Oxidized Phospholipids Regulation of the Monocyte Recruitment Parthway in Human Aortic Endothelial Cells

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OXIDIZED PHOSPHOLIPIDS REGULATION OF THE MONOCYTE RECRUITMENT PATHWAY IN HUMAN AORTIC ENDOTHELIAL CELLS

by

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A thesis submitted to the Graduate College in partial fulfillment of the requirements for the degree of Master of Science in Engineering Chemical Engineering Western Michigan University August 2015

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OXIDIZED PHOSPHOLIPIDS REGULATION OF THE MONOCYTE RECRUITMENT PATHWAY IN HUMAN AORTIC ENDOTHELIAL CELLS

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Western Michigan University, 2015

Low-density lipoprotein particles in blood plasma invade the endothelium and become oxidized, creating risk of CHD associated with atherosclerosis, the main underlying condition in coronary heart disease. Oxidation products of the phospholipid 1-palmitoyl-2-arachidonoyl-sn-glycerol-3-phosphatidylcholine (PAPC) are bioactive components of minimally oxidized LDL that stimulate endothelial cells that line the artery, leading to monocyte binding and atherosclerosis. It is hypothesized that the regulation of the monocyte recruitment pathway by these oxidized phospholipids is mediated by the binding of OxPAPC to one or more mediating proteins. Human recombinant VEGFR2, GRP78, and EP2 bind to OxPNB, a biotinylated analog of OxPAPC, demonstrated using Western blotting. Binding of OxPNB to these proteins in cells remains inconclusive and will need to be investigated in further studies. OxPAPC upregulates IL-8, HO-1 and MCP-1 in HAECs as demonstrated with RT-PCR, agreeing with previous studies. Gene knockdown of GRP78 and EP2 by siRNA yields substantial inhibition of the inflammatory response, including MCP-1 and IL-8. Knocking down VEGFR2 did not inhibit the upregulation of MCP-1 and IL-8. These experiments and future studies on the mechanism of Ox-PAPC-catalyzed inflammation in HAEC will contribute to the development of a new and more effective treatment for heart disease.
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ATHEROSCLEROSIS AN UPRISING CONCERN

Atherosclerosis is the underlying condition that can lead to CHD. In the human population, atherosclerosis is basically absent in the human body under normal physiological state (1), but the disease may develop in the pathophysiological state. CHD is generally considered a modern disease, but has shown its symptoms since ancient times (2) and is now one of the leading causes of death in men and women in the United States (3). Atherosclerosis, a form of arteriosclerosis occurs when artery walls harden by the accumulation of dead cells, white blood cells, triglycerides and cholesterol forming multiple atherosclerosis plaques within the aorta (4). There is high mortality rate from CHD, especially in the lower and middle income families or even countries. Every year, there about 610,000 people die from heart diseases, which 370,000 are from CHD (5).

Although rate of death from CHD is declining, the prevalence of the disease is increasing due to several factors, including diet changes, and obesity prevalence. However, modern medicines have improved the control of cholesterol with statins, niacin, intestinal cholesterol absorption-inhibiting supplements (6), and ACE inhibitors (7) and beta blockers for blood pressure control (8). Among those, statins is one of the most widely used as it is both safe and effective in lowering high risk patients (9).

Statins inhibit HMG-CoA reductase. These drugs are used to treat hypercholesterolemia and other cardiac patients to reduce the risk of CHD-related morbidity and fatality (10, 11). Statins lower LDL-C plasma levels, also resulting in pleiotropic effects including anti-inflammatory response, improvement of endothelial function, stabilization of atherosclerotic plaques (12) and increase in nitric oxide bioavailability that leads to
relaxation of vascular smooth muscle cells (12, 13). However, usage of statins in wide range of patient population yields some mild adverse effects, including headache, rash, and gastrointestinal systems. These adverse effects are more likely with high statins dosages, in addition to possible asymptomatic increases in liver transaminases and myopathy (11, 14). Although statins lowers the risk rate in patients from heart attack (54% lower) and strokes (48% lower), they may also increase patient blood sugar levels resulting in increased risk of diabetes mellitus, a choric and lasting condition that affects the body to utilize energy (15).

ATHEROSCLEROSIS EARLY LESION BY ENTRY OF LOW-DENSITY LIPOPROTEIN

Figure 1: Model of OxPAPC regulation of MCP-1 in endothelial cells

The model of OxPAPC regulation of atherosclerosis in Figure 1, modified from Lee at al. (16) shows that in early atherosclerotic lesions, LDL migrates into the blood vessel wall through the endothelial cell and is oxidized into minimally oxidized LDL (mm-LDL) by
the enzyme MPO and 12/15 LO, along with other mechanisms. MM-LDL stimulates endothelial cells, resulting in monocyte recruitment to the vascular wall, resulting from the upregulation of several genes, including MCP-1 (17, 18). Several of the active components of mm-LDL are oxidation products of the endogenous phospholipid PAPC.

Figure 2: PAPC and its endogenous oxidation products

Oxidation products of PAPC as depicted from Figure 2 are among the most active components of mm-LDL and were shown to regulate over 1,000 genes in HAEC (19). While low OxPAPC treatments decrease the permeability of the endothelial cell monolayer by tightening adherent junctions, high OxPAPC levels cause a strong increase in monolayer permeability resulting in increased entry of LDL into the vessel wall (16). MM-LDL then stimulates endothelial cells leading to an upregulation of the monocyte recruitment pathway, including MCP-1 (16). One study also concluded that smaller molecular weight LDL is associated with hyperlipidemia (20) and plays a role as a risk factor in atherosclerosis (21, 22, 23, 24), as size of LDL may be a factor in endothelial permeability and eventual contribution to lesion formation.
BORDER CONTENT OF STUDIES: CONTRIBUTION OF NOVEL TREATMENTS TO INHIBIT OXPAPC-REGULATED CHRONIC INFLAMMATION IN ARTERIES

During normal physiological functioning, monocytes in the blood continuously bind to the endothelium, “roll” along the arterial wall, and disengage from the wall. It has been reported using radioactively labeled lipid that OxPAPC may binds to the EP2, causing the CS-1 fibronectin to undergo a change in structure and inhibiting disengagement from the arterial wall. OxPAPC also regulates the expression of several chemokines such as specific metalloproteinases known as ADAMs, releasing active HBEGF, EGFR and VEGFR2 and leading to synthesis of IL-8 and MCP-1, two important proteins in the progression of chronic inflammation (16).

Among the oxidation product of PAPC is PEIPC, which is active in HAECs at the lowest concentration (25). This lipid is generally considered as pro-inflammatory at most treatment concentrations and binds cysteines, subsequently regulating biological pathways in HAEC (19). Previously, a biotinylated analog of OxPAPC, OxPNB (oxidized of PAPE-N-Biotin, Figure 3), was developed, as OxPAPC interaction with cysteines inhibits the binding of OxPNB to HAEC proteins (26). This demonstrates strong competition of OxPAPC and PEIPC with OxPNB binding to proteins, showing that OxPNB binds to similar sites as OxPAPC. Furthermore, OxPNB and OxPAPC have similar biological activity, demonstrating OxPNB as an appropriate biotinylated analog for our binding studies (27).
Figure 3: PAPE-N-biotin, biotinylated tagged analog of PAPC and its oxidation product

PEIPC is cleaved to EI, a fatty acid, by PLA2, an endogenous enzyme in human physiology that is concentrated in HDL. Treatment of HAECs with this lipid elicits an anti-inflammatory response, down-regulating several genes that are generally upregulated by PEIPC, including MCP-1 (monocyte recruitment) (25). It has been suggested that mice have higher Ox-PL level than human (28) and accumulation of OxPAPC in the carotid arteries increases atherosclerotic related genes (29). Overexpression of MPO was shown to increase atherosclerosis (30) and knockdown of 12/15 LO diminishes atherosclerosis (31). These are true as MPO and 12/15 LO are both enzymes that are involved in the development of Ox-PL (31, 32).

Various effects of OxPAPC in endothelial cells were either reduced, attenuated or inhibited by binding of several proteins such as VEGFR2 (29), GRP78 (33) and EP2 (34), but the details and mechanism of OxPAPC regulation of the monocyte recruitment
pathway remain elusive. In this study, it is hypothesized that there are one or more mediating proteins that are involved in the OxPAPC upregulation of MCP-1, a key chemokine that regulates monocyte binding, which is a key event in the development of heart disease (35, 36).

IDENTIFIED CANDIDATE MEDIATORY PROTEINS THAT MAY REGULATE OXPAPC REGULATION OF THE MONOCYTE RECRUITMENT PATHWAY

As mentioned, candidate mediatory proteins include VEGFR2, GRP78 and EP2, as there is a variety of evidence that each may mediate OxPAPC effects on HAEC function, including barrier protection and monocyte recruitment. A major goal of the proposed studies is to run a parallel set of experiments to determine if one or more of these candidates are a key mediating protein in OxPAPC action on HAEC function. Current evidence has been summarized below suggesting why each has been chosen as a candidate.

A) Vascular endothelial growth factor receptor (VEGFR2)

Several papers have reported various dose-dependent responses in HAECs to OxPAPC (32, 37, 38, 39). With respect to VEGFR2 activity, lower OxPAPC concentration treatment (5-10 µg/mL) enhances the barrier endothelial monolayer barrier properties in vitro and reduces inflammation in models of acute lung injury in mice caused by LPS intravenous injection (37, 38). Higher OxPAPC concentration treatments (50-100µg/mL) disrupt the barrier function in HAEC (33). Gene specific inhibition of VEGFR2 was shown to reduce OxPAPC upregulation of IL-8 and LDL receptor expression in HAEC (39) and attenuates transendothelial electrical resistance (measurement for EC monolayer
permeability) decline in ECs-induced by OxPAPC with depleted VEGFR2 (33). HAEC treatment with VEGFR2 siRNA also decreases the certain effects of high OxPAPC concentration in this system, bringing about the increase of stress fiber and paracellular gaps in cells (33).

B) **Glucose regulated 78 protein (GRP78)**

GRP78 is an ER chaperone protein that associates with a cofactor, HJT-1 to form an active complex. HTJ-1 is not only responsible in translocation and anchoring of GRP78 to the cell plasma membrane; it also acts as a co-chaperone for endothelium stress involved in the unfolded protein response. GRP78 is also referred to as immunoglobulin heavy chain binding protein (BiP) or Heat shock 78 kDa protein 5 (HSPA5) and is a member of the 70 kDa heat shock protein (HSP70) family of proteins (33, 40, 41, 42). Many studies have shown involvement and/or applications of GRP78 in cellular processes, including the translocation of synthesized polypeptides across the ER membrane to regulates calcium homeostasis, stress response by releasing transmembrane sensors (protein kinase-like ER kinase (PERK), inositol-requiring kinase 1 and transcriptional factor activating transcription factor 6) and initiation of unfolded protein response signaling (33, 40, 43, 44). Alteration of GRP78 tissue levels or activity may be useful in the treatment of cancer (41), Alzheimer’s disease (45) and other ER stress-associated diseases. It was shown that knocking down of HTJ-1 eradicated GRP78 recruitment to the cell membrane of OxPAPC-induced cell and suppressed Rac1 (regulatory role specifically in cell motility, cancer cell growth (33). In 2014, Birukova et. al. showed that blocking GRP78 attenuates the
increase of TER and demonstrated OxPAPC barrier enhancement function in HUVEC (46, 47). The importance of the involvement of the HTJ-1-GRP78 mechanism in the ECs was shown by demonstrating that inhibition of HTJ-1 expression eradicated barrier enhancement by OxPAPC in HAECs (33).

C) Prostaglandin E2 receptor subtype 2 (EP2)

It was reported in 2006 by Li et al. that specific G protein-coupled receptors (GPCR) that are responsible for the activation of the signal transduction pathway and cellular responses play an important role in OxPAPC action by increasing the intracellular levels of cAMP in HAECs and stimulating monocyte binding. By three screening procedures, they were able to identify GPCRs involved in the OxPAPC response. First, they screened for the candidates of GPCR that are expressed in HAEC using RT-PCR. Then the GPCRs candidates with a known ligand were analyzed to see whether monocyte binding is regulated by the ligand or the effect of OxPAPC on monocyte binding is blocked by this known ligand. Lastly, the remaining receptors were screened for OxPAPC activation with the Pathdetect CREB reporter gene assay system. These receptors were treated with OxPAPC and, by means of reporter luciferase activity; the activation of various GPCRs by OxPAPC was measured. With the reporter gene assay, two G-coupled OxPAPC responsive receptors were identified, EP2 and prostaglandin D2 receptor (DP). DP, the closest homolog to EP2 (41% identical to EP2) was also activated by OxPAPC, however no expression of DP was found in HAEC. PEIPC but not POVPC was also demonstrated to activate EP2 with treatments as low as 10 ng/mL concentration. OxPAPC and PEIPC were observed to compete for binding
to EP2 with PGE2, the native ligand to EP2 in EC, dose-dependently (inhibition level: 10% at 0.1 µg/mL OxPAPC; 92% at 50 µg/mL OxPAPC; 22% at 80 nmol/L PEIPC; 57% at 800 nmol/L PEIPC), demonstrating that PEIPC and PGE2 most likely bind the same active site (34).

METHODOLOGY

Materials and reagents

HAECs will be purchased from Life Technologies. VEC complete media was purchased from VEC Technologies; medium 199 (M199) from MediaTech and FBS were purchased from Thermo Scientific. Primers for MCP-1, IL-8, HO-1, ATF-3, VEGFR2, GRP78 and EP2 were purchased from Integrated DNA Technologies. PAPC and PAPE were obtained from Avanti Polar Lipids. High capacity streptavidin-HRP and Neutravidin beads (Pierce Biotechnologies) were purchased from Thermo Scientific. Lipofectamine 2000 reagent and Opti-MEM medium were purchased from Invitrogen. The four siRNA for VEGFR2 (KDR) and EP2 (PTGER2) were purchased from Qiagen. Other alternate siRNA were purchased from Life Technologies for VEGFR2, GRP78 and EP2. Anti-Goat antibody was purchased from R&D systems. Anti-Mouse and Anti-Rabbit antibodies were purchased from Cell Signaling Technology. Primary antibodies anti-VEGFR2, anti-GRP78, anti-EP2 and streptavidin-HRP were purchased from R&D Systems. Tween 20 was purchased from ThermoFisher.

Human aortic endothelial cell culture and treatment

HAECs will be isolated as described previously (48, 49) or purchased from Life Technologies. HAECs were grown to complete confluence in a media of in 100% VEC
complete media or 50% VEC complete media and 50% media of 80% M199 media and 20% FBS overnight before use in experiments. HAECs were then pretreated in M199 media containing 1% FBS for cell treatment. HAECs were then incubated in 37 °C with 5% CO₂ with or without lipid for 1 or 4 hours depending on the lipid used for the experiment.

**Western Blotting**

1) **Preparing to run the gel**

1300 mL running buffer is made from a solution of 130 mL running buffer solution with 1170 mL Millipore water. The gel plates needs to be air bubble free. The gels are placed into the gel apparatus main frame and loaded into the gel running container. Running buffer is poured onto the gel apparatus main frame to check for leakage before pouring the rest to fill up the container. Excess preserve solution of each well in the gel plate is cleaned out carefully with 50 µL of air and then 50 µL of running buffer with a gel-loading pipette tip. Samples are boiled for 5-10 minutes before loading into the gel well. 3 µL of magic marker was loaded into the 1st well, 3-10 µL of benchmark on the 10th well and 20-40 µL samples in between the magic marker and benchmark well. The gel was run at 20 V for 20 minute and then 100-120 volts for 60-90 minutes or until the dye from the sample buffer (under 1 kDa molecular weight) runs near to the bottom of the gel.
II) Preparing membrane to transfer

An appropriate edge (top right) is cut to differentiate or indicate the direction of the membrane will be transferred to. The membrane is wet by soaking in methanol and then rinsed with TBST. The membrane is then immersed in transfer buffer until it is ready to be used.

III) Transferring the gel

1300 mL Transfer buffer was made by mixing 130 mL transfer buffer solution with 260 mL methanol and 910 mL Millipore water. 2 sponges and 2 filter papers are soaked in the transfer buffer and layered on the transfer cassette, having the sponges to be at the bottom of the filter papers at each side. The gel is separated from its gel case and excess gel such as the well walls is cut off. The gel is placed on top of the filter paper and the prepared membrane on top of the gel slowly to minimize air bubble formation between the gel and the membrane, all the while having the black side of the cassette facing the gel side. The other filter paper is placed on top of the membrane and rolled with a test tube to get ensure removal of potential air bubbles. The sponge is then placed on top of the filter and the transfer cassette is closed and locked. The transfer cassette is then placed in the transfer carrier, while ensuring the black part of the transfer cassette faces the black part of the transfer carrier. The transfer is run at 100 mA overnight and then cranked up to 200 mA around 20 minute to ensure the transfer of larger proteins to the membrane. To prevent overheating while at 200 mA, an ice pack stored at -80 °C is placed into the transfer carrier and the solution is magnetically
stirred throughout the process of transfer to ensure that the gel doesn’t melt and proteins are not damaged during transfer.

**IV) Imaging the blot**

The membrane is taken out of the transfer cassette and the gel is discarded. The membrane is briefly washed with TBST on a square plate and washed for 3 more times for 5 minute each while swirling. The protein is then blocked with 5wt% milk solution in TBST for an hour swirling. The milk is then removed and is washed with TBST as stated previously. Primary antibody, according to the manufacturer’s specifications, is added and swirled in 10 mL of 1wt% BSA or 5wt% milk in TBST at room temperature for 1 hour. The primary antibody solution is then removed and washed in TBST 3 times for five minute each, as before. Secondary antibody (i.e. 5 µL anti-rabbit or 5µL anti-mouse) is added and swirled in 10 mL of 5wt% milk in TBST for an hour. The secondary antibody solution is then removed and the membrane is washed in TBST again, as stated previously. ECL solution of 1 mL of solution A and 1 mL of solution B (as purchased from GE Healthcare or Biorad) is dribbled across the membrane to fully wet its surface and the membrane is incubated for 5 minute before imaging. Imaging was done on a Kodak CF440 Image Station that was set to obtain and average images.

**Synthesis of PAPE-N-biotin (PNB)**

PAPE was biotinylated and oxidized as previously described (27). 50 mg of PAPE in 5 mL of dry dichloromethane solution was added drop-wise to a solution of 17 mg biotin,
29 mg of dicyclohexylcarbodiimide and 17 mg of dimethylaminopyridine in 3 ml of dry dichloromethane under argon at room temperature and was mixed with a magnetic stirrer overnight. Solvent was dried under argon, and the residue was purified by solid phase extraction (SPE) to produce PNB. The SPE fractions were dried and tested for PNB with ESI-MS.

**Solid phase extraction of PAPE-N-biotin**

PAPE-N-biotin and OxPAPE-N-biotin were analyzed by ESI-MS in negative mode on a Thermo Electron LCQ Advantage Mass Spectrometer. Lipids were loaded onto C8 columns from Phenomenex in 50% methanol and 50% H₂O, washed with 70% methanol and 30% H₂O and eluted with 90% methanol and 10% H₂O. Fractions enriched in tagged lipid were then identified using MS as fractions that contain the molecular ion m/z (mass to charge ratio). The mass spectrometer was configured to scan in the range of m/z 500 to 1200 in the negative ion mode and samples peak are averaged over each flow injection.

**Affinity purification of lipid bound protein**

Neutravidin beads (10 µL) are added to the cell lysate of each 100 mm dish of lipid treated HAECs and incubated at room temperature with continuous mixing overnight at 4 °C. Non-biotinylated proteins were washed off using PBS with 0.1% Tween 20. The biotinylated lipid-protein adducts retained on the beads are eluted by boiling the beads in reducing Laemmli sample buffer for 10 minute or by boiling in other stringent conditions. The protein content was then analyzed with western blot. The proteins were blocked by a 5wt% milk solution wash for 1 hour, specific primary antibody incubation at 4°C overnight and incubated with a 5% milk solution with the secondary antibody. The secondary antibody used for VEGFR2 is anti-goat antibody and anti-rabbit antibody.
(ladder). The secondary antibody used for GRP78 and EP2 are anti-mouse antibody and anti-rabbit antibody (ladder).

**Synthesis of OxPAPC**

1) **Lipid oxidation protocol**

Burdick and Jackson (B&J) chloroform is used to dilute stock of PAPC by 2 into 12.5 mg/ml. This specific B&J chloroform is used because chloroform from other suppliers may contain additives or contamination that inhibits PAPC oxidation such as ethanol. Eight aliquots of diluted lipid solution are made by pipetting the lipid stock into borosilicate glass tubes. Two precautionary steps are taken. First, the lipid stock is pipette up and down 3 to 4 times before aliquoting to “condition” the pipette tip; otherwise chloroform will leak out. Lipid aliquots are then dried with argon by circular swirling pattern using a pipette tip to create as thin a lipid layer as possible on wall of borosilicate tube to increase the oxidation rate. This is important as it is essential to have rapid oxidation since slow oxidation generally results in over-oxidation of desired components such as PEIPC, POVPC and PGPC into less bioactive components such as lysoPC. The second precautionary measure is the drying of aliquots is done in sets of 8 to prevent premature drying of lipid, which results in inefficient oxidation. Since argon is denser than air and will prevents oxidation, it is removed from all tubes via Pasteur pipette connected to a vacuum line prior to oxidation. The Pasteur pipette is lowered near the bottom of each tube to ensure all argon is removed during vacuuming. The aliquots are allowed to sit in the fume hood for approximately
48 hours for oxidation, and the progression of lipid oxidation is monitored with MS flow injection.

II) MS flow injection

Detection of the oxidized lipid is performed on a Thermo Electron LCQ Advantage Mass Spectrometer. The system is equipped with electrospray and atmospheric chemical ionization interface, which allow for the analysis of a wide range of analytes. Its MS/MS functionality enables extensive structural analysis. The LCQ Advantage Mass Spectrometer is equipped to enable several methods, including chromatographic separation, compound detection, mass analysis, two-stage mass analysis, wideband activation and ZoomScan analysis. Electrospray ionization (ESI) is useful for analyzing polar compounds such as amines, peptides and proteins; Atmospheric pressure chemical ionization (APCI), which is also available, is generally used for non-polar compounds such as steroids, although it is not used in these studies.

III) Checking lipid oxidation with ESI-MS

The MS must be warmed up for about 2 hours before sample analysis. Nitrogen and helium must be turned on and the MS power is turned on to start warm up. The MS is generally fitted with 20µL injection loop for flow injection. During loading, with the injection loop status on load, flush the injection loop on the MS injection port with 500µL methanol with a 500µL Hamilton syringe. Then flush 25µL Hamilton syringe with 25µL methanol for 5 times to ensure that it is clean
from contamination. Removing one aliquot of lipid (0.2-0.5 mg) from oxidation, add 200 µL B&J chloroform and vortex for about 5 seconds. Aliquot 25µL into a microcentrifuge tube and dry it with argon. Add 200µL methanol and vortex again for about 5 seconds. Using the 25µL Hamilton syringe, take up 25µL of the lipid solution and inject it into the MS injection port. In the MS software, open the LCQ tune program and select the most recent tune file for OxPAPC (or PNB for negative mode analysis) and click start button to start data acquisition. If desired, filename/folder can be changed to the preferred filename and location. There is generally the presence of background/noise intensity is usually on the order of $10^4$ to $10^5$ absorbance unit. Wait around 10 seconds to obtain a good background signal and then change the injection port status to ‘inject’, to release the oxidized lipid into the MS for data acquisition. To view the MS spectrum for each sample, click the ‘View’ button and average over the total ion current for the sample peak. (A flat baseline signal followed by an OxPAPC peak and then return to the baseline should be seen throughout the time period of OxPAPC detection.)

**OxPAPC or OxPNB collection protocol**

Add 1mL of B&J chloroform into one aliquot with a “conditioned” pipette tip and vortex it for about 20 seconds. Vortexing produces swirls that allow for the efficient mixing of chloroform and the lipid aliquot without destroying it. Next, with a “conditioned” pipette, pipette chloroform from the current aliquot and add to the next aliquot until 8 aliquots are collected. The last aliquot will be used as a stock tube for OxPAPC (or OxPNB). Add 1mL chloroform into the last aliquot.
without the stock OxPAPC and vortex it for about 10 seconds. Remove the chloroform from the current aliquot and add it to the previous aliquot (reverse direction from the first collection) until all 8 aliquots are done and add it to the stock OxPAPC. Repeat all the steps above for the remaining aliquots and collect all oxidized lipid into the stock OxPAPC tube. Sets of 8 tubes are collected for each subsequent collection to maximize the efficiency and yield of lipid recovery. To store the stock OxPAPC, argon is added to the top with low argon flow rate. Avoid pointing it directly at the solution to prevent evaporation of the stock and changes in concentration of the stock solution. Shoot the argon at the tube wall and allow the argon to flow down since argon is denser than air. The tube is then capped with a yellow cap and sealed with a parafilm to avoid or reduce argon loss and chloroform evaporation during storage. The OxPAPC stock is stored preferably at -40 °C or -20 °C and must not be stored below the chloroform freezing point (-64.5 °C) as if OxPAPC freezes, the lipid components are degraded and biological activity is modified.

**Transfection of siRNA**

Transfections of ECs with siRNAs will be performed as previously described (26, 50). 90-95% confluent endothelial cells are treated with siRNA and Lipofectamine 2000 reagent in Opti-MEM Reduced Serum medium for 4 hours, after which media was changed to normal growth media. Cells were incubated for 48 hours to allow for cell growth, stabilization and protein expression. Transfected cells are harvested for RT-PCR for measurement of gene expression (51).
**Real-time polymerase chain reaction (RT-PCR)**

The model of RT-PCR machine used was the 7900HT Sequence Detector System. Total RNA was isolated from treated cells using the Total RNA extraction kit from Bio-rad. RNA concentrations were measured with SpectraMax384 Plus Absorbance Reader (at a wavelength of 260 nm, using an extinct coefficient of 40 ug/ml for single-stranded RNA) and equable amount of RNA were withdrawn for cDNA synthesis. cDNA were synthesized using a high capacity cDNA kit (Applied Biosciences/Life Technologies). SYBR® green master mixture from ThermoFisher was used for PCR quantification and amplification. GAPDH was measured along with the experimental group as a control ‘housekeeping’ gene that does not change in HAECs with oxidized lipid treatment, and is used to normalize mRNA levels and fold changes of other genes between untreated and treated cells (52). The primer sequences used for RT-PCR were

- **GAPDH**: Forward: 5′ - CCT CAA GAT CAT CAG CAA TGC CTC CT-3’, Reverse: 5’ -GGT CAT GAG TCC TTC CAC GAT ACC AA-3’;
- **VEGFR2**: Forward: 5’ -CTT GGA CTG GCT TTG GCC CAA TAA TC-3’, Reverse: 5’-AAG CAC TTG TAG GCT CCA GTG TCA-3’;
- **GRP78**: Forward: 5’-GGA AAG AAG GTT ACC CAT GC-3′, Reverse: 5’-AGA AGA GAC ACA TCG AAG GT-3’;
- **EP2**: Forward: 5’-ATG GGC AAT GCC TCC AAT GAC TCC C-3’, Reverse: 5’-ACA CCA GCT CGG TCA CCA GCA CGT-3’;
- **MCP-1**: Forward: 5’ -TGC TCA TAG CCA CCT TCA TTC-3’, Reverse: 5’ -GAC ACT TGC TGC TGG TGA TTC TTC-3’;
- **IL-8**: Forward: 5’ -ACC ACA CTG CGC CAA CAC AGA AAT-3’, Reverse: 5’ -TCC AGA CAG AGC TCT CTT CCA TCA GA-3’;
- **HO-1**: Forward: 5’ -ATA GAT GTG GTA CAG GGA GCC CAT CA-3’, Reverse: 5’ -GTC AGA GAA TGC TGA GTT CAT GAG GA-3’ (25, 26, 27, 51, 55).
**OXPNB vs OXPAPC**

Some of the experiments were done using OxPNB and some with OxpAPC. OxPNB is used to determine specific binding interactions. OxPNB specifically binds to streptavidin and its binding can be seen with western blotting by using streptavidin-HRP. OxPAPC is used in biological activity experiments. Since we are measuring gene expressions, we do not need the biotinylated tagged lipid for the experiment and OxPAPC is easier to make and is a moiety of the exact molecules in our body.

**SYNTHESIZING LIPID**

The mass spectrums of PNB and PAPC are shown in Figure 4 below. These lipids were used in the experiments. PNB and PAPC showed consistent MS spectra and activity results between batches.

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**Figure 4: Mass spectrum of synthesized lipid. A) Mass spectrum for PAPE-N-biotin and its oxidation products B) Mass spectrum for PAPC and its endogenous oxidation products**

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EXPERIMENTS AND RESULTS

Binding of commercial human recombinant candidate proteins with OxPNB

Commercially available purified human recombinant VEGFR2, GRP78 and EP2 were treated with or without (50µg/mL) OxPNB (the tagged OxPAPC analog) for 30 minutes at 37 °C. 200ng of each protein was used for each incubation. Binding to each protein was determined by western blotting as described. This experiment was done to determine if the suggested candidate protein would actually bind oxidized lipid.

![Western Blot Image](image)

**Figure 5: VEGFR2 recombinant protein binding with PNB and OxPNB using western blotting**
In Figure 5-7, all three candidate proteins, VEGFR2, GRP78 and EP2 showed binding to OxPNB and none at PNB. All results were repeated at least 3 times for reproducibility. Binding for VEGFR2 to OxPNB can be seen around 82 kDa while GRP78 at around 78 kDa.
kDa and EP2 at around 53 kDa. With the confirmation of OxPNB binding to the candidate protein, we can proceed in determine binding in HAEC.

**Determining OxPNB binding to HAEC protein**

In both in vivo (mice) and in vitro (human) studies, it was found that OxPAPC dose-dependently (0 µg - 100µg) induces an increase of Cx 43 expression in EC and VMSC in carotid artery using immunoblot. However, in advance atherosclerotic lesion in mice, Cx 43 expression is down regulated (53). The phosphorylation of Cx 43 was also correlated to up-regulate HO-1 and this may be vital step in determining the pathological development in atherosclerotic disease (29, 54). Nonetheless, OxPAPC interaction to inflammatory mediators remains the utmost importance in this study. Since it has been shown that OxPAPC is dose dependently active in several studies (29, 32, 34, 54), it is important to determine the molecular weights of proteins that most specifically bind OxPAPC at physiological (lower concentration treatments) and pathological concentrations (higher concentration treatments) of oxidized lipid.

Cultured HAECs cells were treated with or without PNB or OxPNB to screen for the binding of HAEC proteins to OxPNB at different concentration treatments and effectively see which proteins bind under different levels of OxPNB treatment (50µg/mL, 5 µg/mL and 0.5 µg/mL). This experiment will also be used to determine which dose is sufficient for the most specific lipid-protein binding and eventually, pull down of tagged protein with Neutravidin beads. For subsequent experiments, we determined the binding to specific proteins candidates that bind OxPNB at the lowest treatment concentration with the most specific binding.
In Figure 8, it was found that the lowest concentration possible for the candidate protein to specifically bind to OxPNB is 50 µg/mL. Instead of manipulating the concentration in factor of 10, it would be helpful to determine the lowest concentration range near the determined concentration of 20-50 µg/mL. The experiment was repeated for the concentration 50 µg/mL, 20 µg/mL and 5 µg/mL of OxPNB.
Figure 9: Concentration binding of OxPNB to HAEC at 50 µg/mL, 20 µg/mL and 5 µg/mL.

Figure 9 shows that there is some binding in 20 µg/mL of OxPNB to HAEC. This does not only tell us that the protein specifically binds to the lipid but also potentially helps save some cost in purchasing the protein and time synthesizing and oxidizing the lipid.

**Affinity pull down of tagged protein**

Since OxPAPC reacts dose-dependently and based on the reports having higher OxPAPC being disruptive (33), we expect OxPAPC to bind one or more of the candidate proteins in the range of OxPAPC treatment concentration tested.

After determining the concentration needed as described above, it is necessary to check if the candidate protein is bound by OxPAPC. Affinity pull down of HAECs cells were treated with 20 µg/mL OxPNB concentration as determined above and affinity pull down of tagged protein were done overnight with 10 µL Neutravidin beads. Proteins were then
eluted from the beads and Western blots were performed to determine if protein candidates bound the lipid treated in HAECs.

Figure 10: HAEC affinity pull down, followed by western blotting with VEGFR2 antibody

Figure 11: HAEC affinity pull down, followed by western blotting with GRP78 antibody
Figure 10 shows an empty western blot of VEGFR2 affinity pull down. High molecular
proteins of molecular weight 200 kDa and above are notoriously difficult to transfer to
membranes, and it is possible that the protein was able to run through the gel but were not
able to transfer through to the membrane even though the incubation for transfer were ran
at 200 mA for 10 minute and then 300 mA for 30 minute. Figure 11-12 confirmed that
the affinity pull down of the protein that binds to OxPNB were indeed the candidate
proteins which are GRP78 and EP2. However, EP2 blot show bands in control (32 kDa)
and in OxPNB lane (80 kDa). The bands that show were believed as unspecific binding
to the beads.

With this, we can proceed in determining the biological activity of these proteins in
HAEC with OxPAPC while also knocking the proteins down with SiRNA.
Biological activity of OxPAPC binding to HAEC

Cultured HAECs were pretreated with or without siRNA of each candidate protein and co-treated with or without OxPAPC (50 µg/mL) for 4 hours. Treatment with siRNA results in ‘knockdown’ of the expression of each candidate protein. Typically optimal knockdown results in reduction of mRNA level of 80-90 %. After treatment, cells were then analyzed with RT-PCR for mRNA levels of MCP-1 along with IL-8 to check for possible overlap in pathway and compare results with published works.

Figure 13: Upregulation of MCP-1, IL-8 and HO-1 mRNA levels in HAEC with OxPAPC, as measured by RT-PCR; First trial with MCP-1 upregulated as expected

Figure 13 shows the upregulation of MCP-1, IL-8 and HO-1 in HAEC cells treated with OxPAPC. All the genes shows fold increase in comparison to controlled cells that has no lipid in it. MCP-1 shows a fold change of 1.5, IL-8 shows a fold change of 18 and HO-1 shows a fold change of 14 with GAPDH as a normalizing gene.
Although some trials demonstrated that MCP-1 was downregulated by OxPAPC by 80% in Figure 14, previous studies showed transient regulation of MCP-1 by OxPAPC in endothelial cells, and it is anticipated that MCP-1 is strongly upregulated by OXPAPC at earlier time points of the incubation of HAECs. Further studies must be completed to determine the transient effects of MCP-1 regulation by OxPAPC and PEIPC in HAECs at various time points. With these results, we proceeded to knockdown of the candidate proteins with siRNA to investigate the role of each protein in the regulation of OxPAPC in endothelial cells.
Figure 15: Knockdown of VEGFR2 with siRNA transfection in both controls and OxPAPC

Figure 15 shows gene expression of VEGFR2 knockdown with siRNA. VEGFR2 was successfully knocked down at a rate of 50-75%.

Figure 16: Knockdown of GRP78 with siRNA transfection in both controls and OxPAPC

Figure 16 shows gene expression of all candidate proteins, VEGFR2, GRP78, and EP2 knockdown with siRNA. GRP78 were successfully knocked down at a rate of 50-75%.
Figure 17: Knockdown of EP2 with siRNA transfection in both controls and OxPAPC

Figure 17 shows gene expression of EP2 knockdown with siRNA. EP2 were successfully knocked down at a rate of 15-70%.

Figure 18: Influence of siRNA transfection gene knockdown expression of VEGFR2 protein on the regulation of MCP-1 by OxPAPC
The knockdown of VEGFR2 seems to have no effect on inhibiting the regulation of MCP-1 or IL-8 by OxPAPC in this experiment. MCP-1 and IL-8 gene expression were seen to increase in Figure 18-20 which suggests that knocking down VEGFR2 leads to an increase in vascular inflammation. In 2007, Zimman et. al has demonstrated that gene specific knockdown of VEGFR2 with siRNA reduces the gene expression level of IL-8, with lipid-protein cell treatment of 2 hours instead of 4 hours and primer sequence used for VEGFR2 was different.
With the knockdown of the GRP78, the mRNA regulation by OxPAPC of MCP-1 was substantially inhibited by 60% as shown in Figure 20. Upregulation of MCP-1 and IL-8 by OxPAPC was reduced as a result of this knockdown as shown in Figure 20-21. This
suggests that GRP78 may be involved in the regulation of the inflammatory and monocyte recruitment pathways by OxPAPC and that knockdown of this gene leads to reduced inflammation.

**Figure 22:** Influence of siRNA transfection gene knockdown expression of EP2 protein on the regulation of MCP-1 by OxPAPC

**Figure 23:** Influence of siRNA transfection gene knockdown expression of EP2 protein on the regulation of IL-8 by OxPAPC
Figure 22 -23 shows that knocking down EP2 inhibited the upregulation of MCP-1 and IL-8 by 75 % and 35 % respectively. This suggests that EP2 might also be involved in the regulation of both inflammatory and monocyte recruitment by OxPAPC in HAECs.

It was expected that knockout of one or more of the candidate proteins (with siRNA) will inhibit OxPAPC regulation of MCP-1 in HAECs as stated in the hypothesis. These results show similarity some of the recently published results of genes regulated by PEIPC and its fatty acid analog, EI (25). In the future, the siRNA used for the proteins may be purchase from another company which contains not only a single but multiple sequences which might increase the knockdown and thus increases the inhibition of the inflammatory genes. Also treatment times for Transfection may be extended, possibly leading to more extensive knockdown.

**CONCLUSION**

By Western blot, we were able to demonstrate that OxPNB, a biotinylated analog of OxPAPC binds to all candidate human recombinant proteins, VEGFR2, GRP78 and EP2 at 82 kDa, 78 kDa and 53kDa respectively. With these confirmations, HAECs were treated with several concentrations of OxPNB in addition to affinity pull of lipid-bound proteins with Neutravidin beads to determine if it truly was the candidate protein that bound the lipid. The determined lowest concentration of 20 µg/mL was used for the experiment. Although 20µg/mL was the next lowest concentration found, 50 µg/mL were used for the biological activity experiments as previous studies have shown positive inflammation results. EP2 shows positive results, however the control and OxPNB lipid shows bands of unspecific binding to the beads where as GRP78 shows positive results
and VEGFR2 shows absolutely nothing. Further studies are required to clarify the binding. Treatment of HAEC with OxPAPC shows upregulation of all inflammatory genes by 1.5 fold for MCP-1, 18 fold for IL-8 and 14 fold for HO-1 in comparison to the controls. Some trials however shows downregulation of MCP-1 by 80 % which are akin to previous studies indicating that MCP-1 may have transient properties which necessitate further investigation. Knocking down of these candidate proteins, GRP78 and EP2 shows considerable amount of inhibition on OxPAPC regulation. Knockdown was successful at a rate of 40-75% for VEGFR2 and GRP78, and 15-70 % for EP2. VEGFR2 knockdown did not inhibit any upregulation of OxPAPC which suggests that knocking down VEGFR2 may not reduce OxPAPC pro-inflammatory effects. However, knockdown of both GRP78 and EP2 shows substantial inhibition of both MCP-1 and IL-8 upregulation by OxPAPC.

The use of different siRNA which contains multiple gene sequence instead of a singular gene sequence may be purchased from another company and used in these experiments to improve knockdown and inhibition of the inflammatory gene.

This study was geared towards the understanding of the mechanism under which OxPAPC induces inflammation genes that are involved in heart disease. These experiments contribute to an understanding of the underlying mechanisms that will hopefully contribute to the development of new and more effective treatment methods to inhibit chronic inflammation, targeting either OxPAPC activity or through these inflammatory mediators.
FUTURE STUDIES WHEN POSSIBLE

Determining the specific binding site of human recombinant protein with ligand

It may be possible to determine the binding site of OxPAPC on identified lipid-bound proteins. In addition to the Western blot, untreated and OxPAPC-treaded human recombinant protein would be denatured with 1mM dithiolthreitol and alkylated with 100 mMiodoacetamide. The protein would then be acetone-precipitated, pelleted by centrifuge at 1000 RPM for 5 seconds and was re-suspended in pH 7.8 mM NH₄HCO₃. For complete digestion of protein, trypsin would be added according to the manufacturer specifications and incubated at 37 °C. Samples would then be desalted with Millipore (C₁₈ZipTip) and analyzed with MS/MS and database searching. We could then determine the binding site of OxPAPC on the proteins by identifying the lipid modification on bound cysteines residues. We could then determine the binding site of OxPAPC on the proteins by identifying the lipid modification of cysteine residues.

Determining additional proteins bound to lipid in HAEC

After affinity pull down of lipid bound to HAEC, the eluted protein would potentially be digested with trypsin and analyzed with LC-MS/MS. With these experiments, we may be able to identify additional protein candidates that mediate OxPAPC regulation of genes in HAEC.

Investigation on the transient properties of MCP-1 in OxPAPC-regulation in HAEC

MCP-1 is suspected to have transient properties in OxPAPC regulation and further exploration of incubation time points in incubation are needed. Incubation time points may vary from 0 to 24 hours. Previous RT-PCR results are most consistent with
incubation times at or less than 8 hours, possibly due to degradation of OxPAPC or transient cell response.

**Combination knockdown of protein for biological activity**

Mediatory protein knocked down will be done in combination (knocking two or more different protein at once) and mRNA levels of MCP-1 and IL-8 will be measured. It may be that OxPAPC regulates gene expression via binding to several proteins, and the knockdown of several proteins may be necessary in order to completely inhibit pro-inflammatory effects of OxPAPC. These experiments will be performed in the future to test this possibility.
REFERENCES


34. Li R.S., Mouillesseaux K. P., Montoya D., Cruz D., Gharavi N., Dun M., Koroniak L. and Berliner J. A. 2006. Identification of Prostaglandin E2 Receptor Subtype 2 as a Receptor Activated by OxPAPC. *Circulation Research.* **98**:5:642-650


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APPENDIX

Table 1

Non-Standard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Acronyms</th>
<th>Complete Words</th>
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<tbody>
<tr>
<td>12/15 LO</td>
<td>12/15-lipoxygenase</td>
</tr>
<tr>
<td>ACE</td>
<td>Angiotensin-converting-enzyme</td>
</tr>
<tr>
<td>ADAM</td>
<td>Adysintegrin and metalloproteinase</td>
</tr>
<tr>
<td>ATF-3</td>
<td>Activating transcription factor – 3</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CHD</td>
<td>Coronary Heart Disease</td>
</tr>
<tr>
<td>CS-1</td>
<td>Connecting segment 1</td>
</tr>
<tr>
<td>Cx</td>
<td>Connexin</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EI</td>
<td>Epoxyisoprostane</td>
</tr>
<tr>
<td>EP2</td>
<td>E-type prostaglandin receptor</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GRP78</td>
<td>Glucose regulated protein 78kDa</td>
</tr>
<tr>
<td>HAEC</td>
<td>Human aortic endothelial cell</td>
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<tr>
<td>HBEGF</td>
<td>Heparin binding epidermal growth factor</td>
</tr>
<tr>
<td>HDL</td>
<td>High-density lipoprotein</td>
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<td>Acronyms</td>
<td>Complete Words</td>
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<tr>
<td>HMG-CoA</td>
<td>3-hydroxy-3-methylglutaryl coenzyme A</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human Umbilical Vein Endothelial Cells</td>
</tr>
<tr>
<td>IL-8</td>
<td>Interleukin-8</td>
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<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
</tr>
<tr>
<td>LDL-C</td>
<td>LDL cholesterol</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemotactic protein – 1</td>
</tr>
<tr>
<td>mm-LDL</td>
<td>Minimally modified LDL</td>
</tr>
<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
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<tr>
<td>OxPAPC</td>
<td>Oxidized 1-palmitoyl-2-arachidonoyl-\textit{sn}-glycerol-3-phosphatidylcholine</td>
</tr>
<tr>
<td>Ox-PL</td>
<td>Oxidized phospholipids</td>
</tr>
<tr>
<td>OxPNB</td>
<td>Biotinylated analog of OxPAPC</td>
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<tr>
<td>PAPC</td>
<td>1-palmitoyl-2-arachidonoyl-\textit{sn}-glycerol-3-phosphatidylcholine</td>
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<tr>
<td>PAPE</td>
<td>1-palmitoyl-2-arachidonoyl-\textit{sn}-glycerol-3-phosphoethanolamine</td>
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<td>1-palmitoyl-2-donoyl-\textit{sn}-glycerol-3-phosphatidylcholine</td>
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<td>PGE2</td>
<td>Prostaglandin E2</td>
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<td>PGPC</td>
<td>1-palmitoyl-2-glutaroyl-\textit{sn}-glycero-phosphatidylcholine</td>
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<tr>
<td>PLA2</td>
<td>Phospholipase A2</td>
</tr>
<tr>
<td>PNB</td>
<td>1-palmitoyl-2arachidonoyl-\textit{sn}-glycero-3-phosphatidyl-(N-biotinylethanolamine)</td>
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<td>Acronyms</td>
<td>Complete Words</td>
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<td>----------------------------------------------------------</td>
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<tr>
<td>POVPC</td>
<td>1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Real-time polymerase chain reaction</td>
</tr>
<tr>
<td>TER</td>
<td>Transendothelial electrical resistance</td>
</tr>
<tr>
<td>VEC</td>
<td>Vascular Endothelial Cells</td>
</tr>
<tr>
<td>VEGFR2</td>
<td>Vascular endothelial growth factor receptor 2</td>
</tr>
<tr>
<td>VMSC</td>
<td>Vascular smooth muscle cells</td>
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