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Arachidonate Oxidation Metabolite Profiles for Myoglobin and a Structurally Altered Form of Myoglobin Produced in Muscle Disease or Trauma

by

Kathryn Lawrence

Thesis

Submitted to the Department of Chemistry

Eastern Michigan University

in partial fulfillment of the requirements

for the degree of

MASTER OF SCIENCE

In

Chemistry

Thesis Committee:

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Cory Emal, Ph.D.

May 2013

Ypsilanti, Michigan

DEDICATION

TO

MY FAMILY

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to Dr. Hemendra Basu for his patience and wisdom, and for the countless hours he spent mentoring me in the laboratory.

My sincere appreciation is extended to my advisor, Dr. Steven Pernecky, for his guidance, support, and patience throughout this research project.

I would also like to thank my thesis committee members, Dr. Heather Holmes and Dr. Cory Emal, for their guidance and support.

Finally, I would like to thank my family and friends for their encouragement and financial support, and the members of my research group- Lalini Ailaboina, Vasumathi Molugu, and Aditi Munmun Sengupta- for their continuous support.

ABSTRACT

Myoglobin (Mb) and Myoglobin-H (Mb-H) react with glycerophosphocholine (GPC) lipid in the presence of oxygen. This reaction allows the heme group in the protein the chance to induce peroxidation reactions with the lipid. Several aldehyde products of this type of reaction with arachadonic acid have previously been determined, with Mb generating greater quantities of aldehyde products than Mb-H. The focus of this research was to establish the presence of some products from the reactions of Mb and Mb-H with GPC (which contains arachadonic acid) that are more prevalent from Mb-H than Mb, or that only form from the reaction of Mb-H with GPC. A combination of spectrophotometry, TLC, and ion-trapping GC-MS was used to establish the generation of an oxidation product of arachidonic acid metabolism that is generated in larger quantities by the reaction of Mb-H with GPC than by the reaction of Mb with the same lipid.

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CHAPTER 1

INTRODUCTION

1.1. Introduction to Myoglobin

The structure of myoglobin (Mb) was first determined by high-resolution x-ray crystallography in 1958 by John Kendrew and associates.[1] This discovery resulted in Kendrew sharing the 1962 Nobel Prize in Chemistry.[2] Figure 1.1 depicts the tertiary structure of Mb: a 16,700 Dalton globular protein made up of a single polypeptide chain consisting of 154 amino acids. At the center of the eight alpha helixes (purple) that make up myoglobin is an iron-containing porphyrin, the heme prosthetic group (grey). Oxygen reversibly binds to the heme group of Mb, which stores oxygen, acts as a buffer for the partial pressure of oxygen, and facilitates oxygen diffusion.[3]

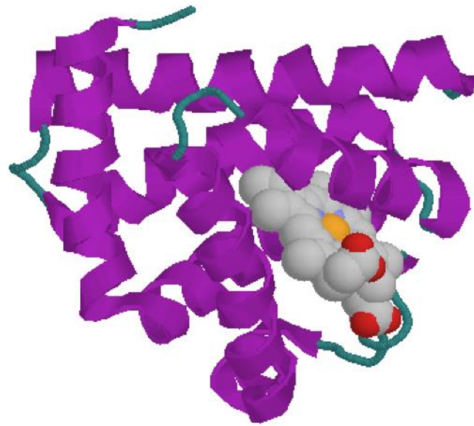

Figure 1.1. Tertiary Structure of Myoglobin

1.2. Introduction to Rhabdomolysis

Rhabdomyolysis is the disintegration of skeletal muscle fibers, resulting in the release of Mb into the bloodstream. There are several causes of muscle injury that result in rhabdomolysis, including major trauma, drug toxicity, infections such as HIV and influenza, severe exertion, and alcoholism.[4] There are two types of causes of rhabdomyolysis: those that are inherited and those that are acquired. Examples of inherited disorders include deficiencies in carbohydrates and fatty oxidation enzymes. Examples of acquired disorders that induce the breakdown of muscle include tissue crush and ischemia, a restriction of blood supply.[5] Rhabdomyolysis results in the leakage of the muscle contents into the surrounding areas and possibly myoglobinuria, or excess Mb in the urine.

Myoglobinuria was first associated with the toxicity leading to acute renal failure during The Battle of Britain of World War II.[6] More recently, it has been noted in substance abuse and drug overdoses.[7] In severe cases of myoglobinuria, this condition can lead to acute renal failure.[6] The release of Mb from skeletal muscle is believed to cause renal vasoconstriction, obstruct the renal tubules, and cause renal cytotoxicity associated with lipid peroxidation within the tubular cells.[8]

When muscular tissue is damaged, intravascular fluids accumulate in the space created as the cells undergo necrosis causing hypovolemia, or a low volume of blood. Hypovolemia activates the sympathetic nervous system and the rennin-angiotensin-aldosterone system to produce vasoconstricting molecules and to limit the production of vasodilators. Myoglobin acts to degrade NO, a major vasodilator in the body. The overall increase in vasoconstrictors along with the decrease in vasodilators leads to renal vasoconstriction.[9] Renal tubular obstructions known as myoglobin casts form from

precipitated myoglobin that is filtered through the glomerulus. The myglobin is deposited in the lumen and causes occlusion as the myoglobin concentrates due to the tubular reabsorption of water.[7,9]

1.3. Modifications of Myoglobin

When myoglobin is allowed to react with H_2O_2, covalent modifications to the hemoprotein occur. As described in Figure 1.2, the three pathways of covalent modification are hypothesized to end with either a covalent alteration on the heme, a covalent alteration on the protein, or a crosslink between the heme and the protein.[10]

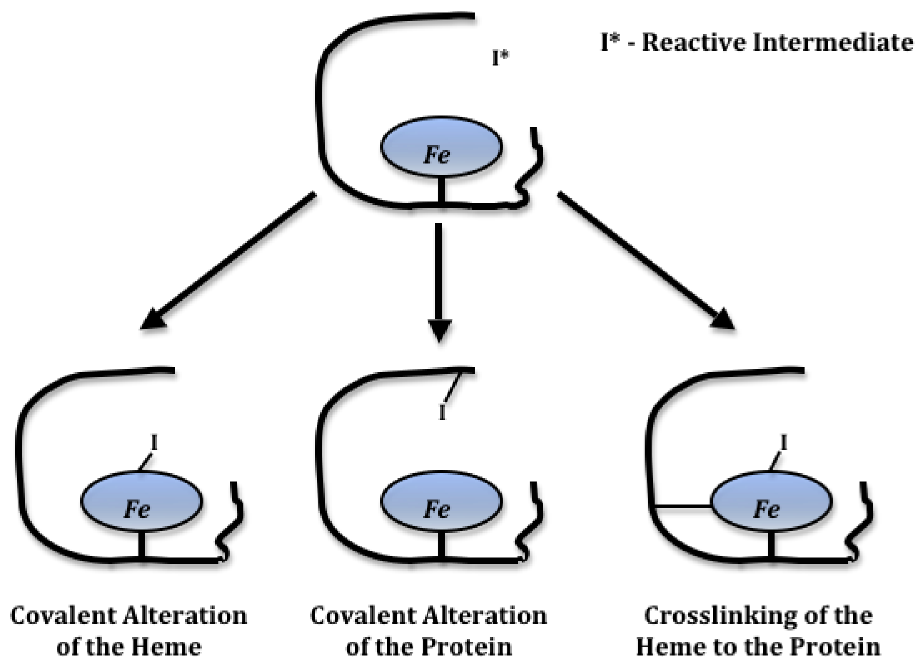

Figure 1.2. Pathways for the Covalent Modification of Hemoproteins.[10]

The crosslinking of the heme to tyrosine residue 103 results in the protein-bound heme adduct of myoglobin (Mb-H) (Figure 1.3).[10]

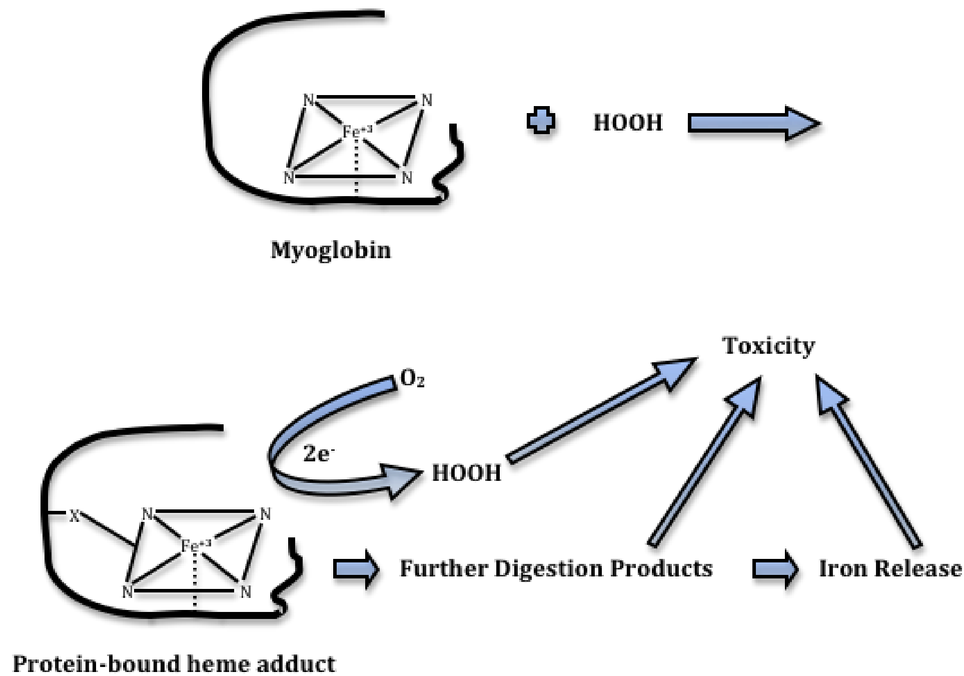

Figure 1.3. Transformation of Myoglobin to an Oxidase.[10]

Radioactive labeling was used to determine that up to 88% of the product of this reaction is Mb-H. It was also demonstrated that within an hour of loading Mb-H into human fibroblasts, 40% of the cells would exhibit cell death, whereas loading of myoglobin or external exposure to Mb-H did not show an increase in cell death compared to untreated cells. Incubation of the cells with N-acetyl-L-cysteine, butylated hydroxytoluene, and extracellular catalase inhibited the toxicity of Mb-H. This indicates that oxidative stress is a contributing factor in Mb-H toxicity.[10]

1.4. Lipid Peroxidation

Cytotoxicity is associated with the release of Mb during rhabdomyolysis. One major consequence of Mb toxicity is lipid peroxidation. The heme center of Mb is thought to

undergo redox cycling between ferrous, ferric, and ferryl oxidation states, which activates oxygen.

Oxygen species with unpaired electrons form a type of free radical known as reactive oxygen species (ROS). When a ROS encounters a cellular membrane, it can initiate a chain reaction known as lipid peroxidation by stealing an electron (often in the form of hydrogen to form water) from the membrane lipids. This results in a lipid radical, a lipid with an unpaired electron. Lipid radicals are very unstable and likely to react with molecular oxygen to generate peroxyl fatty acid radicals. In turn, a peroxyl fatty acid will react with another free lipid to create a hydrogen peroxide and a different lipid radical, which will propagate the peroxidation reaction by reacting with another lipid. The new lipid will continue the cycle, generating more free radicals. The chain reaction is terminated when the radicals are scavenged by the body's antioxidants. Figure 1.4 shows the peroxidation cycle and some of its products.

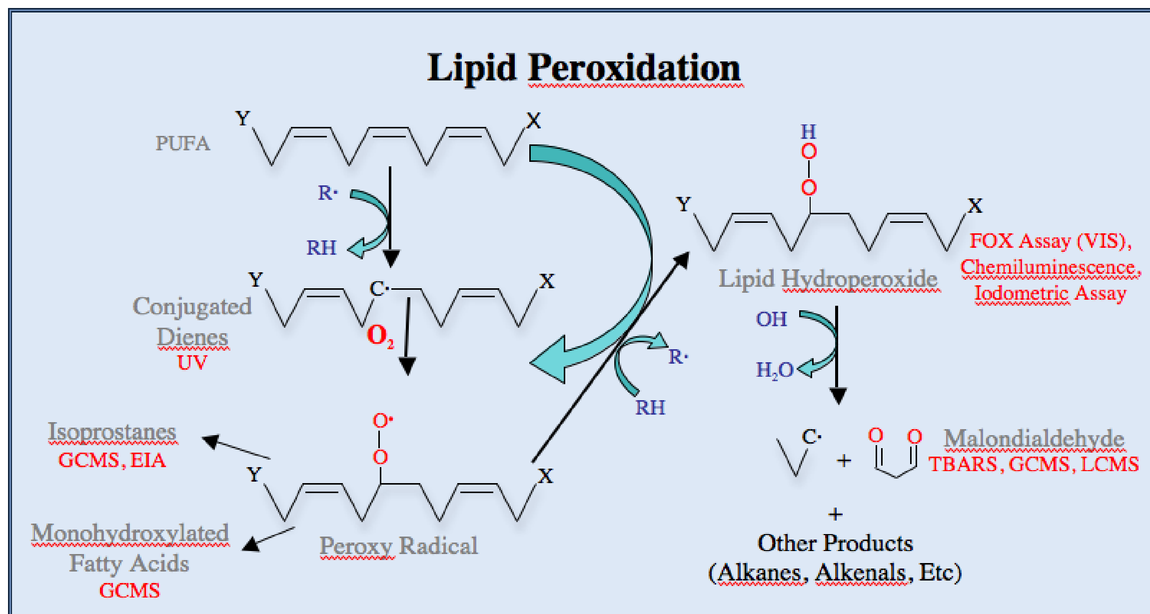

Figure 1.4. Lipid Peroxidation. The lipid peroxidation cycle starts with a polyunsaturated fatty acid. Throughout the cycle of peroxidation, several products and intermediates are

formed, including conjugated dienes, isoprostanes, monohydroxylated fatty acids, lipid hydroperoxide and aldehydes. The various methods of analytical detection of these products are noted in red alongside the products they detect. Definitions of all acronyms can be found in Appendix A.

One of the main cellular polyunsaturated fatty acids attacked by ROS is arachidonic acid, which contains 20 carbon atoms and four double bonds. Glycerophospholipids contain fatty acids such as arachidonic acid attached to the first two carbons of a glycerol backbone and a charged or polar group attached to the third carbon via a phosphodiester linkage. Glycerophosphocholine (GPC) has a positively charged choline attached to the third carbon of the glycerol backbone. Figure 1.5 depicts GPC, where R1 and R2 represent fatty acids, such as arachidonic acid.

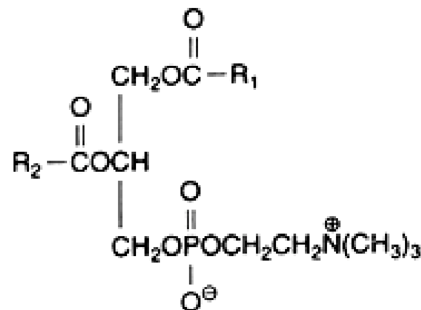

Figure 1.5. Glycerophosphocholine. Glycerophosphocholine has a glycerol backbone with a charged choline at the third Carbon; R1 and R2 represent fatty acids such as arachidonic acid.

The oxidation of arachidonic acid by reactive oxygen species also leads to the formation of F2 isoprostanes, which are not breakdown products of arachidonic acid, but rather molecules that have a structure very similar to that of naturally occurring molecules known as prostaglandins.[11] The F2 isoprostanes are known to be potent renal vasoconstrictors, and patients with rhabdomyolysis are often found to have F2 isoprostanes in the kidneys and urine.[12] The many pathways of enzymatic and non-enzymatic (i.e. lipid peroxidation) oxidation of arachidonic acid are shown in Figure 1.6.

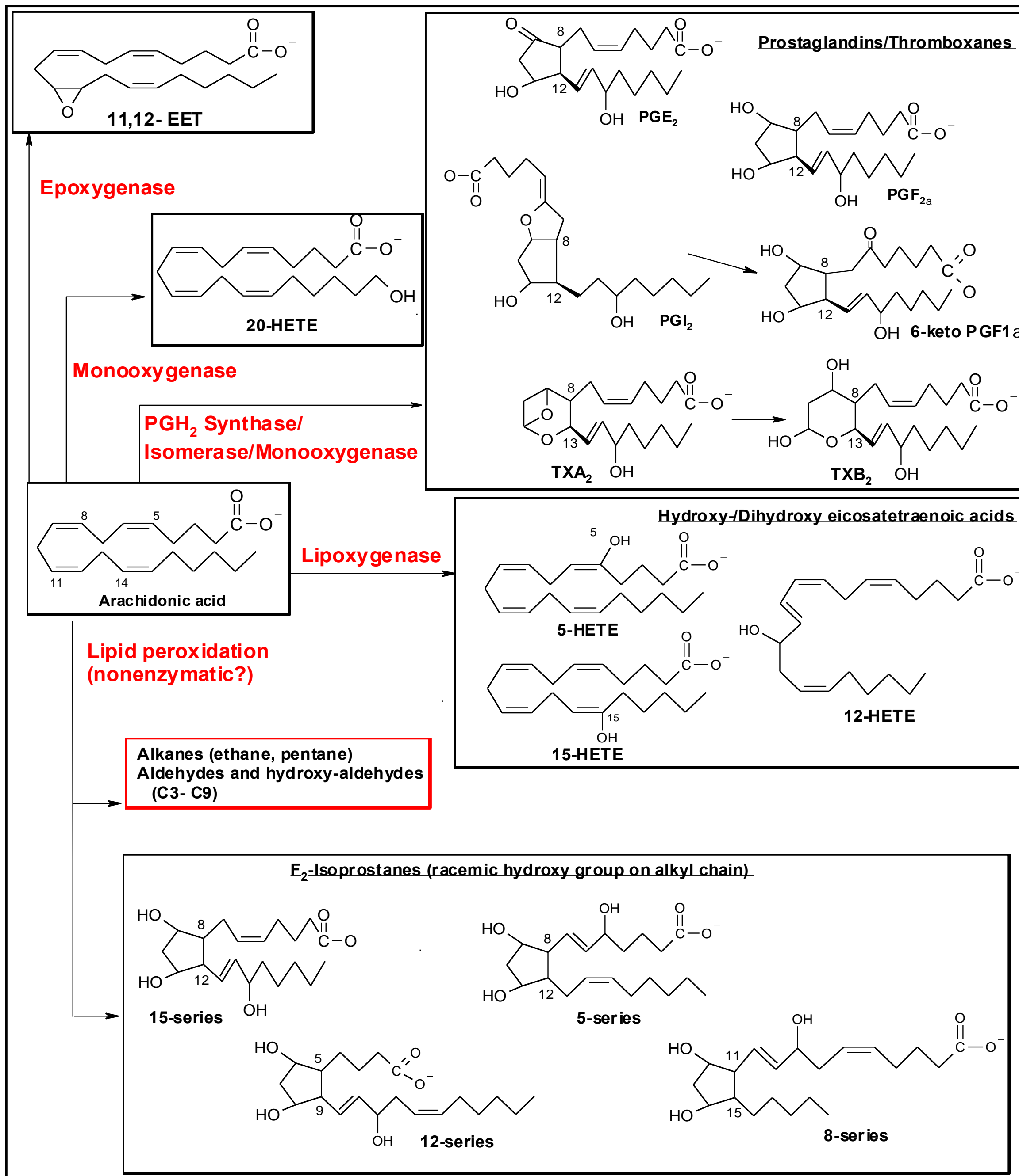

Figure 1.6. Pathways of Arachidonic Acid Oxidation

Conjugated dienes such as arachidonic acid are an oxidized intermediate of polyunsaturated fatty acids (PUFAs), which are produced by hydrogen atom abstraction and migration of double bonds so that they are separated by a single bond. This molecular

feature allows for UV (Ultra Violet) detection of conjugated dienes. These can be detected at a wavelength of 233 nm. Unfortunately, other compounds are also known to absorb light at the same wavelength, which leads to some inconsistencies in using the assay for conjugated dienes as a means to detect lipid peroxidation products.

Lipid hydroperoxides and peroxidase activity can also be detected by chemiluminescence assays in combination with separation techniques such as HPLC and SDS-PAGE.[13] Oxidation of isoluminol by hydroperoxides emits light that allows the distinction between plasma hydroperoxides from cholesterol esters and phospholipids. Immunochemical techniques are also used to detect hydroperoxides. For example, in the iodometric assay iodide reacts with hydroperoxides to form iodine or triiodide. The thiobarbituric acid reactive substance (TBARS) assay is nonspecific, and typically the thiobarbituric acid reacts with substances like malondialdehyde; the products absorbs at 532 nm and fluorescence at 532 and 553 nm. The ferrous oxidation-xylenol orange (FOX) assay, also nonspecific, utilizes xylenol orange to bind to the ferric ions and produces colored complexes with absorbance at 560 nm.[14]

Several aldehyde products of lipid peroxidation can be determined with GC and HPLC assays.[15] These aldehydes include hexanal, malondialdehyde, and 4-hydroxynonenal. In previous studies, the formation of lipid peroxidation products like hexanal, 4-hydroxynonenal, and malondialdehyde from the peroxidation of arachidonic acid by Mb and Mb-H were examined using GC assays.

GC/MS, thin layer chromatography (TLC) and high pressure liquid chromatography (HPLC) have all been used to look at non-aldehyde products of lipid peroxidation of PUFAs. GC/MS has been used to detect peroxidation products such as isoprostanes and

monohydroxylated fatty acids.[16] Thin layer chromatography (TLC) has been used to separate products of lipid peroxidation followed by detection using a reaction with certain acids. Scraping of the TLC plate followed by solid phase extraction has been used to allow further analysis of the material of interest.[17] HPLC has been used to separate peroxidation products using various detection methods including mass spectrometry and UV detection.[14]

1.5. Review of the Literature

In 1996, Drs. Osawa and Williams examined the toxicological effects of treatment of myoglobin with H_2O_2. It was known that treating myoglobin with H_2O_2 resulted in covalent modification of the heme group to the protein. However, the exact nature and the toxicological effects of these adducts were not well defined. When considering the covalent modification of heme proteins, there are three possible pathways that exist: the heme is altered, the protein is altered, or the heme crosslinks to the protein. The alteration of the heme and the alteration of the protein are both well studied and well documented. Osawa and Williams chose to study the crosslinking of the heme to protein. This crosslinking was previously structurally defined in two situations; both resulted in the transformation of the myoglobin protein to an oxidase. The first case involves the histidine residue 93 of myoglobin as a result of treatment with $CBrCl_3$. The complete structure of the adduct in this case has been fully defined. The second case utilizes treatment of myoglobin with H_2O_2; this involves the tyrosine residue 103 of myoglobin. The complete structure of this adduct had yet to be established, so they focused their study on developing a better understanding of this adduct and the toxicological implications of its formation.[10]

Of these two crosslinking pathways, the H_2O_2 treatment was supposed to account for about 18% of the heme, based on the amount of chromophore associated with the protein

after extractions. The $CBrCl_3$, on the other hand, was previously shown to account for about 60% of the heme, based on radioactive-labeling. Osawa and Williams utilized radiolabeling to establish that up to 88% of the heme was altered during reactions with H_2O_2. They were also able to demonstrate that upon introduction of the covalently altered myoglobin into human fibroblasts the cells would die. One hour after fibroblast cells were loaded with a partially purified protein-bound heme adduct, 40% of the cells exhibited cell death. Cells loaded with apomyoglobin or myoglobin exhibited only minimal toxicity, as did cells treated extracellularly with the protein-bound heme adduct. This was an indication of potential toxicological effects of covalently altered myoglobin and suggested important implications for the oxidative injury in cells containing large amounts of myoglobon. Incubation of the cells with either N-acetyl-L-cysteine, butylated hydroxytoluene, or extracellular catalase prior to loading of the protein-bound heme adduct inhibited toxicity by nearly 50%, 40%, and 28%, respectively. This indicated a mechanism that involved oxidative stress. [10]

In 1998, Drs. Osawa and Vuletich developed an assay for rapid, sensitive, and efficient detection of protein-bound heme adducts. The protein-bound heme adducts of myoglobin had not only been shown to act as a biomarker for oxidative damage but also had been shown to act as a peroxidase. Utilizing a combination of the peroxidase activity, standard electrophoresis methods, and enhanced chemiluminescence, Osawa and Vuletich were able to develop a sensitive method for the detection and quantification of these protein-bound heme adducts. There were certain conditions that had been established under which the native heme of myoglobin completely dissociated from the protein during electrophoresis. This was used to quantify the protein-bound heme adduct by enhanced chemiluminescence in samples that contained mixtures of heme products. [13] In this method,

SDS-PAGE was utilized to remove the native heme prosthetic group from myoglobin, leaving only the protein-bound heme adduct. Thiol reducing agents such as dithiothreitol interfere with the peroxidase detection of myoglobin by causing a decrease in peroxidase activity and the deterioration of both the heme and the protein-bound heme adduct during sample preparation. The formation and degradation of this adduct during sample preparation and analysis determined the amount of Mb-H present. In this study, Osawa and Vuletich were able to demonstrate that the thiol reducing agent tris(2-carboxyethyl)phosphine hydrochloride did not lead to the degradation of the protein bound heme adduct or the heme itself; rather it seemed to stabilize the Mb-H during electrophoresis so the peroxidase signal was actually enhanced. Tris(2-carboxyethyl)phosphine hydrochloride was compatible with the enhanced chemiluminescence assay used in the study. It was believed that this method could be used to map active sites of the hemoprotein, to help discover new hemoproteins, and to define heme-binding subunits of hemoproteins. [13]

In 2000, Osawa, Vuletich, and Aviram determined the role of the protein-bound heme in lipid oxidation. It was known that the crosslinking of the heme to the protein involved an active site on a tyrosine residue.[10] Upon the formation of the protein-bound heme adduct, myoglobin was transformed from an oxygen storage protein to oxidase catalyzing enzyme. The protein-bound heme adduct of Mb-H catalytically forms hydrogen peroxide which perpetuates the formation of more Mb-H, making the process self-sustaining. In trying to better understand the toxicity associated with the reaction of myoglobin and Mb-H with peroxides, the oxidation of low density lipoproteins (LDL) and purified lipids by Mb-H were studied. The fatty acids in the LDL were oxidized by both Mb-H and native myoglobin; however, Mb-H was shown to oxidize seven times as much lipid as Mb. Both the formation

of lipid peroxides and the TBARS assay were used to demonstrate a concentration-dependent increase in LDL oxidation after incubation of LDL with Mb-H as shown in Figure 1.7A. [18]

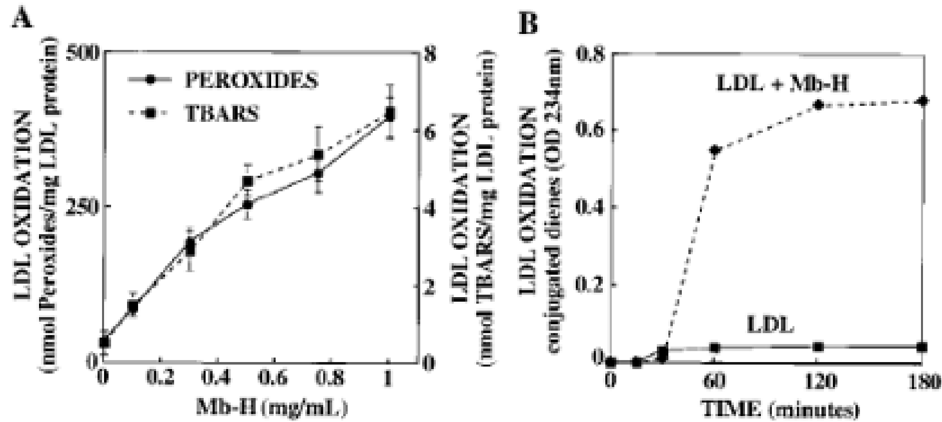

Figure 1.7. LDL Oxidation by Mb-H. (A) Study of purified LDL incubated with various concentrations of Mb-H for 2 hours at 37ºC, then assayed for lipid peroxides (circles) and TBARS (squares). (B) A time-course study of purified LDL incubated with and without Mb-H, alliquots of 1mL were assayed for conjugated diene formation.[18]

Conjugated dienes also increased after an initial lag period of about 30 minutes, as shown in Figure 1.7B. Lipid peroxides were also shown in Figure 1.8 to form from Low Density Lipoprotein (LDL), palmitoyl arachidonyl phosphatidylcholine (PAPC), and cholesteryl arachadonate (CA). PAPC is composed of glycerophosphorylcholine containing the fatty acids palmitate and arachidonate. Although the three substrates form in equal quantities, the TBARS assay indicated slightly less lipid peroxides were formed with CA than with the LDL or PAPC.[18]

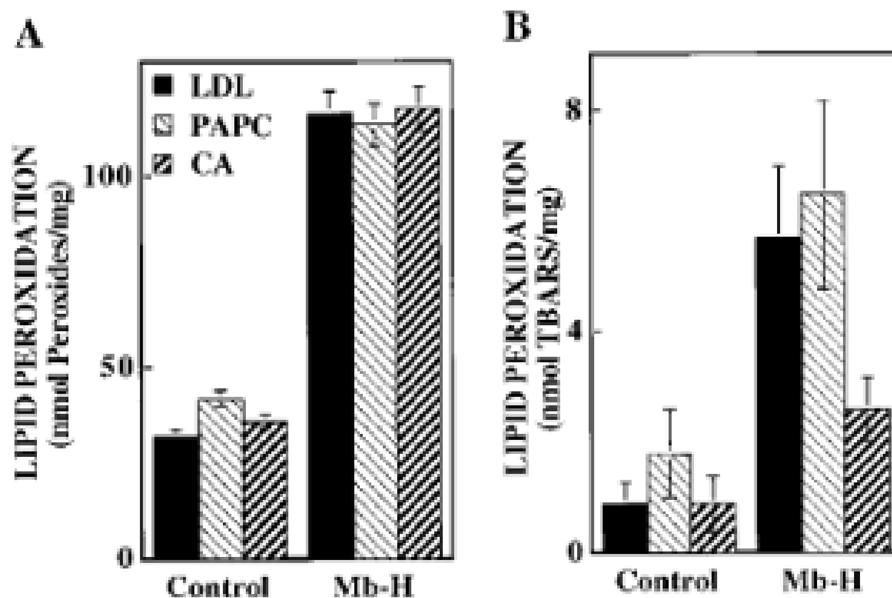

Figure 1.8. Mb-H Induced Phospholipid and Cholesteryl Ester Oxidation.[18] Low Density Lipoprotein (LDL), Palmitoyl Arachidonyl Phosphatidylcholine (PAPC), and Cholesteryl Arachidonate (CA) were independently incubated with and without Mb-H for 2 hours at 37C. (A) Lipid peroxide formation after 2 hour incubation. (B) TBARS formation after 2 hour incubation.[18]

The formation of conjugated dienes was used to compare PAPC oxidation by Mb, Mb-H, and apo-Mb; data are shown in Figure 1.9. The rate of conjugated diene formation from equal concentrations of protein demonstrated Mb-H to be the most effective of the substrates, specifically looking at formation during the first five minutes of reaction.[18]

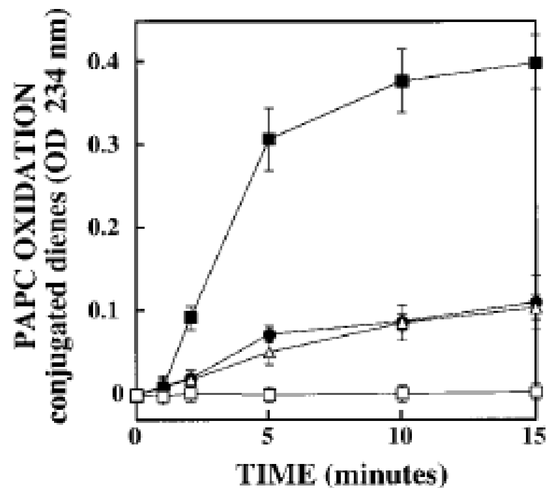

Figure 1.9. PAPC Oxidation.[18] Time-course reactions of PAPC with Mb-H (closed squares), Mb (open triangles), apo-Mb (closed circles), and a control with no protein (open squares). Aliquots were assayed for conjugated diene formation from 4 replicate studies.[17]

The Mb-H preparation actually contained a large amount of apo-Mb, so demonstrating that apo-Mb has a slower rate of conjugated diene formation served to indicate that Mb-H catalyzed the oxidation of PAPC. [18]

1.6. Significance of the Project

The purpose of this study was to examine the reactions of myoglobin and myoglobin-H with arachidonic-containing lipids in search of a product that can serve as a biomarker for these lipid peroxidation reactions. If we can demonstrate the existence of a product and show its formation to be more prevalent in the presence of either Mb or Mb-H, then we can start to have a better understanding of what to look for when dealing with rhabdomyolysis and tissue crush injuries.

EXPERIMENTAL PROCEDURE

2.1. Preparation of Oxidation Reactions

A 200-μL aliquot of glycerophosphatidyl choline containing arachidonic acid (GPC) lipid, 400 μL of Chelex-treated distilled, deionized water (dH_2O), and 200 μL of 0.5 M sodium acetate buffer (pH 6.0) were combined in a test tube and allowed to equilibrate at 37°C for 3 minutes. 200 μL of a 5.7 mg/mL solution of Mb or Mb-H was then added to the test tube, and the reactions were allowed to progress in the presence of oxygen at 37°C with shaking for 0, 7, 15, or 30 minutes. The tubes were then plunged into ice and the reactions were quenched with 200 mL of methanol. The organic layer was extracted three times with 500 mL of ethyl acetate.

Blank samples were prepared in the same manner as the samples using 600 μL of Chelex-treated ddH_2O, 200 μL of GPC lipid, and 200 μL of 0.5 M (pH 6.0) sodium acetate buffer. The proteins were omitted from the blank samples. Negative controls were also prepared using the same procedure. These samples were prepared using 600 μL of Chelex-treated dH_2O, 200 μL of 5.7mg/mL Mb or Mb-H, and 200 μL of 0.5 M sodium acetate buffer (pH 6.0), but no lipid. The oxidation reaction components are summarized in Table 2.1.

Table 2.1. Reaction Components

Components	Mb Reactions (µl)	Mb-H Reactions (µl)	Blank/ Positive Control Reactions (µl)	Negative Control Reactions (µl)
GPC	200	200	200	0
Mb	200	0	0	200 (of either protein)
Mb-H	0	200	0	
0.5 M pH 6.0 Sodium Acetate Buffer	200	200	200	200
ddH_2O	400	400	600	600
Total Volume	1000	1000	1000	1000

2.2 Conjugated Diene Assay

Samples were dried under nitrogen (N_2) gas and re-suspended in 1 mL of cyclohexane before using a UV-VIS spectrophotometer (Shimadzu/UV-1650PC/2.0 nm slit width) to determine the presence of conjugated dienes. The absorption spectrum from 200 nm to 500 nm was recorded; conjugated dienes are detected as a broad band in this region with a maximal absorption at 233 nm.[19]

2.3 Reduction of lipid hydroperoxides with TPP

The solvent was evaporated under nitrogen gas, and the samples were re-suspended in 1 mL of ether. A 100-µL aliquot of 5 mg/mL triphenylphosphine (TPP) in ether was added to each sample. The samples were allowed to react for 40 minutes at room temperature to reduce peroxides to alcohols.

2.4 Saponification of phospholipids

The samples were dried under nitrogen gas. A 2-ml aliquot of 1 M KOH in methanol and 2 mL of deionized, distilled H_2O (ddH_2O) were added to each sample before argon gas was used to displace any oxygen from the tubes. The tubes were capped and then incubated for 90 minutes at 80°C before being put on ice. While on ice, 2 mL of ddH_2O was added to each sample, and 2 M HCl was added drop-wise to bring the pH of each sample solution to 3. Each of the samples was extracted using 1 mL of ethyl acetate:ether solution (1:10 v/v) three times. A minimal amount of anhydrous sodium sulfate was used as a drying agent to remove any water from the samples. Each sample was then transferred to a new tube with ethyl acetate and dried under nitrogen gas. Samples were re-suspended in 200 µL of ethyl acetate.

2.5 Thin Layer Chromatography resolution of lipid analytes

For each sample, a 15-µL aliquot was spotted onto one thin layer chromatography (TLC) plate, and the remainder was put on a second TLC plate. The plates were run simultaneously in a solvent system of hexane:ether:acetic acid (45:5:0.5 v/v/v). The spotted plate was allowed to dry before it was sprayed with phosphomolybdic acid to identify the two retention factor (Rf) values of the compounds of interest. A 1 cm band correlating to the Rf value of the compound was scraped from each plate. The compound of interest was extracted from the scraped silica using 1.0 mL of ethyl acetate, followed by extraction twice with 0.5 mL of ethyl acetate in preparation for analysis by GC/MS.

2.6 Derivatization of lipid analytes

Samples designated for GC/MS were dried under N_2 gas and then derivatized prior to injection onto the column using methods for methyl esters, oximes, and BSTFA (N,O-bis(trimethylsilyl) trifluoroacetamide). The methyl ester derivative was formed by first adding 350 mL of 20% methanol in acetone to each sample followed by 100 mL 0.02 M trimethylsilyldiazomethane (TMSD) in heptane. The sample tubes were capped and wrapped in aluminum foil to block light. They were agitated for 1 minute, incubated at room temperature for 15 minutes, agitated for 1 minute, incubated at room temperature for 15 minutes, agitated for 1 minute, and then dried under nitrogen gas. The oxime derivative was then formed by adding 50 mL of methoxylamine HCl in dried pyridine (2% wt/vol) to each sample. The samples were capped, agitated for 1 minute, incubated at 60°C for 90 minutes, and then dried under nitrogen gas. The BSTFA derivative was prepared by adding 25 μL of dried pyridine followed by 100 μL of BSTFA. Samples were capped, agitated for 1 minute, incubated at 65°C for 60 minutes, and then dried under nitrogen gas. Once the derivatives had been prepared, the samples were re-suspended in 200 μL of ethyl acetate and loaded onto SepPac C18 solid phase extraction columns. The sample tubes and columns were rinsed twice with 100 μL of methyl formate.

2.7 Ion Trapping GC/MS of lipid analytes

The samples were dried under nitrogen gas and then re-suspended in 100 μL of ethyl acetate for injection onto a Varian GC 3800 Gas Chromatograph (GC) coupled to a Saturn 2200 ion trap mass spectrometer (MS). A non-polar VF-5ms 30M x 0.25mm, 0.25 ID capillary column with 5% phenylmetholpolysiloxane phase was used. The injector

tmperature was at 250ºC, and the column flow was set to 1.2 mL/minute. The oven program had a 2-minute hold at an initial temperature of 200ºC, followed by a 40ºC/minute ramp to 240ºC. It then ramped at 8ºC/minute to 275ºC, where it was held for 7.62 minutes. A 2-μL aliquot of each sample was injected onto the GC column. Each sample was analyzed twice, first using chemical ionization, then using electron impact ionization, for molecular fragmentation. Ethyl acetate was used to rinse the system between sample runs.

2.8 Standards

Standard reference materials were prepared for analysis by GC/MS using the same derivatization and analysis procedures as for the samples. The standards tested included 5 μL of 1a,1b-dihomoprostaglandinF2α in methyl acetate (1 mg/100 mL) diluted to 100 μL with ethanol, 10 μL of 8-iso PGE2α in ethanol (1 mg/mL), 100 μL of 2,3-dinor-11b-PGF2α in ethanol (1 mg/10 mL), 50 μL of thromboxaneB2 in ethanol (1 mg/mL), 50 μL of 2,3-dinor-8-iso PGF2α in methyl acetate (1 mg/10 mL), 50 μL of 2,3-dinor PGE1 in ethanol (1 mg/10 mL), and 10 μL of PGF2α methyl ester in methyl acetate (1 mg/100 mL).

RESULTS AND DISCUSSION

3.1 Conjugated Diene Results

The conjugated diene assay was performed to establish a time course for the formation of conjugated dienes in the presence and absence of protein oxidants and oxidizable lipid, palmitoyl arachidonyl phosphatidylcholine (PAPC), hereafter known as GPC. A control mixture, which is referred to as the "GPC blank," contained sodium acetate buffer, GPC lipid, but no protein. This blank was created to determine the extent to which conjugated diene formation occurred in the absence of Mb or Mb-H. Another control contained sodium acetate buffer, and either Mb or Mb-H, with no GPC lipid. This negative control was evaluated to determine whether any conjugated diene formation occurred with either protein, but in the absence of lipid. In samples containing the complete system containing lipid and proteins, Mb or Mb-H were allowed to react with the lipid to establish the time course of conjugated diene formation for each protein. Test reactions were done with the GPC blank or with solutions containing Mb or Mb-H for 0, 7, 15, and 30 minutes. The negative controls containing protein but no lipid were allowed to react for 15 minutes. For all spectra, the absorbance at 500 nm was subtracted from the entire spectrum to normalize the spectra, thereby giving all samples the same background level. The absorption band of interest with a peak at 233 nm served as an identifier of the conjugated diene. Figure 3.1 shows results from the 15-minute reactions that were averaged and normalized.

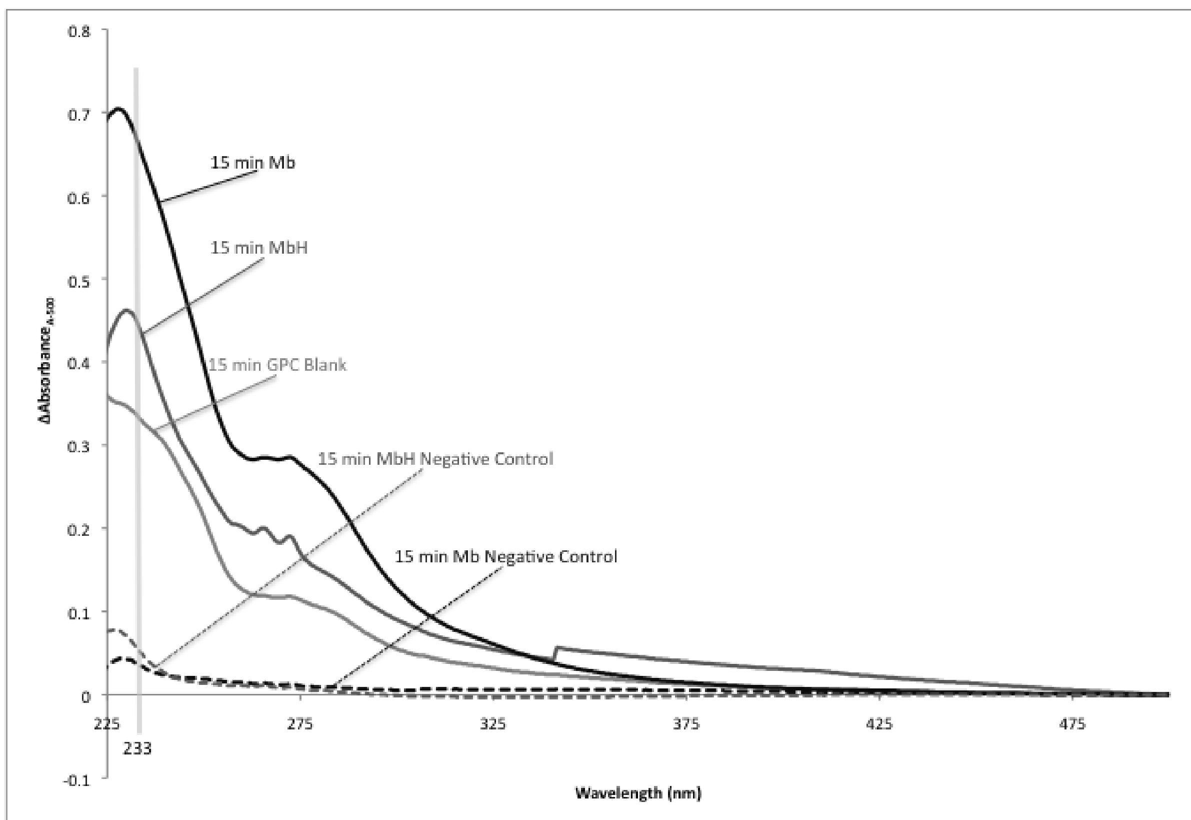

Figure 3.1. Fifteen Minute Conjugated Diene Assay, Averaged and Normalized. Conjugated diene assay results for 15 minute Mb ( ), 15 minute Mb-H ( ), 15 minute GPC Blank ( ), 15 minute negative control Mb ( ), 15 minute negative control Mb-H ( ).

A vertical line at 233 nm shows the wavelength used to assess conjugated diene formation. The Mb, Mb-H, and GPC blank all show some indication of conjugated diene formation. Mb has the highest absorbance, followed by Mb-H, and then the GPC Blank. Both of the negative controls with protein but not lipid indicate negligible formation of conjugated diene.

Although the presence of conjugated dienes can indicate the presence of lipid peroxidation products, this assay is non-exclusive in that other compounds can cause

absorbance at 233 nm. The negative controls demonstrate the absence of conjugated dienes because they have minimal absorption around 233 nm. This indicates that the protein alone is not responsible for the conjugated dienes and that a lipid must be present for these reactions to take place.

The raw data from the time-course reactions was used to calculate the extent of conjugated diene formation as the difference between the absorption maximum at 233 nm and the absorbance corresponding to the isosbestic point at 500 nm. Mb increased the level of conjugated dienes in the presence of GPC when compared to the level produced with GPC alone at the 7, 15, or 30-minute time period. On the other hand, Mb-H had a minimal effect on the conjugated diene level at 7 and 30 minutes with GPC when compared to GPC alone, and a relatively small increase at 15 minutes. Figure 3.2 summarizes the time-course reactions of Mb, Mb-H, and GPC blank. Also included are results from the 15-minute reactions for the negative controls containing Mb or Mb-H but no lipid. As anticipated, these samples generate a negligible absorbance band at 233 nm.

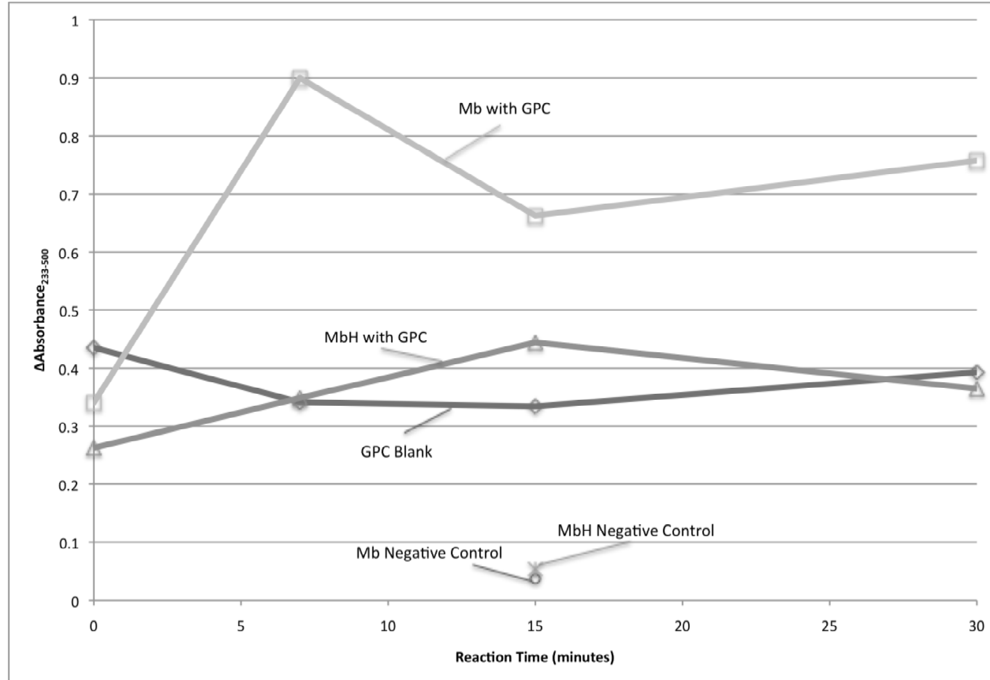

Figure 3.2. Summary of time course of conjugated diene formation in the absence or presence of GPC and protein oxidants. Time course reactions for Mb ( ), Mb-H ( ), GPC Blank ( ), Negative Control Mb (○), Negative Control Mb-H (✳) are shown.

The absorbance at 233 nm was normalized for the reaction times of 0, 7, 15, and 30 minutes for the GPC lipid reactions with and without protein. All time course reactions indicated some conjugated diene formation with the Mb reaction, with GPC indicating the largest production of conjugated diene. Negative controls containing Mb and Mb-H in the absence of lipid are shown at 15 minutes, indicating minimal conjugated diene formation. In summary, the conjugated diene data indicates that there is a difference in the extent to which Mb and Mb-H induce lipid peroxidation, so it will be of interest to determine whether there is also a difference in the type of oxidized arachidonic acid metabolite produced by Mb and Mb-H.

3.2 Thin Layer Chromatography

Thin layer chromatography was used for preliminary evaluation of the types of lipid products generated by incubation of Mb or Mb-H with arachidonic acid-containing GPC (PAPC). A TLC plate was run for each sample as a means of separating out the components of interest. These components responded to the mobile phase in a way expected of oxidized products of arachidonic acid forming a band along the top, hereafter indicated as the Top Spot, and a second band slightly lower than the first band referred to as the 2nd Spot. Figure 3.2 depicts a TLC plate with a Top Spot and a 2nd Spot. In this case, the material spotted on the plate is the incubation of Mb-H with PAPC in buffer. Unreacted arachidonic acid runs near to the origin and is observed as a small, faint spot. The size of the Top Spot and 2nd Spot were considerably smaller in size in negative controls, which contained the lipid but did not contain Mb or Mb-H.

Figure 3.3. A TLC Plate of Extracts from a Reaction of Mb-H with GPC. An example of a typical TLC plate with 2 spots of interest: a Top Spot and a 2nd Spot. Unreacted arachidonate is observed as a small, faint spot just above the origin.

Using TLC as a primary means of separation, it was possible to enrich a preparation for a subset of reaction products that could then be more easily studied by Gas Chromatography/Mass Spectrometry (GC/MS).

3.3 GC/MS Chromatograms and Spectras

Figure 3.4 shows a gas chromatogram of the top spot isolated from a 15-minute Mb reaction with the GPC PAPC after preparation with the reagents used to chemically modify the lipid products prior to analysis by GC/MS. The chromatogram displayed a predominant peak at about 7.5 min and a somewhat lesser peak at 5.5 min. The chromatogram was not monitored beyond 15 min, so it is possible that peaks representing other compounds may have eluted after the end of this monitoring period. However, the focus of the attention was the major peaks generated at about 7.5 min, since this was the region used in prior work was focused on characterization of prostaglandin E2.[20]

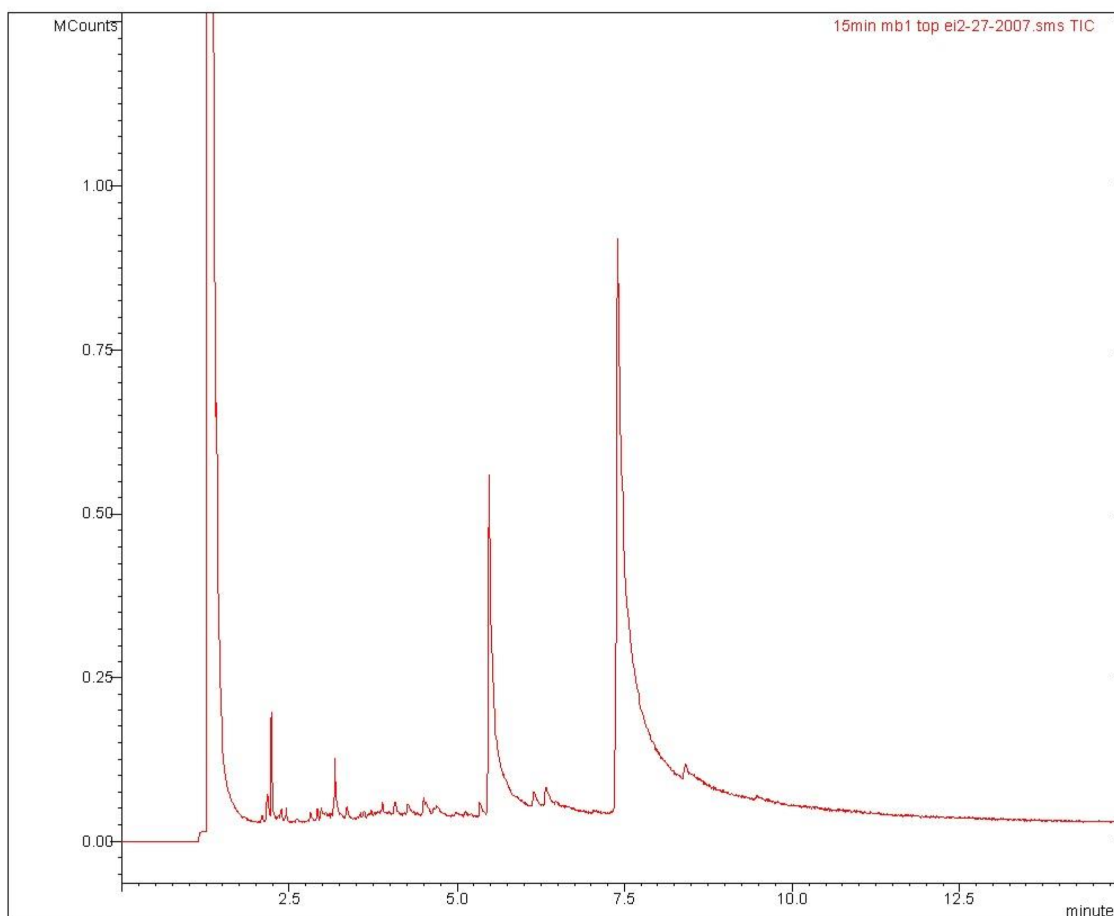

Figure 3.4. Chromatogram for a 15 min reaction of Mb with GPC. A typical chromatogram for the incubations of GPC with Mb.

Figure 3.5 shows the chromatogram obtained from analysis of the top spot from 15-minute reactions of the glycerophosphatidyl choline PAPC with Mb, Mb-H, or no protein after treatment with derivatizing reagents and analysis by GC/MS. The overlay of the three chromatograms gives a more convenient means of qualitatively and semi-quantitatively comparing the reaction products isolated in the top spot of the TLC for the three reactions. All three of the chromatograms have prominent peaks that are detectable at 5.5 and 7.4 minutes.

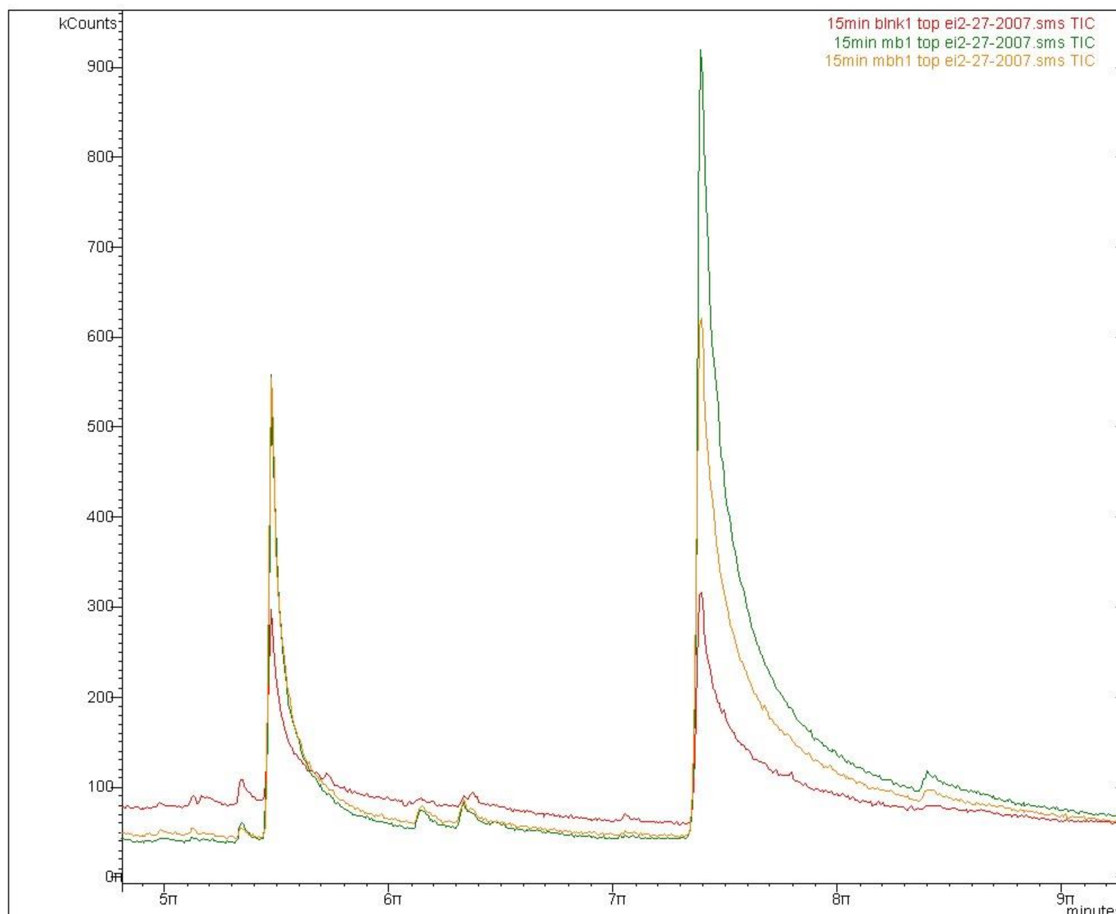

Figure 3.5. Chromatogram for 15 min reactions of Mb, Mb-H, and a Blank with GPC. The overlaid chromatograms demonstrate the consistency of specific peaks generated by the incubation of GPC with and without protein.

Figure 3.6. depicts the mass spectrum from the 7.4-minute peak of the top spot obtained from the 15-minute Mb reaction. The prominent fragments above an *m/z* of 200 were 247, 249, 331, 455, and 512.

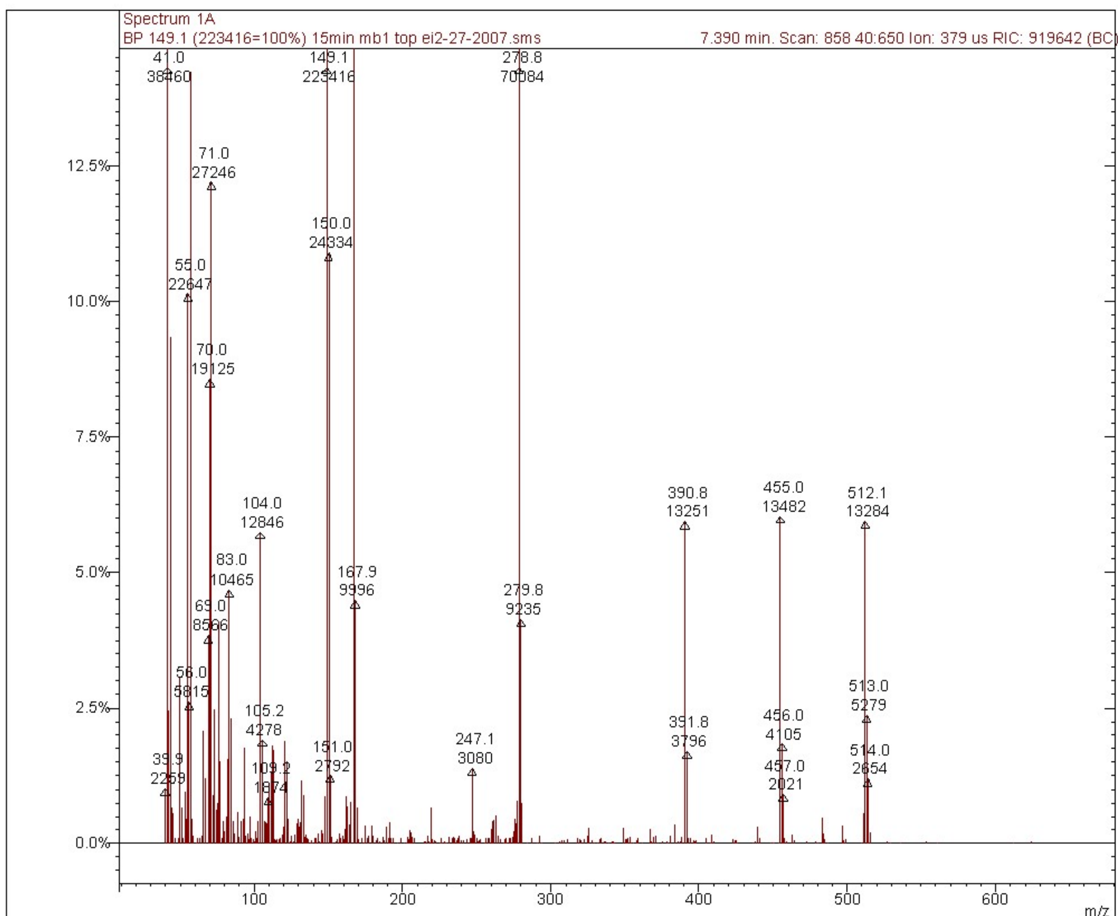

Figure 3.6. Mass Spectra for the 'Top Spot' of the 7.4-Minute Peak of the 15-Minute Reaction of Mb with GPC.

Figure 3.7 shows overlaid gas chromatograms of the standards 8-iso-PGE2, prostaglandinB2, thromboxaneB2, and 8iso-15keto prostaglandin2α, all of which produce a chromatogram with a prominent peak at approximately 7.4 minutes.

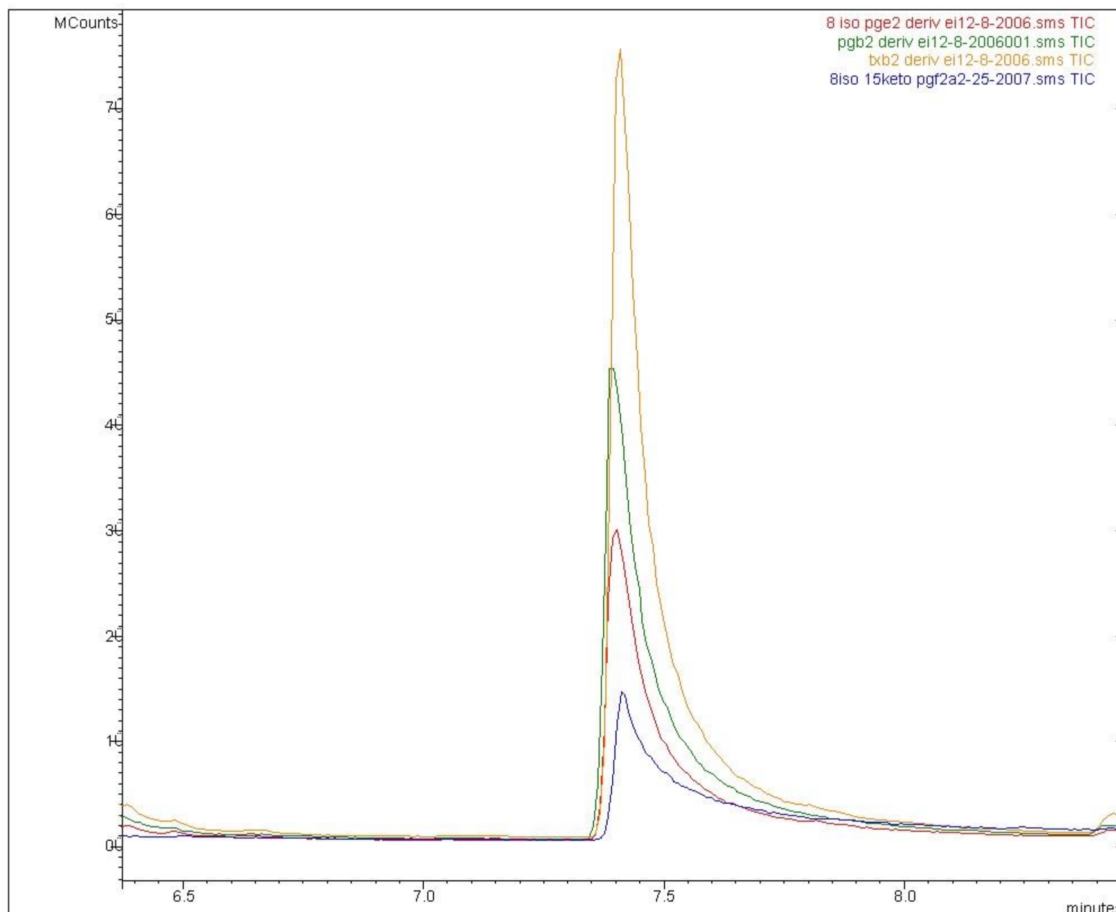

Figure 3.7. Overlaid chromatograms for the derivatized standards 8-iso-PGE2, prostaglandinB2, thromboxaneB2, and 8-iso-15-keto prostaglandin2alpha. The peak at 7.4 min is prominent for these standards.

Figure 3.8 depicts the mass spectrum associated with the chromatographic peak for the 7.4 minute peak for 8-iso-15-keto-prostglandin2alpha. The *m/z* fragments in descending order of abundances are 279 > 512 > 455 > 391. The same *m/z* fragments are present in the 7.4-min peak in the chromatogram for the incubated Mb sample, although the relative abundances of the 512, 455, and 391 *m/z* fragments are different.

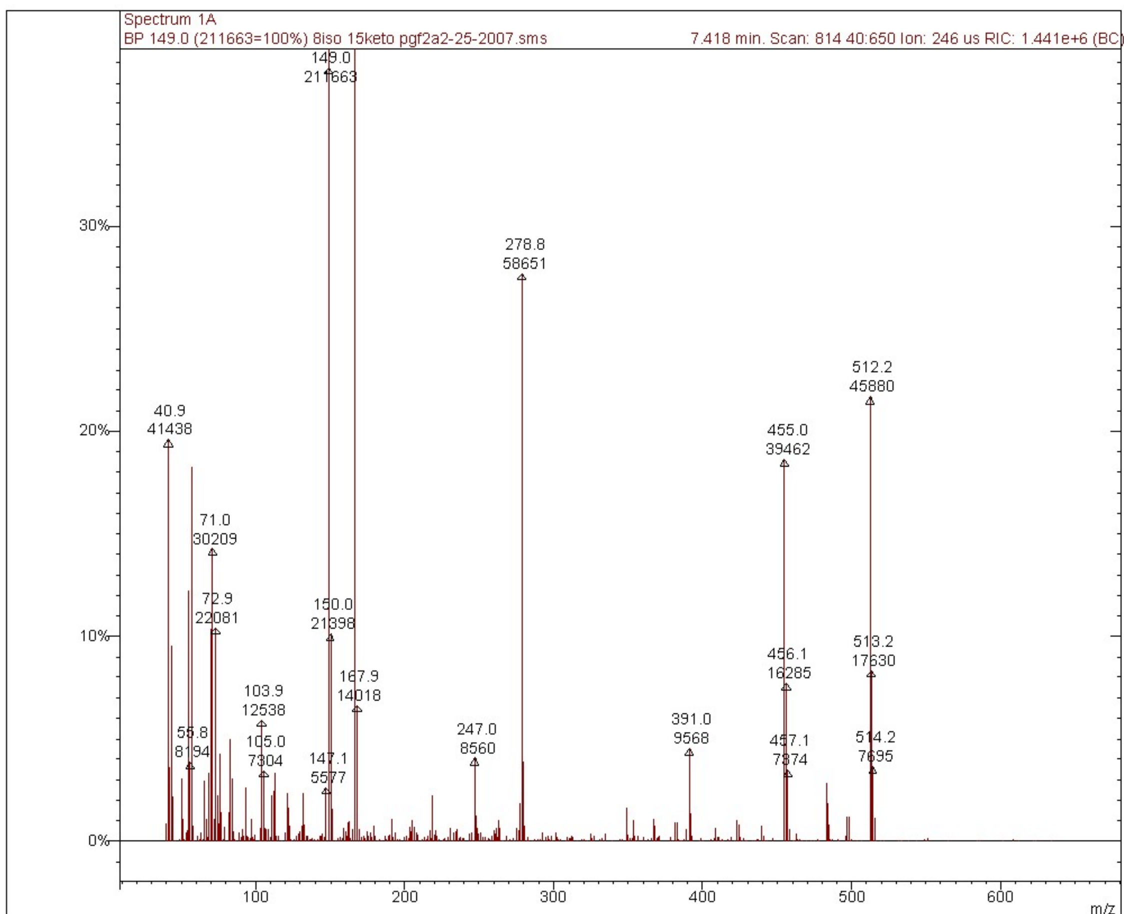

Figure 3.8. Mass Spectra for the derivatized standard 8-iso-15-keto-prostaglandin2a. The *m/z* fragments of 279, 391, 455, and 512 are all prevalent.

The mass spectra for the derivatized standards 8-iso-PGE2, prostaglandinB2, and thromboxaneB2 were all similar to the spectra for 8iso-15keto prostaglandin2a shown in Figure 3.8. The same m/z fragments show up in all of the standards, as well as the reactions of protein with the PAPC lipid.

Areas for the 7.4 min peak of the top spot obtained from the TLC plate from 0 minutes, 15 minutes, and 30 minutes were compared for Mb, Mb-H and a blank reaction in Figure 3.9. All three reactions gave a relatively low level of the 7.4-min peak at zero time, which did not greatly increase after 15 min. Interestingly, the 7.4-min peak for the blank

containing PAPC but no protein was no greater than that for the zero time or 15-min peak, but 7.4-min peak for the reaction containing Mb or Mb-H was significantly above background levels.

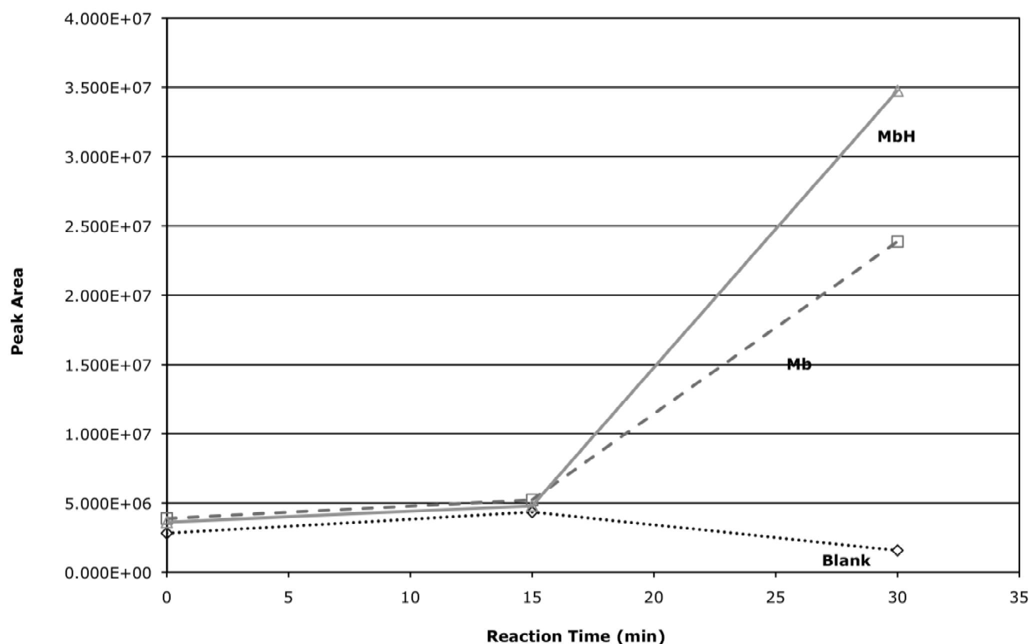

Figure 3.9. Time course reaction of GPC with Mb, Mb-H, and a Blank. The peak area of the 7.4 min peaks were determined and plotted for 0, 15, and 30 minutes. Mb-H has the largest peak area after 30 minutes, indicating a higher amount of product of interest produced by Mb-H ( ) than Mb ( ) or the Blank ( ) reactions.

CONCLUSIONS

4.1 General Conclusions

The data in this study stand in contrast to that originally obtained by Osawa et al.,[17] where conjugated diene formation occurred to a much greater extent with Mb-H than with Mb, which suggested that Mb-H stimulates lipid peroxidation reactions that produce alkanes and oxidized fragments of arachidonate or isoprostanes. Although the experimental conditions were consistent for the 2 studies, the opposite result was obtained in the present study, with Mb producing greater levels of conjugated dienes than with Mb-H or in control reactions with no protein. When either of the proteins was incubated in buffer in the absence of the protein, a relatively small absorption band with a peak at 233 nm was generated, suggesting that the products of interest are coming from the breakdown of lipids. Unfortunately, attempts to use the TBARS assay in the present study (data not presented) to confirm the production of lipid peroxidation did not yield reliable data.

Given the finding that breakdown products of lipid peroxidation did not apparently result from Mb-H incubation with PAPC, an attempt was made to determine whether 20-carbon oxidation products of arachidonic acid could alternatively be produced. The putative products of the GPC-containing reactions that were more polar than the fatty acid components (palmitate and arachidonate) of the phospholipid used in the study were isolated from the TLC plate using solid phase elution techniques. Whereas two spots were revealed during the TLC analysis, only the spot associated with the more polar analytes, i.e. the Top Spot, was characterized. The spots generated from the Mb or Mb-H reactions were considerably larger than that produced during reactions in the absence of the proteins. These products where derivatized and analyzed using GC/MS.

Prominent peaks at 5.5 and 7.4 min were identified in the chromatogram of the derivatized products obtained from the TLC "top spot" prepared from reactions containing Mb, Mb-H or no protein. Several prostaglandin standards were used in an attempt to identify the putative products of the arachidonyl fatty acid. Unfortunately, none of the standards were characterized by NMR, so the structures of the putative derivatives were not confirmed. Theoretically, the mass spectra of the structurally dissimilar derivatized lipid products should yield dissimilar mass spectrum fragmentation patterns, yet the mass fragmentation pattern of the 7.4-min peak were qualitatively similar. Given these results, it is difficult to conclude that these analytes were derived from arachidonic acid and to determine the precise identity of the oxidation product of arachidonic acid. On the other hand, the mass spectra produced by electron impact of the products of diverse prostaglandins that apparently eluted at 7.4 min matched those associated with the 7.4-min peak of the derivatized lipid analyte products of reactions.

Perhaps the most interesting data in the present study come from an experiment in which the time course of the production of the 7.4-min analyte was evaluated. At zero time or 15 min, there was relatively low and constant level of this analyte present in incubations containing the lipid and Mb, Mb-H, or no protein. After 30 min of incubation, however, there was a significantly greater level of this analyte in reactions containing Mb or Mb-H. Interestingly, the level of the analyte in blank samples at 30 min containing the lipid but no protein was no greater than that detected at zero time. These data indicate that the 7.4-min analyte requires Mb or Mb-H, although the identity of this as a lipid analyte remains to be determined.

4.2 Future Analysis

Future work in this area will need to involve successful verification of the structures of prostaglandin derivatives and the more complete characterization of the chromatograms to determine whether relevant peaks are observed beyond the 15-min period established for the chromatographic conditions. The characterization of arachidonic acid analytes by chemical derivatization and gas chromatography and mass spectrometry has proven to be quite difficult in the present study, so alternatively methodologies, perhaps involving the use of ELISA assays to identify prostaglandin products, will provide a more accurate and less time-consuming alternative for identification of oxidation products of arachidonic acid.

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APPENDIX A

BSTFA: N,O-bis(trimethylsilyl) trifluoroacetamide

CA: Cholesteryl Arachadonate

EIA: Enzymatic Immunoassay

FOX: Ferrous Oxidation-Xylenol Orange

GC/MS: Gas Chromatography Mass Spectroscopy

GPC: Glycerophosphocholine

HPLC: High Pressure Liquid Chromatography

LCMS: Liquid Chromatography Mass Spectroscopy

LDL: Low Density Lipoprotein

MB: Myoglobin

Mb-H: Myoglobin-H; Myoglobin with protein-bound heme adduct

PAPC: Palmitoyl Arachidonyl Phosphatidylcholine

PGE1: Prostaglandin E1; Prostaglandin with a ketone at position 9, and a hydroxyl at position 11. The 1 indicates there is 1 double bond.

PGE2α: Prostaglandin E2α; Prostaglandin with a ketone at position 9, and a hydroxyl at position 11. The 2 indicates there are 2 double bonds; the α indicates there is a hydroxyl group at position 15 that is oriented behind the plane.

PGF2α: Prostaglandin F2α; Prostaglandin with a hydroxyl at position 9, and a hydroxyl at position 11. The 2 indicates there are 2 double bonds; the α indicates there is a hydroxyl group at position 15 that is oriented behind the plane.

PUFA: Poly Unsaturated Fatty Acid

Rf: Retention factor

ROS: Reactive Oxygen Species; A type of free radical where Oxygen has unpaired electrons

SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

TBARS: Thiobarbituric acid reactive substance

TLC: Thin Layer Chromatography

TMSD: Trimethylsilyldiazomethane