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Oxidized Phospholipid Regulation of Chronic Inflammation in Endothelial Cells

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OXIDIZED PHOSPHOLIPID REGULATION OF CHRONIC INFLAMMATION IN ENDOTHELIAL CELLS

by

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Atherosclerosis, the primary cause of Coronary Heart Disease (CHD), has become one of the top causes of death in the world. As low-density lipoprotein (LDL) particles become trapped and oxidized inside arterial walls, the primary oxidation products of 1-palmitoyl-2-arachidonyl-*sn*-glycerol-3-phosphatidylcholine (PAPC) induce the binding of monocytes which can potentially lead to fatty plaque build ups. A fragment of one of these oxidation products, OxPAPC, has previously been studied and can act as standard for testing new phospholipids and how they affect protein expression levels. The central hypothesis of this study was that three newly studied classes of oxidized fatty acids exhibit distinct control patterns over specific genes involved in inflammation. The genes of interest are crucial at various points in the pathway: monocyte recruitment (MCP-1), inflammation (IL-8), and oxidative stress regulation (HO-1). Previous studies have shown that other molecules belonging to these classes of fatty acids are able to downregulate certain genes in the inflammation pathway for human aortic endothelial cells (HAECs), likely leading to decreased inflammation levels. The effects of these three groups of novel oxidized fatty acids known as isoprostanes, neuroprostanes, and neurofurans, were investigated and tested to see if there was potential for biological pathway control and identify effects of fatty acid structure on biological activity in HAECs. This research will assist in the development of future treatments for atherosclerosis and CHD.

ACKNOWLEDGEMENTS

I would first like to thank my committee chair, Dr. James R. Springstead, without whose constant encouragement and leadership any of this would have been possible. I would also like to thank my committee members, Dr. Qingliu Wu and Dr. Andrew Kline, for their much-appreciated help in the modeling of this thesis. Thanks also to my fellow graduate student, Piao Jian Tan, as well as the previous graduate students, Abbie Brackman and Gabriel Cole, whose guidance has been invaluable during this time. Also, thank you to all the undergraduate research assistants who have made this a wonderful teaching experience for me as well. Thank you all for your continued support through this thesis in its entirety.

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INTRODUCTION

The primary goal of this Master's thesis was to further understanding of the mechanism through which oxidized fatty acids exert control on the inflammatory pathway genes in human aortic endothelial cells. This was accomplished through defining and understanding the effect of lipid chemistry on regulation of pathways in atherosclerosis. This research will contribute to the development of novel treatments for coronary heart disease and, potentially, other chronic inflammatory diseases.

Importance of Chronic Inflammation/Atherosclerosis Research

Cardiovascular disease is the leading cause of death in fully modernized countries and is on the rise in developing countries. The most recent data shows that cardiovascular disease has claimed approximately 630,000 lives in 2016, accounting for 1 in every 4 deaths.[1] Atherosclerosis is the underlying condition in coronary heart disease (CHD), which involves the accumulation and hardening of fatty deposits on the inside of arterial walls, forming masses that restrict the flow of blood and potentially leading to severe adverse events, such as heart attack and stroke. Certain risk factors, such as smoking, poor diet, and high blood pressure can predispose people to be more at risk for developing atherosclerosis.[2]

Relation to Coronary Heart Disease

Atherosclerosis can occur in any artery inside the body, including but not limited to, the heart, brain, legs, and arms. The main issue is that organs that can be affected by the disease require oxygen-rich blood for proper functioning. Coronary heart disease (CHD), also known as coronary artery disease, occurs when plaque deposits inside of the coronary arteries of the heart

which can lead to various number of diseases if untreated. A pictorial example of this blocking of arteries is shown in figure 1.

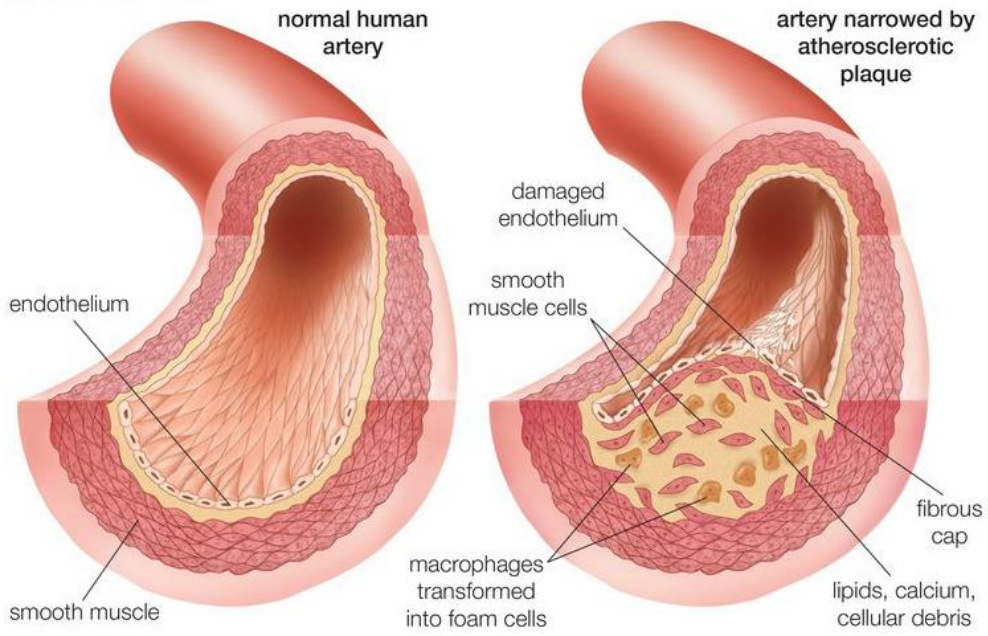

Figure 1. Normal Artery versus Partially Blocked Artery Due to Plaque Build-Up[3]

On the left-hand side of Figure 1, normal artery physiology is shown where no significant amounts of plaque are present and blood flow is unrestricted. As for the right side, plaque build-up is shown which allows for a substantial decrease in the volumetric flow rate of blood that can be allowed through. When referring to specific examples of diseases that can arise from the above pathophysiology, one set of examples are angina and myocardial infarction, otherwise known as chest pain and a heart attack. When the crucial arteries that supply blood to the heart are blocked, there is increased risk of more serious conditions such as heart attack or stroke, both of which can cause morbidity or mortality.[4]

Current Treatments

Presently, there are a number of treatment regimens available for people affected by CHD. These treatment plans include heart healthy eating, maintaining a healthy weight for a given age and height, stress management, as well as physical activity. The goal of these plans is to affect the formation of plaques at the arterial level. This is accomplished through the following overarching points: lowering risk of blood clot formation, preventing development/complications of CHD, reducing risk factors inherent to certain individuals, relieving already present symptoms, as well as widening or bypassing already clogged arteries.[5] Outside of activity-based lifestyle changes, there are also pharmaceutical treatment options that are available. Statins, medications that alter cholesterol concentrations by inhibiting cholesterol production by the body, can cause a decrease in the amount of plaque deposition on coronary arteries. Other medications available are aspirin, beta blockers, calcium channels blockers, and nitroglycerin tablets. More serious methods of treatments involve surgical intervention. Recent procedures that have had fairly significant clinical success are angioplasty/stent placement and coronary artery bypass surgery.[6] While each of these options are viable depending on the stage of blockage that is present in the coronary arteries, future novel treatments must be developed in order to combat the increasing prevalence of heart disease and decrease necessity of surgery, which carries its own risks and cost. In order for this to happen, research science must gain a better grasp on how atherosclerosis works mechanistically.

LITERATURE REVIEW

This literature review will focus on previous research that focused on elucidating how oxidized fatty acids regulate atherosclerosis. Research topics covered in the past that were analyzed are: current theory of atherosclerosis pathophysiology, oxidized phospholipid roles in atherosclerosis, and currently understood role of oxidized fatty acids in atherosclerosis.

Proposed Theory

Recent studies have shown that the formation of atherosclerosis begins with either endothelial injury or by a significant accumulation of low-density lipoprotein (LDL) particles along the inside of the arterial wall. LDL particles may also be oxidatively modified. Once these LDL particles are modified, they take on the form known as minimally modified low density lipoproteins (MM-LDL). Varying levels of inflammation brought about by endothelial injury causes the body to trigger both an innate and adaptive immune response.[7] Figure 2 shows a simplified representation of how these arterial fatty streaks form in the initial stages of the plaque development.

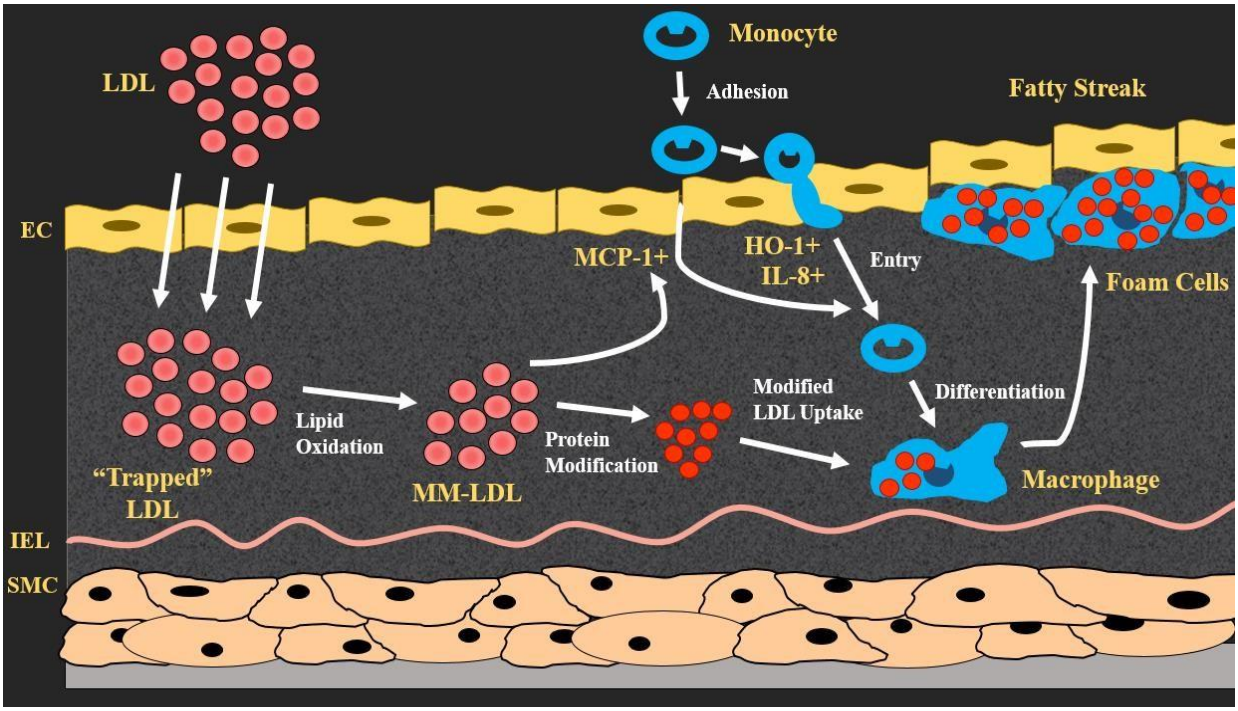

Figure 2. Atherosclerotic Inflammation Pathway Diagram[8]

As seen above, during the development of atherosclerosis, LDL particles pass through the arterial wall and become "trapped" LDL as they undergo oxidation in addition to becoming MM-LDL. These MM-LDL particles contain oxidation products of 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphocholine (Ox-PAPC), as well as other oxidized phospholipids.[9] Referring to the monocyte recruitment pathway, interleukin 8 (IL-8) and monocyte chemotactic protein (MCP-1) are genes that have both been shown to become upregulated in endothelial cells in the presence of Ox-PAPC.[10] MCP-1 is a potent monocyte attractant as well as a member of the CC chemokine family. These chemokines are those with adjacent cysteine amino acid residues near the N-terminus of their protein structure, aiding in their targeting of monocytes as well as T-cells in the body. During inflammation, MCP-1 plays a crucial role in the recruitment of monocytes in order to alleviate infections or in this case, accumulation of oxidized lipids.[11] IL-8 is classified as a

chemoattractant cytokine which aids in the recruitment of neutrophils, a type of white blood cell.[12] In regions where inflammation occurs, IL-8 is produced by a variety of cell types due to inflammatory stimuli and in some studies, has been shown to aid in the recruitment of rolling monocytes onto the arterial wall to combat atherosclerotic lesion formation.[13] Another protein of interest that will be looked at is heme oxygenase-1 (HO-1) which has been shown to exhibit immunosuppressant effects in response to oxidative stress. HO-1 is believed to be involved in this process due to these anti-inflammatory effects. At this point in the pathway, the oxidized LDL particles lead to endothelial dysfunction by attracting monocytes as well as T cells to adhere to the interior of the artery wall, potentially forming foam cells, and eventually fatty, atherosclerotic streaks.[14] These fatty streaks may eventually progress to advanced lesions which often lead to further decrease in the volumetric flow of blood through the artery..

Overview of Ox-PAPC, PEIPC, and EI

The research set forth in this thesis was focused on Ox-PAPC and its major oxidative components. Of the oxidized biomolecules present in Ox-PAPC, one of the most prolific and activity enhancing is 1-palmitoyl-2-(5,6-epoxyisoprostane E_2)-*sn*-glycero-3-phosphorylcholine (PEIPC) and is shown below in Figure 3.[15]

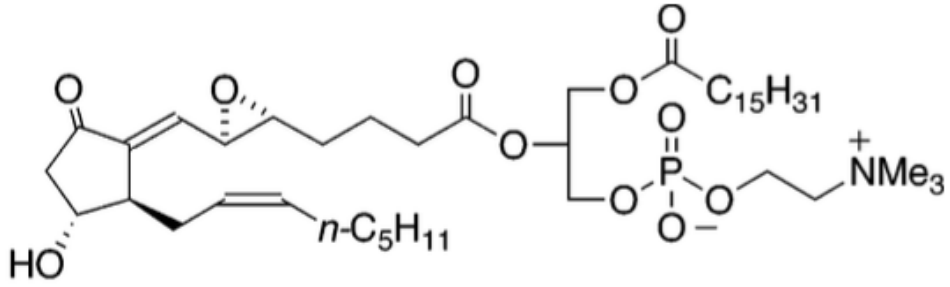

Figure 3. PEIPC Chemical Structure[16]

As shown in multiple studies, PEIPC is a potent inducer of monocyte binding during chronic inflammation. One study in particular involved the collection of endothelial cell cultures from 149 different heart transplant donors. 149 HAEC cultures were obtained from aortic explants of the aforementioned donors and grown to an appropriate confluency level. Significant activity was noted for the cells treated with oxidized phospholipid, including Ox-PAPC and PEIPC. The allylic epoxide group on PEIPC is thought to be the major activity site for the molecule which lends to increased monocyte binding.[17] Microarray analysis showed that of the 2000 genes in question for the study, over 1000 were regulated by Ox-PAPC and at least 80% of these were controlled by PEIPC.[18]

As critical as oxidized phospholipids are for regulation of inflammation, recent studies have shown what may prove to be a key mediator for oxidized phospholipid activity in the form of phospholipases. These phospholipases impact the activity of oxidized phospholipids through hydrolysis of the oxidized fatty acid or the functional head group. This reaction results in the formation of lysophospholipids, phosphatidic acids, and other oxidized fatty acids. Cleavage at each site requires a specific phospholipase. A summary of this activity modulation reaction can be seen below in Figure 4.[19]

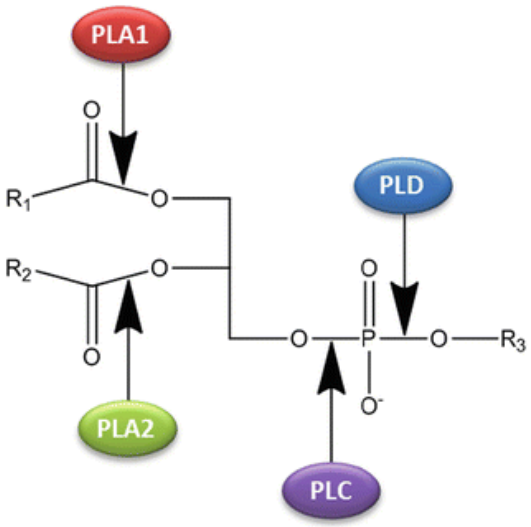

Figure 4. Oxidized Phospholipid Cleavage by Phospholipases[19]

A specific case study of this phospholipase activity involved the culture of HAECs and subsequent treatment with oxidized phospholipids, including PEIPC, that had themselves been treated with snake venom phospholipase A_2 (PLA_2). The results of the study showed that the introduction of PLA_2 decreased PEIPC's capability to stimulate monocyte binding.[20] When treated with PLA_2, the phospholipase reaction produces four regioisomers of PEIPC, the most active of the four is hypothesized to be 5,6-epoxyisoprostane (EI). The reaction and subsequent product can be seen in Figure 5.[18]

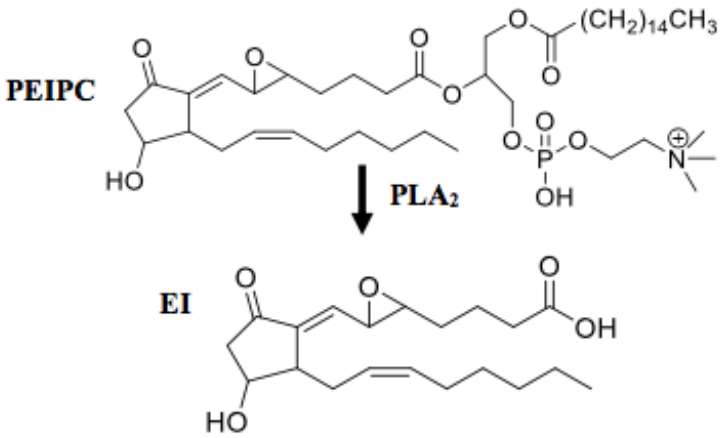

Figure 5. Phospholipase Reduction of PEIPC to EI

From the above previous study that looked at the synthesis of the four analogue structures of EI and their respective effects on genes of interest, Figure 6 shows the structure of each enantiomer of EI, including EI4, which data showed was the most biologically active of the stereoisomers in HAEC as measured by regulation of IL-8, HO-1 and MCP-1.[18]

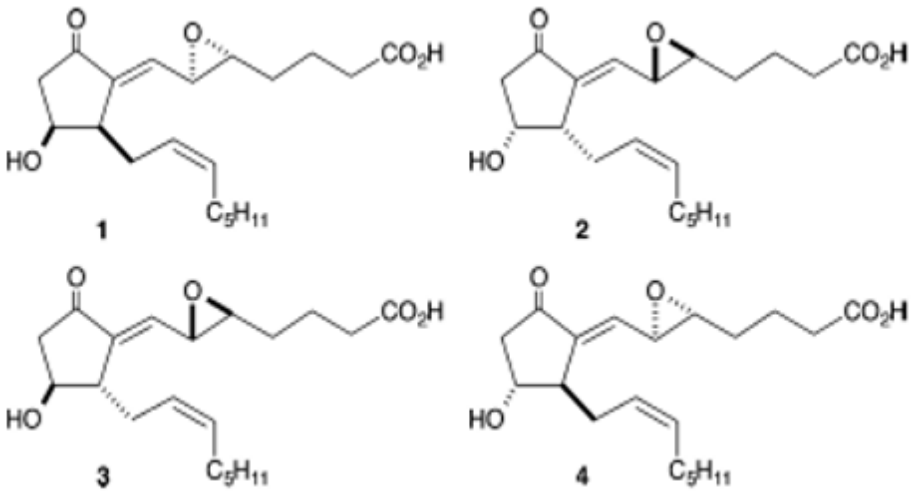

Figure 6: Structure of EI Analogues[18]

Working from previous research that hinted at the importance of EI and its relation to Ox-PAPC and PEIPC in terms of activity, the main hypothesis was that EI would exhibit similar activity levels to PEIPC, be more feasible in terms of synthesis for future studies, and also act as a more stable oxidizing molecule. In relation to the work of this thesis, the effects of EI4 were not directly tested but should be confirmed in future work on the subject. EI4 and PEIPC showed a 5-fold decrease and a 13-fold increase in MCP-1 mRNA levels, respectively. These treatments also resulted in a 3.0-fold increase in IL-8 mRNA for EI4 and a 17-fold increase for PEIPC.

Finally, these treatments also resulted in a 15-fold increase in HO-1 mRNA from EI treatment while PEIPC showed around a 10-fold increase. EI the compound downregulated eight other inflammatory molecules in HAEC, potentially increasing the likelihood that it is involved as an anti-inflammatory regulator. It is vital to look at the effects of EI on IL-1β, which is a cytokine that has been shown to accumulate in atherosclerotic lesions in mice. When this cytokine has been knocked out, atherosclerosis levels decrease in research settings. Results from this study have shown that EI was able to successfully inhibit IL-1β. From these results, it is clear that with respect to certain genes involved in the inflammation pathway, EI and PEIPC regulate similar pathways, but EI shows anti-inflammatory effects, whereas PEIPC often upregulates inflammatory pathways.[18]

Newly Studied Fatty Acids

The purpose of this thesis was to validate and expand previous work results for oxidized lipids, providing a stronger research foundation for future endeavors into the pathway and its intricacies. In this research, regulation of the inflammatory pathway (IL-8), the oxidative stress pathway (HO-1) and the monocyte binding pathway (MCP-1) by an isoprostane, a neuroprostane, and a neurofuran will be compared to regulation by EI. Isoprostanes are molecules produced as a result of free radical damage to arachidonic acid (AA), which is a fatty acid that is heavily distributed throughout both the white and grey matter of the brain. Shown below in Figure 7 is the specific isoprostane that was used in this study.[21]

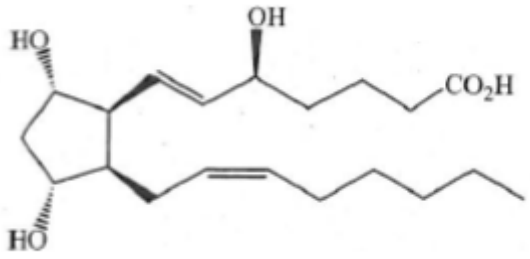

Figure 7: Isoprostane from AA

Neuroprostanes are created in an analogous manner from the molecule docosahexaenoic acid (DHA). In contrast to AA, DHA is mostly present in the grey matter where its main concentration lies in neurons of the region. It has been hypothesized that neuroprostanes may be more sensitive biomarkers for damage caused by free radicals due to DHA being more prone to peroxidation than its counterpart AA. Figure 8 shows the neuroprostane that was involved in this study.[21]

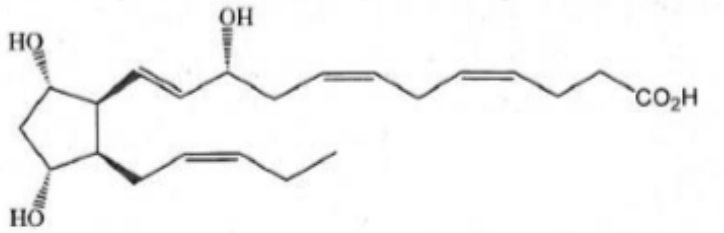

Figure 8: Neuroprostane from DHA

Similarly to neuroprostanes, neurofurans are derived from DHA and can be used as biomarkers to test for levels of oxidative damage in certain areas of the body where these molecules are concentrated. One caveat of these DHA derived molecules is that using them as biomarkers is currently more complicated than using a molecule derived from AA because DHA

is more unsaturated than AA. Shown below in Figure 9 is the neurofuran that was tested in this thesis.[22]

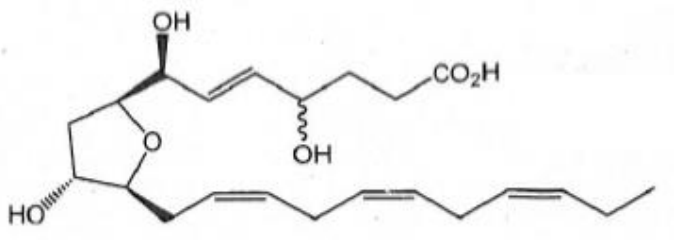

Figure 9: Neurofuran from DHA

The use of isoprostanes and neuroprostanes in the detection of neurological diseases, such as Alzheimer's disease, is currently on the rise as the synthesis rate of these molecules in the brain is noticeably increased when the disease is present.[23] More work is currently being done in the field of neurofurans to if detection of these fatty acids can be detected early enough and used as a risk factor measurement.

MAIN OBJECTIVES

Building on the above-mentioned work, the goal of this research was to further elucidate the mechanism by which oxidized fatty acids, including neurofurans, neuroprostanes, and isoprostanes, affect inflammation in HAECs. This objective was accomplished through the treatment of HAECs with the novel fatty acids and the use of RT-PCR to determine which gene levels are upregulated or downregulated by each treatment. Because the effects of neurofurans,

neuroprostanes, and isoprostanes have not been extensively studied, the results achieved from these molecules were compared to OxPAPC regulation.

Broader Impact

The broader impact of this thesis was to aid in the understanding of how atherosclerosis functions mechanistically. Uncovering this mechanism required experimental data that demonstrated how novel oxidized fatty acids influence inflammation in HAECs, further expanding on previous research in the field. Acquiring this data has served two main purposes: to compare against previous research data acquired in other studies and to act as a comparison against the data for the oxidized fatty acids that have not been tested to date in other work. Overall, the long-term goal of this project will be to help future research efforts to be able to develop more efficient treatment plans for patients affected by atherosclerosis or even reveal potential novel biomarkers for coronary heart disease.

Experimental Methods

The purpose of this experimental methods section is to detail techniques used in the treatment of HAECs with oxidized fatty acids as well as the subsequent data collection methods and procedures. To determine the effect of oxidized fatty acids on inflammation levels for HAECs, several techniques were employed. The HAECs had to be grown to an appropriate level of confluency on tissue culture plates and then treated with 3 μM isoprostane, neuroprostane, or neurofuran. The goal of this stage was to measure gene expression levels based on specific treatments, while comparing fatty acid regulation with previous results. For lipid treatment, the standard exposure time of HAECs to each lipid is approximately 4 hours, but this time was

altered as needed to ensure optimal results. Each 6 well treatment plate was split up into three groups: the first being control (no lipid treatment), the second with 20 µM Ox-PAPC, and the final group with 3 µM of one of the three oxidized fatty acid types of interest. Once the treatment time had lapsed, the cells were exposed to an RNA lysis solution to lyse the cells. From here the RNA were extracted and the concentration was determined to know if enough exists for cDNA preparation in the next step. Finally, RT-PCR was employed to determine the genetic regulation effect of the fatty acids on GAPDH (normalization gene), MCP-1, IL-8, and HO-1. The results obtained for the novel oxidized fatty acids (isoprostanes, neuroprostanes, and neurofurans) were compared to collected results for OxPAPC to show if they are involved in upregulation or downregulation of the above genes, and by what magnitude.

METHODOLOGY

The purpose of this methodology is to outline the experimental procedures used to meet the research objectives set forth in this thesis. This section will include the following sections: materials and reagents, endothelial cell culture growth, oxidized fatty acid treatment, RNA extraction and cDNA synthesis, and RT-PCR.

Materials and Reagents

HAECs and Fetal Bovine Serum (FBS) were purchased through Thermo Scientific. Media 199 (M199) was purchased through MediaTech. RNA Primers for GAPDH, MCP-1, IL-8, and HO-1 were purchased through Integrated DNA Technologies. OxPAPC used during experimental procedures was synthesized beforehand using organic chemistry methods and was

used in the procedure of a previous publication.[18] The three types of novel oxidized fatty acids used were synthesized and provided to us by collaborators at Institut des Biomolecules Max Mousseron (IBMM) (Jean-Marie Galano and Thierry Durand).

HAEC Culture Growth

This section will detail the procedure of growth and transfer of HAECs between appropriate growth and treatment settings. To begin with, 100 mm tissue culture dishes were coated with 5 mL of premade gelatin solution (need concentration) along the entire bottom of the plate to create a scaffolding for cells to later adhere to. Once allowed to incubate at 37 ^{o}C for ~30 minutes, the gelatin was aspirated and the plates were washed with 10 mL of Phosphate Buffer Solution (PBS) and appropriately mixed. The PBS was aspirated, and the plates were then filled with 19 mL of endothelial cell basal medium (ECBM) to provide a nutrient source for the HAECs. Cells (known as Passage 1 or P1 cells) were then transferred in 1 mL of ECBM to the 100 mm plate and sufficiently spread out, ensuring the cells were not overly agitated.

HAEC Passage Splitting

Once HAECs had approached a critical level of confluency, the present ECBM was aspirated from the 100 mm plate. The cells were washed with 10 mL of PBS and then aspirated. Using a 1:3 dilution rate, a trypsin/PBS solution was created, and 3 mL were applied to each plate in order to remove the cells from their scaffolding. Trypsin is an enzyme that cleaves attachment proteins on the cells, allowing for lifting and transfer. After appropriate washing with the trypsin/PBS solution, the 3 mL of cell solution was transferred to a 15 mL test vial and placed in the centrifuge at a speed of 1000 rpm for approximately 10 minutes. The residual

media supernatant above the cell pellet was subsequently aspirated and the remaining pellet was rinsed with 1 mL of ECBM for each plate that the cells would be split to. Most often a 1:3 splitting was desired, so from the previous passage of n number, 3n next passage plates would be produced until passage 5 or 6 was reached, depending on confluency levels.

HAEC Treatment with Oxidized Fatty Acids

This section explains how cells were treated with oxidized fatty acid once the appropriate passage number was achieved and cells were transferred to 6 well plates. HAECs were washed with PBS a total of three times, which was followed by treatment with 3 µM EI, 20 µM Ox-PAPC, or 3 µM isoprostane/neuroprostane/neurofuran in M199 medium containing 1% FBS for four hours.[9] HAECs will be later washed with PBS, lysed in order to extract the RNA, and RT-PCR analysis was run.

Real-Time Polymerase Chain Reaction

For RT-PCR analysis, the 7900HT Sequence Detector System model was utilized. Complete RNAs from the HAECs was extracted from the fatty acid treated cells using the Total RNA extraction procedure from the modified Bio-rad protocol. cDNA synthesis was performed using the Applied Biosciences kit. The SYBR® green master mix bought from ThermoFisher was used in PCR quantification and amplification. For the genes being measured in the PCR analysis, GAPDH was measured to normalize mRNA as well as fold change levels of the other genes between both treated and untreated cells. Each specific primer sequence used in the qPCR analysis was as follows:

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′; HO-1: Forward: 5′ - ATA GAT GTG GTA CAG GGA GGC CAT CA - 3′, Reverse: 5′ - GGC AGA GAA TGC TGA GTT CAT GAG GA - 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′: Forward: 5′ - ACT CTC GCC TCC AGC ATG AA - 3′, Reverse: 5′ - TTG ATT GCA TCT GGC TGA GC – 3’.

PREVIOUS EXPERIMENTAL RESULTS

Referring back to work completed by previous graduate students, a trend of expected results can be obtained to use as a measure for any experimental readouts. The data sets were divided into four main groups, gene regulation levels for OxPAPC, isoprostane, neuroprostane, and neurofuran. For each of these lipids, regulation of MCP-1, IL-8, and HO-1 mRNA levels will be measured.

Gene Regulation by Novel Oxidized Fatty Acids in HAECs

The next three data sets focus on the effect on MCP-1, IL-8, and HO-1 by the novel oxidized fatty acids in the form of isoprostane, neuroprostane, and neurofuran. Through these data sets, it is the hope of this thesis that more information can be deduced about the chemical pathways and reasoning behind the function of oxidative stress in inflammation. Figures 11 through 13 summarize the results of the three groups and their effects on mRNA fold change levels.

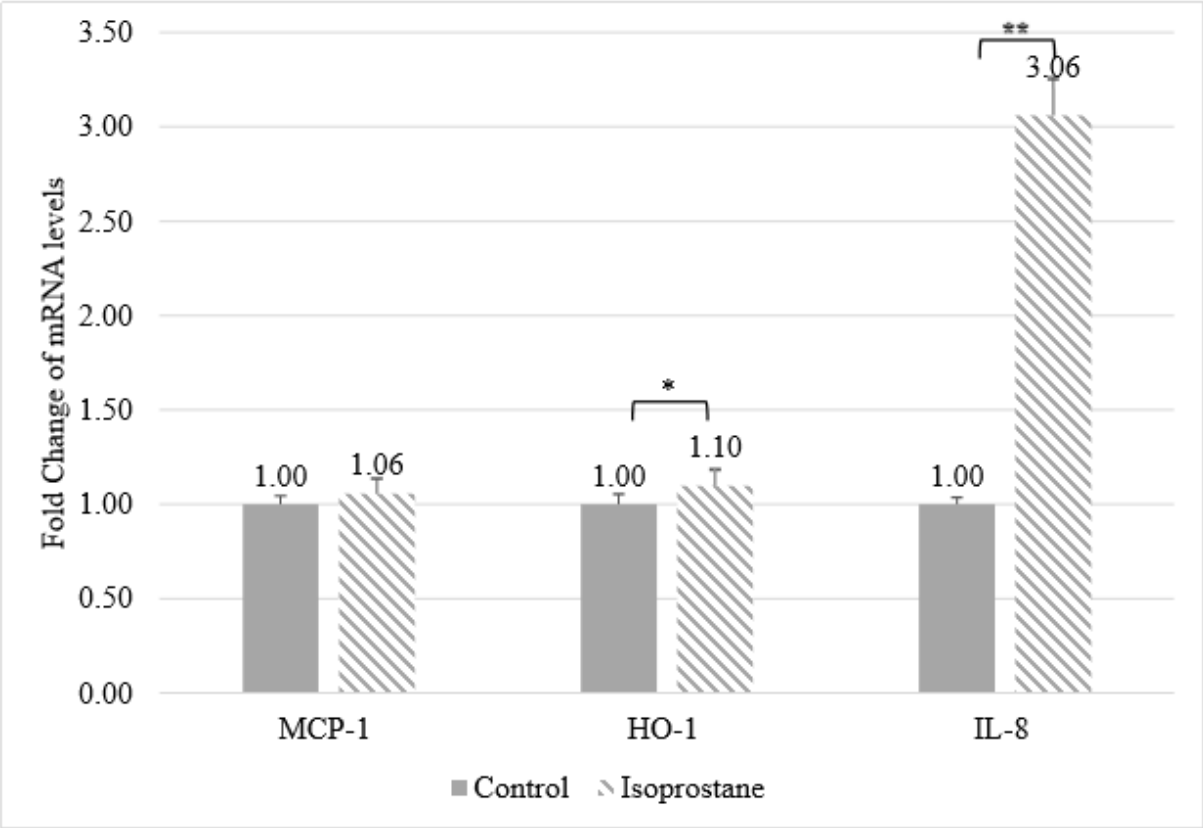

Figure 10. Previous Results from 3μM Isoprostane Treatment Testing Gene Regulation Levels of HAECs

Figure 10 serves to graphically represent regulation of MCP-1, HO-1 and IL-8 mRNA levels by the isoprostane in HAECs, named 15-F_{21}-IsoP ($C_{20}H_{34}O_5$). Both MCP-1 and HO-1 show minimal increases in fold change with values of 1.06 and 1.10 respectively. IL-8, however saw an incredibly significant increase in fold change with an associated value of 3.06. In the above graph, the double asterisk will represent a p-value less than 0.01, but the single asterisk is indicative of a p value less than 0.05. This value of 0.05 is still able to reject the null hypothesis, but with less statistical significance. These results may be indicative of certain isoprostanes upregulating inflammatory (IL-8), oxidative stress (HO-1) and monocyte recruitment (MCP-1) gene pathways in HAECs.

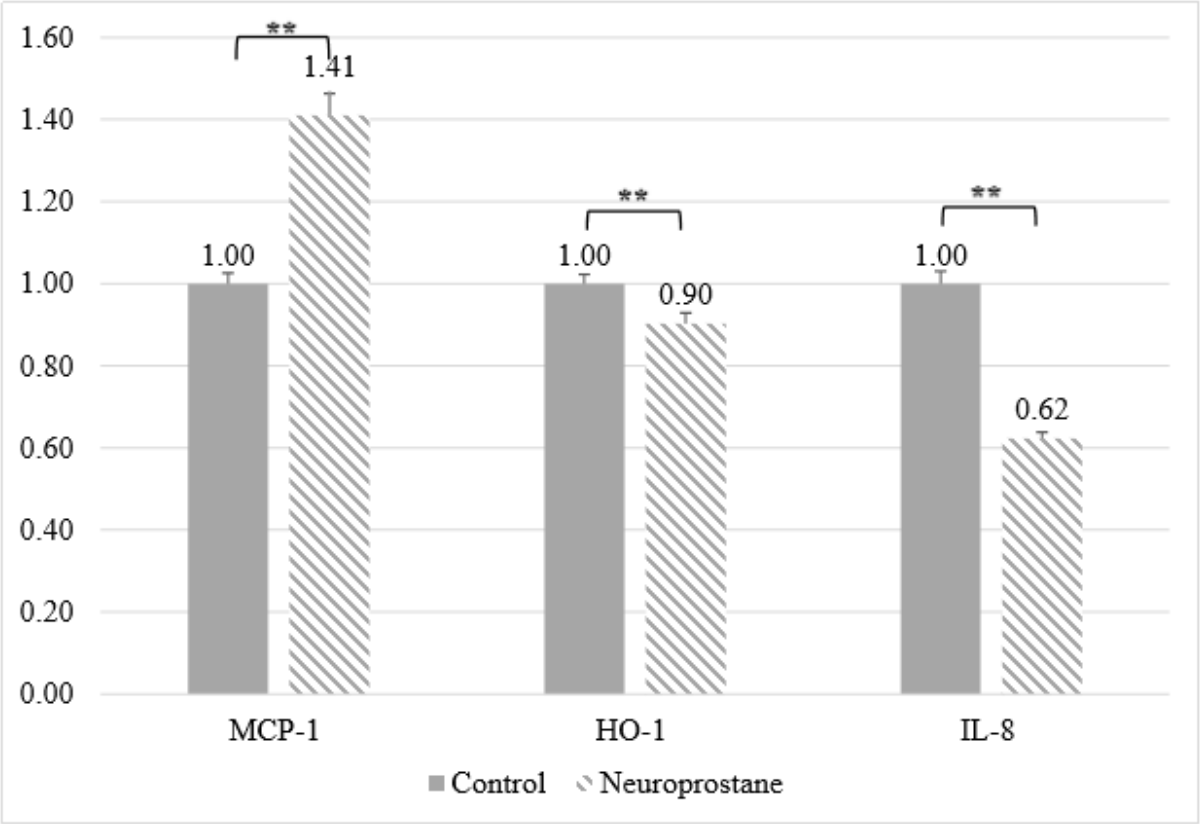

Figure 11. Previous Results from 3μM Neuroprostane Treatment Testing Gene Regulation Levels of HAECs

Figure 11 shows gene regulation levels for the neuroprostane, named 4-epi-4-F_{31}-NeuroP ($C_{22}H_{36}O_5$) has on the mRNA fold change levels of the three genes of interest. MCP-1 mRNA was upregulated by 1.41-fold in HAECs while both HO-1 and IL-8 were both downregulated, with fold changes 0.90 and 0.62, respectively after neuroprostane treatment.

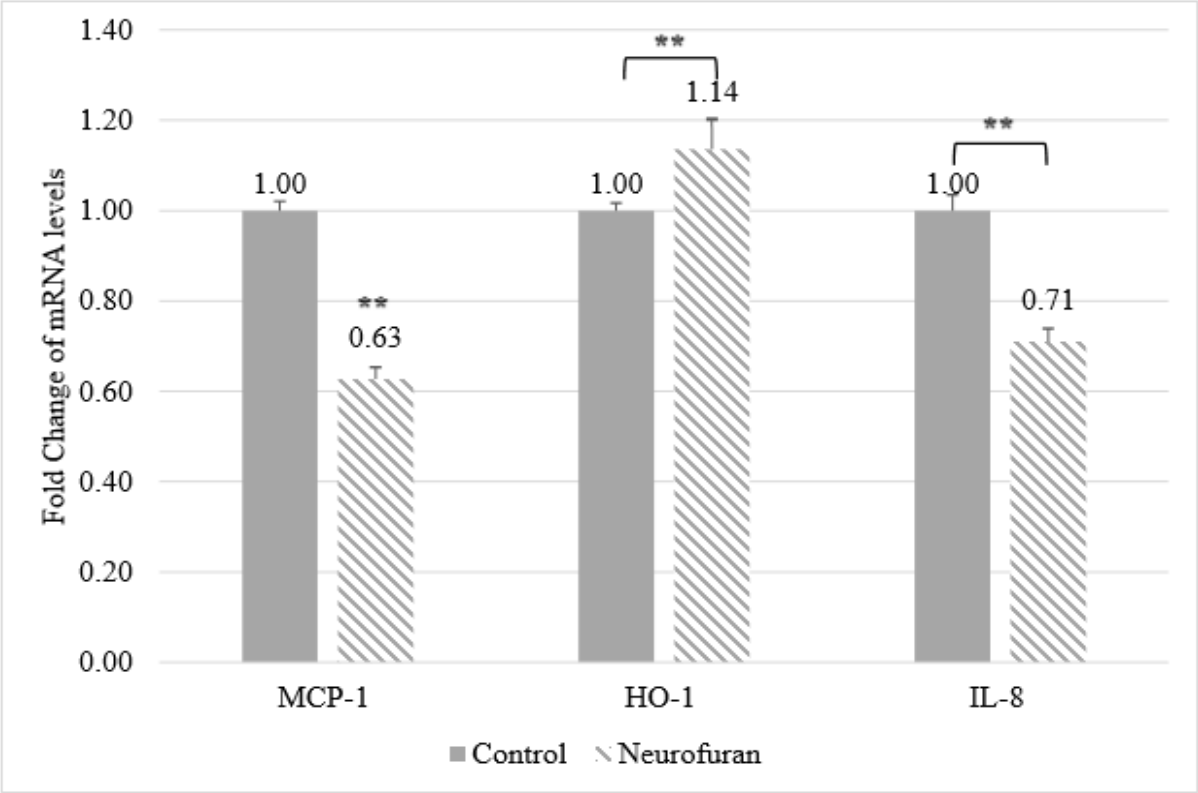

Figure 12. Previous Results from 3μM Neurofuran Treatment Testing Gene Regulation Levels of HAECs

Figure 12 shows gene regulation levels for the neuroprostane, named 4-epi-4-F_{31}-NeuroP ($C_{22}H_{36}O_5$) has on the mRNA fold change levels of the three genes of interest. HO-1 mRNA levels were significantly upregulated by neuroprostane treatment of HAEC, with a fold change of 1.14, while both MCP-1 and IL-8 were downregulated, with fold changes of 0.63 and 0.71, respectively.

EXPERIMENTAL RESULTS

Using the methodology outlined above, the effects of the three types of oxidized fatty acids on the inflammatory pathway were observed in HAECs and validated using two methods. First, the effects of OxPAPC and its subsequent up- or downregulation of the three proteins must be considered. The reasoning for analyzing OxPAPC along with the lipid and control wells is to ascertain whether the results are valid based on previous research which shows the appropriate effects of OxPAPC on the expression levels. From previous work, it has been shown that OxPAPC upregulates both HO-1 and IL-8 but downregulates MCP-1. Based on this the results can be given an extra measure of their validity.

Calculation Methods

Working into the second validation method, it is necessary to explain how both the fold change levels were calculated as well as the p-value. GAPDH was analyzed along with HO-1, IL-8, and MCP-1 for the purpose of being a housekeeper gene. This specific gene acts as the control baseline from which the other value can subtract this baseline to yield the true fold change expression levels. This was completed with the following data points obtained through the 7900HT Sequence Detector System and is shown in equations one through three.

$$\Delta Ct = \text{Average Ct Value of Sample} - \text{Average Ct Value of GAPDH} \quad \textbf{(1)}$$

$$\Delta\Delta Ct = \Delta Ct_{\text{ Experiment}} - \Delta Ct_{\text{ Control}} \quad \textbf{(2)}$$

$$\Psi \text{ (Fold Change of mRNA Levels)} = 2^{(-\Delta\Delta Ct)} \quad \textbf{(3)}$$

The average Ct value is the average cycle number where the fluorescence created from the PCR reaction goes beyond the fluorescence threshold.[24] This subtracted from the GAPDH average cycle number, as mentioned above, gives a more realistic idea of when this number occurs for the gene in question. The $Ct_{Experiment}$ and $Ct_{Control}$ are the ΔCt values for samples treated with lipid and those that were not, respectively. Finally, the fold change level represents how much the lipid causes the up- or downregulation of the specific gene when normalized against the control sample.

After figuring out the fold change levels, the second method of result validation is utilized in the form of the p-value method. The p-value is the calculated probability of the null hypothesis of a given experiment being found true. For most cases, the null hypothesis, H_o is a statement of no difference. This means that there is no significant difference between the data sets of two (or more) groups.[25] For this thesis, H_o is that there is no significant difference between the control level of fold change regulation and the levels for the lipids being tested. Using a 95% confidence interval, is the p-value for the experiment is less than 0.05, then this is a rejection of H_o. This means that there is statistically significant difference between the control and lipid results. Calculation of the p-value was accomplished through the two sample T-test shown below in equations four through seven.

$$d.f. = n_1 + n_2 - 2 \tag{4}$$

$$s_p = \sqrt{\frac{(n_1-1)s_1^2+(n_2-1)s_2^2}{n_1+n_2-2}} \tag{5}$$

$$t = \frac{\Psi_1-\Psi_2}{s_p\sqrt{\frac{1}{n_1}+\frac{1}{n_2}}} \tag{6}$$

p-value = TDIST(t-test, d.f., number of tails) (7)

As shown above, n is the number of duplicate samples for a given test set. s_p is the standard deviation which was calculated by the 7900HT Sequence Detector System. Equations six and seven yield the t-test and subsequent p-value for each experiment. From the graphs that follow, a single asterisk above a result bar is indicative of a p-value less than 0.10 (rejection of H_o if the confidence interval was 90%). A double asterisk symbol above a test result bar indicates the p-value was below 0.05 and the result was statistically significant, rejecting H_o.

Results

For the isoprostane molecule tested in this thesis, the results obtained were relatively similar to previous work as shown below in Figure 13.

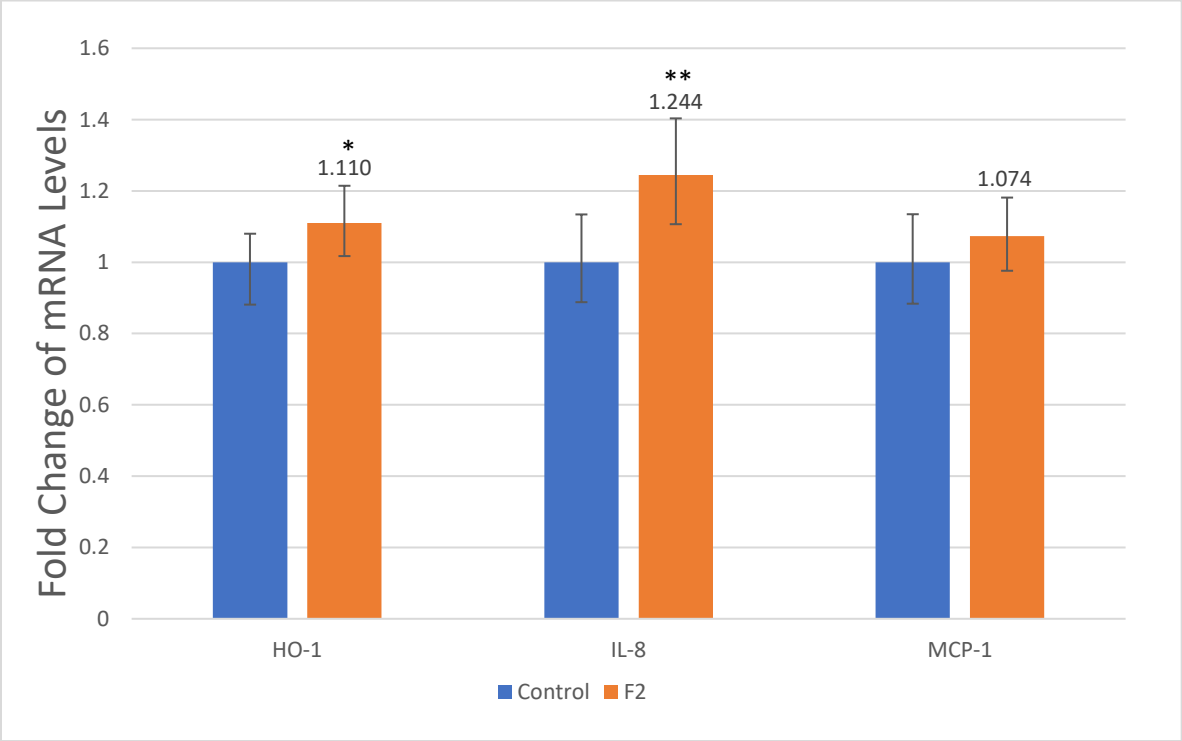

Figure 13. Control and Lipid Results from 3μM Isoprostane Treatment Testing Gene Regulation Levels of HAECs

All three proteins aw slight upregulation compared to the control wells, with IL-8 showing the highest level of upregulation among them. The p-values for HO-1, IL-8, and MCP-1 were 0.0659, 0.0048, and 0.2388 respectively. This indicates that the results for HO-1 are significant, but the results for IL-8 and MCP-1 were not able to say with certainty that they are statistically significant. The issue with this data set however, comes in the form of how OxPAPC expression levels with the three proteins resulted. Both HO-1 and IL-8 saw upregulation as was expected, however, MCP-1 also showed significant upregulation which contradicts previously established results and present a possible source of error for the isoprostane test results. This can be seen graphically below in Figure 14.

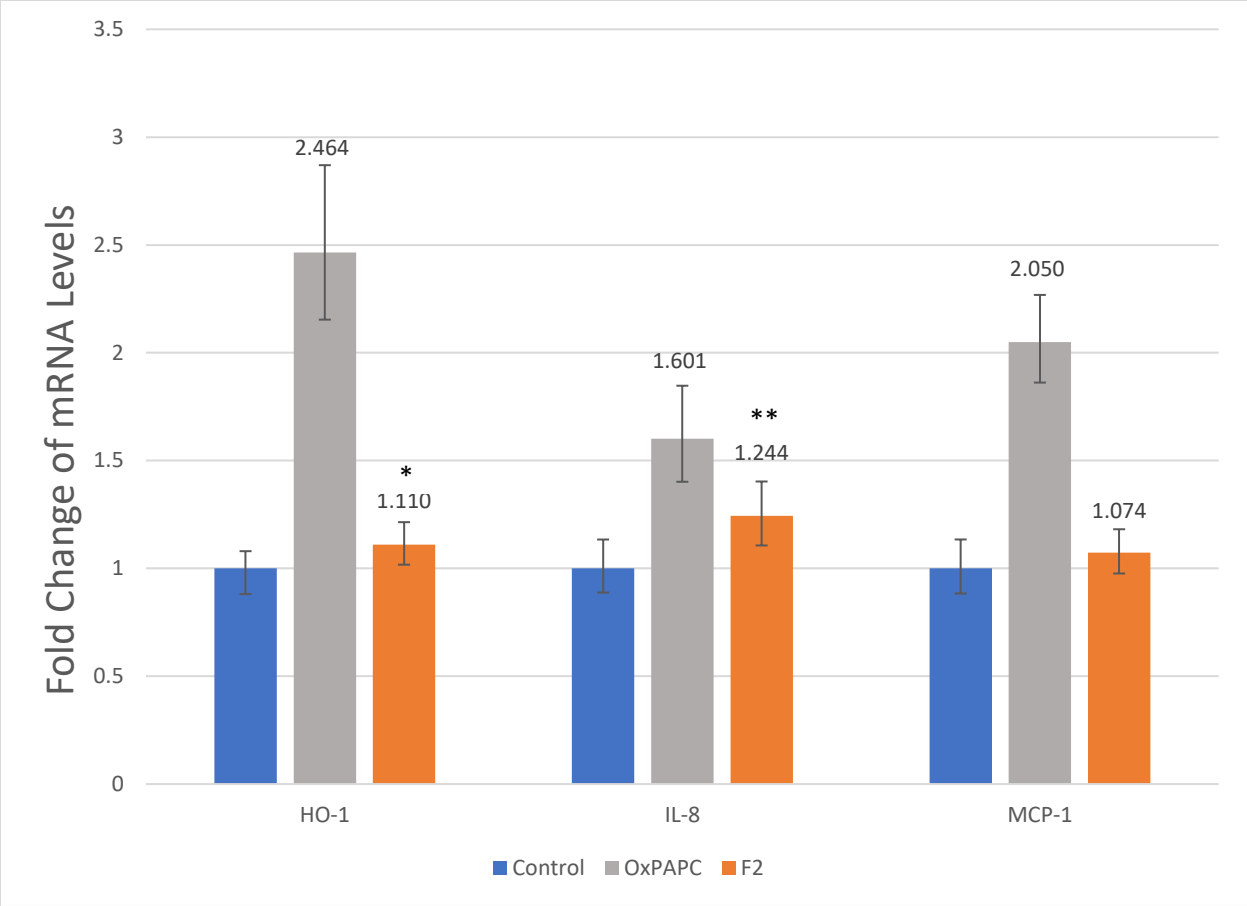

Figure 14. All Results from 3μM Isoprostane Treatment Testing Gene Regulation Levels of HAECs

Next to discuss is the neuroprostane. When comparing the control wells to those treated with neuroprostane, the results for HO-1 and IL-8 were near identical with only slight upregulation being noted in both cases. In the case of MCP-1, there was significant down regulation of the protein when the neuroprostane was present, indicating a potential for regulation of the monocyte recruitment pathway. The p-values for this set were 0.2673, 0.4967, and 0.0639, mimicking the trend from the isoprostane results for MCP-1 but giving different interpretations for HO-1 and IL-8. The graphical results can be seen below in Figure 15.

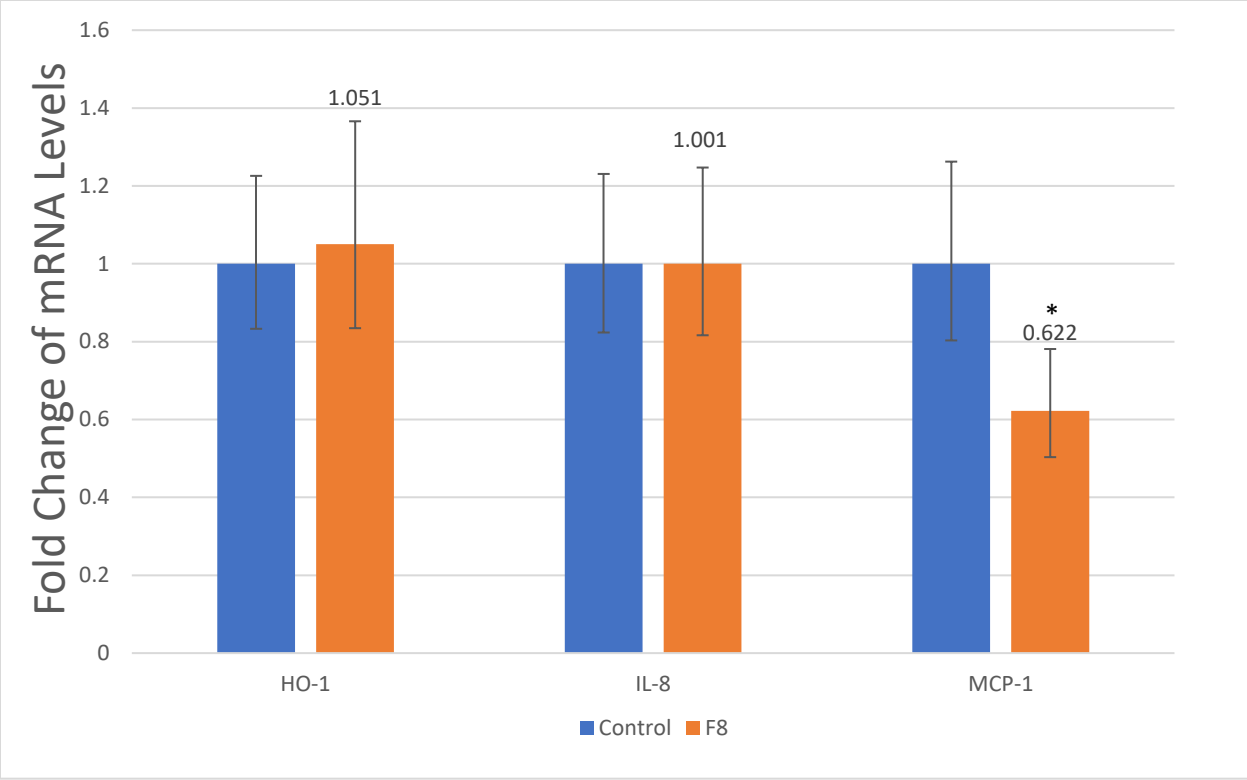

Figure 15. Control and Lipid Results from 3µM Neuroprostane Treatment Testing Gene Regulation Levels of HAECs

When comparing the neuroprostane data for validity, the comparison of results against OxPAPC seem to be slightly more valid than the isoprostane data. The tests show upregulation for HO-1 and downregulation for MCP-1, which are consistent with previous findings, however the issue comes when looking at IL-8 data. As seen in Figure 14, the levels shown in Figure 16 below show similar levels for HO-1 but the upregulation for IL-8 takes a moderate dip.

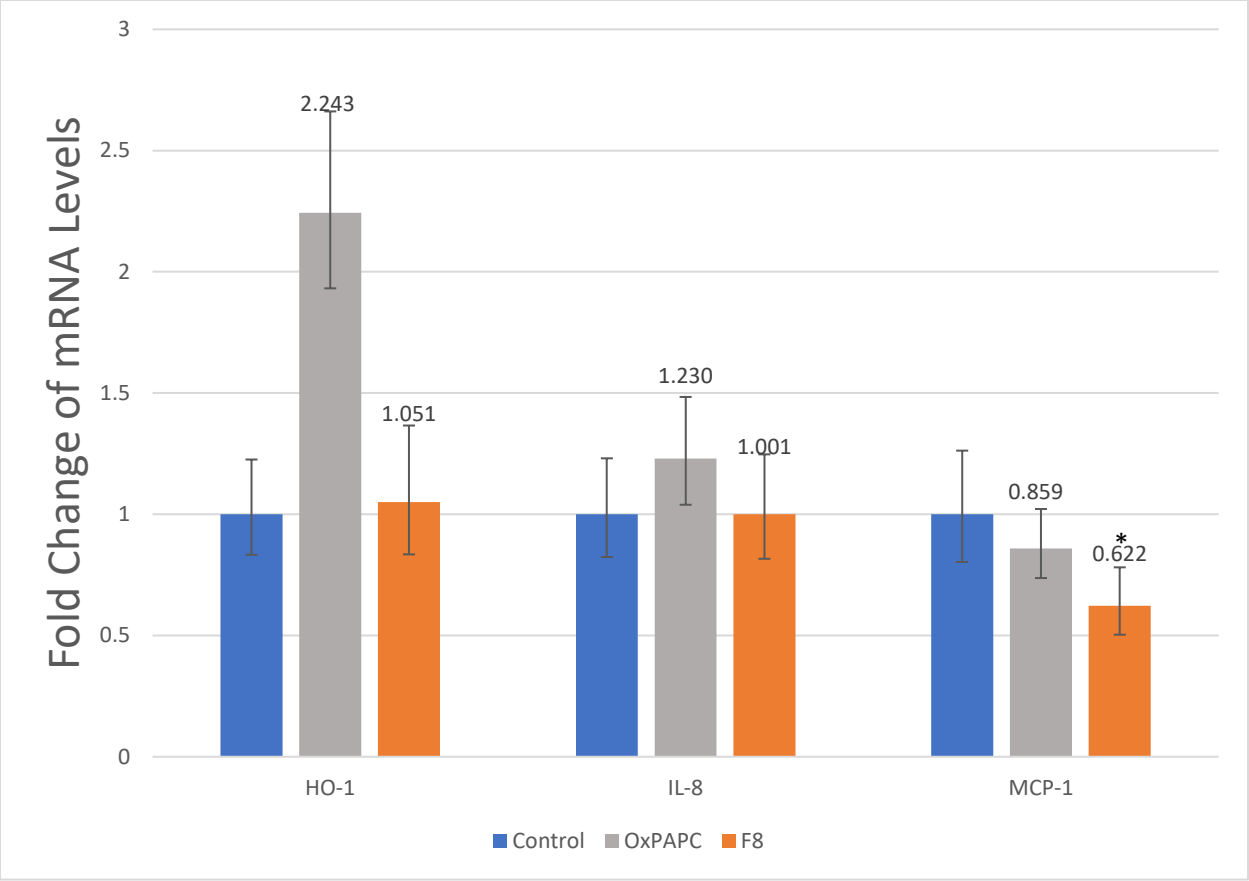

Figure 16. All Results from 3µM Neuroprostane Treatment Testing Gene Regulation Levels of HAECs

Finally, the expression level results for the neurofuran of interest must be noted. From Figure 17, upregulation can be observed for both HO-1 and IL-8, while expression for MCP-1 is downregulated, but not to a major extent. Again, looking to the p-values, the results were as follows: 0.1678, 0.294, and 0.195. This set of p-values indicates that none of the protein expression levels could be said to be statistically different from the control groups, bringing the validity of the neuroprostane as a test article into question.

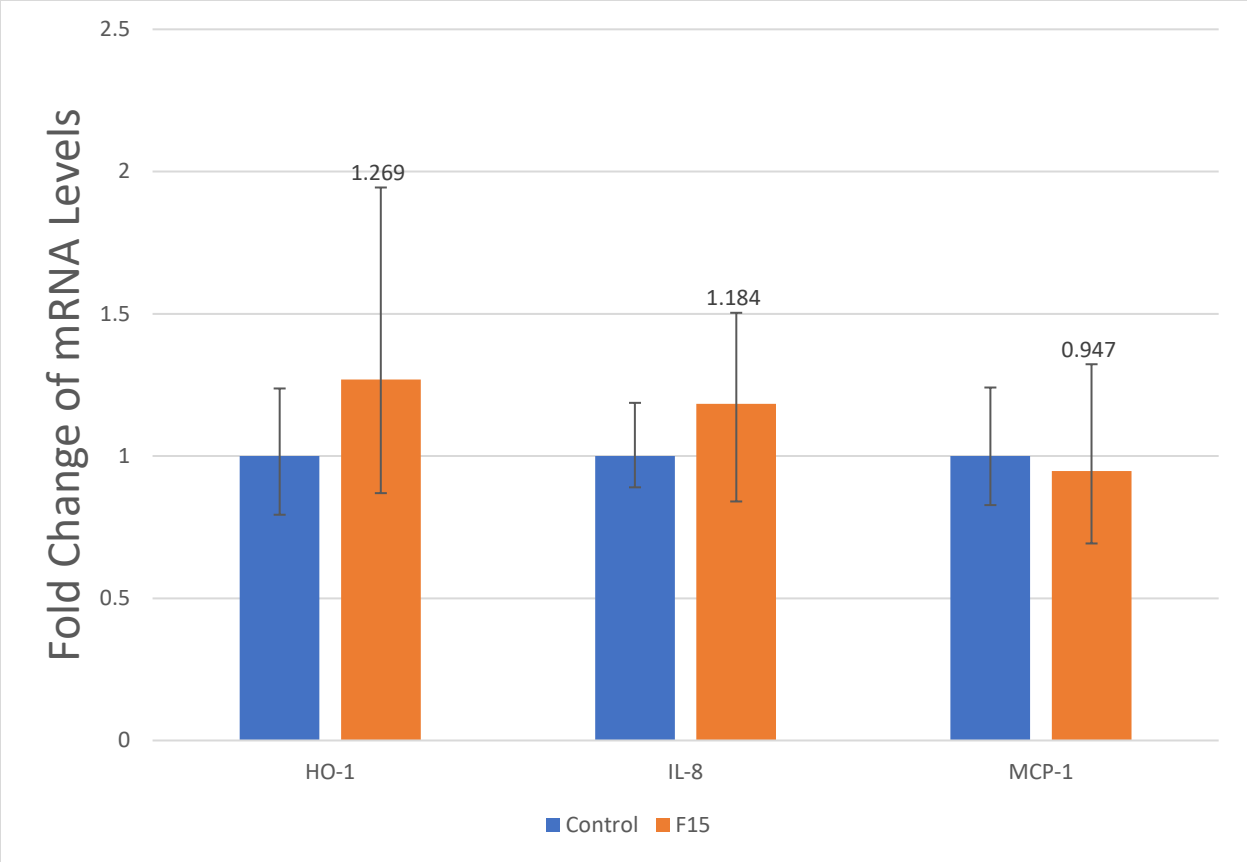

Figure 17. Control and Lipid Results from 3μM Neurofuran Treatment Testing Gene Regulation Levels of HAECs

Looking to Figure 18 for the control and OxPAPC levels compared to neurofuran expression, the data is unique when compared with the other two lipids. Upregulation is noted for all but MCP-1, suggesting potential for future experiments to see if the data skews any more in the direction of downregulation or if this attempt was an outlier for the data.

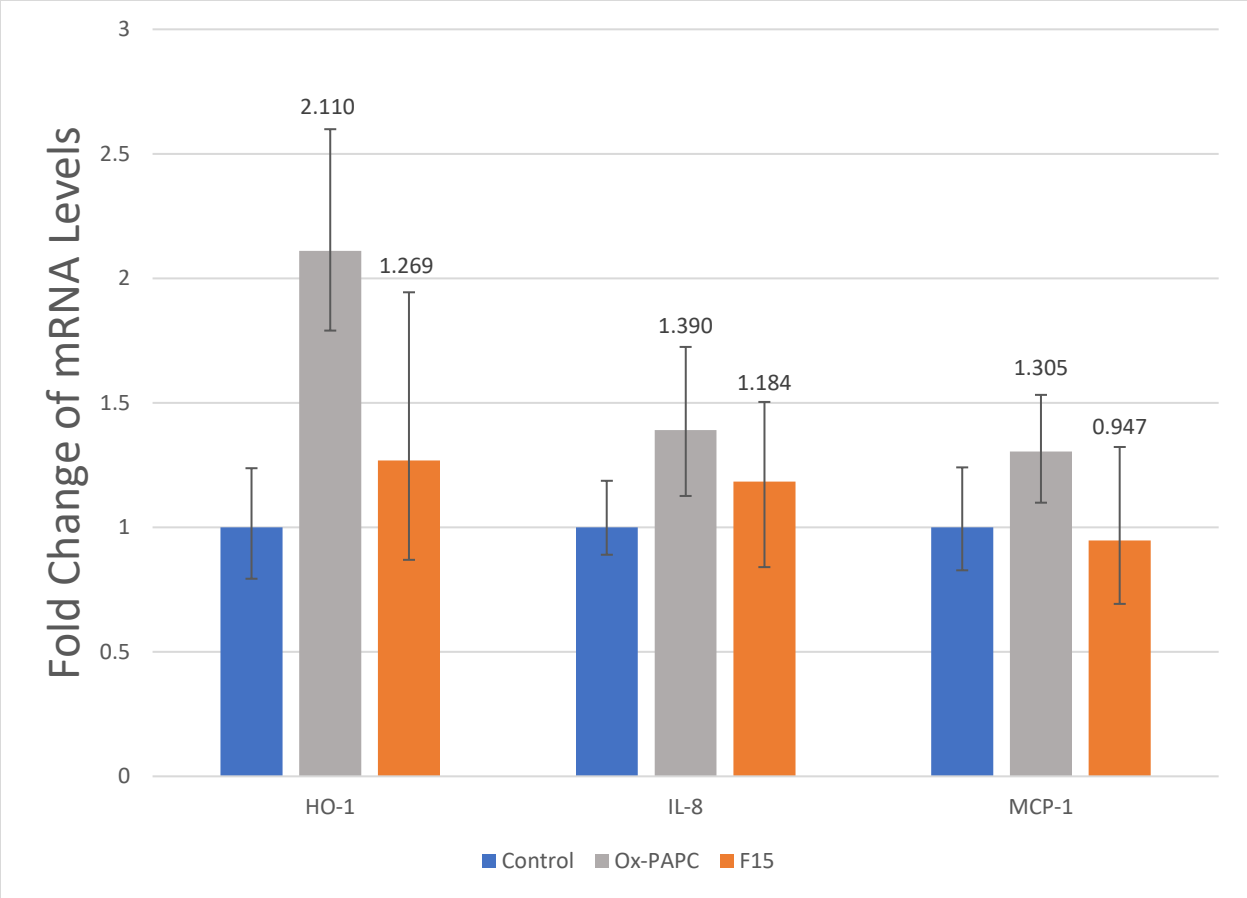

Figure 18. All Results from 3μM Neurofuran Treatment Testing Gene Regulation Levels of HAECs

DISCUSSION

One of the main purposes of this thesis was to compare the results obtained from the above set of experiments with previous work and see if the results are significantly similar. If they are, then the results can be counted as replicates, further strengthening the results. If the results contradict each other, then further sets of experiments mist be conducted to discern which results are valid. Beginning with the isoprostane lipid results, previous work noted upregulation for all three proteins, with the highest levels noted in IL-8. Across the board upregulation was noted in current results, but the lowest levels of upregulation were noted for IL-8 instead of the

highest. Potential reasons for this could be due to experimental variability due to human error or outliers in the data that were not excluded from consideration. As mentioned previously, the validity of the most recent results may be questionable due to the upregulation in IL-8 noted for OxPAPC, but with the other two proteins agreeing with established results, further replicates should be carried out to confirm or deny if the results are valid or a case of skewed data points. Next to consider is the result comparison for the neuroprostane test set. For Previous results, HO-1 and IL-8 showed downregulation while MCP-1 exhibited upregulation. Current results showed polar opposite results in the form of HO-1 and IL-8 showing upregulation (incredibly small margin for IL-8) while MCP-1 showed downregulation. At first glance, these results seem invalid due to their drastic difference from previous work, but the comparison of OxPAPC regulation levels is more consistent with established results, lending itself to the idea the current results are valid. All this is to say that more experiments should be conducted into the neuroprostane to see which way the results will tend toward.

Neurofuran results from previous work showed the trend of upregulation for HO-1 and downregulation for both IL-8 and MCP-1. The current results agreed with HO-1 and MCP-1, showing up- and downregulation respectively. Il-8 however showed opposite result, skewing toward upregulation instead of down. OxPAPC comparison for current results agreed with established values of upregulation for HO-1 and IL-8, and downregulation for MCP-1. This aids in suggesting the current results are valid, once again, pointing to the fact that further experimental repeats are warranted.

On an individual note, the issue regarding neurofuran p-values must be addressed. With all three sets bearing substantially high p-values, the issue becomes figuring out where the problem originates from. All three sets of genes in each set of experiments based on lipid, bore

approximately similar size error bars and each had their own issues where they did not agree with the OxPAPC standard as much as they should have. A potential solution could be to limit the window of data that is included for post experimental analysis, or to complete more sets of experiments all together in conjunction with this to see if the issue subsides at all. As it currently stands, the problem seems to be arising from outlier data points and further results should be obtained to confirm or deny this hypothesis.

Overall, of the three sets of experiments conducted, none completely agreed on all three protein levels with the previous work completed. For the results compared, on average two of the three protein results would agree between past and present results. This is indicative that previous results were valid to some degree, but further testing is required to make sure that the results can be concretely confirmed.

FUTURE STUDIES

With the results of the effects of the three novel types of oxidized fatty acids having been obtained, two main objectives still exist at this point. Reproduction of results to determine experimental data validity is the first priority. If previous work and current results can agree on a trend for the data, then further work can be continued to expand upon achieved results. Second, work done on more oxidized fatty acids in the categories of isoprostanes, neuroprostanes, and neurofurans need be considered to further demonstrate importance of these lipids in the regulation of inflammation, also potentially contributing to the development of novel therapeutics. With all of this in mind, this research will hopefully lead to the development of novel treatments for heart disease in the future.

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