2006

Lipid metabolism in A549 and 9HTE cells quantitation of lipid peroxidation

Elizabeth Quintus

Lipid metabolism in A549 and 9HTE cells quantitation of lipid peroxidation

LIPID METABOLISM IN A549 AND 9HTE CELLS

QUANTITATION OF LIPID PEROXIDATION

by

Elizabeth Quintus

A Senior Thesis Submitted to the

Eastern Michigan University

Honors Program

in Partial Fulfillment of the Requirements for Graduation

with Honors in Chemistry

2006

TABLE OF CONTENTS

List of Figures

List of Tables

INTRODUCTION

Cell membranes are made up of a phospholipid bilayer. They serve as reservoirs for molecules, which, when released from the membrane, become oxidized to produce metabolites. These, in turn, serve as signals to recruit the immune system to the site of inflammation. Arachidonic acid is an important membrane component and is found in several of the phospholipids that compose the lipid bilayer. There are three major

pathways for arachidonic acid metabolism, which are shown in their biochemical context and in relation to one another in Figure1.

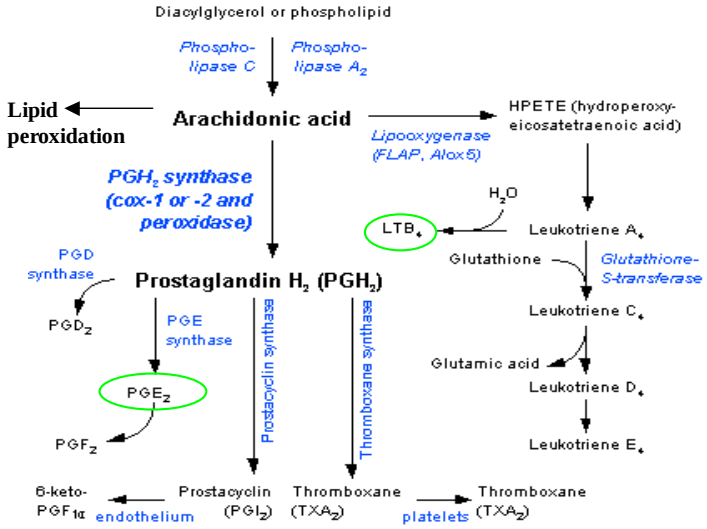

Fig.1. Diverse pathways of arachidonic acid metabolism.(1)

The oxidation pathway that has received the most attention recently involves the metabolism of arachidonic acid by cycloxygenase (COX) to form PGH_2, which is further metabolized by incompletely characterized enzymes to other prostaglandins including PGI_2, PGD_2, PGE_2, $PGF_{2\alpha}$. Prostaglandins are paracrine signaling molecules that serve diverse physiological roles including regulation of smooth muscle and inflammation. One of the prostaglandins studied by this lab, prostaglandin E2 (PGE_2), is involved in the inflammatory response(2).

A second possible fate of arachidonic acid involves its oxidation via lipoxygenase to form the hydroperoxide-containing metabolite HPETE, which is then metabolized to LTA_4. At this point, LTB4 is formed upon addition of a water molecule to the structure. Alternatively, if the cellular reductant, glutathione, is added to the structure , LTC_4 is formed, followed by enzymatic removal of glutamic acid to produce LTD_4 and LTE_4.

Leukotrienes act to sustain inflammatory reactions, leading to asthmatic and allergic reactions. LTB_4 helps recruit macrophages to affected tissue(3).

The third pathway of arachidonic acid oxidation involves the non-enzymatic pathway of lipid peroxidation that produce multiple metabolites. This is a radical reaction occurring at one of the double bonds in arachidonic acid and in other poylunsaturated fatty acids (PUFA). Lipid peroxidation is a radical reaction and proceeds through the three steps known as initiation, propagation, and termination as highlighted in Figure 2. There are several products of this reaction, including conjugated dienes, isoprostanes, lipid hydroperoxides, and the hydroperoxide breakdown products 4-hydroxynonenal and malondialdehyde. These products have been used to monitor lipid peroxidation, and involve analysis by GC-MS, LC-MS, enzyme-linked immunosorbent assays (EIA), and fluorometric and spectrophotometric methods. Malondialdehyde is the easiest and most common way to measure lipid peroxidation, since it involves a simple reagent and a UV/VIS spectrophotometer(4).

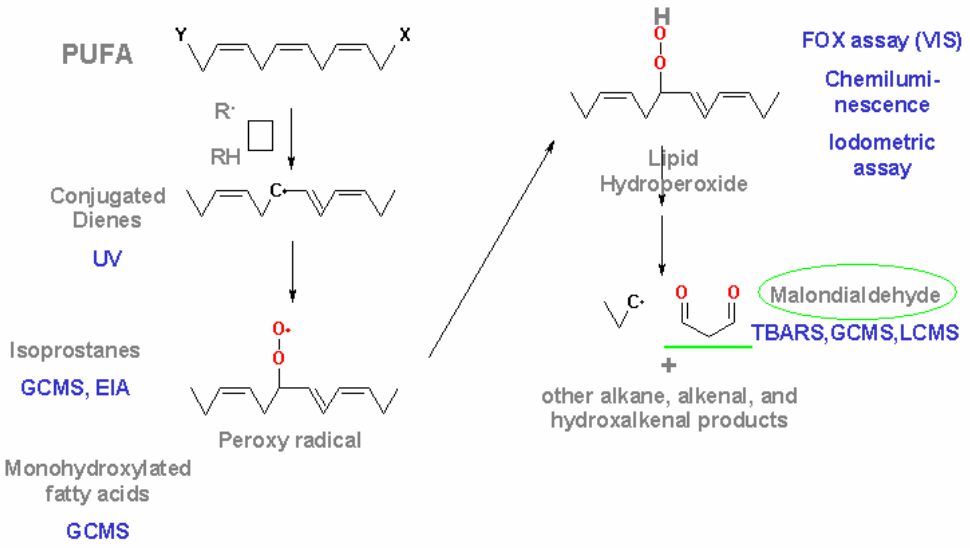

Fig. 2. The metabolites and methods of analysis of lipid peroxidation.

Lipid peroxidation has been linked to many conditions and diseases, including renal failure after rhabdomyolysis (muscle injury), further impaired kidney function, atherosclerosis, chronic gastritis, liver damage, heart disease (myocardial infarction), and cancer (3). Lipid peroxidation has been studied using the TBARS assay, which involves derivatization of malondialdhyde with thiobarbituric acid to produce a pink product that is quantified in a UV-VIS spectrophotometer. This assay, while quick, is not always accurate, since other aldehydes may react with the reagent(2). Gas chromatography and mass spectrometry are more reliable methods to quantify MDA. Unfortunately, malondialdehyde is too unstable to be analyzed directly and must be derivatized first. In the current study, we have tested two different derivatization procedures, which involve the use of pentafluorophenyl hydrazine (PFPH) or the synthesis of pentafluorobenzyl oxime (PFB) derivatives. PFPH modifies the aldehyde groups to form a Schiff base, whereas the other compound modifies carbonyl groups of aldehydes and ketones.

After resolution by gas chromatography, the derivatized compounds are analyzed using both electron impact and chemical ionization. In the present study, the PFPH derivative was initially analyzed using time-of-flight mass spectrometry with electron impact chromatography. Electron impact promotes extensive fragmentation of molecules by using a beam of electrons, whereas chemical ionization is a gentler means of fragmentation that frequently preserves the molecular ion of the compound for analysis. Less fragmentation occurs in chemical ionization because the electron carrier transfers less energy than the traditional electron beam. In time-of-flight mass spectrophotometry, the fragments travel down the mass spectrometer following fragmentation; all of the fragments have the same kinetic energy as they progress in the flight tube, and so the

order in which they reach the detector is inversely proportional to their mass. In another method involving the use of an ion trap, the fragments are analyzed following their removal in a specified orbit in the ion trap from low to high mass. When used in combination with ion trap, the voltage of the electron beam in chemical ionization can be varied to isolate the molecular ion, and then increased to promote cleavage to products in a process known as MS/MS or MS^2. This product may, in turn, be selected in the ion trap and cleaved for further analysis in an MS^3 program, and so on. This approach can be used to study the fragmentation pattern of a molecule, and to develop sensitive methodology that will aid in the identification and analysis of the product of interest. Chemical ionization in combination with MS/MS functions can be used to decrease the limit of detection, thereby allowing small amounts of analytes to be detected in biological samples.

Whereas the combination of a stable derivative and GC/MS can provide good results in a cell free system, to study lipid peroxidation cell models must be employed. The two cell models for inflammation in the lungs and airways are A549 cells, which model bronchial cells, and 9HTE cells, which model tracheal cells. In the present study, lipid peroxidation was investigated through the use of PFPH or PFB oxime derivatives in combination with GC/MS methods, and the COX-dependent and lipoxygenase-dependent pathway were assessed by an enzyme-linked immunosorbent assay (ELISA) to quantify the leukotriene LTB_4 or the prostaglandin PGE_2. Collectively, this methodology will give us a picture of lipid oxidation in these cells in response to a pro-inflammatory stimulus, in this case, the protein interleukin 1β (IL1β), which is administered to the cells in culture.

METHODS

PFPH Derivatization of Malondialdehyde and Chromotagraphic Conditions

Malondialdehyde (MDA), and, when approriate, deuterated MDA, were present in a volume of 250 mL. 10 mL of 2 mM BHT was added to prevent oxidation, and 10 mL 6.6 N H_2SO_4 was added to hydrolyze protein-bound MDA. The samples were incubated at room temperature for 10 min. Protein was then precipitated with 75 mL of 0.3 M Na_2WO_4 and the samples were clarified by centrifugation. Citrate-phosphate buffer was added to the supernatant fraction until the pH was between 3 and 5. 25 µL of 5 mg/mL PFPH was then added to derivatize the malondialdehyde. The samples were allowed to react at room temperature for 30 minutes, then the reaction was quenched with 9 N H_2SO_4. The samples were extracted into the solvent, then dried down with nitrogen gas, and resuspended in 60-70 µL of ethyl acetate. Finally, the samples were resuspended and loaded into GC-bottles. Samples of 1 µL were injected onto a capillary column containing a stationary phase of 250 mm film thickness consisting of 5% diphenyl-95% dimethylpolysiloxane, 30 meters in length, and 0.25 mm in diameter. The column was pre-heated at 50 ^{o}C at a column flow of 2.5 mL/min. The column was maintained for 1 min at 50 ^{o}C for the Leco, 2 min for the Varian, and the oven temperature was increased to 280 ^{o}C at a rate of 20 ^{o}C/sec. The peak areas were obtained by integration performed by the software or where there was at least 75% of the 234 mass ion. The conditions for GC included automated method development. This was used to fragment the molecule over a range of voltages. Several runs were performed to obtain a mass spectrum for 0

volts to 1.4 volts. All masses used were taken from the peak at a retention time between 5.5 min and 6.6 min.

PFB Derivatization and Chromatographic Conditions

400µL samples containing MDA were adjusted to a final concentration in 0.1 M sodium acetate buffer, pH 6, and a final volume of 500 µL in a 1.5mL microcentrifuge tube. PFB solution was added to the sample in 50µL of PIPES buffer. The PFB mixtures contained 10mg of PFB, 100µl of 70% methanol, and 50µL of PIPES buffer. The samples were vortexed for one minute and incubated at room temperature for ten minutes. The MDA derivative was then extracted using hexane, and dried under a stream of nitrogen gas. 25 µL of pyridine and 1mL of TMS, for silylation of hydroxyl groups, were added, and the sealed samples incubated at 65 ^{o}C for one hour (this step was only required for biological samples). The samples were concentrated by evaporation under nitrogen gas and then resuspended in 70uL ethyl acetate prior to analysis by gas chromatography. Samples of 1 µL were injected onto a capillary column containing a stationary phase of 250 mm film thickness consisting of 5% diphenyl-95% dimethylpolysiloxane, 30 meters in length, and 0.25 mm. The column was pre-heated at 40 ^{o}C at a column flow of 1.4 mL/min. The column was maintained for 2 min at 40 ^{o}C and the oven temperature was increased to 140 ^{o}C at a rate of 10 ^{o}C/sec. The injector type 1177 was held at 250 ^{o}C. The ionization method was by chemical ionization. The auto sampler used was the Varian CP 8410 using a sample depth of 90%. The peak areas are obtained by integration preformed by the software or where there was at least 75% of the parent ion.

ELISA Assays

The ELISA assay used for PGE_2 was from Cayman Chemical company, the prostaglandin E2 EIA-kit monoclonal (catalog No. 5140010). The results were read at 450nm. The ELISA assay used for LTB_4 was from assay designs, Leukoriene B_4 correlate EIA-kit (catalog No. 10140512A). The results were read at 405nm. For each samples the data was worked up as described by the assay.

RESULTS

A simple spectrophotometric assay was first used to quantify malondialdehyde after derivatization with thiobarbituric acid. The standard curve for malondialdehyde in the TBARS assay is shown in Figure 3. The TBARS method was used to characterize the time course of lipid peroxidation in the cell-free system involving the pro-oxidant protein myoglobin, and arachidonic acid. The results of the time course experiment shown in Figure 4 indicated that 30 minutes were sufficient to produce the MDA as quantified by the TBARS. The TBARS assay is quick and sensitive; however, this assay can't measure other aldehydes. Also it only indirectly measures lipid peroxidation, and is a difficult assay to use reproducibly in cellular systems. Whereas the TBARS assay was useful for a general idea of the extent of lipid peroxidation in a system, the study of lipid peroxidation biomarkers is more comprehensively evaluated using gas chromatographic methods.

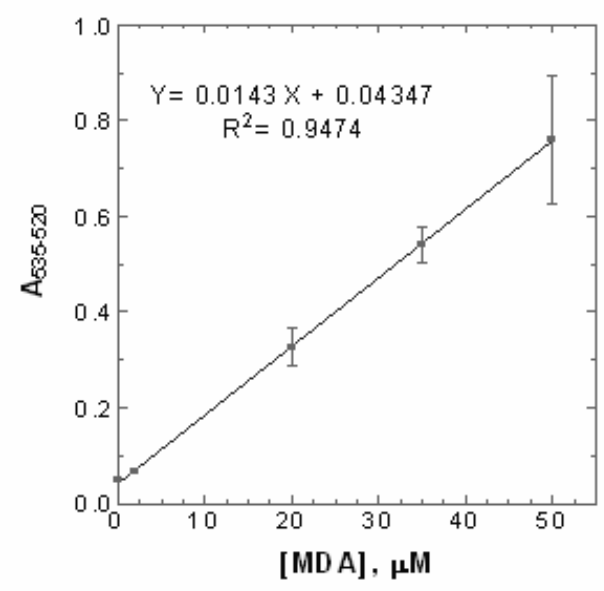

Fig. 3. Standard curve for TBARS assay with malondialdehyde. A high blank precludes the use of this assay for sensitive analysis of TBARS in biological samples.

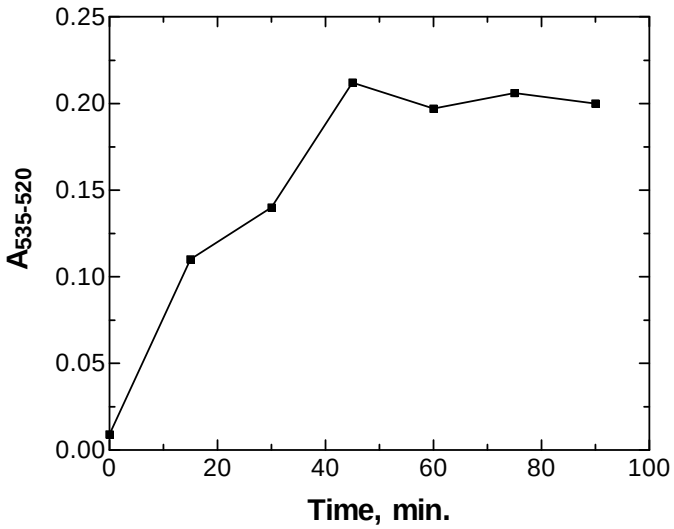

Fig. 4 The time course at 30 ^{o}C for MDA production from arachidonic acid contained in phospholipids in the presence of myoglobin. MDA is produce at a continuous rate for up to 45 minutes of incubation.

In light of the instability of MDA to GC-MS analysis, a prior chemical derivatization of malondialdehyde is required. The first derivative evaluated was pentafluorylphenyl hydrazine. This derivative was analyzed by gas chromatography and time-of-flight mass spectrometry. The standard curve created for MDA-PFPH on the Pegasus II time-of-flight spectrophotometer is shown in Figure 5. The limit of detection for this standard curve and GC/MS equipment is 5.0μM. The MDA-PFPH standards were also analyzed following fragmentation by electron impact (EI) and positive chemical ionization (PCI) and analysis by ion trap mass spectrometry. The standard curve created using EI is shown in Figure 6. The limit of detection for election impact fragmentation was 0.5μM. The need to lower the LOD further lead to using the chemical ionization mode on the Varian. When analyzed using this less extensive fragmentation the limit of detection was 01.μM.

In the interest of preparing the analysis method of a PFPH derivative measured by PCI GC with ion trap MS, an internal standard must be used. The internal standard chosen was the MDA-PFPH compound with two deuteriums added in place of hydrogen atoms. The internal standard was added to the MDA standards in the next experiment. For an internal standard to be useful it is helpful that it is resolved from the external standard on the chromatogram. In this case good separation was seen and an example chromatogram is shown in Figure 7. The resulting standard curve is shown in Figure 8, where varied concentrations of external standard were used with a constant amount of internal standard, and the standard responses are quantified as the ratio of the peak areas for the external standard to that of the internal standard.

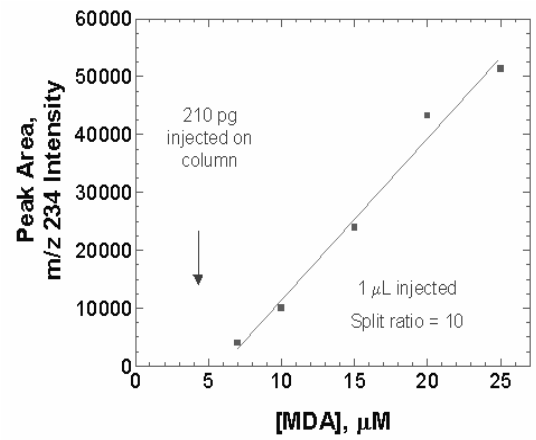

Fig. 5 MDA-PFPH Standard Curve for the Pegasus II time-of-flight mass spectrometer using electron and time of flight mass spectrometry. These results show a limit of detection of 5µM.

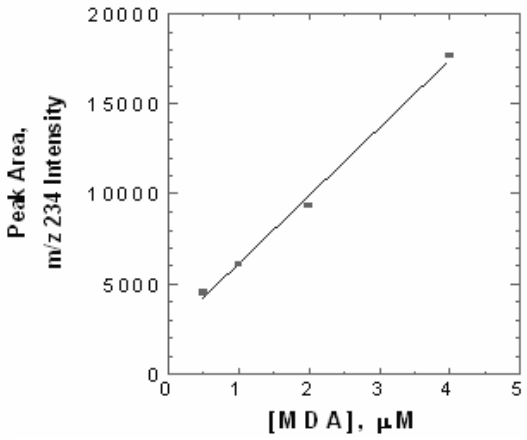

Fig. 6 This figure shows the results for the MDA-PFPH standard curve in electron impact mode on the Varian. This method gave a limit of detection of 0.5 µM.

The mass fragmentation pattern of the MDA-PFPH derivative was investigated more thoroughly in an independent project in the physical chemistry laboratory. The

fragmentation pattern of the MDA-PFPH derivative was determined following PCI in conjunction with an automated method development paradigm in the ion trap mass spectrometer. This approach allowed characterization of the CI-induced spectrum following step-wise changes in excitation amplitude to identify novel ions not observed under electron impact conditions. The resulting graph is shown in Figure 9. This information is valuable in that it can be used in the creation of the MS/MS assay that was alluded to above. The analysis of the fragmentation data gave rise to a proposed fragmentation pattern, which is shown in Figure 10.

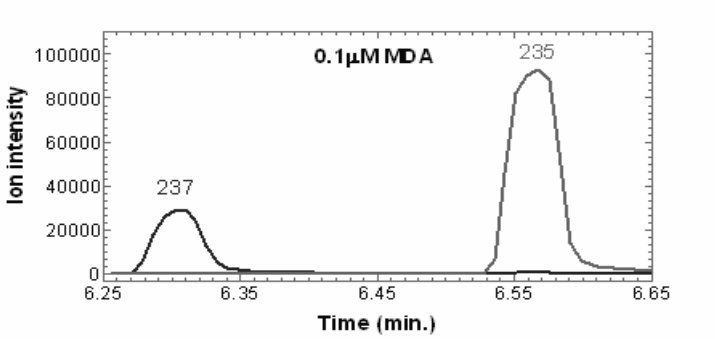

Fig. 7. This figure shows the separation of the two peaks for the internal and external standards when analyzed using chemical ionization. The peaks labeled with the parent ions 237 and 235 represent the internal and external standard respectively.

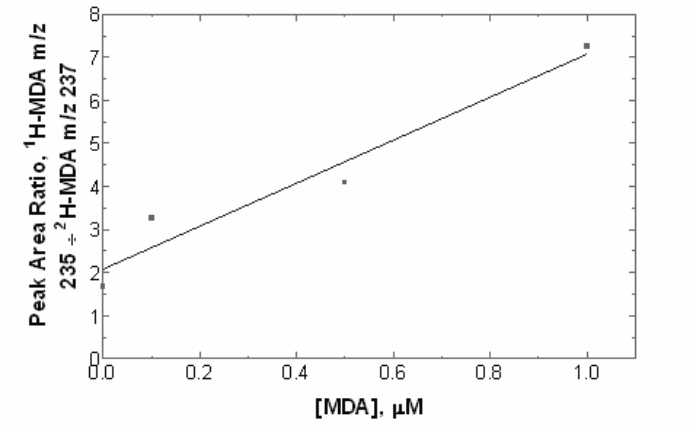

Fig. 8. This figure shows the standard curve obtain for the internal, and external standard method using chemical ionization. This method gave a limit of detection of 0.1 µM

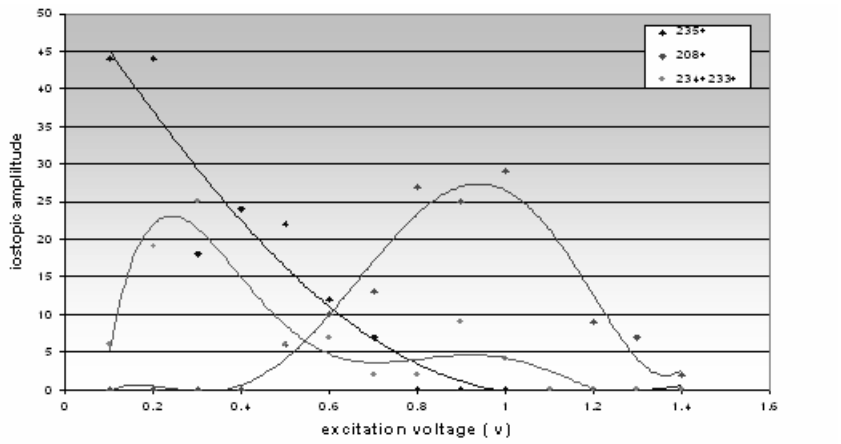

Fig. 9. The dependence of isotopic amplitude on excitation voltage for the MDA-PFPH derivative. Here the ion intensity varies with the excitation amplitude and at different voltages a different ion pattern is produced. These results show that the 208 ion is arising from the fragmentation of the MDA-PFPH moiety.

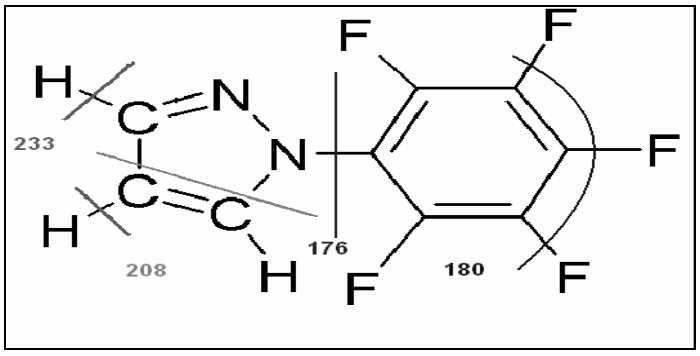

Fig. 10. The proposed fragmentation pattern determined from the AMD study of the 234 parent ion.

Several issues arose that made the PFPH derivation assay more difficult to use than was originally thought. The PFPH-MDA molecule was not as stable as would be desired and the solutions often turned yellow before GS/MS analysis was performed. The yellow color indicated that the derivative had decomposed. The Varian GC is in high demand for many research projects, all of which have different requirements. This means that to study lipid peroxidation in cell systems the samples need to be stored for weeks.

The best results were obtained only when the derivatized samples were analyzed on the same day. The requirement that the samples be analyzed the same day they are derivatized caused this assay to be abandoned in favor of one where the derivatized aldehyde would have a longer shelf life. A slightly different assay was investigated where the aldehyde group forms an oxime linkage to a pentafluorobenzyl moiety (PFB). The initial results using this new method have been promising.

The standard curve for the PFB derivatives of MDA is shown in Figure 11. The limit of detection for this assay is 1.0 μM , although further experiments can be carried out in hopes of identifying a lower limit of detection. Originally, it was difficult to quantify the MDA-PFB derivative since the chromatographic conditions were such that it eluted in the void volume. After adjusting the GC method to a lower temperature so that the MDA-PFB was retained for a short time prior to its elution, the peaks were much better resolved, thus resulting in a standard curve as shown in figure 11.

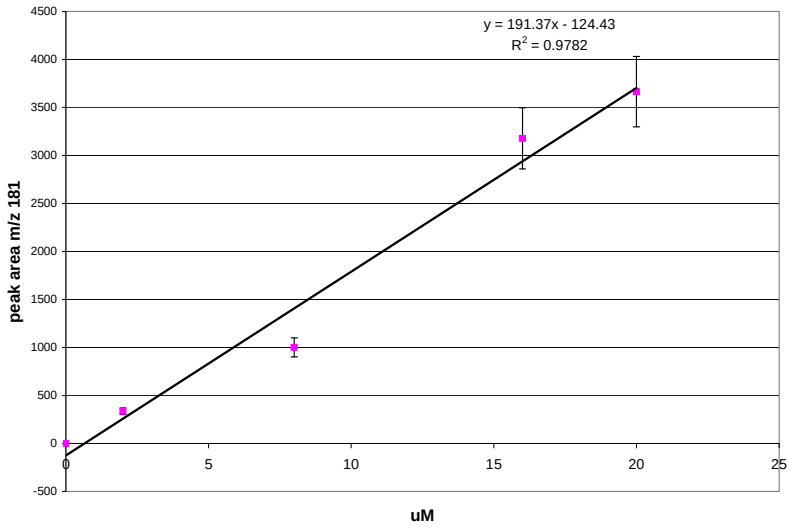

Fig. 11. MDA standard curve using the PFB oxime derivative. This method gave a limit of detection of 1.0 μM

This project's goal is to analyze the response to various inflammatory stimuli in several cell models. A549 cells and 9HTEo- cells were used to model inflammation in bronchial epithelial cells and in tracheal epithelial cells, respectively. These cells were grown in small cultures and stimulated with IL1β to ascertain an initial metabolic profile. The cell media and the cells were separated and frozen at 0, 6, and 12 hours after stimulation. An unstimulated cell culture was allowed to grow for 12 hours and was separated and frozen along with the stimulated samples in the time course. These samples were than derivatized using the PFB oxime procedure and analyzed using GC/MS. The graphs of the peak area, or amount of derivatized MDA, versus time for the A549 cells and 9HTE cells are shown in Figures 12 and 13 respectively. This experiment was run in duplicate from the tissue cultures to the GC analysis so, these graphs show both sets of samples.

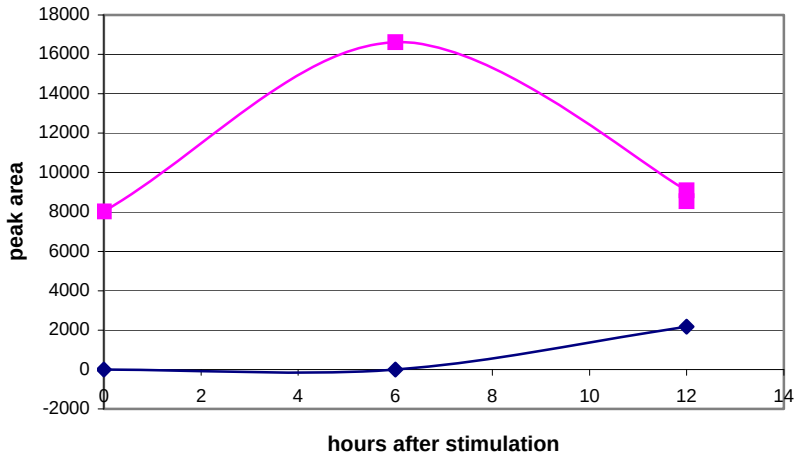

Figure 12. A549 cell media time course after stimulation with IL1-β

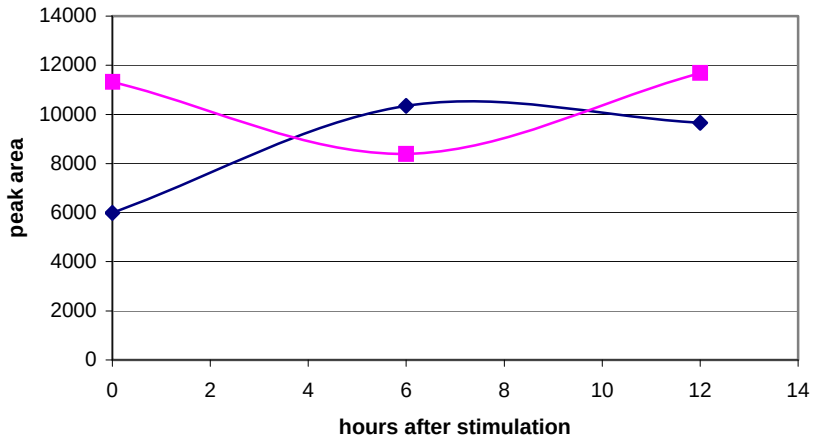

Figure 13. 9HTE cell media time course after stimulation with IL1-β

In response to the initial appearance of low levels of MDA production, the samples from the cellular stimulation study were analyzed for PGE_2 and LTB_4 production. These compounds are produced in the cyclooxygenase and lipoxygenase pathways respectively. These experiments were performed to examine the possibility that other pathways are being activated by the IL1β stimulation, since it needs to be established before the cell lines are stimulated with macrophages or bacteria that express putative pro-oxidant proteins. The samples were analyzed using ELISA kits for PGE_2 and LTB_4. Using an ELISA assay, the results with the A549 cells after 0, 6 and 12 hours of stimulation with IL1β, and 12 hours without stimulation indicated that PGE_2 was expressed after 6 and 12 hours of stimulation; only low levels of PGE2 were produced by the 9HTE cells as determined from the data shown in Table 1. The standard curve for the PGE_2 ELISA assay is shown in Figure 14. It is evident from these results that the A549 cells show that the cyclooxygenase pathway is active and that PGE_2 is being produced. The samples were also analyzed for the leukotriene LTB_4 using another ELISA kit,

although no LTB_4 was found with either cell line or at any of the time periods noted above.

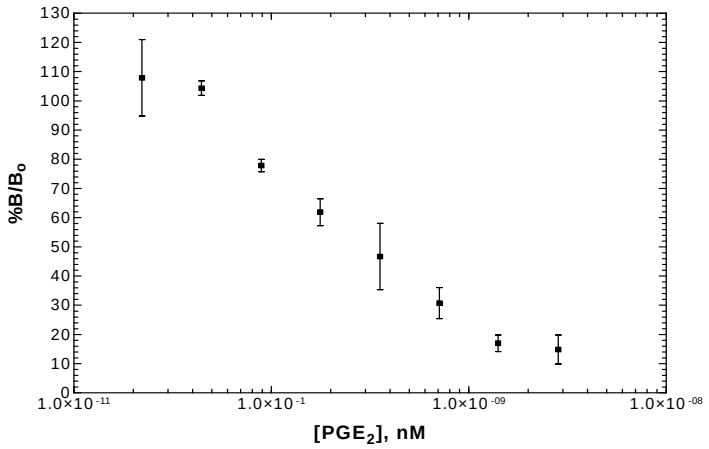

Fig. 14. Standard curve for PGE_2 ELISA assay.

Table 1. PGE_2 results of A549 and 9HTE time course.

sample	avg %B/Bo	Log of [PGE_2]	[PGE2]
A549 0hr	86.7927	1.360	22.887
A549 6hr	14.6227	2.832	678.970
A549 12hr	20.0472	2.721	526.249
A549 12hr un	111.5569	0.854	7.152
9HTEo 0hr	126.6512	0.546	3.520
9HTEo 6hr	100.9436	1.071	11.774
9HTEo 12hr	101.4153	1.061	11.516
9HTEo 12hr un	116.9814	0.744	5.543

Tng the logarithm of the standard concentrations was plotted against the their corresponding %B/Bo. %B/Bo is ratio of the corrected absorbance to that of the maximum binding well. The linear fit gave a line with the equation, %B/Bo = -49.02*(log[PGE_2]) + 153.44, with a R^2 value of 9.747.

DISCUSSION

The TBARS assay is known to be a sensitive, but non-selective means of measuring lipid peroxidation. It can overestimate the amount of MDA by over ten-fold. This overestimation is thought to occur from cross reactivity with other aldehydes (2). These issues lead to the use of a GC/MS procedure to analyze MDA following its derivatization. The PFPH assay had good initial results and, by changing the GC method from electron impact in time-of-flight or ion trap to positive chemical ionization with ion trap, the limit of detection was decreased to a level that was suitable for detection of MDA in cells from tissue culture. Even though the method was developing well, issues of reproducibility and a low shelf life of the MDA-PFPH compound lead it to be set aside in favor of the PFB oxime procedure.

The failure of an assay is an unwelcome, but unavoidable, occurrence. This investigation has merit in that the quantitation of MDA is a highly characteristic product of lipid peroxidation. With the development of a robust assay to measure MDA, many systems may be easily studied. The effort to complement such studies with an examination of PGE_2 and or LTB_4 provides a good basic profile of lipid metabolism in any given system. The PFB assay has proved much easier to utilize and currently provides reasonable limit of detection and range of linearity. The limit of detection listed in the results is 1.0μM. This is a little above where the linear average intersects the x-axis. The lowest concentration used was 2.0μM. The limit of detection should decrease further when lower concentrations of the standard are used. To improve the PFB derivative assay further an internal standard will eventually be needed. In the PFPH experiments the internal standard used was the MDA with two deteriums incorporated.

This caused a high background amount of MDA. Better results could be obtained by using a compound similar to the MDA-PFB derivative as the internal standard, which could be measured separately. In the literature one group used 1,3-[2H8]propanediol as their internal standard for MDA, since they were reducing aldehydes to their alcohols as a way to derivatize.(6) This type of complementary internal standard with a similar structure but different enough to be easily separated, would be a better choice for the PFB assay. This assay requires an internal standard for use on biological samples such as the cell media time course.

The cellular stimulation study shows the lipid peroxidation profile of the two cell lines A549 and 9HTE. The media samples were analyzed by a PFB oxime detivatization and GC/MS analysis. The results for the A549 duplicate sample sets are contradictory. The results of the PGE2 assay show that high levels of PGE_2 were produced in response to IL1-β. If it is assumed that these results mean that MDA is not being produced, then the lipid peroxidation pathway is being turned off, and the cyclo-oxygenase pathway is turned on. The cellular time course of the A549 cells appears to be signaling with PGE_2 in response to the inflammatory stimuli.

The initial results show no apparent change in the amount of MDA for the 9HTE cells, while the results of the ELISA assay showed small amounts of PGE_2 produced. The minimal amount of PGE_2 and MDA leads to the question of whether something else is being produced in the 9HTE cells in response to the stimulation. From the LTB_4 experiment it appears that leukotrienes are not being produced either. Another possibility currently being examined by others in the lab is thromboxanes. When discussing the results for the 9HTE cells it is important to remember that these and the A549 cells are

very different types of cells. While the increase in the amount of PGE_2 produced in the 9HTE cells may seem small when compared to the data for the A549 cells, the amount at 6 and 12 hours is four fold the amount at 0 hours. This means that both cell lines are actually producing PGE_2.

The reformation of the chromatographic conditions for the PFB derivative will provide new data for MDA in the time course studies, revealing the changes in MDA concentration over the 12 hours of the time course. To complement this, the time course study will benefit from the use of more time points. This means that future stimulation studies should give a clearer picture of the MDA production in the two cell lines. All these experiments work together to provide a picture of lipid metabolism. The picture is not yet fully developed but promises to provide new insight to inflammation and the inflammatory response.

REFRENCES

1. Wolff, J.F.D.(2004) *Wikipedia.*
http://en.wikipedia.org/wiki/Image:Eicosanoid_synthesis.png
2. Moore, K., and Roberts, L.J..(1998) *Free RadicRes.* **6**, 659-71.
3. Janero, D.R.. (1990).*Free Radic Biol Med.* **9(6),** 515-40. 4. Moore, K., and Roberts, LJ.. (1998) *Free RadicRes.* **6,** 659-71.
4. Yeo, H.C., Liu, J., Helbock, H.J., and Ames, B.N. (1999) *Methods Enzymol*. **300**, 70-78
5. Yeo, H.C., Helbock, H.J., Chyu, D.W., and Ames, B.N.(1994) *Analytical Biochemistry*. **220**, 391-396
6. Des Rosiers C, Rivest MJ, Boily MJ, Jette M, Carrobe-Cohen A, Kumar A.(1993) Anal Biochem. **208(1),** 161-70.