angiogenesis, cell cycle, and apoptosis.[25-27]

In addition to free radical production, cytosolic Ca^{2+} overloading that began under ischemia is exacerbated after reperfusion because the re-energizing of the mitochondrial electrochemical gradient forces Ca^{2+} out of the mitochondria into the cytoplasm.[28] Mitochondrial re-activation causes Ca^{2+} overloaded mitochondria to open their mPTP which is a critical step in apoptosis, and cytosolic calcium overload occurs which causes hypercontracture of the sarcomeres.[29, 30] The mPTP is a non-specific pore that is found in mitochondria composed of various proteins from the inner and outer membranes of the mitochondria, which has been investigated as a target for IR injury and is modulated by cyclosporine A (CsA).[31] The opening of the pore releases cytochrome C, a pro-apoptotic cell signaling molecule, and result in a decrease in $\Delta\Psi_m$ which has been shown to be part of hypoxic injury within neonatal ventricular rat cardiomyocytes.[32] The cytochrome C molecule is integral to the activation of the intrinsic pathway of programmed cell death called apoptosis and will be discussed later. Ion exchanges also contribute to the overloading of Ca^{2+}. The Na^+/H^+ exchanger and the Na^+/HCO_3^- co-transporter on the plasma membrane are activated to remove Na^+ from the cytoplasm and cause the rapid reversal of acidosis, however, this process also reverses the action of the Na^+/Ca^{2+} exchanger, which responds to decreased levels of lactate.[25, 33 34] Due to the increased cytosolic Ca^{2+} concentration cardiomyocytes undergo hyper contraction. Ca^{2+} ions are required to trigger actin-myosin head interactions which allows for sarcomere contraction. The protein troponin C, has the ability to bind to Ca^{2+} and then disrupt tropomyosin and troponin I within myofibrils from shields the actin fibers from the myosin head group in

the dense band of the sarcomere. Upon binding to Ca^{2+}, troponin C causes the tropomyosin-troponin I to lose affinity for the actin fiber and dissociate, allowing the myosin head to interact with actin and complete myosin-actin cross-bridging causing contraction. At high levels of Ca^{2+}, there is continual inhibition of the ability of tropomyosin-troponin I from being able to disrupt the actin-myosin binding after the power phase to allow for cross-bridge cycling of myosin head group with actin chain. As well the increased levels of Ca^{2+} increase the affinity of the myosin head group for actin, however, if there is no ATP there will be no release by the myosin head from the actin fiber.

Reperfusion accelerates inflammation that is initiated during ischemia. Chemokines and cytokines are released by damaged cells in the muscle that attract immune cells that engulf cell debris. Monocytes of a $Ly6C^{hi}$ phenotype peak 1 day after MI and are replaced later by $Ly6C^{low}$ monocytes.[35] Further analysis of the acute response was that the $Ly6C^{hi}$ phenotype cells attract macrophages and dendritic cells in the acute setting after MI due to their ability to infiltrate inflammatory sites effectively. This monocyte phenotype increases in the acute phase of an infarction 4.5 fold and they remain the dominant cell type within the myocardium until day 4 post infarct.[36] They create a pro-inflammatory environment that is sensitive to inflammatory signaling molecules MCP-1 and MIP-1α which are increased in the acute phase. The response to MCP-1 is due to the $Ly6C^{hi}$ monocyte expressing the MCP-1 receptor, CCR2. The receptor appears to be integral to the over-expression of this monocyte. These monocytes also express TNFα in a much higher proportion than the other monocytes. A subsequent study on B lymphocytes also noted that recruitment of the $Ly6C^{hi}$ monocytes could be from Ccl7. Ccl7 is a protein that produced in B cells in response to MI and when mice are B cell-depleted there is reduced

production of this protein and concurrently there are fewer Ly6C^{hi} monocyte mobilized to the area of cardiac remodelling and adaptive immune response.[36] The recruitment of different cell phenotypes are important because macrophages will engulf apoptotic and necrotic debris, and necessary cardiac remodeling by fibroblasts will occur. Secondary to this is increased inflammation which can cause more cell death and scar formation that is not caused directly by the ischemic period. This contributes to the AAR that can sustain additional damage even though blood supply is resupplied to the ischemic muscle.[37]

Reperfusion can be viewed as a trigger to exacerbate damage to the AAR constituted of numerous cell types that respond adversely either through inflammatory or apoptotic triggers. The rapid shift from anaerobic to aerobic respiration in the mitochondria causes production of ROS that increases oxidative stress initiating inflammatory responses when TLRs recognize DAMPS, and monocytes excrete TNFα.[38-40] B lymphocytes that are surrounding the area of infarct generate CCR2 ligands, MCP-1 and Ccl7, to be recognized and attract monocytes that induce inflammation and cardiac remodeling.[36] Calcium, sodium, and hydrogen ion gradients are disrupted and the ion exchangers lead to hyper contraction of cardiomyocytes or fibrillation due to increased cytoplasmic calcium ions. The mPTP opening signals cardiomyocytes to undergo apoptotic cell death by action of cytochrome C. Even though these processes are part of the normal response after MI and reperfusion, the environment induces inadvertent cell death and scar formation in the AAR of cardiomyocytes that were functional immediately before reperfusion of the ischemic area. The injury that results is multifaceted involving many pathways. Therefore by pro-actively protecting these cardiomyocytes to maintain their functionality before reperfusion, it may be possible to prevent the significant exacerbation of IR injury.[41] Strategies that

have had some success include post-conditioning where there was significant reduction in MI size after four sixty second cycle of low-pressure inflation and deflation of angioplasty balloon, in three of six prospective studies of patients undergoing primary PCI.[42-47] Some pharmacological therapies have been investigated which had been promising.[18] For example adenosine, showed promise *in vitro* where the right atrial appendages of patients undergoing cardiac surgery were cultured and showed increased post ischemic contractile force if pre-treated with adenosine.[48] Another study showed that stimulation of the A_3 adenosine receptor (A_3AR) in dogs could reduce the myocardial reperfusion injury through stimulation of immune cells by low doses of an A_3AR agonist.[49] However, in the AMISTAD II trial which administered adenosine post-PCI, there was no significant benefit, unless the patients presented under 4 hours post MI.[50] Another study, the CIRCUS trial investigating the benefits of CsA post MI, aimed to block the opening of the mPTP and potentially act as an immunosuppressive treatment. However, CsA has well known adverse side effects like nephrotoxicity.[18, 51] None of these studies however, have adequately addressed targeting a more proximal signaling step in IR injury. Antioxidant therapy was once thought of as being a useful treatment for IR injury, however, the efficacy of inhibitors and scavengers of free radicals, like edaravone, have been used clinically but have not had the expected results.[52, 53] One theory is that once the free radicals have interacted with other cellular components there is rapid reactions that lead to bioactive compounds. They react with membrane phospholipids and cause structural and chemical changes of the normal phospholipids to generate novel molecules call oxidized phospholipids (OxPL) which may represent a newly identified target to mitigate IR injury.

Oxidized Phospholipids

For many years, phospholipids (PLs) were considered only to be cellular building blocks with very little biological activity. Due to their susceptibility to oxidation, they are modified in the presence of ROS. Apart from impairment of their structural function, oxidized phospholipids (OxPL) acquire novel biological activities that are not characteristic of their unoxidized precursors. In cardiac IR injury, ROS production causes oxidation of cellular macromolecules including phospholipids. This interaction with oxygen-derived free radicals activates phospholipids to become potent signaling molecules within cells.[54, 55] The most prevalent class of OxPL are novel compounds called oxidized phosphatidylcholines (OxPC) and are involved in inflammation and cell signaling pathways.[56] They are produced after oxidation of phosphatidylcholines (PCs) which are the most abundant phospholipid species within cardiomyocyte membranes.[57] Oxidation of the sn-2 fatty acid results in both fragmented and non-fragmented moieties (Figure 1.2).[38] The structural change, causes both chemical, polar, and functional changes that allow OxPC to acquire novel biological activities not seen in native PC molecules.[58-60] The effects of OxPCs described *in vitro* and *in vivo* suggest their potential relevance in different pathologies including atherosclerosis, acute inflammation, lung injury, and diseases where oxidative stress plays significant role in the pathophysiology.[61, 62] The actions of OxPC vary depending upon the specific species of phospholipid being oxidized and the resulting OxPC. For instance, when a peroxidized PC has a polyunsaturated fatty acid (PUFA) chain that is 18 carbons with 3 unsaturated (double) bonds at carbon 6, 9 and 12 (abbreviated as 18:3) at the sn-2 position undergoes a cleavage, it can produce 1-palmitoyl-2-(5'-oxo-valeroyl)-sn-glycero-3-phosphocholine (POVPC) but a similar molecule that has an 18:2

molecule with the double bonds at C9 and C12 upon cleavage would produce 1-palmitoyl-2-(9'-oxo-nonanoyl)-sn-glycero-3-phosphocholine (PONPC) (Figure 1.2 Green Aldehyde box). However, from this same cleavage, the product could have a carboxylate moiety on the end (Figure 1.2 Green Carboxylic acid box).

Recently, OxPC have been shown to exert their biological activity through multiple pathways. They have been shown to be potent stimulators of platelet activating factor (PAF) receptor, prostaglandin receptors and PPARγ receptors resulting in platelet aggregation, induction of the coagulation cascade and apoptosis and cell death.[63, 64] Moreover, the structural change to OxPC causes increased permeability of membranes and they become immunogenic. Several antibodies have been found that have specific reactivity towards them.[65, 66] The class of antibodies are called EO^- autoantibodies, derived from mice lacking apolipoprotein E. They are monoclonal IgM antibodies capable of binding to oxidized low-density lipoprotein (OxLDL), however with different affinities and cross-reactivity with native phospholipids.[67] OxPCs have been shown to be toxic to cultured macrophages and, in human aortic endothelial cells, oxidized lipids cause a dramatic increase in the gene transcription of over a thousand genes affecting many cell functions.[68, 69] In addition, POVPC and 1-palmitoyl-2-glutaryl-sn-glycero-3-phosphocholine (PGPC) treatment of smooth muscle cells resulted in increased apoptotic signaling.[70] Bioactive OxPC molecules have also been shown to be present in porcine heart tissue after significant oxidative stress in an analysis of *ex vivo* perfusate and heart transplant correlating as a marker of increased damage.[1, 71, 72]

The nascent field of lipidomics has allowed for the detection of these molecules and has allowed for an improved understanding of the conditions that produce these

molecules. The generation of the OxPC in pathology of disease and how they are produced within tissues and cells has become essential to understanding their biological functions.

Phospholipids represent the major component of lipid bilayers due to their amphipathic structure. Polar head groups are able to interact with the aqueous environment and cytoplasm, and the fatty acid chains sequester to form the lipid core of the membrane acting as a semi-permeable barrier. During inflammatory, apoptotic, and necrotic responses of the cell to tissue injury or aberrant signaling in diseases like IR injury, atherosclerosis, and diabetes, the structural integrity of the phospholipid bilayer becomes compromised and also chemical modification of the phospholipids both through enzymatic and non-enzymatic pathways alters their function. As described above, inflammation is a common mechanism to these pathological processes and is characterized by a major feature with respect to oxidative stress which is the increased production of ROS.[20, 22] Significantly, the superoxide radicals quickly interact with targets that are readily oxidized and have the ability to stabilize a free electron. Membranes close to superoxide radical production, like the mitochondrial membrane are likely to have proteins and lipids that could become oxidized resulting in generation of molecules that have potent biological activity and could induce apoptotic or necrotic cell death. [26, 27]

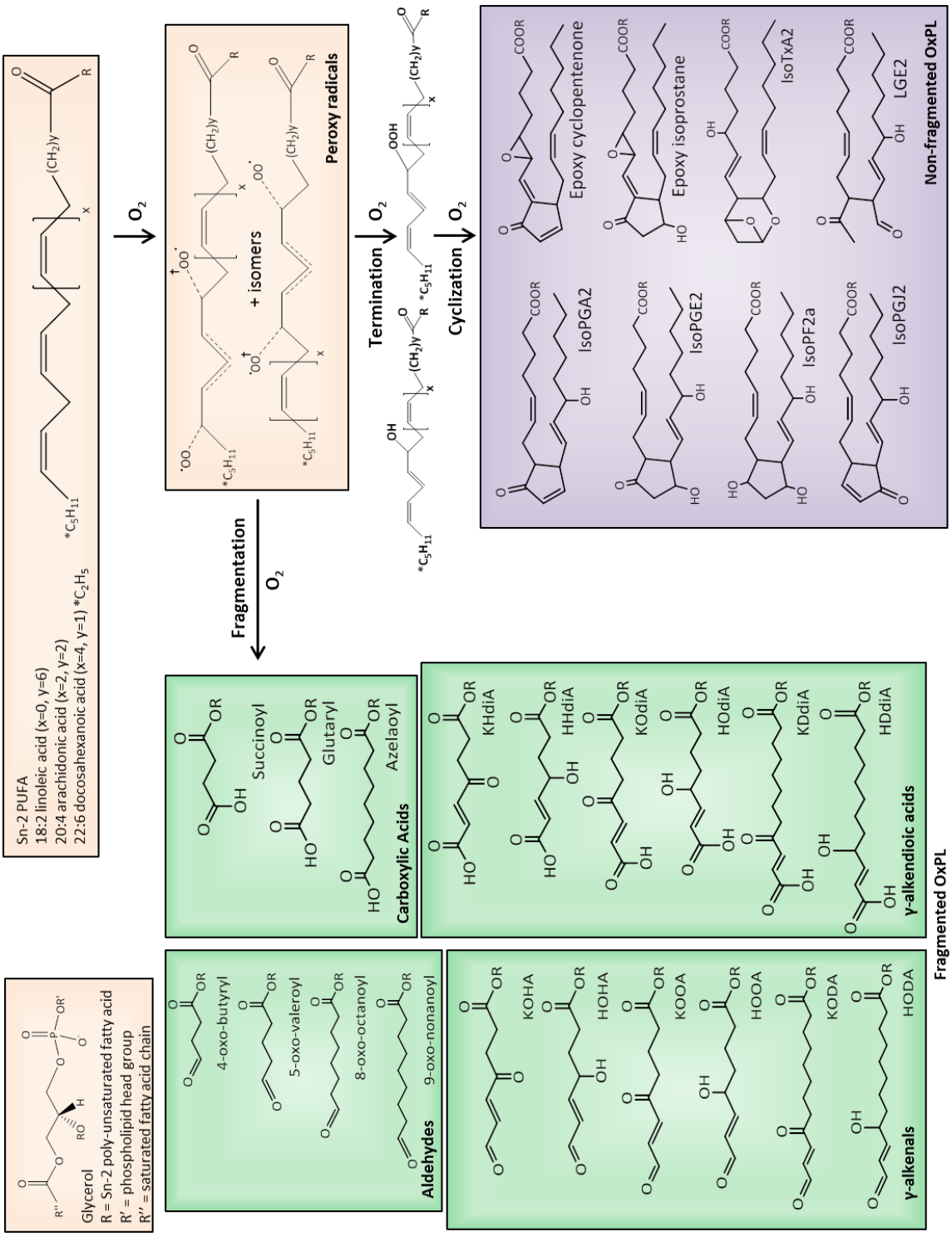

Figure 1.2: Oxidized fatty acyl moieties that could be produced after oxidation of the *sn*-2 PUFA.

Sources of ROS include the mitochondria of apoptotic cells, which produce a significant amount of ROS, and neutrophils during inflammation.[73] Neutrophils have two enzyme systems, the NADPH oxidase and myeloperoxidase systems, which produce superoxide anions and hypochlorous acid that are then released to induce cell death of target cells.[74-76] During reperfusion, this means that apoptotic cells produce ROS, and recruit neutrophils that also release ROS capable of producing bioactive OxPC from PUFA containing PC that are susceptible to oxidative modification. However, this process and the products currently have never been described in cardiomyocytes. The location and number of double bonds in addition to the formation of stabilized intermediates by hydrogen transfer to neighboring carbon molecules will determine the final structure. The initial oxidation of a conjugated diene allows for cleavage of carbon-carbon bonds after hydrogen removal that produces shorter chain, lower mass, fragmented species.[77] If the conjugated diene becomes stabilized and remains intact, further oxidation yields longer chain, higher mass, non-fragmented oxidized species. The ROS-based oxidation of PL forms a heterogeneous pool of OxPL in which the oxidized fatty acid remains esterified to the glycerol backbone.[78] The OxPLs can be broadly categorized into two groups: the fragmented OxPLs and the non-fragmented OxPLs. Fragmented OxPLs generally comprise of terminal aldehyde or carboxylic acid species. Non-fragmented species have hydroxide and/or peroxide additions and rearrangement by cyclization may generate other end-products like the eicosanoids.

OxPLs represent a heterogeneous group of oxidized lipids with multiple functional groups present at the *sn-2* position. The generation of specific OxPL's and their physiological effects are tissue specific. For instance, in the setting of rat lung oxidative

injury, the most abundant OxPC is an isoprostane containing PC whereas in human atherosclerotic tissue, the fragmented OxPC molecule, POVPC is the most abundant.[79, 80] Not only is the structure of OxPC tissue specific but their biological roles are also cell and tissue specific. For example, POVPC acts as an anti-inflammatory molecule by inhibiting LPS-induced intracellular signaling and the expression of adhesion molecules in human umbilical vein endothelial cells (HUVECs), while in mouse lung macrophages POVPC produce IL-6 production resulting in a pro-inflammatory effect.[81, 82] OxPL have been shown to play a role in multiple disease processes where oxidative stress and inflammation are known mechanisms. These include atherosclerosis,[78] diabetes,[83] malignancy,[84] chronic heart failure,[59] cystic fibrosis,[85] and neurodegenerative diseases,[86] such as Parkinson's disease.

Detection of Oxidized Phospholipids

Over the last 20 years, there has been a revolution in the understanding of lipids and their biological activity.[87] This has been driven by the advent of new mass spectrometric tools that allow us to identify and quantitate complex lipid mixtures.[88] With the softer methods of ionization, we can identify single phospholipid molecules as a whole structures and this allows us to follow their chemical modifications through pathological processes.

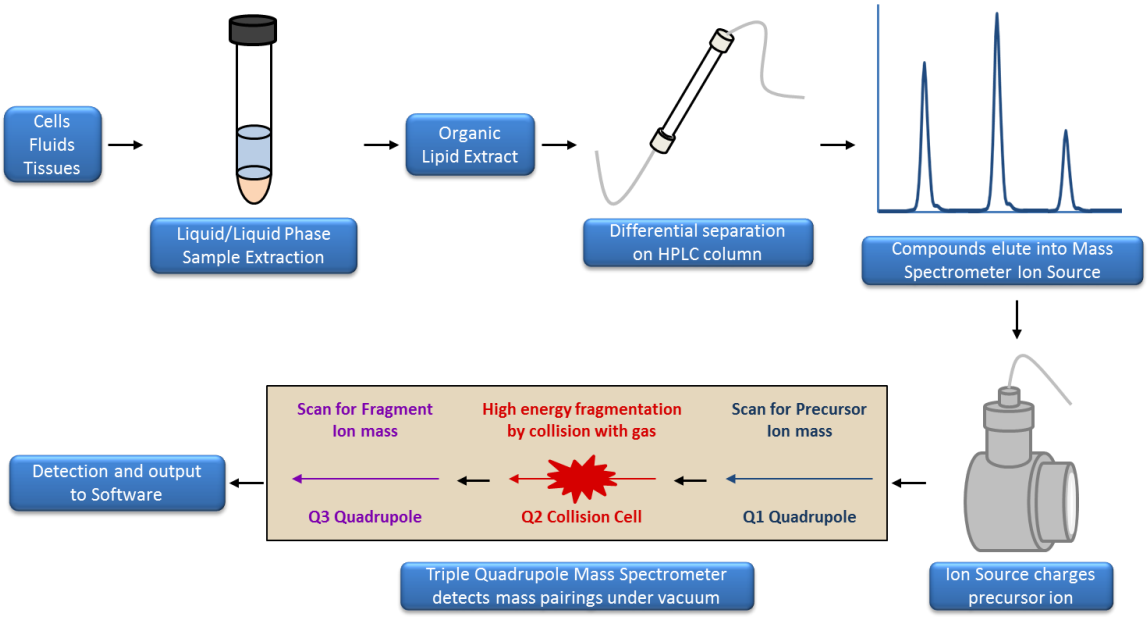

Figure 1.3: Phospholipid extraction workflow. Procedure from sample to data output established for phospholipid extraction, separation, and detection with a HPLC column linked to an electrospray ionization triple quadrupole tandem mass spectrometer. HPLC (high performance liquid chromatography)

Electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) mass spectrometry allows for ionization of PL molecules without causing fragmentation permitting for identification of whole molecules within heterogeneous samples.[87] Mass spectrometry is being used to determine comprehensive lipid profiles in cells, tissues, and pathological samples. These lipidomic analyses usually follow the same work flow and employ extraction, separation, and detection methodology to establish the lipid profile (Figure 2).[89] There has been great progress in applying this methodology to understand the oxidative changes that occur within the phospholipidome;[90, 91] not only of OxPC which are the most abundant but also other OxPL species generated from phosphatidylserine (PS), phosphatidylethanolamine (PE), cardiolipin (CL), and phosphatidylinositol (PI). With these novel techniques, both with a targeted approach and

a wide spectrum approach, such as a shotgun lipidomics analysis, we can follow the changes that occur within a specific phospholipid class during disease processes.[87] Given that PC represents the largest phospholipid group in mammalian cells, the majority of our understanding of oxidative modification comes from studies on OxPC molecules.

The understanding of the normal physiology relating to PCs and PL in general, and the types of lipids circulating in the body is being studied in different ways. A joint research study conducted by National Institute of Diabetes and Digestive and Kidney Diseases, the National Institute of Standards, and the LIPID MAPS Consortium, generated a comprehensive profile of the human plasma lipidome encompassing all of the major lipid classes.[92] The study was able to identify over 500 individual lipid species from a pooled reference plasma sample. Recent follow up studies correlated sex, smoking status, body mass index (BMI) and age with changes in the lipid classes in plasma, with BMI and age showing significant changes in PL amounts.[93] Even though phospholipid represented 43% of the plasma lipidome by mass, the report did not address the identity of OxPL within plasma. Lipidomics has also made strides in identifying phospholipids from cell specific samples such as macrophages, which have been shown to play a major role in the inflammatory cascade. Macrophage activation by TLR-4 agonists led to changes within the lipid profiles identified by mass spectrometry at the cellular and subcellular levels.[94, 95] Therefore, PLs are important for a rapid inflammatory response before and after activation of macrophages.

There are fewer studies that have looked at the OxPL profile within tissues since they represent only 1% of the total phospholipid pool. The majority of the studies investigating the role of OxPL have been related to vascular pathology and atherosclerosis

in particular since there is a larger body of research correlating OxLDL with initiation and progression of atherosclerotic plaques. Lipidomic profile of atherosclerotic plaques at different stages of development have shown the presence of both fragmented and non-fragmented OxPCs within carotid endarterectomy plaque material.[80] PCs represent the largest class of phospholipids within plaques with PC aldehydes, being the largest OxPC fraction. Both fragmented and non-fragmented OxPC were present through all stages of plaque progression which indicated continual generation and catabolism of these bioactive molecules within atherosclerotic plaques.

Biological Activity of Oxidized Phospholipids

Due to their fatty acid's susceptibility to oxidation, phospholipids can be modified in the presence of ROS. Once PL molecules are oxidized, they generate a multitude of oxidation products that remain esterified to the parent PL molecule (Figure 1.4). OxPLs gain bioactive properties that were not attributed to their precursors as a result of oxidation. OxPL are able to induce cell signaling pathways and cause an active cell response. Studies of human aortic endothelial cells (HAECs) indicate that just a brief exposure to a small number of OxPC's that are generated *in vivo* will affect the transcription of >1,000 genes involved in multiple pathways including inflammation, unfolded protein response, pro-coagulant activity, cell cycle, and angiogenesis.[69] Likewise phenotypic changes of cells are also observed and were demonstrated within macrophage populations within atherosclerotic plaques.[96] One of the first defined OxPCs were the fragmented platelet-activating factor (PAF)-like lipids that through a G protein-coupled receptor (GPCR) mediated pathway resulted in cellular activation.[97] Since the initial discovery of OxPC molecules, there have been other classes of phospholipids that have been shown to undergo

oxidative modification forming homologous products as well as being important in normal cellular function. Examples of OxPLs in normal function include OxPS molecules involved with macrophage recognition of apoptotic cells, as well as integral roles in apoptosis and mitochondrial dysfunction.[98] Oxidized ethanolamine phospholipids were shown to be involved in prostanoid formation and also mediate platelet activation.[99] Unpublished data from our oxolipidomic studies have also demonstrated that OxPI molecules form a significant portion of OxLDL and therefore could play a role in atherosclerosis.

Due to their increased solubility, OxPLs can be sequestered within phospholipid bilayers allowing increased interaction with membrane proteins resulting in binding to a wide variety of inflammatory receptors.[100-102] Signal-transducing receptors can be stimulated by OxPCs through either cell membrane receptors or nuclear receptors. Such receptors are GPCRs,[103] the receptor tyrosine kinases,[104] TLRs,[105, 106] and nuclear-localized transcription factors like PPARs which are ligand-activated.[107] Overexpression of cardiomyocyte specific PPARα in a mouse model of IR injury showed that there was increased injury sustained after 7 days. The amount of damage was increased over normal IR injured hearts and represents an important mediator of IR injury.[108] The specificity of OxPL receptor binding is likely a result of the chemical similarity of the OxPL to the receptor ligand, this was demonstrated in with the PAF-like response from OxLDL.[103] Production of long chain OxPCs which have been cyclized, like those containing the esterified isoprostaglandins like 1-palmitoyl-2-(5,6-epoxyisoprostane E2)-*sn*-glycero-3-phosphocholine (PEIPC), activate receptors recognizing prostaglandins E2 and D2 by GPCRs, EP2 and DP, respectively.[81] The EP2 receptor is expressed in all cell types relevant to atherosclerosis including endothelial cells (ECs), monocytes, macrophages, and

vascular smooth muscle cells (VSMCs).[81] Activation of EP2 receptor on ECs results in activation of β1 integrin and increased binding of monocytes to ECs similar to that induced by OxPC, while EP2 receptor antagonists inhibit action of OxPC.[81]

Innate immune responses to OxPL are mediated by natural antibodies (N-Ab), C-reactive protein (CRP), and CD36 on macrophages.[38] The PAF receptor and TLRs are well studied initiators of OxPL signaling and impact cascades like PI3K, Akt, JAK, ERK1/2 and MAPK signaling.[38, 60] Multiple other receptors exist to mediate cellular activity of OxPL including EP2, VEGFR2 and SR-B1.[105, 109, 110] The N-Ab against OxPL are encoded in germ-line tissue and are produced by B-cells as IgM immunoglobulins.[111, 112] They are able to bind antigens that represent pathogens and stress-induced self-antigens as part of the humoral arc of innate immunity.[113, 114] N-Ab have showed affinity for OxPL in studies that used T15/EO6 N-Ab to block the effects of OxPL on macrophage uptake of OxLDL.[67, 115] Complement response to OxPL is mediated by interaction with the defense molecule CRP. High levels of CRP are used to identify an active inflammatory response.[60] CRP has been shown to bind specifically OxPL within OxLDL.[116] The complex of CRP bound to OxLDL, by the cleaved product of OxPC, lysoPC, was shown to mediate suppression of inflammation in macrophages via reduced activation of the inflammatory transcription factor NF-κB.[117] Macrophage activation is central to inflammation. OxPL bind the macrophage by scavenger receptors specifically by CD36 which is the primary scavenger receptor capable of binding OxLDL and has been shown to bind OxPL.[118] CD36 binding OxLDL is integral to the development of 'foam cells' which are macrophages with large depositions of OxLDL including a lipid rich core. These foam cells are believed to be the initial step in generation of fatty streak resulting in atherosclerotic plaque formation.[119]

OxPLs have also been shown to play a role in the thrombosis and the clotting cascade through two particular receptors tissue factor pathway inhibitor (TFPI) and PAF receptor. The accumulation of the PAF-like (alkyl-acyl) OxPLs and lysophospholipids (alkyl-hydroxyl) in plaques, leads to platelet aggregation.[64, 120] OxPL induce increased expression of P-selectin causing a change in platelet shape which favors aggregation of platelets. In concert with ADP and other agonists of platelet accumulation, the diacyl-OxPLs appear to be active in inducing significant platelet aggregation, but by themselves are only weak inducers of clotting factors.[121] Other OxPLs are able to increase transcription of tissue factor protein which is able to cause clotting in response to injury, and also binds its inhibitor TFPI, causing clotting signaling to be activated.[38] Importantly this activity causes increase in the thrombogenic environment after plaque rupture, so in the setting of IR injury, there may be thrombi that are released after reperfusion and can cause stroke or deep vein thrombosis.

Transmission of signaling cascades initiated by OxPL have widespread effects. Inflammation, cell cycle and cell death pathways can be up-regulated or down-regulated when OxPL bind to the cell.[122] There are multiple secondary messengers, like cAMP and Ca^{2+}, increased by OxPL. Transcription factors, like NF-κB and STAT3, and modifying enzymes, like kinases and phosphatases, are also activated by OxPL. Together these influence diverse tissue and cell specific responses.[38] OxPL have been shown to influence PI3K/Akt signaling, mediating inflammation by nitric oxide production by NADPH oxidases and endothelial nitric oxide synthase.[123] This study also demonstrated up-regulation of IL-8, a pro-inflammatory cytokine, was generated in endothelial cells by this process. The *Jun* N-terminal kinase (JNK) pathway can be up-regulated by OxPC while

there is simultaneous down-regulation of phosphorylated-Akt signaling during oxidative stress within rat oligodendrocytes.[124] These pathways are influenced specifically by POVPC inducing neutral sphingomyelinases. The downstream apoptotic signaling up-regulates caspase-8 and caspase-3 which are important for completion of apoptosis, and is discussed below. Inflammatory genes and the unfolded protein response are pathways in which transcriptional activation occurs in response to OxPL. Activating transcription factor-6 (ATF-6) and X-box binding protein-1 (XBP-1) are transcription factors activated by OxPL that target inflammation genes. ATF-6 induces XBP-1 mRNA and splicing is mediated by the ER membrane protein Inositol requiring 1 (IRE1) allowing modulation in the nucleus.[125] Another mechanism described is the phosphorylation of eIF2α catalyzed by double-stranded RNA-dependent protein kinase (PKR)-like ER kinase (PERK) leading to presence of ATF-4 acting as a transcription factor.[69] XBP-1 and ATF-4 bind to promoter regions upstream of the target IL-6 and IL-8 inflammatory signals causing them to be up-regulated. TLR signaling modulates the inflammatory pathways relating to the innate immune system. OxPL were able to initiate TLR-4 signaling through MAPK cascade to NF-κB and influence lipid metabolism and inflammation.[126] When TLR-4 is activated Bcl-2 family proteins in the mitochondria, Bid, Bad, Bax and the nuclear transcription factor NF-κB shut down oxidative phosphorylation within the mitochondria and act together to increase expression of pro-inflammatory cytokines.[127, 128] This process induces the pathways of inflammation through a caspase-1-mediated mechanism to increase active IL-1β and IL-18 in the extracellular spaces.[127] This pro-inflammatory and pro-apoptotic environment catalyzed by OxPL catapults the cells into cell stress culminating in inflammation or apoptosis if not reversed. Cells exposed to modified and OxLDL

demonstrate up-regulation of two adhesion molecules, β1-integrin[129] and P-selectin,[130] that specifically promote monocyte adhesion. Infiltration of macrophages past adjacent endothelial cells is also promoted during lung injury by disruption of adherens junctions. A short chain fragmented PC produced during oxidative stress, PGPC was demonstrated to modulate the phosphorylation of VE-cadherin via activation of Src kinase that phosphorylated tyrosine residues important for adherens junctions stability.[131]

Chemokines are important modulators of inflammatory response and OxPL are able to target several chemokines that modulate the immune system. The chemokines MCP-1, MCP-3, MCP-5, MIP-1α, MIP-1β, MIP-2β, IL-6, IL-8, and GROα are influenced upon

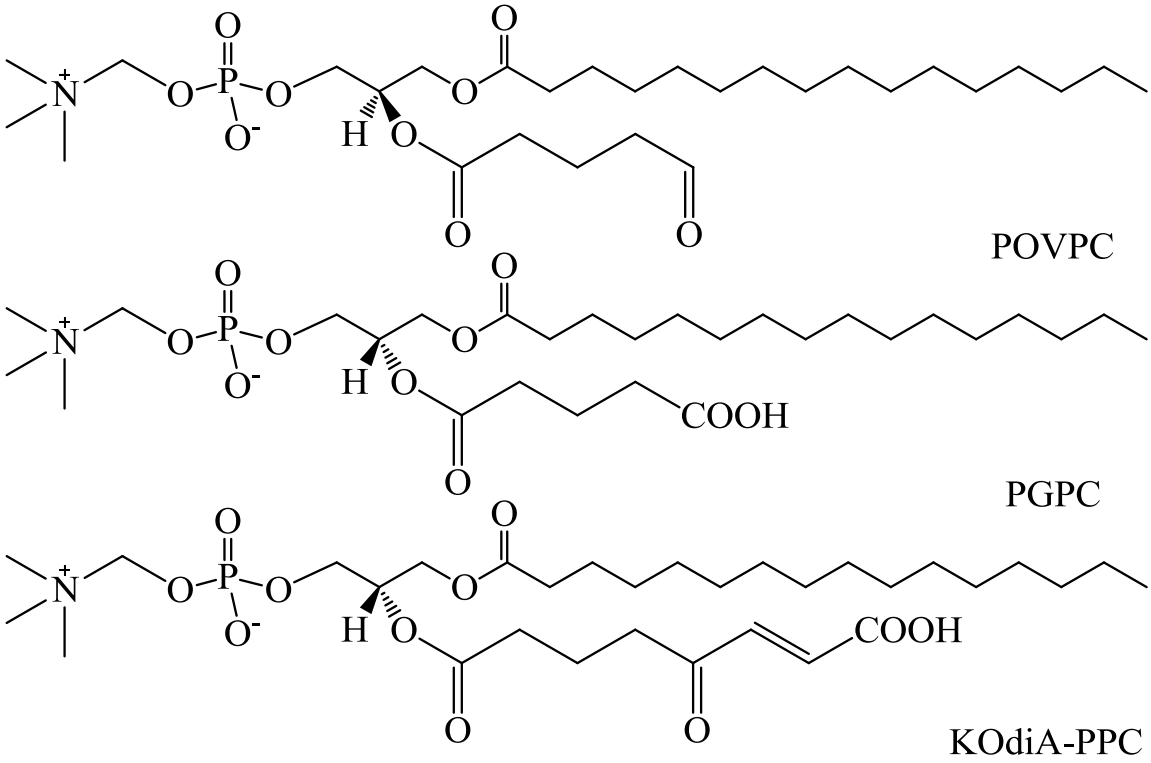

Figure 1.4: Oxidized phosphatidylcholine standard structures used in the study. POVPC, 1-palmitoyl-2-(5'-oxo-valeroyl)-sn-glycero-3-phosphocholine. PGPC, 1-palmitoyl-2-glutaryl-sn-glycero-3-phosphocholine. KOdiA-PPC, 1-palmitoyl-2-(5-keto-6-octene-dioyl)-sn-glycero-3-phosphocholine.

exposure to OxPL.[38, 69, 132, 133] MCP and MIP proteins are able to attract and activate macrophages causing sustained IL-8 production causing positive feedback to the inflammatory response induced by OxPL. IL-6 is particularly important in the acute phase inflammation as *Il-6*$^{-/-}$ knock-out mice demonstrate impaired immune response.[134] These pro-inflammatory signals cooperate to modulate other cell types in response to these stresses.

Apoptosis

Apoptotic cell death is defined as a programmed sequence of cellular events that result in controlled cellular death.[17] There are multiple triggers for apoptosis induction which include receptor-dependent or –independent, which lead to extrinsic or intrinsic apoptotic activation respectively.[135]

Extrinsic apoptotic activation relies on ligand binding of receptors that have a particular cytoplasmic death domain (DD), that trimerize and recruit cytosolic adaptor proteins forming the death-inducing signaling complex (DISC) that activates the initiator caspases of apoptosis which include caspase-8 or caspase-10 (Figure 1.5).[136] The receptors include the TNF receptors (TNFR) 1 and 2, the Fas cell surface death receptor, and TNF-related apoptosis-inducing ligand (TRAIL) receptors 1 and 2 which are all part of the TNF receptor superfamily of proteins. The recruited adaptor proteins TNFR-associated DD (TRADD), Fas-associated DD (FADD), and receptor-interacting protein 1 (RIP1) activate caspase-8 directly that initiates caspase-dependent apoptosis. This type of apoptosis hinges on cell signaling resulting in effector caspase activation.[137] One of these caspases is caspase-3, which is an effector caspase that cleaves proteins leading to cell death. Both the

extrinsic and intrinsic pathways converge at the effector caspases and caspase-3 itself is considered to be a central player in apoptosis.[137-139] In a murine model of IR injury, cardiac-specific caspase-3 overexpression exacerbated IR injury including increased infarct size and decreased left ventricular function.[140] Caspase-3 cleaves a variety of proteins ranging from structural proteins, to DNA repair proteins, to transcription factors. An *in vitro* study of adult rat cardiomyocytes investigating the effect of hypoxia on caspase activation demonstrated that caspase-3 was a downstream signal of cytochrome C release, but an upstream signal to DNA fragmentation as inhibition of caspase-3 cleavage by N-acetyl-Asp-Glu-Val-Asp-acid aldehyde (Ac-DEVD-CHO) reduced cleavage of the DNA repair protein poly-ADP-ribose-polymerase (PARP), but did not reduce cytochrome C release from the mitochondria.[141] The researchers concluded that since there are multiple caspases activated through hypoxia, however caspases upstream are activated sequentially and in a hierarchical order since caspase-1 inhibition, prevented cytochrome C release in addition to PARP cleavage, but caspase-3 inhibition only prevented PARP cleavage while cytochrome C release still occurred.[141]

The second pathway that activates apoptosis is the intrinsic pathway (Figure 1.5). This pathway becomes activated under cell stress conditions like hypoxia, exposure to UV light, chemotherapy, or mitochondrial dysfunction.[142] The focal point of intrinsic activation are the mitochondria. Increased intracellular calcium, activation of JNK pathway as noted above, or cross-activation by the extrinsic system influence the mitochondria during apoptosis.[143]

Apoptotic mitochondria release multiple proteins including, cytochrome C, apoptosis-inducing factor (AIF), second mitochondria-derived activator of caspases/direct

inhibitor of apoptosis (IAP)-binding protein with low pI (SMAC/DIABLO), and endonuclease G. SMAC/DIABLO prevents the IAP proteins from blocking caspase activation thereby increasing the likelihood of apoptotic induction. More importantly, the protein cytochrome C leads to direct activation and accumulation of apoptotic peptidase-activating factor-1 (Apaf-1) leading to formation of the apoptosome.[144, 145] This is important for the activation of the initiator caspase-9 which then follows the conserved pathway to activate the effector caspases seen in extrinsic activation.[146]

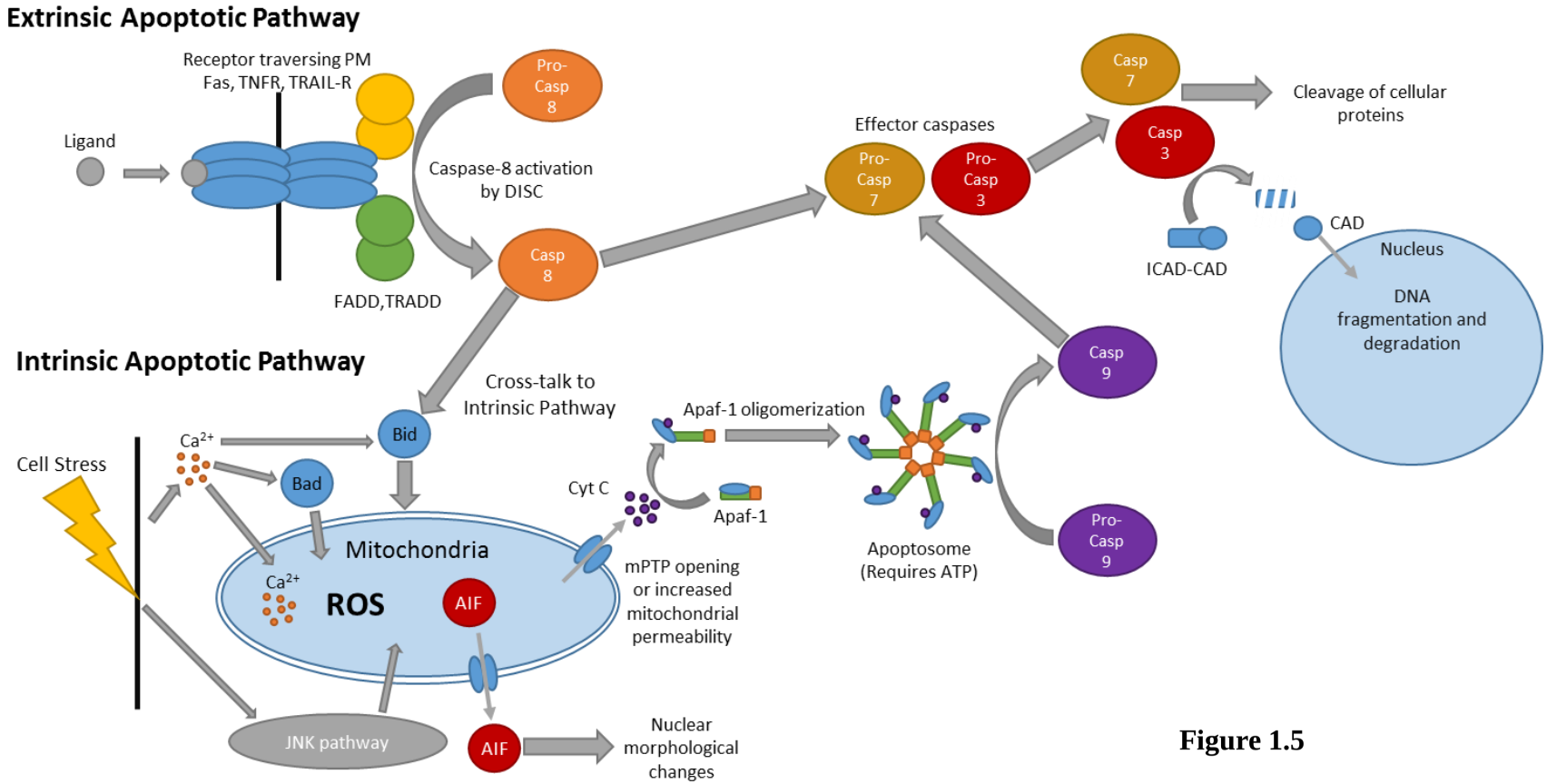
Extrinsic Apoptotic Pathway
Receptor traversing PM
Fas, TNFR, TRAIL-R
Ligand
Pro-
Casp
8
Caspase-8 activation
by DISC
Casp
8
FADD,TRADD
Effector caspases
Pro-
Casp
7
Pro-
Casp
3
Casp
7
Casp
3
Cleavage of cellular
proteins
ICAD-CAD
CAD
Nucleus
DNA
fragmentation and
degradation
Intrinsic Apoptotic Pathway
Cross-talk to
Intrinsic Pathway
Bid
Ca2+
Cell Stress
Bad
Mitochondria
Ca2+
ROS
AIF
Cyt C
Apaf-1 oligomerization
Apaf-1
Apoptosome
(Requires ATP)
Casp
9
Pro-
Casp
9
mPTP opening
or increased
mitochondrial
permeability
JNK pathway
AIF
Nuclear
morphological
changes

Figure 1.5

Figure 1.5: Apoptotic pathways within cardiomyocytes. Extrinsic activation of cell death receptors recruits cytoplasmic death domain (DD) proteins (FADD, TRADD) forming the DISC complex that cleaves pro-caspase-8 to activated caspase-8. Caspase-8 then activates the effector caspases, caspase-3 and caspase-7 which cleave a variety of death substrates, and can activate the intrinsic pathway via cross-talk through a Bcl-2 protein family protein, BH-3 interacting-domain death agonist (Bid). Inhibitor of caspase-activated deoxyribonuclease (ICAD) is cleaved by casp-3 activating CAD, which translocates to the nucleus and fragments the DNA. Poly-ADP-ribose polymerase (PARP), used in DNA repair, is cleaved and inactivated (not shown). Intrinsic activation of apoptosis by cell stress causes calcium signaling or *Jun* N-terminal caspase sequestration in the mitochondria and ROS production. This along with other cell stresses can induce the mitochondrial permeability transition pore (mPTP) to open releasing cytochrome C and apoptosis-inducing factor (AIF). Cytochrome C binds to apoptotic peptidase-activating factor-1 (Apaf-1) leading to the formation of the apoptosome a hallmark of the intrinsic apoptotic pathway. This also leads to downstream activation of initiator casp-9 which cleaves casp-3 which acts as above. AIF translocates to the nucleus where morphological changes of membrane and DNA fragmentation occurs.

The mPTP is an important concept within IR injury. It has been established as the critical mediator of IR injury within the heart and has been investigated as a target for therapeutic intervention. There have been conflicting reports about the identity of the mPTP, however the major components are considered to be cyclophilin-D interacting with the mitochondrial ATP synthase inner membrane '*c*' subunit which is essential for calcium activated opening of the pore.[147, 148] The mPTP opening causes loss of $\Delta\Psi_m$, which reduces the ability of the inactivated ATP synthase molecules to function. This triggers mitochondrial dysfunction and release of the key components cytochrome C and AIF that are known to lead to apoptosis. It is understood that functional loss of $\Delta\Psi_m$ is most likely mediated by mPTP opening, however the components and mechanism is not adequately understood.

Mitochondrial dysfunction and induction of apoptosis in mitochondria appear to be influenced by OxPC in a few different ways. OxPC have been shown to bind proteins that are important regulators in mitochondria such as the Bcl-2 family proteins. One of these proteins is the Bax protein. Bax is a mitochondrial pro-apoptotic protein, which has been shown to interact with OxPL during oxidative stress activating the intrinsic apoptotic pathway.[149] This was confirmed as the application of hexadecyl azeloyl glycerophosphocoline led to mitochondrial depolarization and dysfunction of mitochondria isolated from rat liver homogenates as well as in HL-60 cells analyzed by flow cytometry.[40, 128] Another truncated OxPC, 1-palmitoyl-2-azelaoyl-*sn*-glycero-3-phosphocholine (PAzPC) induced apoptotic changes in cell morphology including phosphatidylserine flipping release of mitochondrial cytochrome C leading to apoptosis-inducing factor release culminating in the activation of caspase-3.[40] These pathways

together show that OxPCs interacting with Bcl-2 family proteins cause induction of cellular apoptosis in multiple cell types.[149] Macrophages, VMSCs, and dendritic cells demonstrate increased apoptotic signaling in the presence of OxPL.[69, 124, 150, 151]

Currently, the OxPC have not been investigated within cardiomyocytes. It is believed that the apoptotic pathway contributes to the majority of cardiomyocyte cell death induced within IR injury and therefore OxPC that cause cell death in other cells may have similar effects.[152] In a mouse model of IR injury, the extent of dysfunctional cardiac muscle post-MI was correlated to the amount of cardiomyocyte apoptosis.[153] Additionally, permeability changes to the outer and inner mitochondrial membrane result in mPTP opening and cell death resulting from the intrinsic death pathway.[154-156] These results are consistent with OxPC effects in other cell types.[70, 151]

Preliminary results of this study, qualitatively demonstrated that exogenously added OxPCs induced a marked increase in cell death of cardiomyocytes *in vitro*. There is good evidence to support the concept that OxPC could activate the intrinsic apoptotic death pathway via mitochondrial permeability within cardiomyocytes however this has never been shown. If these compounds induce cardiomyocyte apoptosis, pharmacologic intervention remains integral to preventing cell death during IR injury.

Anti-Oxidized Phosphatidylcholine Antibodies

Oxidation of phospholipids cause changes to the polarity of the molecule and as a result new biological activity is imparted to phospholipids. The oxidation of the PUFA moiety changes the structure and function of phospholipids increases their bioactivity, to become immunogenic and increasingly reactive in cell signaling.[109, 133, 157] Due to the

structural change that occurs after oxidation, there has been evidence to suggest that the *sn*-2 moiety swings out of the plasma membrane, and is exposed on the surface of the cell.[158] This change in structure renders OxPC immunogenic by presenting to the innate immune system cells as a DAMP which cross-bridge cell receptors on monocytes inducing potent upregulation of inflammatory cytokines.[159]

The antibodies that are produced against OxPC are reported to be N-Abs that are germ-line encoded recognizing DAMPS that need to be cleared by the immune system; DAMPS themselves have been seen to mediate the immune system from within cardiomyocytes.[160, 161] These antibodies were first described to bind OxLDL and have been actively investigated within the context of atherosclerosis.[67, 162, 163] The description of these antibodies interacting with OxLDL in humans was a seminal event for the understanding that linked oxidation-specific epitopes to heart disease which continues to be investigated.[164-167] The group of antibodies as they related to atherosclerosis were identified from hybridomas of apolipoprotein E-deficient mice B lymphocytes fused to a myeloma cell line.[67, 162] The apoE-deficient mouse strain that were used to clone the antibodies were shown to have an increased risk of atherosclerosis when fed a high cholesterol diet.[162] The group isolated the spleens of these mice and extracted the IgM-secreting cells to be immortalized and cloned to identify the particular antibodies that could bind OxLDL in human plasma.[162] Another group showed that it was B-cells from the spleen of these hypercholestrolemic mice that were protective to splenectomised apoE knockout mice and reduced atherosclerosis by 75% as compared to controls.[168] To determine which antibodies had reactivity against OxLDL, the researchers screened the supernatant of the apoE-deficient hybridomas for antibodies that bound to OxLDL and called the antibodies the EO

antibodies.[162] EO antibodies have since been shown to be IgM N-Abs as they are produced in germ-free mice and in human umbilical cord blood.[169] This means that IgM secreting B-cells in the spleen were responsible for producing N-Abs that were protective against oxidation-specific epitopes contained in OxLDL that led to formation of 'foam' cells and atherosclerotic plaque. It then became important to categorize the different epitopes and the antibodies themselves, as the initial research showed that there were up to 17 of these antibodies.[162]

After discovering the 17 different EO antibodies, the researchers investigated the reactivity to self-antigens and other oxidized components. They found that the EO6 antibody was least reactive to native phospholipids and LDL, but highly sensitive to OxLDL, OxPAPC, and POVPC as compared to other EO antibodies.[67] The EO6 antibody was also shown to recognize OxPC-protein adducts which could be part of the reactivity of OxPC.[67, 170, 171] Shortly afterward, the EO6 antibody was shown to block OxPC activity in apoE-null mice.[172] More rigorous investigations showed that EO6 had no reactivity to native PCs, could only recognize OxPCs after oxidation and was also important in implicating the OxPC in the pathogenesis of atherosclerosis; it was used to demonstrate OxPC aggregation in human atherosclerotic plaque development.[80, 113] As previously mentioned, the EO6 antibody is capable of blocking OxPC activity towards macrophages.[115, 173] This could have significant benefits in preventing the development of the 'foam' cell phenotype leading to plaque progression.[96] The EO6 antibody represents the best EO antibody to block the adverse effects of OxPC-induced inflammation particularly in atherosclerosis. As the least cross-reactive EO antibody with native phospholipids it will most likely have the highest tolerance, yet still have significant

recognition and inhibition of bioactive OxPC lipids. However, as the OxPC are only beginning to be described in cardiac tissue, there have been no investigations into the application of the EO6 antibody with regards to myocardial IR injury.

Summary

There is a growing body of evidence supporting the role of OxPC in inflammation and apoptosis. Both of these processes play an important role in cardiac tissue post-MI, and reduce the ability of the cardiac tissue to maintain cardiac output at a level that sustains blood flow to the coronary vasculature and the other organs of the body. This resulting morbidity and mortality causes a great burden on the healthcare system financially but also causes undue suffering for both patients and their families. In order to establish novel therapies that allow for significant recovery and more lives saved, there needs to be development to address the currently untreated IR injury after an MI.

Our lab has previously shown that the OxPCs are an uninvestigated target for myocardial IR injury by using an oxolipidomics analysis to detect OxPCs from porcine transplant myocardial tissue.[1] Analysis demonstrated increased levels of OxPC species within cardiac transplanted tissue, and that under *ex vivo* perfusion, when levels of OxPC are reduced in both plasma and muscle, the heart has better function.[1, 72] It is also apparent that OxPLs are not bystanders, but are biologically active molecules able to influence a host of diverse signaling pathways, and mediate the inflammatory process and apoptosis. Only recently with the advent of improved experimental techniques such as ESI and MALDI ionization technologies, have we gained a better understanding of the individual characteristics and roles of these molecules. Being able to identify these compounds has

led to the discovery that OxPLs are produced under cell stress conditions found in the pathophysiology behind inflammation and cell death in atherosclerosis, neurodegenerative, and inflammatory diseases and similar patterns of induction, signaling and death are found in IR injury.[174] The OxPCs appear to respond to the physical chemical changes that lead to biological signaling. The previous research has allowed for the understanding that the OxPCs are bioactive and immunogenic, properties that are recognized by the immune system and as a result there are molecules and pathways that have been conserved to mitigate their negative effects. The presence of the EO6 antibody in human umbilical cord blood demonstrates that the human body needs to recognize these molecules as DAMPs that can ramp up the immune response; a response that is beneficial when responding to pathogens or injury, however, harmful for the patient undergoing primary PCI leading to loss of functional cardiac tissue.

As the OxPCs have been coming to the forefront of research in other areas of pathophysiology, understanding the detrimental effects OxPCs have within the cardiomyocyte is integral for developing a more robust understanding of myocardial IR injury and will provide a clear benefit for patients and clinicians in the field of cardiology.

Chapter II: Study Rationale and Methods

Statement of Problem

It is well established that OxPCs are bioactive molecules in vascular and immune cells; however there has not been an investigation into the effect of OxPC on cardiomyocytes. In addition, it is not known if cardiomyocytes produce OxPC during pathological stress, like IR injury. There is currently no therapy for IR injury that sufficiently reduces morbidity and mortality post-reperfusion.

Aims and Rationale

Aim 1: Investigate if cardiomyocytes produce OxPCs under simulated IR injury.

Aim 2: Determine if short-chain OxPC induce cardiomyocyte cell death.

Aim 3: Characterize mitochondrial permeability changes induced by short-chain OxPC molecules.

Aim 4: Determine if the EO6 antibody will block OxPC-mediated cell death.

The determination of OxPC production during a simulated IR injury *in vitro* model using post-natal rat cardiomyocytes will demonstrate if a change in the OxPC lipid profile occurs within these cells and will establish that these molecules are part of the toxic environment within cardiomyocytes. Furthermore, investigating the action of particular fragmented OxPC on cardiomyocytes with respect to cell viability and apoptotic signaling, providing a mechanism by which the compounds exert their bioactive properties on these cells. As these are immunogenic compounds, the EO6 antibody, against oxidized moieties, could bind these molecules effectively preventing the bioactivity of these molecules in pathological ischemic stress.

Hypotheses

We hypothesize that;

(1) there is significant production of fragmented OxPC in simulated IR injury of cardiomyocytes,

(2) when cardiomyocytes are incubated with fragmented OxPC the bioactive compounds will induce decreased cardiac cell viability and increased mitochondrial permeability verifying the plausibility of an intrinsic cell death mechanism;

(3) EO6 antibody co-treatment with OxPC will prevent OxPC apoptotic action by blocking the cell death induction as well as blocking OxPC-induced mitochondrial permeability providing a plausible treatment option.

Methods

Chemicals

Chemicals used for HPLC were HPLC-grade and were purchased through Millipore and included chloroform, methanol, water, acetonitrile, isopropyl alcohol and hexane. Oxidized phospholipid standards were purchased through Avanti Polar Lipids and Cayman Chemicals and are indexed in Table 1. Detection of phospholipids in the following lipidomics platform needed calibration and was achieved using the DNPC standard, and analysis of OxPC standards were identified using the HPLC-ESI-MS/MS technology described. EO6 Monoclonal antibody was purchased through Avanti Polar Lipids.

Table 1: Chemical name of lipid standards used in experiments

Chemical Name	Abbr.	Source
1,2-Dinonanoyl-*sn*-glycero-3-phosphocholine	DNPC	Avanti Polar Lipids
1-palmitoyl-2-stearoyl-*sn*-glycero-3-phosphocholine	PSPC	Avanti Polar Lipids
1-palmitoyl-2-(5-oxo-valeroyl)-*sn*-glycero-3-phosphocholine	POVPC	Avanti Polar Lipids
1-palmitoyl-2-(9-oxo-nonanoyl)-*sn*-glycero-3-phosphocholine	PONPC	Avanti Polar Lipids
1-palmitoyl-2-azelaoyl-*sn*-glycero-3-phosphocholine	PAzPC	Avanti Polar Lipids
1-palmitoyl-2-glutaryl-*sn*-glycero-3-phosphocholine	PGPC	Cayman Chemicals, USA
1-palmitoyl-2-(5-keto-6-octene-dioyl)-*sn*-glycero-3-phosphocholine	KOdiA-PPC	Cayman Chemicals, USA
1-palmitoyl-2-(4-keto-dodec-3-enadioyl)-*sn*-glycero-3-phosphocholine	KDdiA-PPC	Cayman Chemicals, USA

Post-natal Cardiomyocyte Cell Culture

Post-natal rat cardiomyocytes (NCMC) were isolated from 1-2 day old Sprague-Dawley rat pups as previously described.[175] Whole hearts were excised from rat pups by midline sternotomy. Hearts were collectively washed and minced to adequately break up macroscopic structures before re-washing with cold filter sterilized PBS containing 10g/L of glucose (PBS^{-1}) to remove RBC and debris. Repeated enzymatic digestion of heart fragments was performed with collagenase (740U), trypsin (370U), and DNase (2880U) (Worthington Biochemical) agitating at 35˚C for three 10-minute and three 7-minute, for six total, digestions. Digested supernatant solutions were centrifuged into a cell pellet and then separation of cell types using a Percoll® (GE Healthcare) gradient of 1.05, 1.06, and 1.082g/mL allowed for a layer enriched with myocytes to be isolated. To remove fibroblasts, the cells were pre-plated on non-coated 150mm culture plates for 45min. Purified NCMC were then transferred to sterile tissue culture plates at the particular cell

density of 1.75 x 10^6/35-mm plate. Collagen-coated glass coverslips in 24-well tissue culture plates were used for microscopy analysis and NCMC were plated at 3.2 x 10^5/well. Cells were incubated overnight in Dulbecco's Modified Eagle Medium/Ham's nutrient mixture F-12 (1:1) containing 2mM glutamine, 3mM $NaHCO_3$, 15mM HEPES, and 50mg/mL gentamycin (DMEM/F12) plus 10% fetal bovine serum (FBS). DMEM/F12 with 10% FBS was changed to serum-free DMEM/F12 (DFSF) the following day.

Adult Cardiomyocyte Cell Culture

Ventricular myocytes were isolated from 12-week old male Sprague-Dawley rats (250-300 g) as described previously.[176] In brief, an intraperitoneal injection of a mixture of ketamine (90 mg/kg) and xylazine (10 mg/kg) was used to anesthetize the animal. Hearts were excised and transferred to a Langendorff perfusion apparatus and perfused with calcium (Ca^{2+}) free buffer containing 90 mM NaCl, 10 mM KCl, 1.2 mM KH_2PO_4, 5 mM $MgSO_4.7H_2O$, 15 mM $NaHCO_3$, 30 mM taurine and 20 mM glucose for 10 min. The perfusion medium was then switched to Ca^{2+} free buffer containing collagenase (0.05%) and bovine serum albumin (BSA) (0.2%). After 30 minutes, ventricles were minced into pieces, incubated in a 37°C water bath and separated into individual cardiomyocytes by gravitational centrifugation. Cardiomyocytes were then suspended in buffer containing Ca^{2+}. The supernatant was then replaced with Ca^{2+} buffers containing a higher concentration of calcium (150 µM). This step was repeated twice to increase the extracellular Ca^{2+} concentration to 500 µM and then to 1.2 mM. Cells were finally re-suspended in M199 and transferred to laminin coated culture dishes.

Simulated Ischemia Reperfusion Treatments

Cardiomyocytes were cultured in 6-well plates and exposed to simulated ischemia reperfusion. Control solution consisted of 140mM NaCl, 6mM KCl, 1.25mM $CaCl_2$, 6mM HEPES, and 10mM D-glucose buffered to pH 7.4, ischemic solution was buffered to pH 6.0, and consisted of the same components, except 8mM KCl was added and no D-glucose was added. The ischemic buffer was purged of oxygen by bubbling 95% N2 and 5% CO_2 gas into the medium for 1 hour before being applied to cells.[177] A hypoxic chamber designed for storage in a 37˚C water jacketed tissue culture incubator was used to maintain an atmosphere of 95% N_2 and 5% CO_2 gas conditions for 18 hours overnight. Reperfusion was achieved by applying control buffer to cells for 4 hours in a normal culture incubator. After treatment cells were scraped off into a small portion of PBS without glucose (PBS^{-2}).

Phospholipid Extraction

OxPC phospholipids were extracted from ischemia reperfusion treated NCMC by modification of a previously described protocol.[91] Cell media was removed, and cells were washed with PBS^{-2}. Each well was scraped into 1mL of methanol/acetic acid (3% v/v) solution containing 10mg/100mL of the antioxidant butylated hydrotoluene (BHT) and transferred to a 10mL glass conical tube and capped under nitrogen gas. A known amount (10ng) of 1, 2-dinonanoyl-sn-glycero-3-phosphocholine (DNPC) was added as internal standard into each sample for quantitation. Two millilitres of hexane containing BHT is added to the tube, capped under nitrogen gas, vortexed for five seconds, and then centrifuged for 5 minutes at 3500 rpm at 4˚C. The upper hexane layer is then siphoned off

using a glass Pasteur pipette and discarded. The hexane/BHT wash is repeated three times, capping under nitrogen gas, vortexing for five seconds, and centrifuging after each wash. After the final hexane/BHT wash, 2mL of chloroform containing BHT and 750µL of PBS^{-2} were added to the tube then vortexed and centrifuged as above. The lower organic layer is removed using a glass Pasteur pipette to a clean 15mL glass conical tube where the solution was aspirated off using a nitrogen evaporator, and then reconstituted into 300µL of chloroform/methanol (2:1 v/v) for storage at -80˚C. Before reverse-phase (RP) analysis, 100µL of extracts were reconstituted into RP solvent consisting of acetonitrile/isopropanol/water (65:30:5 v/v/v).

High Performance Liquid Chromatography for OxPC Lipid Profiling

The HPLC system used for analysis included two Shimadzu (Columbia, MD) LC-20AD high performance pumps interfaced with a Shimadzu SIL-20ACHT Prominence UFLC® Autosampler. The autosampler applied 30µL of extracts per injection to a Supelco Analytical Ascentis® Express C18 (2.7µm, 15cm x 2.1mm) column in duplicate. Detection of oxidized PCs were carried out in reverse-phase (RP) chromatography and the samples were reconstituted in an RP solvent system consisting of Acetonitrile-Isopropanol-Water (65:30:5 vol/vol). Elution was performed by linear gradient of solvent A (Acetonitrile-Water, 60:40 vol/vol) and solvent B (Isopropanol- Acetonitrile, 90:10, vol/vol) both the solvents containing 10 mM Ammonium formate and 0.1% formic acid. The time program used was as follows: initial solvent B at 32% until 4.00 min; switched to 45% B; 5.00 min 52% B; 8.00 min 58% B; 11.00 min 66% B; 14.00 min 70% B; 18.00 min 75% B; 21.00 min 97% B; 25.00 min 97% B; 25.10 min 32% B until the elution was stopped at 30.10

min. A flow rate of 260 µl/min was used for analysis, and the column and sample tray were held at 45C and 4C, respectively.

Mass Spectrometry for OxPC Lipid Profiling

The eluate flowed into an AB Sciex 4000 Q-TRAP® quadrupole linear ion trap hybrid mass spectrometer with MS/MS conditions applied as a positive MRM electrospray ionization (ESI) method. Detection of compounds is based on Q1 masses for various predicted OxPCs and Q3 scanned for a product ion 184 m/z corresponding to the PC-specific head group described in Table 2.[91] Detection software was the Analyst 1.6 Software (AB Sciex) that controlled the autosampler, solvent pumps, and mass spectrometer transitions. Standard OxPC compounds were run to determine expected retention time and DNPC peak data was used for quantitation using MultiQuant 2.1 Software (AB Sciex) for data calculations.

Table 2: Transitions used for mass spectrometry with retention times

Q1 m/z	**Head Group Q3 transition**	**Oxidized *sn-2* Q3 transition**	**Proposed Structure**	**Retention times(min)**
538.6	184.3	157.1	di-9:0-PC (external standard)	3.45
580.6	184.3	101	4-oxo-butyryl-PPC	3.49
594.6	184.3	115	POVPC	3.63
596.6	184.3	117	Succinoyl-PPC	3.35
610.6	184.3	131	PGPC	3.39
622.6	184.3	115	SOVPC	6.02
632.6	184.3	153	Furylbutanoyl-PPC	3.39
634.6	184.3	155	KOHA-PC	3.32
636.6	184.3	157	8-oxo-octanoyl-PPC	3.08
638.6	184.3	131	SGPC	5.6
640.6	184.3	161	Acetal-POVPC	3.96
648.6	184.3	169	KOOA-PPC	3.44
650.6	184.3	171	PONPC	5.22
660.6	184.3	153	Furylbutanoyl-SPC	5.62
664.6	184.3	185	KOdiA-PPC	6.87

666.6	184.3	187	PAzPC	4.35
676.6	184.3	169	KOOA-SPC	5.33
678.6	184.3	171	SONPC	7.38
688.6	184.3	207	Furyloctanoyl-PPC	3.75
692.6	184.3	185	KOdiA-SPC	5.29
694.6	184.3	187	SAzPC	6.68
696.6	184.3	217.1	Acetal-PONPC	5.21
704.6	184.3	225.1	KODA-PPC	4.67
706.6	184.3	227.1	HODA-PPC	3.86
710.6	184.3	209.1	12-oxo-8,10-dodecendienoyl-PPC	3.04
716.6	184.3	207	Furyloctanoyl-SPC	4.4
720.6	184.3	241.1	KDdiA-PPC	3.55
722.6	184.3	243.1	HDdiA-PPC	3.93
724.6	184.3	217.1	Acetal-SONPC	6.43
732.6	184.3	223.1	10-OH-5,8,11-tridecatrienoyl-PPC	3.23
734.6	184.3	227.1	HODA-SPC	6.23
748.6	184.3	241.1	KDdiA-SPC	3.95
750.6	184.3	243.1	HDdiA-SPC	4.8
760.6	184.3	223.1	10-OH-5,8,11-tridecatrienoyl-SPC	5.57
772.6	184.3	294.2	PLPC-keto	9.35
774.6	184.3	296.2	PLPC-OH	9.36
788.6	184.3	310.2	PLPC-epoxy,keto	8.2
790.6	184.3	312.2	PLPC-OOH	9.34
794.6	184.3	317.2	15-deoxy-?12,14-isoPGJ2-PPC	7.88
796.6	184.3	318.2	PAPC-keto	9.55
798.6	184.3	320.2	PAPC-OH	10.65
800.6	184.3	294.2	SLPC-keto	10.88
802.6	184.3	296.2	SLPC-OH	10.13
804.6	184.3	326.2	PLPC-OOH,keto	7.01
806.6	184.3	328.2	PLPC-OOH,OH	8
808.6	184.3	330.2	PLPC-triOH	8.01
812.6	184.3	333.2	isoPG(A2,J2)-PPC	8.22
814.6	184.3	336.2	PAPC-OOH	9.54
816.6	184.3	310.2	SLPC-epoxy,keto	9.92
818.6	184.3	312.2	SLPC-OOH	10.81
820.6	184.3	342.2	2,3-dinor-isoTxB2-PPC	7.76
822.6	184.3	317.2	15-deoxy-?12,14-isoPGJ2-SPC	9.21
824.6	184.3	318.2	SAPC-keto	11.05
826.6	184.3	320.2	SAPC-OH	11.09
828.6	184.3	350.2	PEIPC	7.31
830.6	184.3	352.2	isoPG(E2,I2,D2)-PPC	7.3
832.6	184.3	354.2	isoPGF2?-PPC	7.62
834.6	184.3	328.2	SLPC-OOH,OH	9.29

836.6	184.3	330.2	SLPC-triOH	9.4
840.6	184.3	333.2	isoPG(A2,J2)-SPC	9.2
842.6	184.3	336.2	SAPC-OOH	11.03
844.6	184.3	366.2	PAPC-OOH,OH,keto	6.01
846.6	184.3	368.2	PAPC-diOOH	7.13
848.6	184.3	370.2	iso-TxB2-PPC	7.68
850.6	184.3	344.2	SLPC-diOOH,epoxy	9.16
852.6	184.3	346.2	SLPC-OOH,OH,keto	8.37
856.6	184.3	350.2	SEIPC	8.64
858.6	184.3	352.2	isoPG(E2,I2,D2)-SPC	8.64
860.6	184.3	354.2	isoPGF2?-SPC	8.99
862.6	184.3	384.2	PAPC-diOOH,OH	5.83
864.6	184.3	358.2	SLPC-diOOH,keto,epoxy	8.77
866.6	184.3	360.2	SLPC-diOOH,OH,epoxy	8.25
870.6	184.3	364.2	SAPC-OOH,diketo	8.43
872.6	184.3	366.2	SAPC-OOH,OH,keto	7.88
874.6	184.3	368.2	SAPC-diOOH	8.51
876.6	184.3	370.2	Iso-TxB2-SPC	8.47
878.6	184.3	400.2	PAPC-triOOH	5.68
882.6	184.3	376.2	SLPC-triOOH	8.22
888.6	184.3	382.2	SAPC-OOH,OH,epoxy	7.44
890.6	184.3	384.2	SAPC-diOOH,OH	7.69
894.6	184.3	416.2	PAPC-triOOH,OH	5.04
906.6	184.3	400.2	SAPC-triOOH	7.64
922.6	184.3	416.2	SAPC-triOOH,OH	7.03

Oxidized Phosphatidylcholine treatment

Fresh DFSF is applied to NCMC on glass cover-slips and supplemented with 1, 2, 5, and 10μM concentrations of OxPC lipids sonicated into PBS without glucose (PBS^{-2}) forming unilaminar micelles. Six OxPC species, 1-palmitoyl-2-(5'-oxo-valeroyl)-sn-glycero-3-phosphocholine (POVPC), 1-palmitoyl-2-(9'-oxo-nonanoyl)-sn-glycero-3-phosphocholine (PONPC), 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphocholine (PGPC), 1-palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine (PAzPC) were applied to NCMC

plates for 4 hours. Plates were then washed with PBS^{-2} before NCMC were analyzed for cell viability and mitochondrial permeability as described below.

EO6 Antibody Treatment

The IgM EO6 antibody was used at a concentration of 10µg/mL within cell culture. The antibody was purchased from Avanti Polar Lipids as 100µg suspended in 100µl of PBS suitable for direct application into cell culture. Co-treatment with OxPC was at 5µM of each OxPC molecule and PSPC. Cell viability was assessed as described below.

Fluorescence Microscopy

All microscopy was done on an Olympus AX70 microscope, with pictures taken using a CoolSnap® camera and Image Pro-Plus 5.1.2 Software. Images were assessed using Adobe Photoshop CS5.1 Software.

Cell Viability Assay using Fluorescence Microscopy

Cells treated with OxPCs were stained using vital dyes, calcein-acetoxymethyl ester (AM) and ethidium homodimer-1 (Invitrogen) as previously described.[178] Calcein-AM is able to cross the membranes of cells, and cytosolic AM esterase enzymes are able to cleave the AM group off of calcein. Calcein by itself is now unable to pass through the membranes and will fluoresce. These processes only occur in live cells, which will be stained green, indicating active hydrolysis of acetoxymethyl esterases. However, in dead cells, acetoxymethyl esterase enzymes are not produced and therefore, calcein-AM is not cleaved and the cells will not fluoresce green. The counter stain used in this case is ethidium homodimer-1 which is unable to cross intact membranes of live cells. In contrast, dead

cells have compromised membranes and as a result the dye is able to enter the nucleus of the cell and bind to DNA, resulting in red fluorescence. Utilizing these two fluorescence stains, cell viability is represented as the percentage of the number of red stained cells over the sum of red stained cells and green stained cells. Cell counts were performed in Adobe Photoshop CS5.1 Software.

Mitochondrial Permeability Transition Pore Opening

Opening of the mitochondrial PTP after exposure to OxPC molecules was monitored by fluorescence microscopy using a solution of calcein-AM fluorescent dye with cobalt chloride ($CoCl_2$) applied to ventricular cardiomyocytes cultured on glass coverslips as previously described.[178] Unlike calcein-AM which can infiltrate the mitochondria, $CoCl_2$ is unable to pass through the inner and outer membranes of the mitochondria. $CoCl_2$ quenches the fluorescence of calcein, so the cytoplasmic fluorescence will be inhibited while still staining the mitochondria. However, if the mitochondrial permeability is increased there will be a reduction in fluorescence because $CoCl_2$ will be able to enter the mitochondria and quench the calcein inside the membranes. After staining NCMC will be immediately washed with PBS^{-2} and treated with fresh serum free DMEM/F12 cell culture medium. Coverslips will then be inverted on glass slides and immediately visualized at the same exposure. Reduced fluorescence compared to control cells will denote mitochondrial permeability. Fluorescence was quantitated using Adobe Photoshop CS5.1 Software.

Statistical Analysis

Data collection from mass spectrometry analysis was through the Analyst 1.6 Software (AB Sciex), and quantitation using MultiQuant 2.1 Software (AB Sciex). Fold

changes were determined by dividing the average OxPC mass of the treatment groups by the average OxPC mass of the control group standardized to the total protein extracted using a Micro BCA™ Protein Assay Kit from Thermo Scientific ™. Further data analysis was performed using Microsoft Excel®. Cell counts and fluorescence data were analyzed by Microsoft Excel functions. Student's *t*-tests were performed comparing each treatment group to control by two-tailed analysis with significant *p*-value of 0.05. All data is represented as mean ± standard error of mean (SEM).

Chapter III: Results

Oxolipidomics Analysis

Simulated ischemia reperfusion of NCMC showed that there was significant generation of OxPC by NCMC under ischemia reperfusion conditions. Excised ion chromatographs of short-chain OxPCs, Succinoyl-PPC, PONPC, SOVPC, and KOOA-PPC showed increased detection in ischemia reperfusion samples (Figure 3.1). The total amount of OxPC increased from 17.1±1.37 picograms of OxPC per microgram of protein (pg/µg) in control to 112.3±20.9 pg/µg after reperfusion from approximately $1.75x10^6$ NCMC in one $35mm^2$ culture plate ($p<0.05$) (Figure 3.2). Ischemic conditions also showed an increase in the OxPC to 68.7±4.13 pg/µg ($p<0.05$). Fragmented OxPC increased from 1.48±0.14 pg/µg in control to 17.4±2.60 pg/µg after ischemia and 60.6±13.4 pg/µg after reperfusion ($p<0.05$) (Figure 3.3). Analysis of the 6 OxPC standards showed increases in each species with significant increases in PONPC and KDdiA-PPC ($p<0.05$) (Figure 3.4). Analysis of adult rat cardiomyocytes for OxPC showed an increased content to 10.2±0.67 mg/mL in ischemia reperfusion that was significantly increased over control (1.79±0.26 mg/mL, $p<0.05$) (Figure 3.5). Further analysis of the fragmented OxPCs generated in ischemia and reperfusion of adult rat cardiomyocytes showed significant increases (156.3±10.6 ng/mL, 146.6±12.5 ng/mL respectively) compared to control (77.5±0.76 ng/mL control, $p<0.05$) (Figure 3.6). These results are in agreement with previous results of oxolipidomics analysis of porcine post-transplant myocardium with increasing OxPC content after transplant (Figure 3.7). Fold changes demonstrates significantly increased

levels of OxPCs with increased oxidative stress after reperfusion that is significantly increased over baseline (Figure 3.8).[1]

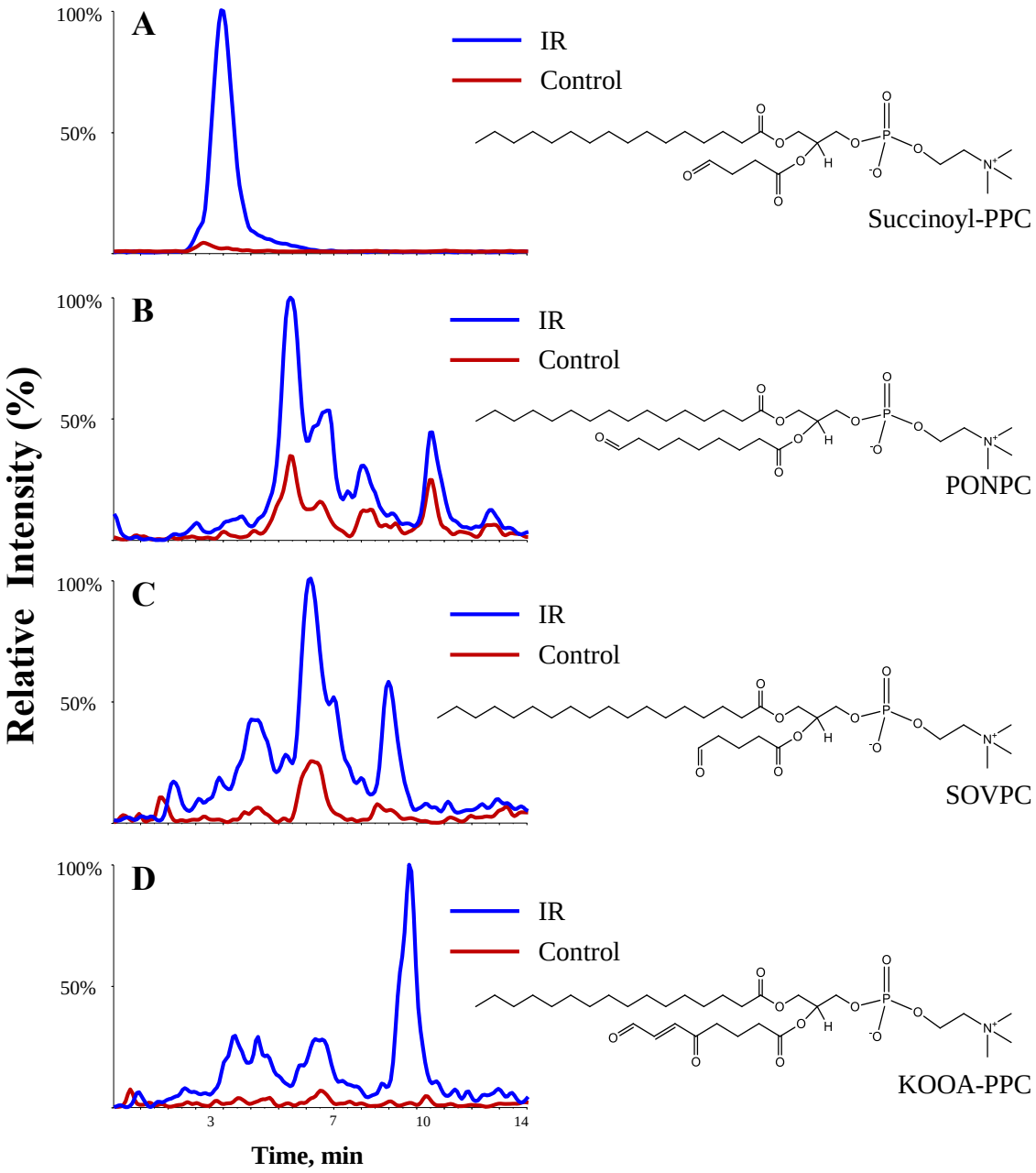

Figure 3.1: Excised Ion Chromatograms generated using Analyst 1.6 Software (AB Sciex), for the detection of oxidized phosphatidylcholine species detected by oxolipidomic analysis of post-natal rat cardiomyocytes. Blue chromatogram illustrates the detected ion transition for ischemia reperfusion conditions and red chromatogram is the same ion transition in control conditions.

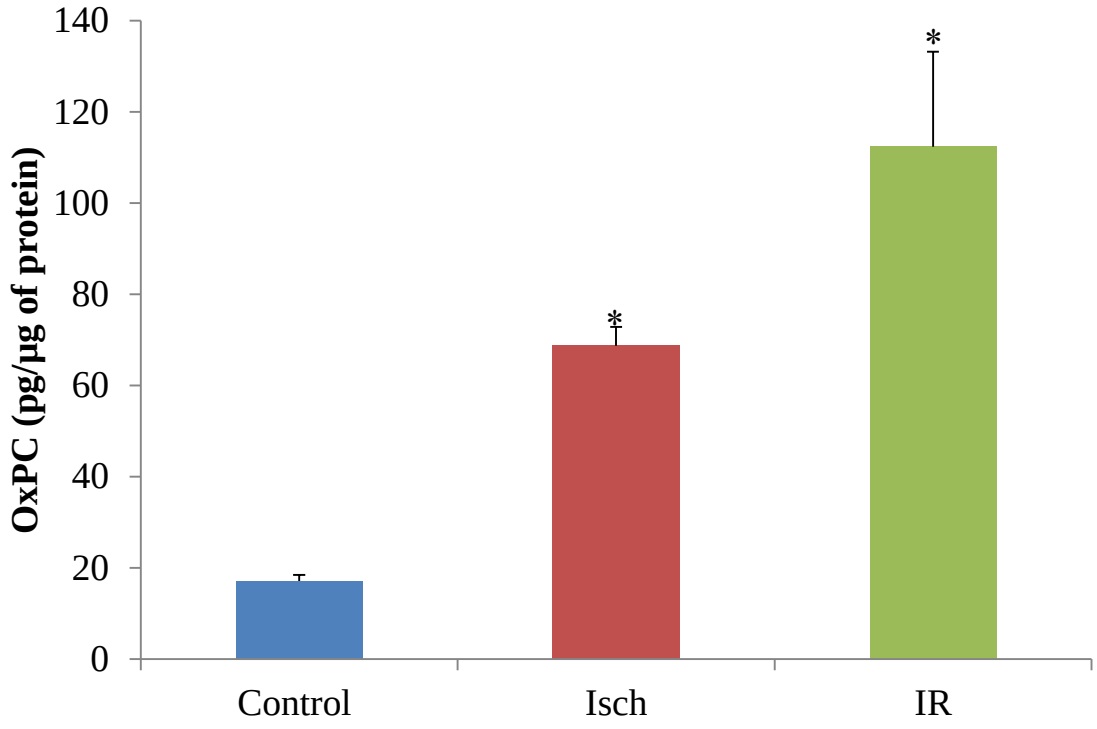

Figure 3.2: Oxidized phosphatidylcholine mass detected after oxolipidomic analysis of post-natal rat cardiomyocytes over the amount of protein extracted from a 35mm^2 culture dish. The detected mass of OxPC was increased significantly after both ischemia and IR compared to control ($p<0.05$) (n=3).

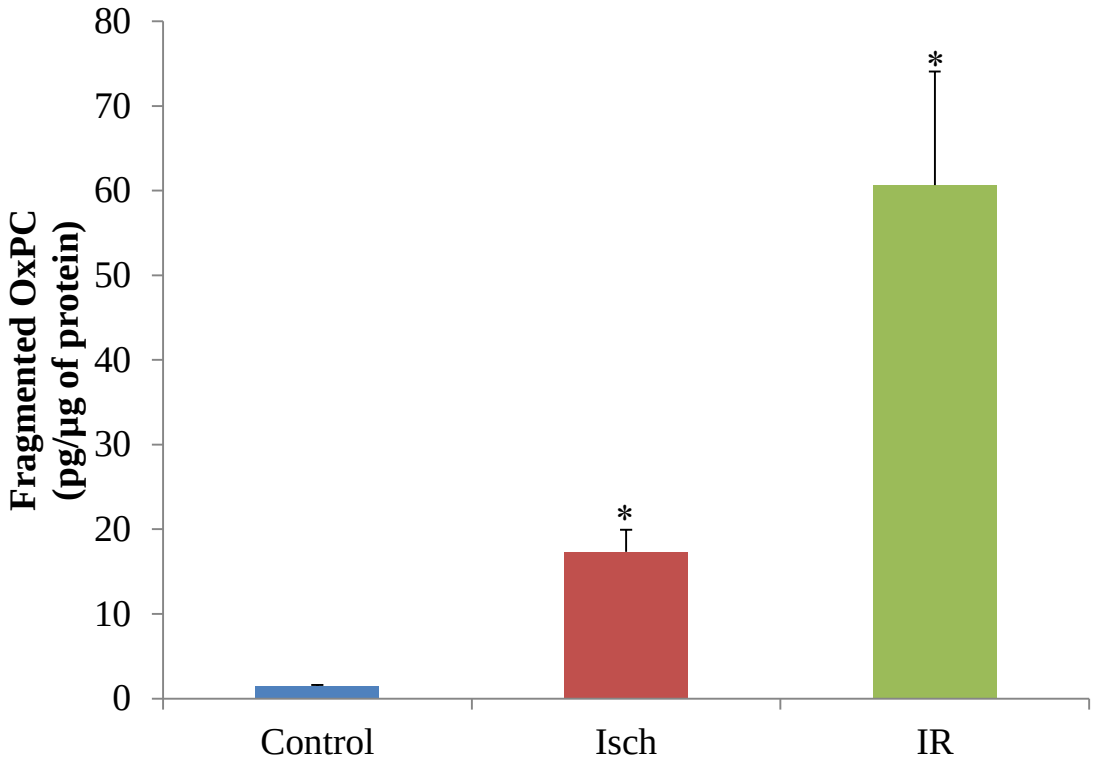

Figure 3.3: Fragmented oxidized phosphatidylcholine mass detected after oxolipidomic analysis of post-natal rat cardiomyocytes over the amount of protein extracted from a 35mm^2 culture dish. The detected mass of OxPC was increased significantly after ischemia and reperfusion (17.4±2.60pg/µg, 60.6±13.4pg/µg respectively) compared to control (1.48±0.14pg/µg, $p<0.05$) (n=3).

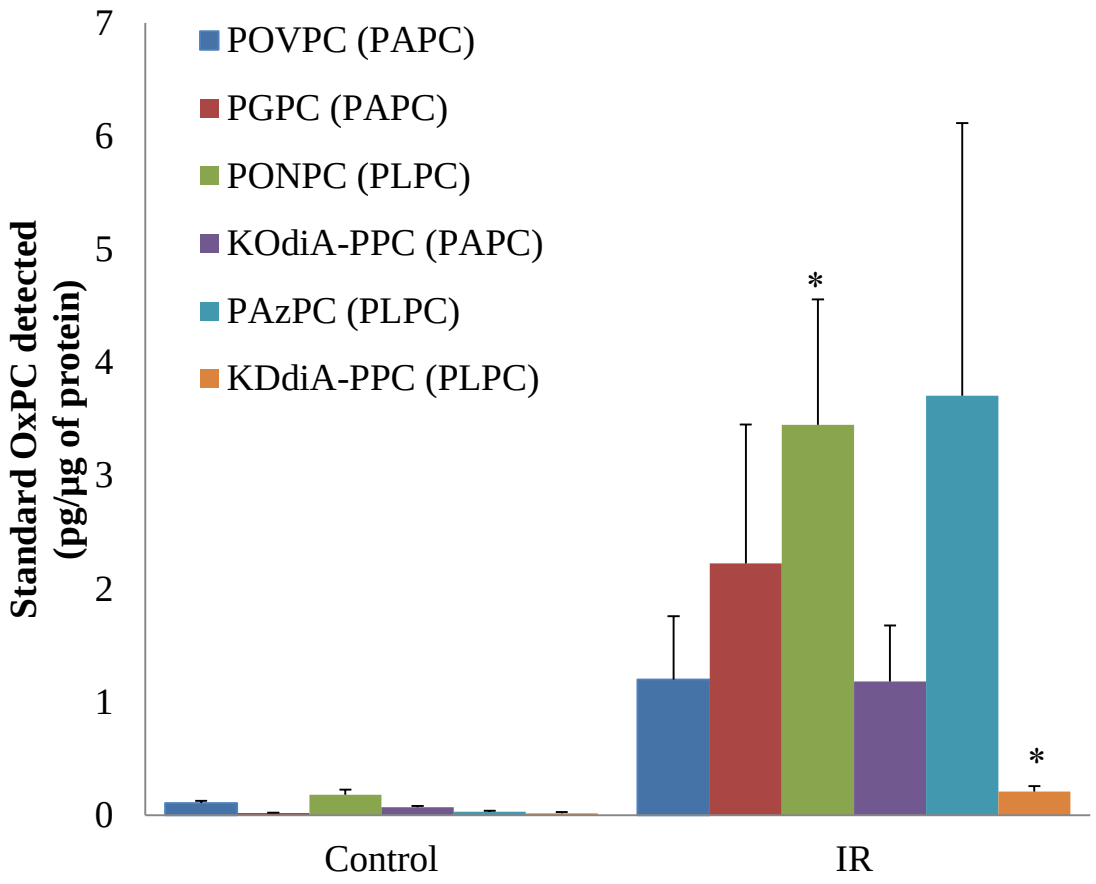

Figure 3.4: Oxidized phosphatidylcholines standards detected after oxolipidomic analysis of post-natal rat cardiomyocytes after simulated IR. All species were increased, PONPC and KDdiA-PPC were significantly increased after IR compared to control ($p<0.05$) (n=3).

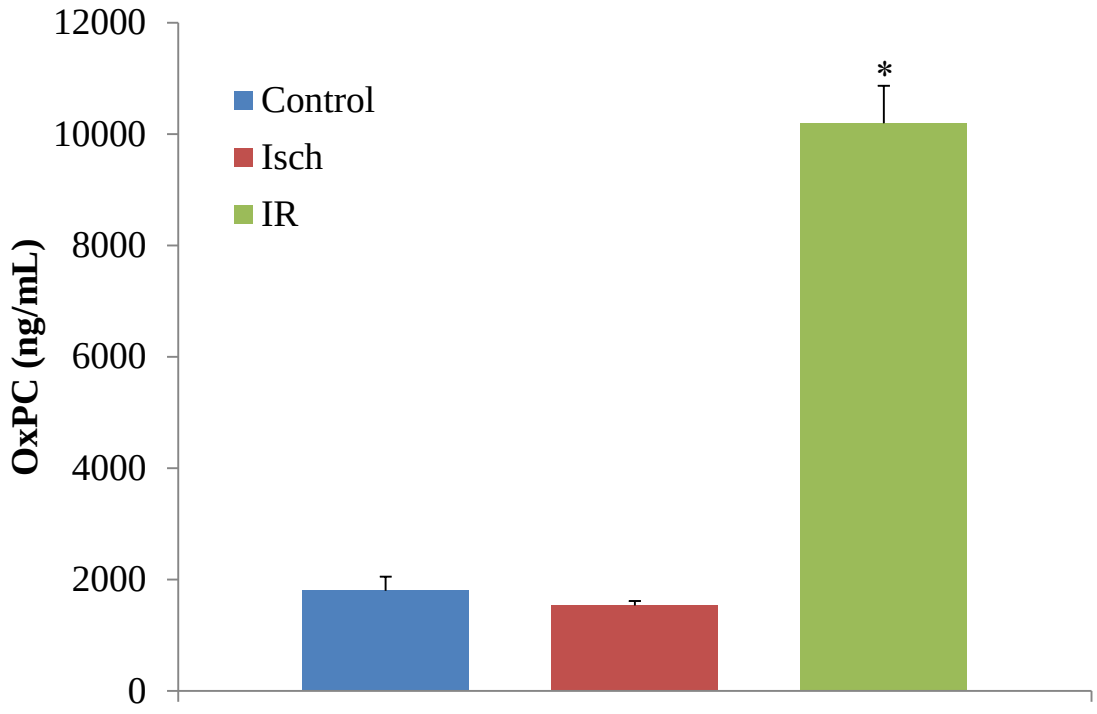

Figure 3.5: Bioactive oxidized phosphatidylcholine content detected using electrospray ionizing tandem mass spectrometric analysis of adult rat cardiomyocytes under simulated control, ischemia or reperfusion conditions. Quantitation was performed by previously described methods of 82 oxidized phosphatidylcholine compounds. Adult cardiomyocytes generate increased amounts of OxPC after reperfusion (10.2±0.67mg/mL) compared to control (1.79±0.26mg/mL control, $p<0.05$) (NB – ng to mg converted units) (n=3).

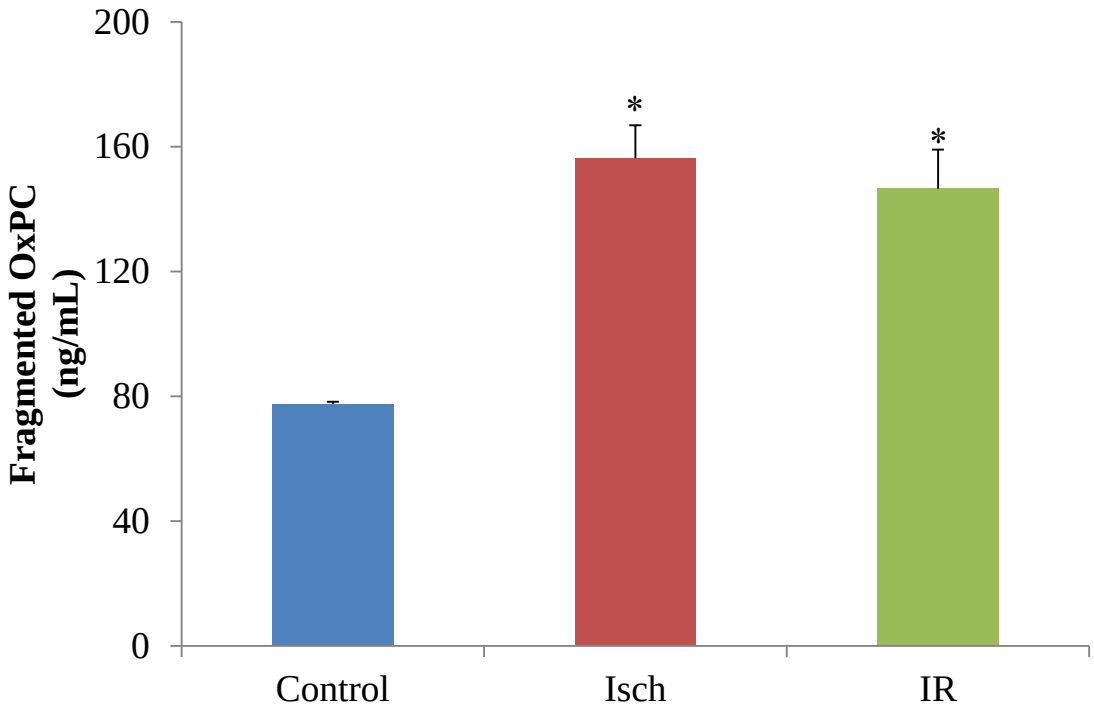

Figure 3.6: Fragmented oxidized phosphatidylcholine content detected using electrospray ionizing tandem mass spectrometric analysis of adult rat cardiomyocytes under simulated control, ischemia or reperfusion conditions. Adult cardiomyocytes generate increased amounts of OxPC after ischemia and reperfusion (156.3±10.6ng/mL, 146.6±12.5ng/mL respectively) compared to control (77.5±0.76ng/mL control, $p<0.05$) (n=3).

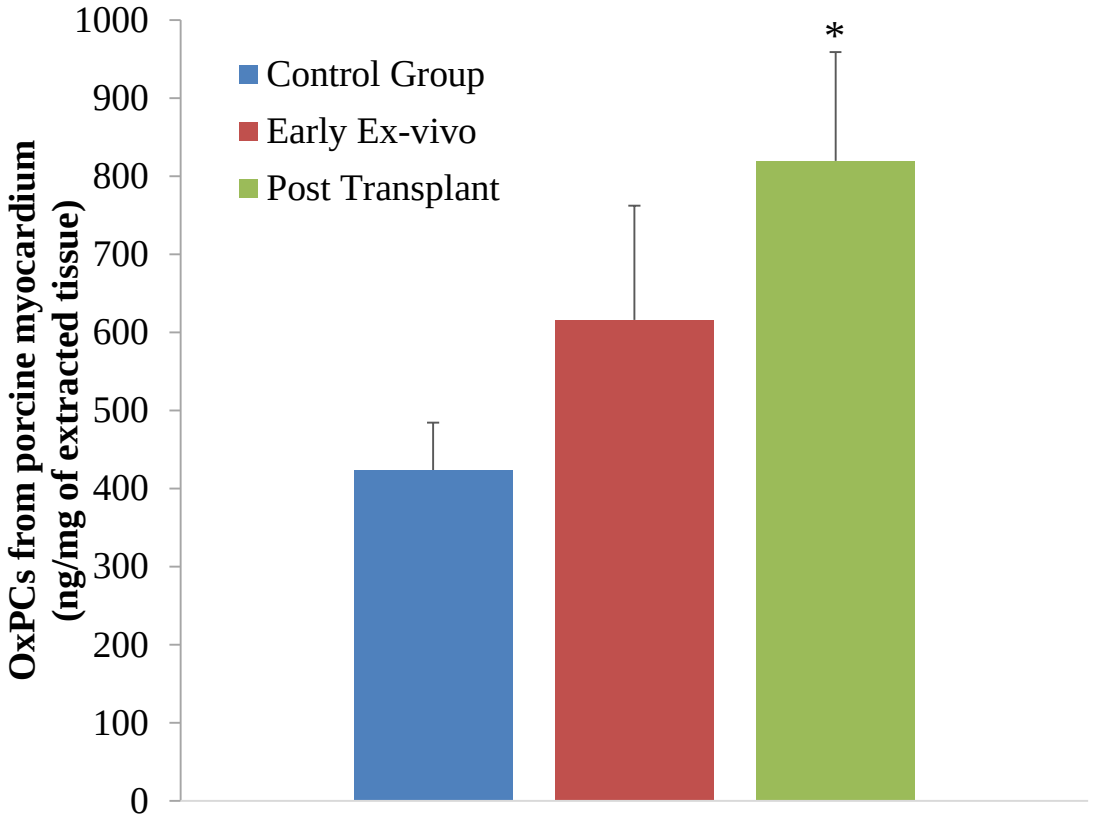

Figure 3.7: Total mass of oxidized phosphatidylcholine species in extracted porcine myocardial tissue. Each group contained 6 samples that were extracted and analysed through HPLC-ESI/MS/MS scanning for product ion of m/z 184. A statistically significant difference was demonstrated between the control group and post-transplant group ($p<0.05$) (n=3).

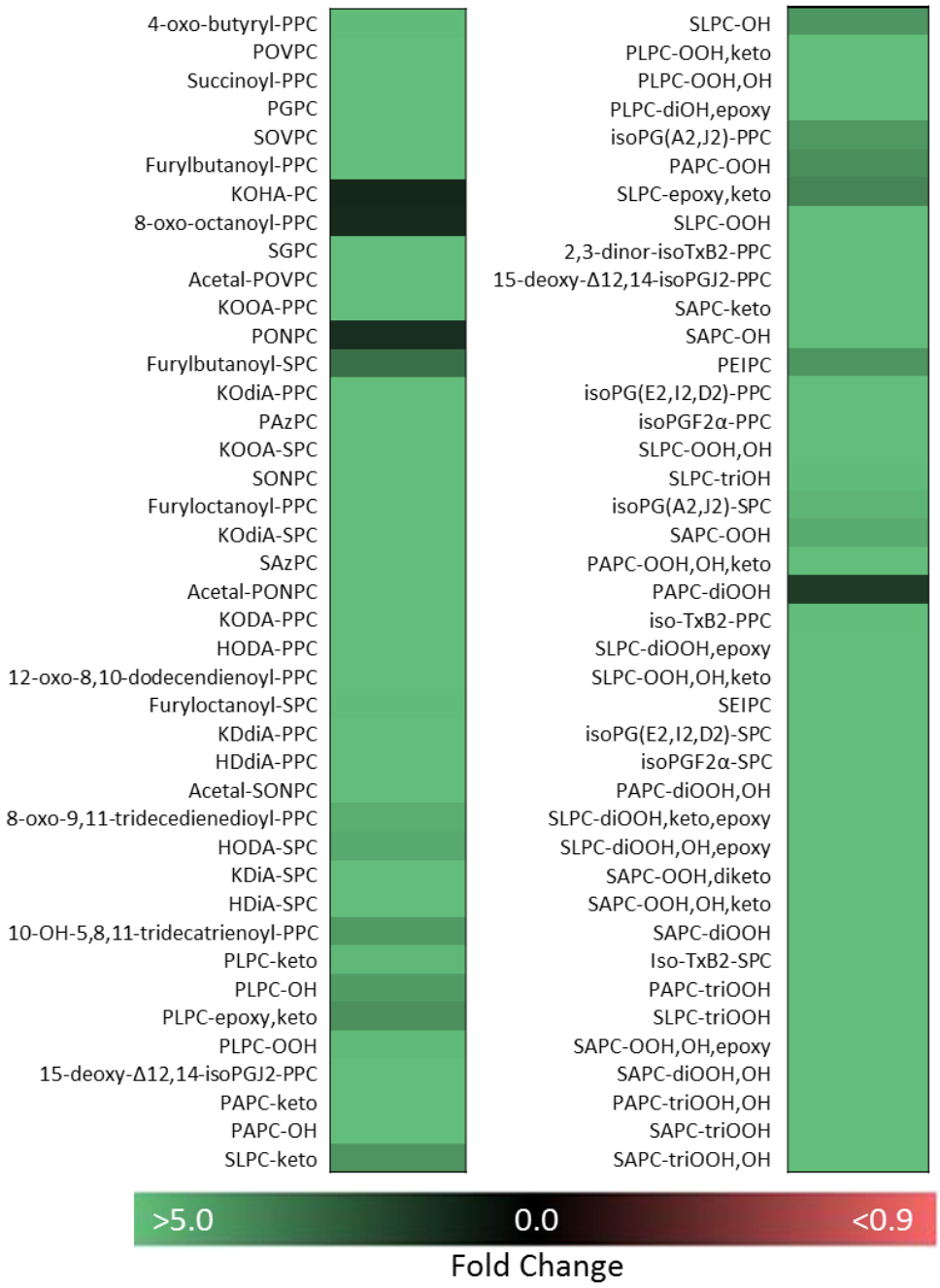

Figure 3.8: Oxolipidomic analysis of post-transplant pig myocardium fold-change between post-reperfusion and post-transplant myocardium of 82 OxPC compounds. Heat map illustrates green fold-change increases in the majority of compounds.[1]

Cell Viability Analysis

Exogenous administration of fragmented phosphatidylcholines demonstrated dose-dependent cell death induction in NCMC compared to non-oxidized control from the cell viability assay in representative images (Figure 3.9A). Increased number of ethidium homodimer-1 (red-dead) stained cells over fewer calcein-AM (green-live) stained cells showed percent cell death for POVPC was 20.0±4.46%, 26.0±5.41%, 46.4±4.86%, and 68.6±4.56% for 1, 2, 5, and 10μM respectively ($p<0.05$). PONPC induced 22.3±2.58%, 15.5±1.61%, 40.2±5.37%, and 46.8±4.06%; PGPC caused 18.6±3.27%, 33.9±2.81%, 45.3±3.26%, and 50.1±4.38%; PAzPC stimulated 27.1±5.33%, 44.4±4.56%, 74.5±5.03%, and 68.7±6.63% cell death respectively ($p<0.05$) (Figure 3.9B). The non-oxidized control PSPC treated NCMC showed non-significant increases in cell death at 1, 5, and 10μM, 7.58±2.10%, 9.11±3.16%, and 10.5±1.58% respectively (vs 6.95±1.08% control, $p>0.05$). However at 2μM there was a significant reduction of cell death 1.82±0.55% ($p<0.05$).

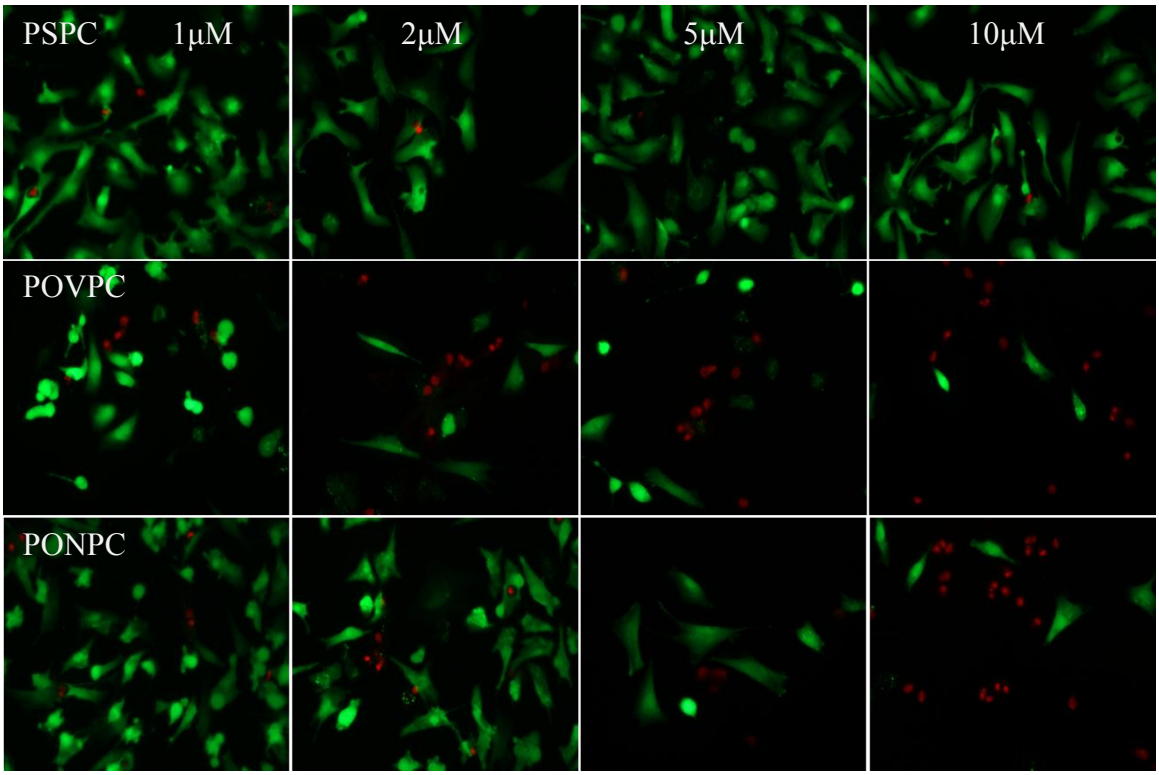

Figure 3.9A: Representative images of post-natal rat cardiomyocytes stained using the vital dyes, calcein-AM (green - live) and ethidium homodimer-1 (red - dead), grown on glass coverslips exposed to increasing concentrations of POVPC and PONPC for cell viability assay comparing to non-oxidized control PSPC. Similar images were observed after cardiomyocytes were exposed to PGPC and PAzPC.

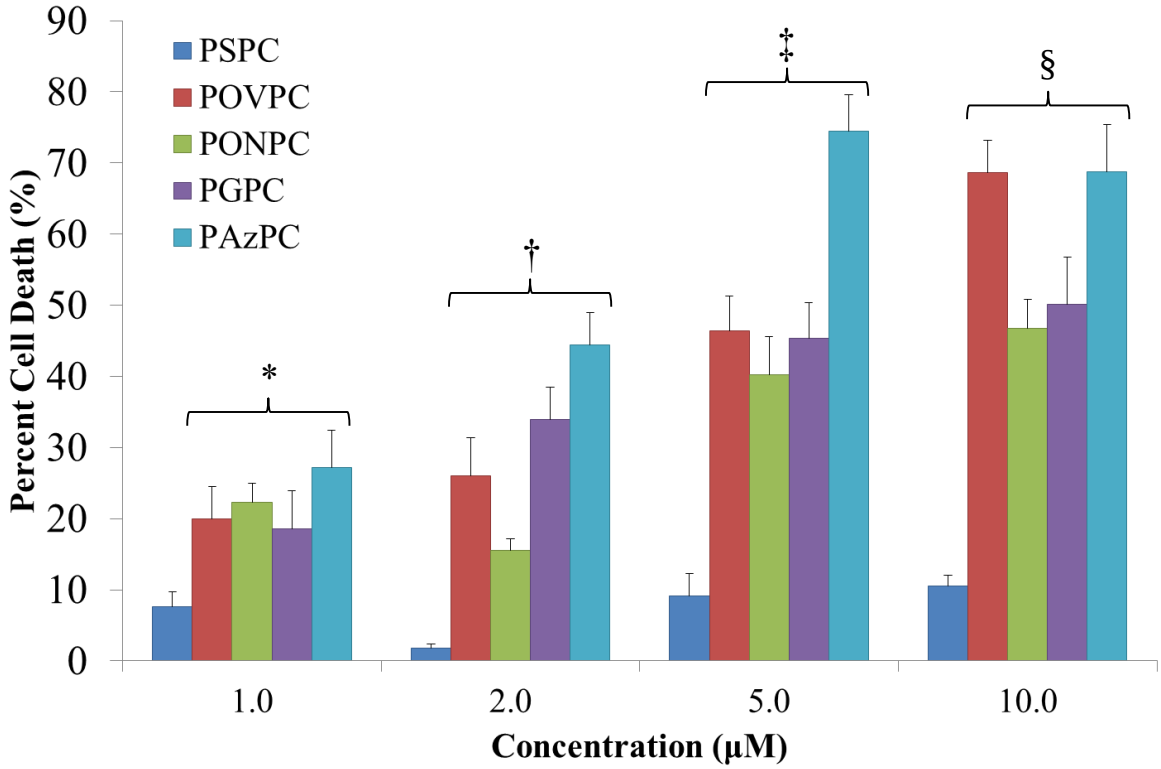

Figure 3.9B: Cell viability of post-natal rat cardiomyocytes exposed to increasing concentrations of aldehyde and carboxylic acid OxPCs compared to cell viability after treatment with non-oxidized control PSPC. OxPCs induce dose dependent cell death in cardiomyocytes and are increased compared to PSPC (blue bar). *$p<0.05$ vs 1µM PSPC, †$p<0.05$ vs 2µM PSPC, ‡$p<0.05$ vs 5µM PSPC, §$p<0.05$ vs 10µM PSPC, (n=3)

Mitochondrial Permeability Analysis

There was also significant changes to the mitochondrial permeability within OxPC exposed cardiomyocytes demonstrated in representative images (Figure 3.10A). Increased mitochondrial permeability is demonstrated by a decrease in fluorescence and is represented as a fold reduction compared to non-oxidized PSPC (Figure 3.10B). Significant fold reduction was seen in all compounds at 5μM (POVPC 0.50±0.05 fold, PONPC 0.38±0.02 fold, PGPC 0.39±0.04 fold, and PAzPC 0.34±0.05, $p<0.05$). Interestingly, POVPC demonstrated significantly higher fluorescence compared to PSPC at 1μM (1.48±0.09 fold, $p<0.05$).

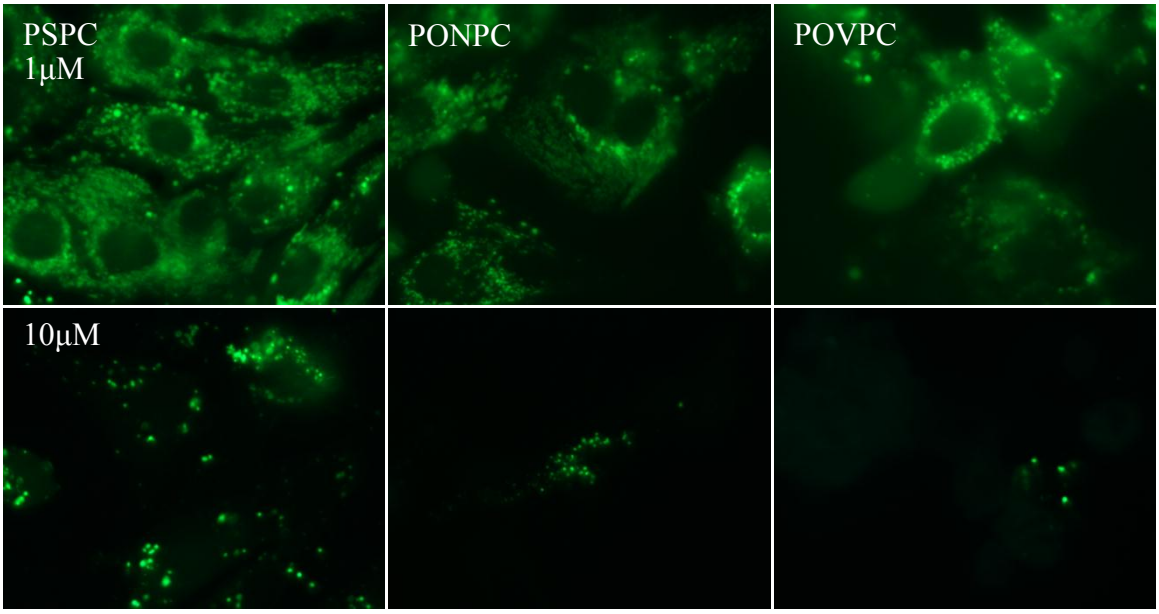

Figure 3.10A: Representative images of post-natal rat cardiomyocytes stained with calcein-AM and $CoCl_2$ grown on glass coverslips exposed to increasing concentrations of POVPC and PONPC for mitochondrial permeability assay comparing to non-oxidized control PSPC. Similar images were observed after cardiomyocytes were exposed to PGPC and PAzPC.

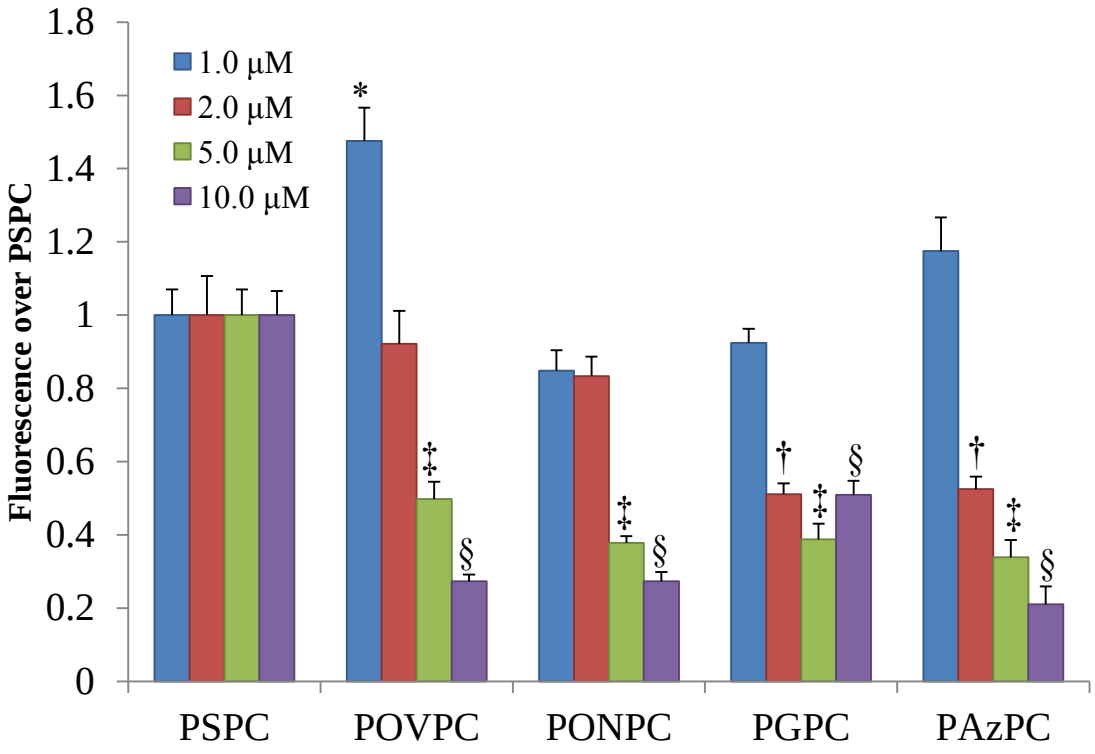

Figure 3.10B: Mitochondrial permeability of post-natal rat cardiomyocytes exposed to increasing concentrations of aldehyde and carboxylic acid OxPCs is depicted as the fold change over the fluorescence of non-oxidized control PSPC treated cardiomyocytes stained with calcein-AM and $CoCl_2$ at equal concentration. OxPCs show significantly decreased fluorescence compared to PSPC beyond 5µM. *p<0.05 vs 1µM PSPC, †p<0.05 vs 2µM PSPC, ‡p<0.05 vs 5µM PSPC, §p<0.05 vs 10µM PSPC, (n=3)

EO6 Antibody-mediated OxPC Inhibition Analysis

Cardiomyocytes exposed to OxPC and EO6 antibody were analyzed by vital dye staining to determine if the antibody could significantly block the actions of OxPC on cardiomyocytes. Representative images show that there was a significant reduction in cardiomyocyte cell death to levels equivalent to non-oxidized control PSPC, and non-treated control levels (Figure 3.11A). Each compound was significantly inhibited from inducing cell death at 5μM with addition of 10μg/mL of EO6 antibody (POVPC 22.6±4.14% vs 8.02±1.58% with EO6, PONPC 25.3±3.40% vs 6.14±1.02%, PGPC 43.4±4.95% vs 10.3±1.58%, PAzPC 23.4±3.10% vs 6.74±1.54%, $p<0.05$) (Figure 3.11B). The EO6 antibody treatment of PSPC and control conditions were not different (PSPC 6.15±1.60% vs 6.27±1.11% with EO6, and control 3.56±1.32% vs 4.81±2.06%, $p>0.05$).

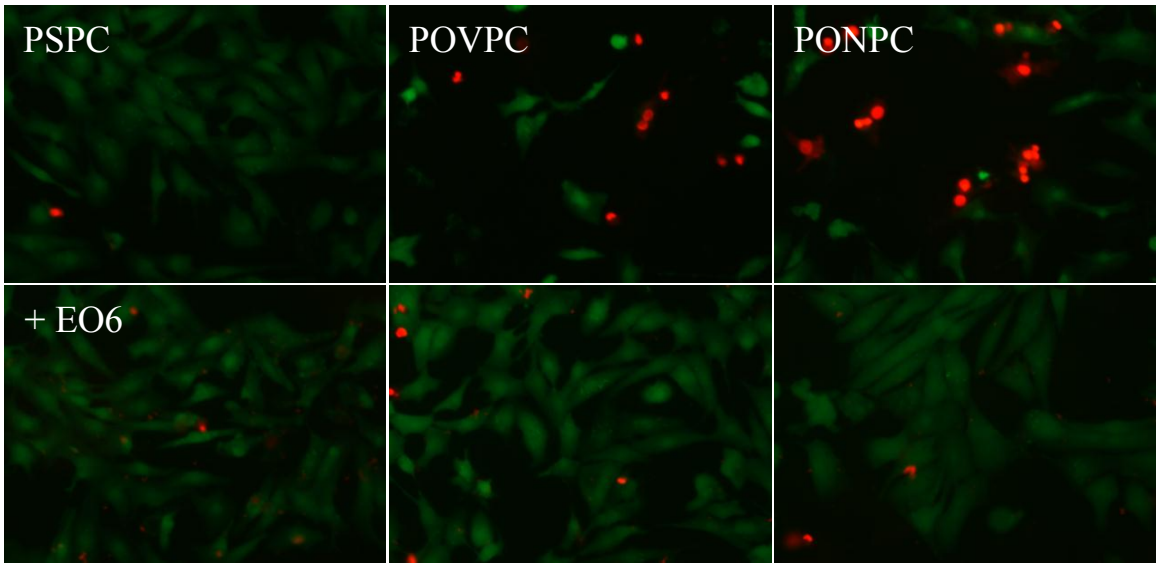

Figure 3.11A: Representative images of post-natal rat cardiomyocytes stained using the vital dyes, calcein-AM (green - live) and ethidium homodimer-1 (red - dead), grown on glass coverslips co-treated with 5μM of POVPC and PONPC and 10μg/mL of OxLDL-specific EO6 antibody. There was significant inhibition of OxPC-induced cell death at 5μM concentration. Similar images were observed after cardiomyocytes were exposed to PGPC and PAzPC with 10μg/mL of the EO6 antibody.

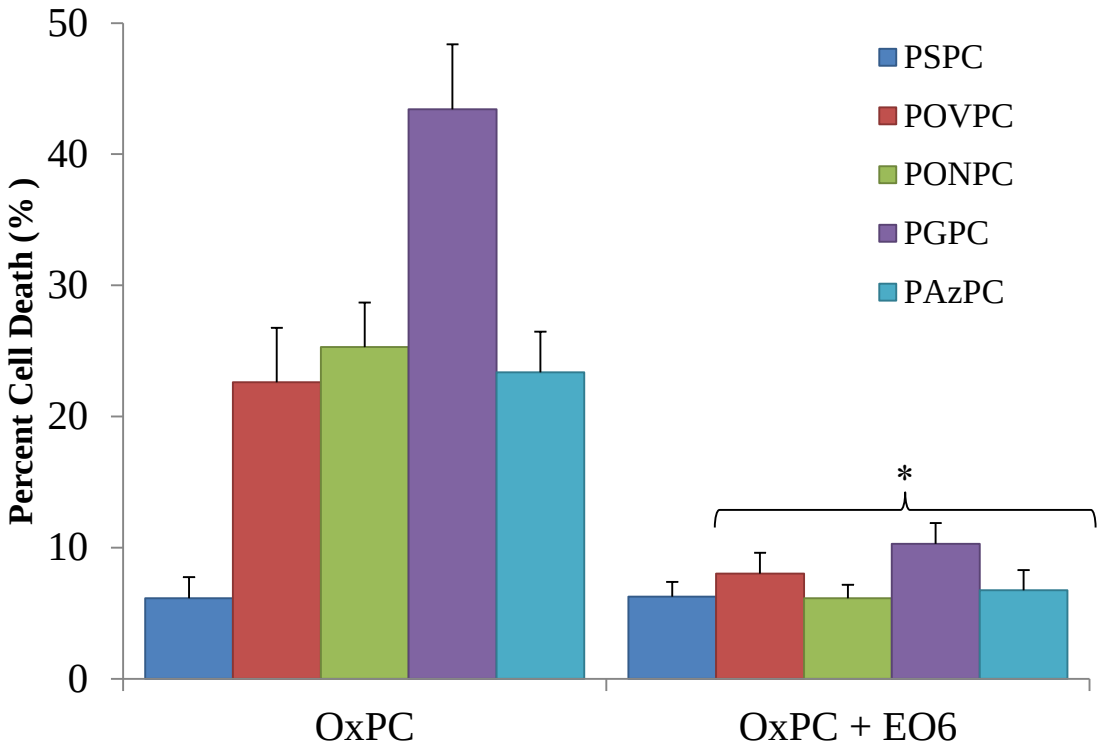

Figure 3.11B: Cell viability of post-natal rat cardiomyocytes co-treated with 5μM of aldehyde and carboxylic acid OxPCs and 10μg/mL of the OxLDL-specific EO6 antibody. OxPC-induced cell death was significantly inhibited to levels similar to PSPC and non-treated controls. *$p<0.05$ vs OxPC treated, (n=3)

Chapter IV: Discussion

This study has conclusively shown that there is a significant increased production of bioactive OxPC species in post-natal rat cardiomyocytes under *in vitro* simulated ischemia reperfusion. This is significant because it has been previously determined that OxPC are important bioactive compounds that are produced during oxidative stress in other cell types and mostly described in atherosclerosis.[61, 62, 179] We have previously shown that increased oxidative stress in a porcine transplant model leads to elevated OxPC production.[1] OxPC are important signaling molecules after membrane phospholipids become oxidized by ROS.[26, 180] Previous investigations demonstrate that macrophages, vascular smooth muscle cells, endothelial cells, and nerve cells are impacted either through inflammatory pathways or in apoptotic cell death pathways when exposed to OxPC.[69, 70, 124, 151] Within the heart, each of these cell types are present, as well as fibroblasts and importantly cardiomyocytes.[181]

This study has demonstrated a significant increase in OxPC species, in post-natal and adult cardiomyocytes under IR, and importantly within myocardial tissues post-transplant (Figure 3.2-3.8). In particular, it is shown that a significant portion of this increase can be attributed to an increase in fragmented OxPC species (Figure 3.3, 3.4, 3.6) and are therefore important when cardiomyocytes undergo stress. The lipidomics analysis has made it possible to identify OxPL from biological samples utilizing this novel HPLC and mass spectrometry technology. This methodology allows us to investigate each OxPC species by generating chromatograms to compare their presence as they relate to disease development and progression (Figure 3.1).[90, 91, 182] Over the past 20 years lipidomics technology has allowed for more lipids to be identified and particularly with oxidized

phospholipids, their impact in disease has begun to be elucidated. The *in vitro* model that we have employed is useful for answering the particular question about endogenous OxPC generation by cardiomyocytes, which leads us to believe that there are two ways that the OxPC can then induce cellular response. The first is by intracellular signaling when produced inside cellular membranes, OxPC are able to bind receptors within the cell either nuclear or cytosolic; an example of each would be PPAR or PI3K. The signaling cascades could then be activated to result in apoptosis or release of inflammatory cytokines and chemokines that are increased during ischemia reperfusion like TNF-α, IL-6, or MCP-1.[133, 183]

The second mechanism that OxPC could be produced is that upon release of OxPC into the extracellular environment. OxPC receptors present on cardiomyocytes could bind them and transduce the signal into the cell. Previous studies have shown that the stimulation of the OxPC receptor TLR-4 by LPS, which has been shown to compete with OxPC for TLR-4 binding, leads to reduced contractility of cardiomyocytes.[26, 184] Additionally, the toxicity of OxPC has been shown in macrophages where exogenous POVPC and PGPC induced apoptosis which was significantly blocked by NB19 an acid sphingomyelinase inhibitor.[151] These two particular fragmented OxPC, POVPC and PGPC were shown to induce apoptosis in vascular smooth muscle cells marked by DNA fragmentation and morphological changes.[150] This suggests that an extrinsic mechanism of cell signaling can be induced by fragmented OxPC and could cause detrimental effects in cardiomyocytes.

Since there are a few fragmented OxPC that were commercially available, it was important to determine what type of cell response is induced in cardiomyocytes. Although it appears that the overall generation of OxPC may be detrimental to cells, the particular

cell response to each of the chosen standards, POVPC, PONPC, PGPC, and PAzPC could be cell specific. It is also important to rule out a non-specific non-oxidized phosphatidylcholine response in cardiomyocytes which was why the non-oxidized PSPC species was selected to treat the cells with as a negative control. In order to treat cells with these lipids, lipid micelles were generated of each lipid species that could be suspended in a non-toxic solution. Many of the standards are received in either ethanol or a mixture of chloroform-methanol solvents that could give confounding results when looking at viability studies. In order to counteract this, sonication of the lipid film into PBS^{-2} after inert nitrogen evaporation of the solvent avoids this. This step also allows for mixing with culture media in order to treat cardiomyocytes with the increasing concentrations of these lipids in a controlled manner.

The cell viability assay demonstrates that there is significant induction of cell death in cardiomyocytes when exposed to the fragmented aldehyde and fragmented carboxylic acid species of OxPC (Figure 3.9A,B). The increased cell death can be seen in two ways first is that there is an increase in the number of red stained cells which indicate the condensation of the chromatin and the permeability of the nuclear envelope to ethidium homodimers-1 which chelates the nuclear DNA and fluoresces in the red spectrum.[139, 185] Also the second indication is that there is increased cell death is the significant reduction in the confluency of the cardiomyocytes when exposed to OxPC, this infers that there are unaccounted cells which could be washed away during the procedure. This would seem therefore that the amount of cell death induced could be underestimated by this analysis.

As previously mentioned, OxPC-induced cell death has been confirmed in multiple cell types including smooth muscle cells and macrophages. For the first time, it has been

shown within cardiomyocytes that OxPCs induce cardiomyocyte cell death and could suggest a potential link between atherosclerosis, heart failure and ischemia reperfusion injury.[166] There have been many investigations into the bioactivity of OxLDL and the role lipoproteins have in the progression of atherosclerosis and the formation of the 'foam cell' phenotype of macrophages.[80, 186] The OxPCs are able to instigate TNF-α signaling which is part of the aberrant cell signaling in macrophages, endothelial cells and vascular cells leading to plaque progression.[187] It was demonstrated in Jurkat cells that there is an important link between TNF-α and OxPC as TNF-α treatment generated endogenous OxPC that led to mitochondrial permeability and apoptotic cell death.[180] This mechanism could also be present in cardiomyocytes as increased levels of cardiomyocyte cell death seen in heart failure correlate with the increased levels of TNF-α.[187] However, this study proposes that exogenous OxPC can bypass this TNF-α dependent mechanism but lead to a positive feedback for OxPC production that causes increased cardiomyocyte cell death.

This study sought to further describe the specific mechanism of OxPC-action which was suspected to be intrinsic apoptosis. In order to investigate this, mitochondrial permeability was assessed. Mitochondrial permeability transition, or loss of $\Delta\Psi_m$, was a phenomenon that was previously observed during apoptosis, and was attributed to the mPTP.[188, 189] The pore is currently believed to consist of the mitochondrial ATP synthase which is the energy producing enzyme of all aerobic life and the most important functional protein of mitochondria; opening causes release of the aforementioned pro-apoptotic components, CytC and AIF, and permeability leads to cell death.[147]

Investigations into OxPC effects on the mitochondria have shown that there are direct interactions with the mitochondrial proteins causing physiological changes. Studies

into the action of OxPC on mitochondrial function showed that the B-cell lymphoma 2 (Bcl-2) proteins Bid (pro-apoptotic) and Bcl-X_L(anti-apoptotic) act as co-factors modulating mitochondrial sensitivity to PAzPC.[128] These two proteins appear to be in a state of flux that either exacerbates or suppresses OxPC action, respectively. This Bcl-2 family of mitochondrial proteins seem to be key to the mechanism of action that OxPC act through. Bax, which is another pro-apoptotic Bcl-2 family protein, has also been shown to interact with PAzPC.[149] As previously described the OxPCs capability to interact with these proteins and the mitochondria cause the mitochondria to swell, release CytC and AIF, and reduce the electrochemical gradients across the mitochondrial membrane.

These three factors influence the $\Delta\Psi_m$ halting ATP production, and the significant release of CytC induces apoptosis.[29] CytC release triggers the activation of caspases, resulting from apoptotic stimuli at the mitochondrial level.[190, 191] With the understanding that reperfusion injury was influenced by cell death, the mPTP became a target for cardioprotection.[192] The conditions of reperfusion where there are increased levels of Ca^{2+} in the mitochondria, oxidative stress, lowered ATP production and pools favor the opening of the mPTP.[193] It was well known that CsA and sanglifehrin A were inhibitors of the pore and administration of both of these compounds onto human atrial trabecula under oxidative stress prevented the loss of contractile function seen in the control group.[192] This previous study also demonstrated that blockage of the mPTP at the time of reperfusion improved cell viability of human atrial myocytes.[192] These results indicated that there is significant benefit to preventing this pore from opening as a means of reducing ischemia reperfusion injury. Two small studies have shown that there appears to be a benefit to the use of CsA after PCI, however the potent immunosuppressive effects of the drug are concerning.[194, 195]

The small size of these studies make generalizations difficult and the latter study demonstrated that if the angiography indicated significant acute coronary syndrome (ie >60% blockage) the infarct size was not reduced in three patients treated with CsA.[195] There continues to be other landmark studies underway including the CYCLE, CIRCUS, and CLOTILDE trials (ClinicalTrials.gov NCT01650662, ClinicalTrials.gov NCT01502774, and ClinicalTrial.gov NCT01901471) that are investigating the action of CsA during acute response in ST-elevation myocardial infarction (STEMI), prognosis after STEMI and the response of patients to CsA after STEMI complicated by cardiogenic shock respectively.[148]

Using this working knowledge of the mPTP, if OxPC induce mitochondrial permeability then they could be part of the proximal signal that leads to induction of cell death seen within ischemia reperfusion injury. When OxPC are applied to NCMC, there is significant reduction in the fluorescence of the mitochondria indicating increased permeability of mitochondrial membranes (Figure 3.10A,B). This observation has important implications for treatment as it may be useful to block the mPTP from opening, but OxPC could be a more proximal target to prevent the exacerbation of both the apoptotic and inflammatory damage that is increased in ischemia reperfusion injury due to the previously described toxic activity on macrophages.[68] By blocking the OxPC proximally in both cardiomyocytes and macrophages, we may be able to significantly restore cardiac function by limiting cell death and inflammation due to the significant production of fragmented OxPC species at reperfusion.

As mentioned above, there is a N-Ab that can recognize OxLDL, OxPAPC, and POVPC, called the EO6 antibody, of the T15 idiotype.[115] Determining if the EO6 antibody

could block OxPC-induced cell death within cultured cardiomyocytes would demonstrate that EO6 can block the exogenous action of OxPC, but also can block the positive feedback that could be induced by the presence of OxPC molecules. If there is any induction of cell signaling by OxPC leading to internal OxPC being generated, EO6 could act as an external sink to bind the OxPC outside the cell preventing the activation of cell signaling cascades in a local autocrine fashion. After co-applying the EO6 antibody there was significant reduction of cardiomyocyte cell death in a cell viability assay (Figure 3.11A,B). The antibody reduced cell death induction to similar levels when exposed to non-oxidized PSPC as well as non-treated controls. Other studies have shown that this antibody is able to bind OxPC and may prevent the development of atherosclerosis.[116, 196] Interestingly, immunization with modified LDL, termed MDA-LDL, increased the titre of the EO6 antibody in hypercholesterolemic rabbit and mouse models, that were being produced in T cells and B cells.[162] When the splenocyte cultures of normocholesterolemic mice that had been immunized against MDA-LDL, were incubated overnight with MDA-LDL, there was significantly more cells that secreted IL-5.[196] The authors also showed that immunizing $LDLR^{-/-}$ mice against MDA-LDL could reverse the atherosclerotic phenotype, most likely because this immunization led to increased levels of the T15/EO6 natural IgM antibody titre produced by B-1 cells.[196] These observations indicate that there is a protective role of IL-5 against OxPCs due to the increased production of the EO6 antibody in the case of atherosclerosis. Our results suggest that this may also be possible in the case of ischemia reperfusion injury. If the direct infusion of the EO6 antibody at time of reperfusion is not feasible, perhaps using a cytokine therapy of IL-5, or inducing IL-5 production may lead to beneficial recovery within the heart muscle.

It is well established that cardiac IR injury is a complex stress within the myocardium and previous trials have been relatively underwhelming in both effectively reducing cellular damage and injury, and restoring function.[18] Part of this is most likely because IR injury is a multifaceted stress culminating in an extensive cell response in all the cell types of the heart, culminating in the loss of functional cardiomyocytes. It is important to break down our understanding of the injury into parts that are manageable and understandable. The present study targeted the cardiomyocyte as the *in vitro* cell type because it represents the functional unit of contraction within the heart and is therefore arguably the most important cell type with regards to cardiac function which suffers during ischemia reperfusion injury. OxPCs analyzed from cardiomyocytes exposed to simulated IR injury showing that there were a number of species produced. Since this model used primary cultures of post-natal rat cardiomyocytes, the study may underestimate the response to simulated ischemia as the post-natal myocytes are more resilient to cell culture and pathological stress than adult cardiomyocytes.

The significant OxPC production within cardiomyocytes indicates that cardiomyocytes are a source of these bioactive compounds in the setting of the ischemic myocardium. The OxPCs induce cell death of different cell types in the heart including macrophages, vascular smooth muscle cells and, as demonstrated in this study the cardiomyocytes themselves. As we propose that there is a mitochondrial-based mechanism for the pro-apoptotic effects of OxPC in cardiomyocytes, it becomes very important to characterize the mitochondrial response. The four standards of fragmented aldehyde species and carboxylic acids cause the mitochondria of the cardiomyocytes to become

permeable potentially activating the intrinsic mechanism of apoptotic cell death by cytochrome C release.

Together the results suggest that during IR injury there is increased production of fragmented OxPC molecules that induce apoptotic signaling pathways via increased permeability of the mitochondrial membrane or by endogenous OxPC generation in the mitochondrial membranes making them porous. This study reports that the EO6 antibody blocks the apoptotic activity of fragmented OxPC molecules; potentially the antibody acts as a sink outside of the cells to clear away bioactive fragmented OxPC therefore dampening the loss of cardiomyocytes by cell death.

Future Directions

This is the first description of the production and activity of OxPC within rat cardiomyocytes and the results are promising and hypothesis-generating as there is incomplete information about the cell signaling that occurs within cardiomyocytes when exposed to OxPC and IR treatments. Certain experiments that would need to be conducted would include characterizing the type of cell death further. One such technique could be the analysis of nuclear DNA fragmentation to determine if the cell death is apoptotic. It may also be useful for further studies to characterize if the process is caspase-dependent, which is most likely induced upon cytochrome C release.

A useful experiment would be to determine if the action of OxPC is mediated only by the mPTP. This could be determined by incubating cells with CsA at the same time as OxPC treatment to see if the inhibition of mPTP opening sufficiently reduces cell death and mitochondrial permeability induced by the aldehyde and carboxylic acid OxPCs.

The data presented in this thesis provides information about the production of the OxPCs at the whole cell level, determining the subcellular structures that produce these compounds could further describe the mechanism by which the OxPCs exert their bioactivity. This could be done by immunocytochemistry utilizing the mouse EO6 antibody as the primary and a fluorescent goat anti-mouse IgM secondary antibody, using a previously described protocol for dendritic cells.[124] A second, more quantitative approach could be to run lipidomics analysis of subcellular fractionations of the mitochondria, Golgi, nuclei, and endoplasmic reticulum of cardiomyocytes.

Protein studies are also important in the case of OxPCs as they have been previously shown to form Schiff bases with protein residues.[197, 198] It has also been shown that the ability of the EO6 antibody to bind to free OxPC can be significantly influenced by the amount of proteins modified by OxPC.[170] Protein modification by OxPC is an important aspect that is beyond the scope of this thesis. Determining if there are important proteins that are modulated during IR by OxPC may be part of the inflammatory and dys-regulated processes that underlie IR injury. Particular proteins of interest might be TNF-α modulation as a result of exogenous OxPC exposure which has been shown to be important in IR, as well as PPARγ which has been shown to be responsive to OxPCs.[199, 200] Another possible avenue of biomarker research could be to correlate the production of OxPC with TNF-α in IR injured cardiomyocytes and cardiac tissue. This would be important to link the amount of pro-apoptotic signaling within the muscle with the generation of the bioactive OxPCs inducing that response.

There are changes in gene response that are regulated by OxPC treatment, which has been previously shown in VSMC.[69] A similar analysis of the gene regulation environment

in cardiomyocytes may be important in narrowing down key mediators of OxPC bioactivity for each compound. As well determining the regulation of particular cell signaling pathways like PI3K, ERK, JAK-STAT, MAPK, and NFκB signaling could help to determine which pathways appear to be upregulated the most during OxPC-mediated cell death.

The study also leads us to consider how EO6 treatment may benefit cells during IR, even though OxPCs are still produced. IR injury is multifaceted in nature and there are other factors that could induce OxPC-independent cell death which makes the model used important for the future study.[201] Utilizing a relevant animal model of IR injury, ideally the murine model to reduce cross-reactivity, to determine the efficacy of the mouse IgM EO6 antibody in reducing IR injury would yield important information about the organ level response to EO6 and to identify any inflammatory side effects that may be altered after EO6 treatment. As well, the method of application could be compared to determine if systemic or localized administration of the antibody is most feasible, practical, or effective. This type of study would have to be performed in a large animal model, such as the porcine model, to address the technical aspects of the procedure.[201]

One of the more intriguing ideas in cardiac disease modeling is the use of human induced-pluripotent stem cells (iPSC) to produce human cardiomyocytes.[202] This technique is promoted as an useful model that more accurately models the human cell type that could be used for drug discovery.[203] Perhaps the techniques of this study could be applied to these human iPSC-derived cardiomyocytes to recapitulate the results of this study and to confirm efficacy in human cells. This could help to avoid the lack of success previously seen from IR therapy trials in the past.[18]

Limitations

Lipidomics is a relatively nascent field and as a result there are limitations to the supporting technologies and sciences, like organic chemistry. The precise identity of the compounds cannot be established without a doubt as the technical aspects of organic chemistry have not been able to produce all of the species of OxPC that have been previously hypothesized to exist. Until this is possible and we can inject pure samples of each phospholipid species for analysis, we are limited in the complete clarity of the structure we have identified; however, based on the chromatography and fragmentation analysis we can be reasonably confident that the compounds are oxidized phosphatidylcholines, and we can detect POVPC, PONPC, PGPC, and PAzPC reliably in cell extracts.

We are also limited by the small amount of cells and only one cell type found in this analysis. We know that during IR the response in each cell type is most likely different and is dependent on minimizing the ischemic time before an individual is reperfused. Temporal and environmental factors from patient to patient are difficult to control in patients and therefore, an *in vivo* animal model or human cardiomyocyte cell culture from induced pluripotent stem cells is needed to provide strong evidence to move forward. There could be a larger reservoir of oxidized phospholipids in another cell type that may contribute to more cell death within the heart. As well our study is unable to comment on the fibroblasts within the heart that would influence healing after IR injury and importantly, the extracellular matrix that would be produced in these circumstances. The extracellular matrix may also protect cardiomyocytes from the direct effects of OxPCs, even though we

have shown that they respond to them. The ability of the heart's scaffolding to modulate cell response is always important to consider here.

The scope of this thesis was to characterize endpoints of cell signaling like cell viability and mitochondrial permeability. A more rigorous approach could be taken to further describe the particular proteins that are being up-regulated in response to OxPCs and IR, like described above for TNF-α, to characterize the particular pathways that are induced within cardiomyocytes. This would give us better understanding of how to prevent cardiomyocyte cell death in the myocardium after IR.

A key limitation is that the EO6 antibody is uncharacterized for the OxPCs that they are capable of binding. For that matter, although the EO6 antibody appears to be able to non-specifically block any oxidized phosphatidylcholines that are fragmented; we cannot comment on its reactivity with the hydroxide or hydroperoxides that are produced as the seminal step in fragmented oxidized phosphatidylcholine synthesis. There could be beneficial OxPC species that are required for recovery processes. The antibody's recognition ability of other types of oxidized phosphatidylcholines should be investigated as well as if it can interact with other phospholipid classes if it has not already been done. From a clinical level, the non-specific effects of the antibody in other organs within the body could be concerning especially if the process of humanizing this particular antibody proves to be a challenge in the future. This would have to be investigated in an *in vivo* model of ischemia reperfusion injury or in the previously described iPSC-derived cardiomyocyte system described above.

Chapter V: Study Conclusion

This novel study, for the first time described the unknown role of OxPC within post-natal rat cardiomyocytes in a model of simulated IR injury. During IR, oxidative stress causes production of OxPC within cardiomyocytes, which influences cellular signaling. The fragmented OxPCs, in particular, have significant negative influence on cardiomyocyte cell viability and mitochondrial permeability. Further research is needed to confirm that the intrinsic pathway of apoptotic cell death is induced, as these results show increased mitochondrial permeability a marker of this mechanism. Our crucial finding is that the EO6 antibody significantly inhibits fragmented OxPC-induced cell death of cardiomyocytes *in vitro*. This antibody could be part of a future treatment program used in the clinical setting to ameliorate the continued loss of heart function post-reperfusion during life-saving therapies, like PCI or heart transplant. The OxPCs are a novel therapeutic target for therapy against IR injury attenuating their potent biological activity.

Literature Cited

1. White CW, Ali A, Hasanally D, Xiang B, Li Y, Mundt P, Lytwyn M, Colah S, Klein J, Ravandi A, Arora RC, Lee TW, Hryshko L, Large S, Tian G, Freed DH. A cardioprotective preservation strategy employing ex vivo heart perfusion facilitates successful transplant of donor hearts after cardiocirculatory death. *The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation*. 2013;32:734-743
2. Mackay J, Mensah GA, Mendis S, Greenlund K. The atlas of heart disease and stroke. *World Health Organization*. 2004
3. Statistics-Canada. Mortality, summary list of causes. 2012;2009:125
4. Sponder M, Fritzer-Szekeres M, Marculescu R, Litschauer B, Strametz-Juranek J. A new coronary artery disease grading system correlates with numerous routine parameters that were associated with atherosclerosis: A grading system for coronary artery disease severity. *Vascular health and risk management*. 2014;10:641-647
5. Glass C, Witztum J. Atherosclerosis. The road ahead. *Cell*. 2001;104:503-516
6. Rezkalla S, Kloner R. Coronary no-reflow phenomenon. *Current treatment options in cardiovascular medicine*. 2005;7:75-80
7. Gottlieb R. Mitochondrial signaling in apoptosis: Mitochondrial daggers to the breaking heart. *Basic research in cardiology*. 2003;98:242-249
8. Piper H, García-Dorado D, Ovize M. A fresh look at reperfusion injury. *Cardiovascular research*. 1998;38:291-300

9. Prasad V, Cheung M, Cifu A. Chest pain in the emergency department: The case against our current practice of routine noninvasive testing. *Archives of internal medicine*. 2012;172:1506-1509

10. Weaver W, Simes R, Betriu A, Grines C, Zijlstra F, Garcia E, Grinfeld L, Gibbons R, Ribeiro E, DeWood M, Ribichini F. Comparison of primary coronary angioplasty and intravenous thrombolytic therapy for acute myocardial infarction: A quantitative review. *JAMA : the journal of the American Medical Association*. 1997;278:2093-2098

11. Keeley E, Boura J, Grines C. Primary angioplasty versus intravenous thrombolytic therapy for acute myocardial infarction: A quantitative review of 23 randomised trials. *Lancet*. 2003;361:13-20

12. Harrison RW, Aggarwal A, Ou F-S, Klein LW, Rumsfeld JS, Roe MT, Wang TY, American College of Cardiology National Cardiovascular Data R. Incidence and outcomes of no-reflow phenomenon during percutaneous coronary intervention among patients with acute myocardial infarction. *The American journal of cardiology*. 2013;111:178-184

13. Kolh P, Windecker S, Alfonso F, Collet J-PP, Cremer J, Falk V, Filippatos G, Hamm C, Head SJ, Jüni P, Kappetein AP, Kastrati A, Knuuti J, Landmesser U, Laufer G, Neumann F-JJ, Richter DJ, Schauerte P, Sousa Uva M, Stefanini GG, Taggart DP, Torracca L, Valgimigli M, Wijns W, Witkowski A, Zamorano JL, Achenbach S, Baumgartner H, Bax JJ, Bueno H, Dean V, Deaton C, Erol C, Fagard R, Ferrari R, Hasdai D, Hoes AW, Kirchhof P, Lancellotti P, Linhart A, Nihoyannopoulos P, Piepoli MF, Ponikowski P, Sirnes PA, Tamargo JL, Tendera

M, Torbicki A, Pepper J, Anyanwu A, Badimon L, Bauersachs J, Baumbach A, Beygui F, Bonaros N, De Carlo M, Dobrev D, Dunning J, Eeckhout E, Gielen S, Luckraz H, Mahrholdt H, Montalescot G, Paparella D, Rastan AJ, Sanmartin M, Sergeant P, Silber S, Tamargo J, Ten Berg J, Thiele H, van Geuns R-JJ, Wagner H-OO, Wassmann S, Wendler O. 2014 esc/eacts guidelines on myocardial revascularization: The task force on myocardial revascularization of the european society of cardiology (esc) and the european association for cardio-thoracic surgery (eacts)developed with the special contribution of the european association of percutaneous cardiovascular interventions (eapci). *European journal of cardio-thoracic surgery : official journal of the European Association for Cardio-thoracic Surgery*. 2014;46:517-592

14. Akar FG, Aon MA, Tomaselli GF, O'Rourke B. The mitochondrial origin of postischemic arrhythmias. *The Journal of clinical investigation*. 2005;115:3527-3535

15. Kloner RA, Bolli R, Marban E, Reinlib L, Braunwald E. Medical and cellular implications of stunning, hibernation, and preconditioning: An nhlbi workshop. *Circulation*. 1998;97:1848-1867

16. Robbers LF, Eerenberg ES, Teunissen PF, Jansen MF, Hollander MR, Horrevoets AJ, Knaapen P, Nijveldt R, Heymans MW, Levi MM, van Rossum AC, Niessen HW, Marcu CB, Beek AM, Royen Nv. Magnetic resonance imaging-defined areas of microvascular obstruction after acute myocardial infarction represent microvascular destruction and haemorrhage. *European heart journal*. 2013;34:2346-2353

17. Gottlieb R, Burleson K, Kloner R, Babior B, Engler R. Reperfusion injury induces apoptosis in rabbit cardiomyocytes. *The Journal of clinical investigation*. 1994;94:1621-1628

18. Hausenloy DJ, Yellon DM. Myocardial ischemia-reperfusion injury: A neglected therapeutic target. *The Journal of clinical investigation*. 2013;123:92-100

19. Dupré-Crochet S, Erard M, Nüβe O. Ros production in phagocytes: Why, when, and where? *Journal of leukocyte biology*. 2013;94:657-670

20. Apel K, Hirt H. Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. *Annual review of plant biology*. 2004;55:373-399

21. Murphy MP. How mitochondria produce reactive oxygen species. *The Biochemical journal*. 2009;417:1-13

22. Lenaz G. The mitochondrial production of reactive oxygen species: Mechanisms and implications in human pathology. *IUBMB life*. 2001;52:159-164

23. Guzy RD, Schumacker PT. Oxygen sensing by mitochondria at complex iii: The paradox of increased reactive oxygen species during hypoxia. *Experimental physiology*. 2006;91:807-819

24. Schofield CJ, Ratcliffe PJ. Oxygen sensing by hif hydroxylases. *Nature Reviews Molecular Cell Biology*. 2004;5:343-354

25. Cour M, Gomez L, Mewton N, Ovize M, Argaud L. Postconditioning: From the bench to bedside. *Journal of cardiovascular pharmacology and therapeutics*. 2011;16:117-130

26. Oskolkova O, Afonyushkin T, Preinerstorfer B, Bicker W, von Schlieffen E, Hainzl E, Demyanets S, Schabbauer G, Lindner W, Tselepis A, Wojta J, Binder B,

Bochkov V. Oxidized phospholipids are more potent antagonists of lipopolysaccharide than inducers of inflammation. *Journal of immunology (Baltimore, Md. : 1950)*. 2010;185:7706-7712

27. Lambeth J. Nox/duox family of nicotinamide adenine dinucleotide (phosphate) oxidases. *Current opinion in hematology*. 2002;9:11-17

28. Crompton M. Mitochondrial intermembrane junctional complexes and their role in cell death. *The Journal of physiology*. 2000;529 Pt 1:11-21

29. Regula KM, Ens K, Kirshenbaum LA. Mitochondria-assisted cell suicide: A license to kill. *Journal of molecular and cellular cardiology*. 2003;35:559-567

30. Saini HK, Dhalla NS. Defective calcium handling in cardiomyocytes isolated from hearts subjected to ischemia-reperfusion. *American journal of physiology. Heart and circulatory physiology*. 2005;288:H2260-H2270

31. Leung AW, Halestrap AP. Recent progress in elucidating the molecular mechanism of the mitochondrial permeability transition pore. *Biochimica et biophysica acta*. 2007;1777:946-952

32. Li H-XX, Zhou Y-FF, Zhao X, Jiang B, Yang X-JJ. Gata-4 protects against hypoxia-induced cardiomyocyte injury: Effects on mitochondrial membrane potential. *Canadian Journal of Physiology and Pharmacology*. 2014;92:669-678

33. Piper H, Meuter K, Schäfer C. Cellular mechanisms of ischemia-reperfusion injury. *The Annals of thoracic surgery*. 2003;75:S644-648

34. Avkiran M, Marber MS. Na(+)/h(+) exchange inhibitors for cardioprotective therapy: Progress, problems and prospects. *Journal of the American College of Cardiology*. 2002;39:747-753

35. Nahrendorf M, Swirski FK, Aikawa E, Stangenberg L, Wurdinger T, Figueiredo J-LL, Libby P, Weissleder R, Pittet MJ. The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. *The Journal of experimental medicine*. 2007;204:3037-3047

36. Zouggari Y, Ait-Oufella H, Bonnin P, Simon T, Sage AP, Guérin C, Vilar J, Caligiuri G, Tsiantoulas D, Laurans L, Dumeau E, Kotti S, Bruneval P, Charo IF, Binder CJ, Danchin N, Tedgui A, Tedder TF, Silvestre J-SS, Mallat Z. B lymphocytes trigger monocyte mobilization and impair heart function after acute myocardial infarction. *Nature medicine*. 2013;19:1273-1280

37. Turer A, Hill J. Pathogenesis of myocardial ischemia-reperfusion injury and rationale for therapy. *The American journal of cardiology*. 2010;106:360-368

38. Bochkov V, Oskolkova O, Birukov K, Levonen A-L, Binder C, Stöckl J. Generation and biological activities of oxidized phospholipids. *Antioxidants & redox signaling*. 2010;12:1009-1059

39. McIntyre T. Bioactive oxidatively truncated phospholipids in inflammation and apoptosis: Formation, targets, and inactivation. *Biochimica et biophysica acta*. 2012;1818:2456-2464

40. Chen R, Yang L, McIntyre T. Cytotoxic phospholipid oxidation products. Cell death from mitochondrial damage and the intrinsic caspase cascade. *The Journal of biological chemistry*. 2007;282:24842-24850

41. Yellon DM, Hausenloy DJ. Myocardial reperfusion injury. *The New England Journal of Medicine*. 2007;357:1121-1135

42. Sörensson P, Saleh N, Bouvier F, Böhm F, Settergren M, Caidahl K, Tornvall P, Arheden H, Rydén L, Pernow J. Effect of postconditioning on infarct size in patients with st elevation myocardial infarction. *Heart (British Cardiac Society)*. 2010;96:1710-1715

43. Lønborg J, Kelbaek H, Vejlstrup N, Jørgensen E, Helqvist S, Saunamäki K, Clemmensen P, Holmvang L, Treiman M, Jensen JS, Engstrøm T. Cardioprotective effects of ischemic postconditioning in patients treated with primary percutaneous coronary intervention, evaluated by magnetic resonance. *Circulation. Cardiovascular interventions*. 2010;3:34-41

44. Thibault H, Piot C, Staat P, Bontemps L, Sportouch C, Rioufol G, Cung TT, Bonnefoy E, Angoulvant D, Aupetit J-FF, Finet G, André-Fouët X, Macia JC, Raczka F, Rossi R, Itti R, Kirkorian G, Derumeaux G, Ovize M. Long-term benefit of postconditioning. *Circulation*. 2008;117:1037-1044

45. Staat P, Rioufol G, Piot C, Cottin Y, Cung TT, L'Huillier I, Aupetit J-FF, Bonnefoy E, Finet G, André-Fouët X, Ovize M. Postconditioning the human heart. *Circulation*. 2005;112:2143-2148

46. Tarantini G, Favaretto E, Marra MP, Frigo AC, Napodano M, Cacciavillani L, Giovagnoni A, Renda P, De Biasio V, Plebani M, Mion M, Zaninotto M, Isabella G, Bilato C, Iliceto S. Postconditioning during coronary angioplasty in acute myocardial infarction: The post-ami trial. *International journal of cardiology*. 2012;162:33-38

47. Freixa X, Bellera N, Ortiz-Pérez JT, Jiménez M, Paré C, Bosch X, De Caralt TM, Betriu A, Masotti M. Ischaemic postconditioning revisited: Lack of effects on

infarct size following primary percutaneous coronary intervention. *European heart journal*. 2011;33:103-112

48. Cain BS, Meldrum DR, Dinarello CA, Meng X, Banerjee A, Harken AH. Adenosine reduces cardiac tnf-α production and human myocardial injury following ischemia-reperfusion. *Journal of Surgical Research*. 1998;76:117123

49. Auchampach J, Ge Z-D, Wan T, Moore J, Gross G. A3 adenosine receptor agonist ib-meca reduces myocardial ischemia-reperfusion injury in dogs. *American journal of physiology. Heart and circulatory physiology*. 2003;285:H607-613

50. Ross AM, Gibbons RJ, Stone GW, Kloner RA, Alexander RW, Investigators A-I. A randomized, double-blinded, placebo-controlled multicenter trial of adenosine as an adjunct to reperfusion in the treatment of acute myocardial infarction (amistad-ii). *Journal of the American College of Cardiology*. 2005;45:1775-1780

51. Busauschina A, Schneulle P, van der Woude FJ. Cyclosporine nephrotoxicity. *Transplantation Proceedings*. 2004;36:229S-233S

52. Goldhaber J, Weiss J. Oxygen free radicals and cardiac reperfusion abnormalities. *Hypertension*. 1992;20:118-127

53. Kikuchi K, Tancharoen S, Takeshige N, Yoshitomi M, Morioka M, Murai Y, Tanaka E. The efficacy of edaravone (radicut), a free radical scavenger, for cardiovascular disease. *International journal of molecular sciences*. 2013;14:13909-13930

54. Lucas D, Szweda L. Cardiac reperfusion injury: Aging, lipid peroxidation, and mitochondrial dysfunction. *Proceedings of the National Academy of Sciences of the United States of America*. 1998;95:510-514

55. Reis A, Spickett C. Chemistry of phospholipid oxidation. *Biochimica et biophysica acta*. 2012;1818:2374-2387

56. Greig F, Kennedy S, Spickett C. Physiological effects of oxidized phospholipids and their cellular signaling mechanisms in inflammation. *Free radical biology & medicine*. 2012;52:266-280

57. Post J, Verkleij A, Langer G. Organization and function of sarcolemmal phospholipids in control and ischemic/reperfused cardiomyocytes. *Journal of molecular and cellular cardiology*. 1995;27:749-760

58. Samhan-Arias A, Ji J, Demidova O, Sparvero L, Feng W, Tyurin V, Tyurina Y, Epperly M, Shvedova A, Greenberger J, Bayır H, Kagan V, Amoscato A. Oxidized phospholipids as biomarkers of tissue and cell damage with a focus on cardiolipin. *Biochimica et biophysica acta*. 2012;1818:2413-2423

59. Tsutsui H, Kinugawa S, Matsushima S. Oxidative stress and heart failure. *American journal of physiology. Heart and circulatory physiology*. 2011;301:H2181-2190

60. Weismann D, Binder C. The innate immune response to products of phospholipid peroxidation. *Biochimica et biophysica acta*. 2012;1818:2465-2475

61. Chisolm G, Steinberg D. The oxidative modification hypothesis of atherogenesis: An overview. *Free radical biology & medicine*. 2000;28:1815-1826

62. Fessel J, Porter N, Moore K, Sheller J, Roberts L. Discovery of lipid peroxidation products formed in vivo with a substituted tetrahydrofuran ring (isofurans) that are favored by increased oxygen tension. *Proceedings of the National Academy of Sciences of the United States of America*. 2002;99:16713-16718

63. Weinstein E, Li H, Lawson J, Rokach J, FitzGerald G, Axelsen P. Prothrombinase acceleration by oxidatively damaged phospholipids. *The Journal of biological chemistry*. 2000;275:22925-22930

64. Marathe G, Zimmerman G, Prescott S, McIntyre T. Activation of vascular cells by paf-like lipids in oxidized ldl. *Vascular pharmacology*. 2002;38:193-200

65. Tsimikas S, Lau H, Han K-R, Shortal B, Miller E, Segev A, Curtiss L, Witztum J, Strauss B. Percutaneous coronary intervention results in acute increases in oxidized phospholipids and lipoprotein(a): Short-term and long-term immunologic responses to oxidized low-density lipoprotein. *Circulation*. 2004;109:3164-3170

66. Tsimikas S, Brilakis E, Miller E, McConnell J, Lennon R, Kornman K, Witztum J, Berger P. Oxidized phospholipids, lp(a) lipoprotein, and coronary artery disease. *The New England journal of medicine*. 2005;353:46-57

67. Hörkkö S, Bird D, Miller E, Itabe H, Leitinger N, Subbanagounder G, Berliner J, Friedman P, Dennis E, Curtiss L, Palinski W, Witztum J. Monoclonal autoantibodies specific for oxidized phospholipids or oxidized phospholipid-protein adducts inhibit macrophage uptake of oxidized low-density lipoproteins. *The Journal of clinical investigation*. 1999;103:117-128

68. Stemmer U, Dunai Z, Koller D, Pürstinger G, Zenzmaier E, Deigner H, Aflaki E, Kratky D, Hermetter A. Toxicity of oxidized phospholipids in cultured macrophages. *Lipids in health and disease*. 2012;11:110-123

69. Gargalovic P, Imura M, Zhang B, Gharavi N, Clark M, Pagnon J, Yang W-P, He A, Truong A, Patel S, Nelson S, Horvath S, Berliner J, Kirchgessner T, Lusis A. Identification of inflammatory gene modules based on variations of human

endothelial cell responses to oxidized lipids. *Proceedings of the National Academy of Sciences of the United States of America*. 2006;103:12741-12746

70. Loidl A, Sevcsik E, Riesenhuber G, Deigner H-P, Hermetter A. Oxidized phospholipids in minimally modified low density lipoprotein induce apoptotic signaling via activation of acid sphingomyelinase in arterial smooth muscle cells. *The Journal of biological chemistry*. 2003;278:32921-32928

71. Ali A, White P, Xiang B, Lin HY, Tsui S, Ashley E, Lee T, Klein J, Kumar K, Arora R, Large S, Tian G, Freed D. Hearts from dcd donors display acceptable biventricular function after heart transplantation in pigs. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons*. 2011;11:1621-1632

72. White CW, Hasanally D, Mundt P, Li Y, Xiang B, Klein J, Müller A, Ambrose E, Ravandi A, Arora RC, Lee TW, Hryshko LV, Large S, Tian G, Freed DH. A whole blood-based perfusate provides superior preservation of myocardial function during ex vivo heart perfusion. *The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation*. 2015;34:113-121

73. Zweier J, Talukder M. The role of oxidants and free radicals in reperfusion injury. *Cardiovascular research*. 2006;70:181-190

74. Vinten-Johansen J. Involvement of neutrophils in the pathogenesis of lethal myocardial reperfusion injury. *Cardiovascular research*. 2004;61:481-497

75. Frangogiannis N, Smith C, Entman M. The inflammatory response in myocardial infarction. *Cardiovascular research*. 2002;53:31-47

76. Baines CP. How and when do myocytes die during ischemia and reperfusion the late phase. *Journal of cardiovascular pharmacology and therapeutics*. 2011;16:239-243

77. Schneider C, Porter NA, Brash AR. Routes to 4-hydroxynonenal: Fundamental issues in the mechanisms of lipid peroxidation. *The Journal of biological chemistry*. 2008;283:15539-15543

78. Allen D, Hasanally D, Ravandi A. Role of oxidized phospholipids in cardiovascular pathology. *Clinical Lipidology*. 2013;8:205-215

79. Nonas S, Miller I, Kawkitinarong K, Chatchavalvanich S, Gorshkova I, Bochkov VN, Leitinger N, Natarajan V, Garcia JG, Birukov KG. Oxidized phospholipids reduce vascular leak and inflammation in rat model of acute lung injury. *American journal of respiratory and critical care medicine*. 2006;173:1130-1138

80. Ravandi A, Babaei S, Leung R, Monge J, Hoppe G, Hoff H, Kamido H, Kuksis A. Phospholipids and oxophospholipids in atherosclerotic plaques at different stages of plaque development. *Lipids*. 2004;39:97-109

81. Li R, Mouillesseaux K, Montoya D, Cruz D, Gharavi N, Dun M, Koroniak L, Berliner J. Identification of prostaglandin e2 receptor subtype 2 as a receptor activated by oxpapc. *Circulation research*. 2006;98:642-650

82. Birukova A, Fu P, Chatchavalvanich S, Burdette D, Oskolkova O, Bochkov V, Birukov K. Polar head groups are important for barrier-protective effects of oxidized phospholipids on pulmonary endothelium. *American journal of physiology. Lung cellular and molecular physiology*. 2007;292:35

83. Furukawa M, Gohda T, Tanimoto M, Tomino Y. Pathogenesis and novel treatment from the mouse model of type 2 diabetic nephropathy. *TheScientificWorldJournal*. 2013;2013:1-8

84. Paschos A, Pandya R, Duivenvoorden WC, Pinthus JH. Oxidative stress in prostate cancer: Changing research concepts towards a novel paradigm for prevention and therapeutics. *Prostate cancer and prostatic diseases*. 2013;16:217-225

85. Hammond V, Morgan A, Lauder S, Thomas C, Brown S, Freeman B, Lloyd C, Davies J, Bush A, Levonen A-L, Kansanen E, Villacorta L, Chen Y, Porter N, Garcia-Diaz Y, Schopfer F, O'Donnell V. Novel keto-phospholipids are generated by monocytes and macrophages, detected in cystic fibrosis, and activate peroxisome proliferator-activated receptor-γ. *The Journal of biological chemistry*. 2012;287:41651-41666

86. Hernandes MS, Britto LR. Nadph oxidase and neurodegeneration. *Current neuropharmacology*. 2012;10:321-327

87. Wenk M. Lipidomics: New tools and applications. *Cell*. 2010;143:888-895

88. Han X, Gross RW. Electrospray ionization mass spectroscopic analysis of human erythrocyte plasma membrane phospholipids. *Proceedings of the National Academy of Sciences of the United States of America*. 1994;91:10635-10639

89. Hasanally D, Chaudhary R, Ravandi A. Role of phospholipases and oxidized phospholipids in inflammation. In: Tappia PS, Dhalla NS, eds. *Phospholipases in health and disease*. Springer New York; 2014:55-72.

90. Nakanishi H, Iida Y, Shimizu T, Taguchi R. Analysis of oxidized phosphatidylcholines as markers for oxidative stress, using multiple reaction

monitoring with theoretically expanded data sets with reversed-phase liquid chromatography/tandem mass spectrometry. *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences*. 2009;877:1366-1374

91. Gruber F, Bicker W, Oskolkova O, Tschachler E, Bochkov V. A simplified procedure for semi-targeted lipidomic analysis of oxidized phosphatidylcholines induced by uva irradiation. *Journal of lipid research*. 2012;53:1232-1242

92. Quehenberger O, Armando A, Brown A, Milne S, Myers D, Merrill A, Bandyopadhyay S, Jones K, Kelly S, Shaner R, Sullards C, Wang E, Murphy R, Barkley R, Leiker T, Raetz C, Guan Z, Laird G, Six D, Russell D, McDonald J, Subramaniam S, Fahy E, Dennis E. Lipidomics reveals a remarkable diversity of lipids in human plasma. *Journal of lipid research*. 2010;51:3299-3305

93. Weir JM, Wong G, Barlow CK, Greeve MA, Kowalczyk A, Almasy L, Comuzzie AG, Mahaney MC, Jowett JB, Shaw J, Curran JE, Blangero J, Meikle PJ. Plasma lipid profiling in a large population-based cohort. *Journal of lipid research*. 2013;54:2898-2908

94. Andreyev A, Fahy E, Guan Z, Kelly S, Li X, McDonald J, Milne S, Myers D, Park H, Ryan A, Thompson B, Wang E, Zhao Y, Brown H, Merrill A, Raetz C, Russell D, Subramaniam S, Dennis E. Subcellular organelle lipidomics in tlr-4-activated macrophages. *Journal of lipid research*. 2010;51:2785-2797

95. Dennis E, Deems R, Harkewicz R, Quehenberger O, Brown H, Milne S, Myers D, Glass C, Hardiman G, Reichart D, Merrill A, Sullards M, Wang E, Murphy R, Raetz C, Garrett T, Guan Z, Ryan A, Russell D, McDonald J, Thompson B, Shaw W, Sud

M, Zhao Y, Gupta S, Maurya M, Fahy E, Subramaniam S. A mouse macrophage lipidome. *The Journal of biological chemistry*. 2010;285:39976-39985

96. Moore KJ, Sheedy FJ, Fisher EA. Macrophages in atherosclerosis: A dynamic balance. *Nature Reviews Immunology*. 2013;13:709-721

97. Prescott SM, Zimmerman GA, Stafforini DM, McIntyre TM. Platelet-activating factor and related lipid mediators. *Annual review of biochemistry*. 1999;69:419-445

98. Tyurina Y, Tyurin V, Zhao Q, Djukic M, Quinn P, Pitt B, Kagan V. Oxidation of phosphatidylserine: A mechanism for plasma membrane phospholipid scrambling during apoptosis? *Biochemical and biophysical research communications*. 2004;324:1059-1064

99. Thomas C, Morgan L, Maskrey B, Murphy R, Kühn H, Hazen S, Goodall A, Hamali H, Collins P, O'Donnell V. Phospholipid-esterified eicosanoids are generated in agonist-activated human platelets and enhance tissue factor-dependent thrombin generation. *The Journal of biological chemistry*. 2010;285:6891-6903

100. Podrez E, Byzova T, Febbraio M, Salomon R, Ma Y, Valiyaveettil M, Poliakov E, Sun M, Finton P, Curtis B, Chen J, Zhang R, Silverstein R, Hazen S. Platelet cd36 links hyperlipidemia, oxidant stress and a prothrombotic phenotype. *Nature medicine*. 2007;13:1086-1095

101. Androulakis N, Durand H, Ninio E, Tsoukatos DC. Molecular and mechanistic characterization of platelet-activating factor-like bioactivity produced upon ldl oxidation. *Journal of lipid research*. 2005;46:1923-1932

102. Singleton PA, Chatchavalvanich S, Fu P, Xing J, Birukova AA, Fortune JA, Klibanov AM, Garcia JG, Birukov KG. Akt-mediated transactivation of the s1p1

receptor in caveolin-enriched microdomains regulates endothelial barrier enhancement by oxidized phospholipids. *Circulation research*. 2009;104:978-986

103. Marathe GK, Davies SS, Harrison KA, Silva AR, Murphy RC, Castro-Faria-Neto H, Prescott SM, Zimmerman GA, McIntyre TM. Inflammatory platelet-activating factor-like phospholipids in oxidized low density lipoproteins are fragmented alkyl phosphatidylcholines. *The Journal of biological chemistry*. 1999;274:28395-28404
104. Salvayre R, Auge N, Benoist H, Negre-Salvayre A. Oxidized low-density lipoprotein-induced apoptosis. *Biochimica et Biophysica Acta*. 2002;1585:213-221
105. Walton K, Hsieh X, Gharavi N, Wang S, Wang G, Yeh M, Cole A, Berliner J. Receptors involved in the oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine-mediated synthesis of interleukin-8. A role for toll-like receptor 4 and a glycosylphosphatidylinositol-anchored protein. *The Journal of biological chemistry*. 2003;278:29661-29666
106. Walton KA, Gugiu BG, Thomas M, Basseri RJ, Eliav DR, Salomon RG, Berliner JA. A role for neutral sphingomyelinase activation in the inhibition of lps action by phospholipid oxidation products. *Journal of lipid research*. 2006;47:1967-1974
107. Berliner JA, Subbanagounder G, Leitinger N, Watson AD, Vora D. Evidence for a role of phospholipid oxidation products in atherogenesis. *Trends in cardiovascular medicine*. 2001;11:142-147
108. Duerr GD, Heinemann JC, Arnoldi V, Feisst A, Kley J, Ghanem A, Welz A, Dewald O. Cardiomyocyte specific peroxisome proliferator-activated receptor-α overexpression leads to irreversible damage in ischemic murine heart. *Life sciences*. 2014;102:88-97

109. Bochkov V. Inflammatory profile of oxidized phospholipids. *Thrombosis and haemostasis*. 2007;97:348-354

110. Zimman A, Mouillesseaux K, Le T, Gharavi N, Ryvkin A, Graeber T, Chen T, Watson A, Berliner J. Vascular endothelial growth factor receptor 2 plays a role in the activation of aortic endothelial cells by oxidized phospholipids. *Arteriosclerosis, thrombosis, and vascular biology*. 2007;27:332-338

111. Tsiantoulas D, Gruber S, Binder C. B-1 cell immunoglobulin directed against oxidation-specific epitopes. *Frontiers in immunology*. 2012;3:415

112. Perry H, Bender T, McNamara C. B cell subsets in atherosclerosis. *Frontiers in immunology*. 2012;3:373

113. Binder C, Chou M-Y, Fogelstrand L, Hartvigsen K, Shaw P, Boullier A, Witztum J. Natural antibodies in murine atherosclerosis. *Current drug targets*. 2008;9:190-195

114. Chou MY, Hartvigsen K, Hansen L, Fogelstrand L, Shaw P, Boullier A, Binder C, Witztum J. Oxidation-specific epitopes are important targets of innate immunity. *Journal of internal medicine*. 2008;263:479-488

115. Shaw PX, Hörkkö S, Chang MK, Curtiss LK, Palinski W, Silverman GJ, Witztum JL. Natural antibodies with the t15 idiotype may act in atherosclerosis, apoptotic clearance, and protective immunity. *The Journal of clinical investigation*. 2000;105:1731-1740

116. Chang M-K, Binder C, Torzewski M, Witztum J. C-reactive protein binds to both oxidized ldl and apoptotic cells through recognition of a common ligand:

Phosphorylcholine of oxidized phospholipids. *Proceedings of the National Academy of Sciences of the United States of America*. 2002;99:13043-13048

117. Chang M-K, Hartvigsen K, Ryu J, Kim Y, Han K. The pro-atherogenic effects of macrophages are reduced upon formation of a complex between c-reactive protein and lysophosphatidylcholine. *Journal of inflammation (London, England)*. 2012;9:42

118. Boullier A, Friedman P, Harkewicz R, Hartvigsen K, Green S, Almazan F, Dennis E, Steinberg D, Witztum J, Quehenberger O. Phosphocholine as a pattern recognition ligand for cd36. *Journal of lipid research*. 2005;46:969-976

119. Febbraio M, Hajjar DP, Silverstein RL. Cd36: A class b scavenger receptor involved in angiogenesis, atherosclerosis, inflammation, and lipid metabolism. *Journal of Clinical Investigation*. 2001;108:785-791

120. Haserück N, Erl W, Pandey D, Tigyi G, Ohlmann P, Ravanat C, Gachet C, Siess W. The plaque lipid lysophosphatidic acid stimulates platelet activation and platelet-monocyte aggregate formation in whole blood: Involvement of p2y1 and p2y12 receptors. *Blood*. 2004;103:2585-2592

121. Göpfert M, Siedler F, Siess W, Sellmayer A. Structural identification of oxidized acyl-phosphatidylcholines that induce platelet activation. *Journal of vascular research*. 2005;42:120-132

122. Berliner J, Leitinger N, Tsimikas S. The role of oxidized phospholipids in atherosclerosis. *Journal of lipid research*. 2009;50 S207-S212

123. Gharavi NM, Baker NA, Mouillesseaux KP, Yeung W, Honda HM, Hsieh X, Yeh M, Smart EJ, Berliner JA. Role of endothelial nitric oxide synthase in the regulation

of srebp activation by oxidized phospholipids. *Circulation research*. 2006;98:768-776

124. Qin J, Testai F, Dawson S, Kilkus J, Dawson G. Oxidized phosphatidylcholine formation and action in oligodendrocytes. *Journal of neurochemistry*. 2009;110:1388-1399

125. Yoshida H, Matsui T, Yamamoto A, Okada T, Mori K. Xbp1 mrna is induced by atf6 and spliced by ire1 in response to er stress to produce a highly active transcription factor. *Cell*. 2001;107:881-891

126. Dinasarapu AR, Gupta S, Maurya MR, Fahy E, Min J, Sud M, Gersten MJ, Glass CK, Subramaniam S. A combined omics study on activated macrophages--enhanced role of stats in apoptosis, immunity and lipid metabolism. *Bioinformatics (Oxford, England)*. 2013;29:2735-2743

127. Lartigue L, Faustin B. Mitochondria: Metabolic regulators of innate immune responses to pathogens and cell stress. *The International Journal of Biochemistry & Cell Biology*. 2013;45:2052-2056

128. Chen R, Feldstein A, McIntyre T. Suppression of mitochondrial function by oxidatively truncated phospholipids is reversible, aided by bid, and suppressed by bcl-xl. *The Journal of biological chemistry*. 2009;284:26297-26308

129. Shih P, Elices M, Fang Z, Ugarova T, Strahl D, Territo M, Frank J, Kovach N, Cabanas C, Berliner J, Vora D. Minimally modified low-density lipoprotein induces monocyte adhesion to endothelial connecting segment-1 by activating beta1 integrin. *The Journal of clinical investigation*. 1999;103:613-625

130. Vora D, Fang Z, Liva S, Tyner T, Parhami F, Watson A, Drake T, Territo M, Berliner J. Induction of p-selectin by oxidized lipoproteins. Separate effects on synthesis and surface expression. *Circulation research*. 1997;80:810-818

131. Birukova A, Starosta V, Tian X, Higginbotham K, Koroniak L, Berliner J, Birukov K. Fragmented oxidation products define barrier disruptive endothelial cell response to oxpapc. *Translational research : the journal of laboratory and clinical medicine*. 2013;161:495-504

132. Kadl A, Galkina E, Leitinger N. Induction of ccr2-dependent macrophage accumulation by oxidized phospholipids in the air-pouch model of inflammation. *Arthritis and rheumatism*. 2009;60:1362-1371

133. Furnkranz A, Schober A, Bochkov V, Bashtrykov P, Kronke G, Kadl A, Binder B, Weber C, Leitinger N. Oxidized phospholipids trigger atherogenic inflammation in murine arteries. *Arteriosclerosis, thrombosis, and vascular biology*. 2005;25:633-638

134. Kopf M, Baumann H, Freer G, Freudenberg M, Lamers M, Kishimoto T, Zinkernagel R, Bluethmann H, Köhler G. Impaired immune and acute-phase responses in interleukin-6-deficient mice. *Nature*. 1994;368:339-342

135. Lopez-Neblina F, Toledo AH, Toledo-Pereyra LH. Molecular biology of apoptosis in ischemia and reperfusion. *Journal of investigative surgery : the official journal of the Academy of Surgical Research*. 2004;18:335-350

136. Orogo AM, Gustafsson ÅBB. Cell death in the myocardium: My heart won't go on. *IUBMB life*. 2013;65:651-656

137. Gustafsson A, Gottlieb R. Mechanisms of apoptosis in the heart. *Journal of clinical immunology*. 2003;23:447-459

138. Halestrap AP, Kerr PM, Javadov S, Woodfield KY. Elucidating the molecular mechanism of the permeability transition pore and its role in reperfusion injury of the heart. *Biochimica et biophysica acta*. 1998;1366:79-94

139. Nagata S. Apoptotic DNA fragmentation. *Experimental cell research*. 2000;256:12-18

140. Condorelli G, Roncarati R, Ross J, Pisani A, Stassi G, Todaro M, Trocha S, Drusco A, Gu Y, Russo MA. Heart-targeted overexpression of caspase3 in mice increases infarct size and depresses cardiac function. *Proceedings of the National Academy of Sciences*. 2001;98:9977-9982

141. de Moissac D, Gurevich RM, Zheng H, Singal PK, Kirshenbaum LA. Caspase activation and mitochondrial cytochrome c release during hypoxia-mediated apoptosis of adult ventricular myocytes. *Journal of molecular and cellular cardiology*. 1999;32:53-63

142. Boatright KM, Salvesen GS. Mechanisms of caspase activation. *Current opinion in cell biology*. 2003;15:725-731

143. Dhanasekaran DN, Reddy EP. Jnk signaling in apoptosis. *Oncogene*. 2008;27:6245-6251

144. Kim H-E, Du F, Fang M, Wang X. Formation of apoptosome is initiated by cytochrome c-induced datp hydrolysis and subsequent nucleotide exchange on apaf-1. *Proceedings of the National Academy of Sciences of the United States of America*. 2005;102:17545-17550

145. Riedl SJ, Salvesen GS. The apoptosome: Signalling platform of cell death. *Nature Reviews Molecular Cell Biology*. 2007;8:405-413

146. Reubold TF, Eschenburg S. A molecular view on signal transduction by the apoptosome. *Cellular signalling*. 2012;24:1420-1425

147. Bonora M, Bononi A, Marchi E, Giorgi C, Lebiedzinska M, Marchi S, Patergnani S, Rimessi A, Suski JM, Wojtala A. Role of the c subunit of the fo atp synthase in mitochondrial permeability transition. *Cell Cycle*. 2013;12:674-683

148. Ong S-BB, Samangouei P, Kalkhoran SB, Hausenloy DJ. The mitochondrial permeability transition pore and its role in myocardial ischemia reperfusion injury. *Journal of molecular and cellular cardiology*. 2015;78C:23-34

149. Wallgren M, Lidman M, Pham QD, Cyprych K, Gröbner G. The oxidized phospholipid pazepc modulates interactions between bax and mitochondrial membranes. *Biochimica et biophysica acta*. 2012;1818:2718-2724

150. Fruhwirth G, Moumtzi A, Loidl A, Ingolic E, Hermetter A. The oxidized phospholipids povpc and pgpc inhibit growth and induce apoptosis in vascular smooth muscle cells. *Biochimica et biophysica acta*. 2006;1761:1060-1069

151. Stemmer U, Dunai Z, Koller D, Pürstinger G, Zenzmaier E, Deigner H, Aflaki E, Kratky D, Hermetter A. Toxicity of oxidized phospholipids in cultured macrophages. *Lipids in health and disease*. 2012;11:110

152. Mughal W, Dhingra R, Kirshenbaum LA. Striking a balance: Autophagy, apoptosis, and necrosis in a normal and failing heart. *Current hypertension reports*. 2012;14:540-547

153. Toldo S, Breckenridge D, Mezzaroma E, Van Tassell B, Shryock J, Kannan H, Phan D, Budas G, Farkas D, Lesnefsky E, Voelkel N, Abbate A. Inhibition of apoptosis signal-regulating kinase 1 reduces myocardial ischemia-reperfusion injury in the mouse. *Journal of the American Heart Association*. 2012;1

154. Tait SWG, Green DR. Caspase-independent cell death: Leaving the set without the final cut. *Oncogene*. 2008;27:6452-6461

155. Galluzzi L, Vitale I, Abrams J, Alnemri E, Baehrecke E, Blagosklonny M, Dawson T, Dawson V, El-Deiry W, Fulda S, Gottlieb E, Green D, Hengartner M, Kepp O, Knight R, Kumar S, Lipton S, Lu X, Madeo F, Malorni W, Mehlen P, Nuñez G, Peter M, Piacentini M, Rubinsztein D, Shi Y, Simon HU, Vandenabeele P, White E, Yuan J, Zhivotovsky B, Melino G, Kroemer G. Molecular definitions of cell death subroutines: Recommendations of the nomenclature committee on cell death 2012. *Cell death and differentiation*. 2012;19:107-120

156. Shahzad T, Kasseckert S, Iraqi W, Johnson V, Schulz R, Schlüter K-D, Dörr O, Parahuleva M, Hamm C, Ladilov Y, Abdallah Y. Mechanisms involved in postconditioning protection of cardiomyocytes against acute reperfusion injury. *Journal of molecular and cellular cardiology*. 2013;58:209-216

157. Aldrovandi M, O'Donnell VB. Oxidized pls and vascular inflammation. *Current atherosclerosis reports*. 2013;15:323

158. Greenberg M, Li X-M, Gugiu B, Gu X, Qin J, Salomon R, Hazen S. The lipid whisker model of the structure of oxidized cell membranes. *The Journal of biological chemistry*. 2008;283:2385-2396

159. Gillotte KL, Hörkkö S, Witztum JL, Steinberg D. Oxidized phospholipids, linked to apolipoprotein b of oxidized ldl, are ligands for macrophage scavenger receptors. *Journal of lipid research*. 2000;41:824-833

160. Miller Y, Choi S-H, Wiesner P, Fang L, Harkewicz R, Hartvigsen K, Boullier A, Gonen A, Diehl C, Que X, Montano E, Shaw P, Tsimikas S, Binder C, Witztum J. Oxidation-specific epitopes are danger-associated molecular patterns recognized by pattern recognition receptors of innate immunity. *Circulation research*. 2011;108:235-248

161. Lin L, Knowlton AA. Innate immunity and cardiomyocytes in ischemic heart disease. *Life sciences*. 2014;100:1-8

162. Palinski W, Hörkkö S, Miller E, Steinbrecher U, Powell H, Curtiss L, Witztum J. Cloning of monoclonal autoantibodies to epitopes of oxidized lipoproteins from apolipoprotein e-deficient mice. Demonstration of epitopes of oxidized low density lipoprotein in human plasma. *The Journal of clinical investigation*. 1996;98:800-814

163. Palinski W, Ylä-Herttuala S, Rosenfeld ME. Antisera and monoclonal antibodies specific for epitopes generated during oxidative modification of low density lipoprotein. *Arteriosclerosis, Thrombosis, and Vascular Biology*. 1990;10:325-335

164. Palinski W, Rosenfeld ME, Ylä-Herttuala SG, G. C., Socher SS, Butler SW, Parthasarathy SC, T. E., Steinberg D, Witztum JL. Low density lipoprotein undergoes oxidative modification in vivo. *Proceedings of the National Academy of Sciences*. 1989;86:1372-1376

165. Ylä-Herttuala S, Palinski W, Rosenfeld M, Parthasarathy S, Carew T, Butler S, Witztum J, Steinberg D. Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man. *The Journal of clinical investigation*. 1989;84:1086-1095

166. Tsimikas S, Miyanohara A, Hartvigsen K, Merki E, Shaw P, Chou M-Y, Pattison J, Torzewski M, Sollors J, Friedmann T, Lai N, Hammond H, Getz G, Reardon C, Li A, Banka C, Witztum J. Human oxidation-specific antibodies reduce foam cell formation and atherosclerosis progression. *Journal of the American College of Cardiology*. 2011;58:1715-1727

167. van Dijk R, Kolodgie F, Ravandi A, Leibundgut G, Hu P, Prasad A, Mahmud E, Dennis E, Curtiss L, Witztum J, Wasserman B, Otsuka F, Virmani R, Tsimikas S. Differential expression of oxidation-specific epitopes and apolipoprotein(a) in progressing and ruptured human coronary and carotid atherosclerotic lesions. *Journal of lipid research*. 2012;53:2773-2790

168. Caligiuri G, Nicoletti A, Poirier B, Hansson GK. Protective immunity against atherosclerosis carried by b cells of hypercholesterolemic mice. *The Journal of clinical investigation*. 2002;109:745-753

169. Chou MY, Fogelstrand L, Hartvigsen K, Hansen LF, Woelkers D, Shaw PX, Choi J, Perkman T, Backhed F, Miller YI, Hörkkö S, Corr M, Witztum JL, Binder CJ. Oxidation-specific epitopes are dominant targets of innate natural antibodies in mice and humans. *The Journal of clinical investigation*. 2009;119:1335-1349

170. Friedman P, Horkko S, Steinberg D, Witztum J, Dennis E. Correlation of antiphospholipid antibody recognition with the structure of synthetic oxidized

phospholipids. Importance of schiff base formation and aldol condensation. *The Journal of biological chemistry*. 2002;277:7010-7020

171. Ullery J, Marnett L. Protein modification by oxidized phospholipids and hydrolytically released lipid electrophiles: Investigating cellular responses. *Biochimica et biophysica acta*. 2012;1818:2424-2435

172. Faria-Neto J, Chyu K-Y, Li X, Dimayuga P, Ferreira C, Yano J, Cercek B, Shah P. Passive immunization with monoclonal igm antibodies against phosphorylcholine reduces accelerated vein graft atherosclerosis in apolipoprotein e-null mice. *Atherosclerosis*. 2006;189:83-90

173. Shaw PX, Horkko S, Tsimikas S, Chang MK, Palinski W, Silverman GJ, Chen PP, Witztum JL. Human-derived anti-oxidized ldl autoantibody blocks uptake of oxidized ldl by macrophages and localizes to atherosclerotic lesions in vivo. *Arteriosclerosis, thrombosis, and vascular biology*. 2001;21

174. Bernhard D, Diethard P, Terezia BA, Sasa MM, Walther S, Achim N, Christian FV. Ischemia-reperfusion injury. *European Journal of Trauma and Emergency Surgery*. 2007;33

175. Kirshenbaum LA, Schneider MD. Adenovirus e1a represses cardiac gene transcription and reactivates DNA synthesis in ventricular myocytes, via alternative pocket protein- and p300-binding domains. *The Journal of biological chemistry*. 1995;270:7791-7794

176. Louis XL, Murphy R, Thandapilly SJ, Yu L, Netticadan T. Garlic extracts prevent oxidative stress, hypertrophy and apoptosis in cardiomyocytes: A role for nitric

oxide and hydrogen sulfide. *BMC complementary and alternative medicine*. 2012;12:140

177. Maddaford TG, Hurtado C, Sobrattee S, Czubryt MP, Pierce GN. A model of low-flow ischemia and reperfusion in single, beating adult cardiomyocytes. *American Journal of Physiology-Heart and Circulatory Physiology*. 1999;277:H788-798

178. Weidman D, Shaw J, Bednarczyk J, Regula K, Yurkova N, Zhang T, Aguilar F, Kirshenbaum L. Dissecting apoptosis and intrinsic death pathways in the heart. *Methods in enzymology*. 2008;446:277-285

179. O'Donnell V. Mass spectrometry analysis of oxidized phosphatidylcholine and phosphatidylethanolamine. *Biochimica et biophysica acta*. 2011;1811:818-826

180. Latchoumycandane C, Marathe G, Zhang R, McIntyre T. Oxidatively truncated phospholipids are required agents of tumor necrosis factor α (tnfα)-induced apoptosis. *The Journal of biological chemistry*. 2012;287:17693-17705

181. Xin M, Olson EN, Bassel-Duby R. Mending broken hearts: Cardiac development as a basis for adult heart regeneration and repair. *Nature Reviews Molecular Cell Biology*. 2013;14:529-541

182. Berdyshev E. Mass spectrometry of fatty aldehydes. *Biochimica et biophysica acta*. 2011;1811:680-693

183. Furuichi K, Wada T, Iwata Y, Kitagawa K, Kobayashi K-I, Hashimoto H, Ishiwata Y, Asano M, Wang H, Matsushima K, Takeya M, Kuziel WA, Mukaida N, Yokoyama H. Ccr2 signaling contributes to ischemia-reperfusion injury in kidney. *Journal of the American Society of Nephrology : JASN*. 2003;14:2503-2515

184. Avlas O, Fallach R, Shainberg A, Porat E, Hochhauser E. Toll-like receptor 4 stimulation initiates an inflammatory response that decreases cardiomyocyte contractility. *Antioxidants & redox signaling*. 2011;15:1895-1909

185. Glazer AN, Peck K, Mathies RA. A stable double-stranded DNA-ethidium homodimer complex: Application to picogram fluorescence detection of DNA in agarose gels. *Proceedings of the National Academy of Sciences of the United States of America*. 1990;87:3851-3855

186. Podrez E, Poliakov E, Shen Z, Zhang R, Deng Y, Sun M, Finton P, Shan L, Febbraio M, Hajjar D, Silverstein R, Hoff H, Salomon R, Hazen S. A novel family of atherogenic oxidized phospholipids promotes macrophage foam cell formation via the scavenger receptor cd36 and is enriched in atherosclerotic lesions. *The Journal of biological chemistry*. 2002;277:38517-38523

187. Kleinbongard P, Heusch G, Schulz R. Tnfα in atherosclerosis, myocardial ischemia/reperfusion and heart failure. *Pharmacology & therapeutics*. 2010;127:295-314

188. Zoratti M, Szabò I. The mitochondrial permeability transition. *Biochimica et Biophysica Acta (BBA)* 1995;1241:139-176

189. Crompton M. The mitochondrial permeability transition pore and its role in cell death. *Biochem. J*. 1999;341:233-249

190. Arnoult D, Parone P, Martinou JC, Antonsson B, Estaquier J, Ameisen JC. Mitochondrial release of apoptosis-inducing factor occurs downstream of cytochrome c release in response to several proapoptotic stimuli. *The Journal of Cell Biology*. 2002;159:923-929

191. Martinou JC, Desagher S, Antonsson B. Cytochrome c release from mitochondria: All or nothing. *Nature cell biology*. 2000;2:E41-E43

192. Shanmuganathan S, Hausenloy D, Duchen M, Yellon D. Mitochondrial permeability transition pore as a target for cardioprotection in the human heart. *American journal of physiology. Heart and circulatory physiology*. 2005;289:H237-H242

193. Widgerow AD. Ischemia-reperfusion injury: Influencing the microcirculatory and cellular environment. *Annals of plastic surgery*. 2014;72:253-260

194. Piot C, Croisille P, Staat P, Thibault H, Rioufol G, Mewton N, Elbelghiti R, Cung T, Bonnefoy E, Angoulvant D, Macia C, Raczka F, Sportouch C, Gahide G, Finet G, André-Fouët X, Revel D, Kirkorian G, Monassier J-P, Derumeaux G, Ovize M. Effect of cyclosporine on reperfusion injury in acute myocardial infarction. *The New England journal of medicine*. 2008;359:473-481

195. Mewton N, Croisille P, Gahide G, Rioufol G, Bonnefoy E, Sanchez I, Cung TT, Sportouch C, Angoulvant D, Finet G, André-Fouët X, Derumeaux G, Piot C, Vernhet H, Revel D, Ovize M. Effect of cyclosporine on left ventricular remodeling after reperfused myocardial infarction. *Journal of the American College of Cardiology*. 2010;55:1200-1205

196. Binder C, Hartvigsen K, Chang M-K, Miller M, Broide D, Palinski W, Curtiss L, Corr M, Witztum J. Il-5 links adaptive and natural immunity specific for epitopes of oxidized ldl and protects from atherosclerosis. *The Journal of clinical investigation*. 2004;114:427-437

197. Brame C, Boutaud O, Davies S, Yang T, Oates J, Roden D, Roberts L. Modification of proteins by isoketal-containing oxidized phospholipids. *The Journal of biological chemistry*. 2004;279:13447-13451

198. Kinnunen PKJ, Kaarniranta K, Mahalka AK. Protein-oxidized phospholipid interactions in cellular signaling for cell death: From biophysics to clinical correlations. *Biochimica et biophysica acta*. 2012;1818:2446-2455

199. Davies SS, Pontsler AV, Marathe GK, Harrison KA, Murphy RC, Hinshaw JC, Prestwich GD, Hilaire A, Prescott SM, Zimmerman GA. Oxidized alkyl phospholipids are specific, high affinity peroxisome proliferator-activated receptor ligands and agonists. *Journal of Biological Chemistry*. 2001;276:16015-16023

200. Zhang Q, Southall MD, Mezsick SM, Johnson C. Epidermal peroxisome proliferator-activated receptor γ as a target for ultraviolet b radiation. *Journal of Biological Chemistry*. 2005;280:73-79

201. Ytrehus K. Models of myocardial ischemia. *Drug Discovery Today: Disease Models*. 2006;3:263-271

202. Savla JJ, Nelson BC, Perry CN, Adler ED. Induced pluripotent stem cells for the study of cardiovascular disease. *Journal of the American College of Cardiology*. 2014;64:512-519

203. Matsa E, Burridge PW, Wu JC. Human stem cells for modeling heart disease and for drug discovery. *Science translational medicine*. 2014;6