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Influence of cell differentiation on arachidonic acid metabolism in stimulated and unstimulated HL-60 cells

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INFLUENCE OF CELL DIFFERENTIATION ON ARACHIDONIC ACID METABOLISM IN STIMULATED AND UNSTIMULATED HL-60 CELLS

By

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Influence of Cell Differentiation on Arachidonic acid Metabolism in Stimulated and Unstimulated HL-60 Cells (76 pp).

Director: George L. Card

Human promyelocytic HL-60 cells were differentiated and examined for the influence of arachidonic acid (AA) distribution and patterns of metabolism on products produced in response to stimulation. HL-60 cells were differentiated to macrophage/monocyte type cells with phorbol ester (PMA) or to granulocyte type cells with retinoic acid (RA). Stimulation was achieved with calcium ionophore A23187. Lipids were extracted using a Bligh and Dyer method modification, separated on two dimensional SG81 silicic acid paper or on LK6D silica gel TLCs and visualized by autoradiography.

The relative phospholipid composition of all three cell types was similar: PC accounted for 63%-69% of total added label; PE 9%-16%; PI 2%-7%; and PS 4%-5%. Marked differences occurred in AA distribution within the three cell types. In PMA differentiated cells the neutral lipid contained 40% of the total, 18% in RA differentiated cells and <3% in undifferentiated cells. PE contained the largest portion of AA in the RA treated and undifferentiated cells, 49% and 43% respectively. The PMA treated cells contained 26% AA in their PE fraction.

After differentiation, the RA treated and undifferentiated cells showed a similar pattern of AA uptake with maximum uptake at 6 hours. The PMA treated cells continued AA uptake for 8 hours. During this time the granulocyte-type cells and undifferentiated cells exhibited a turnover between the PE and PC pools while the macrophage/monocyte cells showed a turnover between the neutral lipid and PC pools.

During differentiation, [3H]AA prelabeled cells lost radiolabel from the PC pool but gained in the PE and PI pools of RA treated and undifferentiated cells. PMA treated cells showed no change throughout the differentiation period.

Stimulation of the three cell types generated no net change in the total phospholipid composition of any of the cell types. Stimulation resulted in reorganization of AA in all three cell types.

The differences in AA distribution among the HL-60 cell types and the similarities between the undifferentiated and RA treated cells may help explain the AA metabolite production patterns seen in the three cell types.
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGMENT</td>
<td>iii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>v</td>
</tr>
<tr>
<td>CHAPTER</td>
<td></td>
</tr>
<tr>
<td>1. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>2. MATERIALS AND METHODS</td>
<td>24</td>
</tr>
<tr>
<td>3. RESULTS</td>
<td>29</td>
</tr>
<tr>
<td>4. DISCUSSION</td>
<td>59</td>
</tr>
<tr>
<td>5. SUMMARY</td>
<td>68</td>
</tr>
<tr>
<td>LITERATURE CITED</td>
<td>70</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Oxygenation Reactions Involving Arachidonic Acid</td>
</tr>
<tr>
<td>2.</td>
<td>Conversion of LTA₄ to Other Peptidoleukotrienes</td>
</tr>
<tr>
<td>3.</td>
<td>Model for the Mobilization of Arachidonic Acid from PI and PC in Response to Physiological Stimuli</td>
</tr>
<tr>
<td>4.</td>
<td>Comparison of Phospholipids in Differentiated and Undifferentiated HL-60 Cells</td>
</tr>
<tr>
<td>5.</td>
<td>[³H]Arachidonic Acid Distribution in Differentiated and Undifferentiated HL-60 Cells</td>
</tr>
<tr>
<td>6.</td>
<td>Uptake of [³H]Arachidonic Acid by Differentiated and Undifferentiated HL-60 Cells</td>
</tr>
<tr>
<td>7.</td>
<td>Uptake of [³H]Arachidonic Acid in Lipid Pool of PMA Differentiated (Macrophage/monocyte-type) HL-60 cells</td>
</tr>
<tr>
<td>8.</td>
<td>Uptake of [³H]Arachidonic Acid in Lipid Pool of RA Differentiated (Granulocyte-type) HL-60 Cells</td>
</tr>
<tr>
<td>9.</td>
<td>Uptake of [³H]Arachidonic Acid in Lipid Pool of Undifferentiated HL-60 cells</td>
</tr>
<tr>
<td>10.</td>
<td>Turnover of [³H]Arachidonic Acid During Differentiation of HL-60 Cells by PMA</td>
</tr>
<tr>
<td>11.</td>
<td>Turnover of [³H]Arachidonic Acid During Differentiation of HL-60 Cells by RA</td>
</tr>
<tr>
<td>12.</td>
<td>Turnover of [³H] Arachidonic Acid in Undifferentiated HL-60 Cells</td>
</tr>
<tr>
<td>13.</td>
<td>Turnover of Phospholipid Fractions in PMA Treated HL-60 Cells</td>
</tr>
<tr>
<td>14.</td>
<td>Turnover of Phospholipid Fractions in Stimulated PMA Treated HL-60 Cells</td>
</tr>
<tr>
<td>15.</td>
<td>Turnover of Phospholipid Fractions in RA Treated HL-60 Cells</td>
</tr>
<tr>
<td>16.</td>
<td>Turnover of Phospholipid Fractions in Stimulated RA Treated HL-60 Cells</td>
</tr>
</tbody>
</table>
17. Turnover of Phospholipid Fractions in Undifferentiated HL-60 Cells.....................47
18. Turnover of Phospholipid Fractions in Stimulated Undifferentiated HL-60 Cells...........48
19. \[^{3}\text{H}]\text{Arachidonic Acid Turnover in PMA Differentiated HL-60 Cells}...............50
20. \[^{3}\text{H}]\text{Arachidonic Acid Turnover in PMA Differentiated HL-60 Cells upon Stimulation}.....51
21. \[^{3}\text{H}]\text{Arachidonic Acid Turnover in RA Differentiated HL-60 Cells}...................53
22. \[^{3}\text{H}]\text{Arachidonic Acid Turnover in RA Differentiated HL-60 Cells upon Stimulation}.....54
23. \[^{3}\text{H}]\text{Arachidonic Acid Turnover in Undifferentiated HL-60 Cells}......................56
24. \[^{3}\text{H}]\text{Arachidonic Acid Turnover in Undifferentiated HL-60 Cells upon Stimulation}..57
Chapter 1
INTRODUCTION

Arachidonic acid (5-cis, 8-cis, 11-cis, 14-cis eicosatetraenoic acid) is one of the three most common C\textsubscript{20} polyunsaturated fatty acids in animals. It is the precursor of the important oxygenated eicosanoid family which includes the leukotrienes (LT) and lipoxins (Lx), lipoxygenase products, and the prostaglandins, cyclooxygenase products (Fig. 1).

Nonesterified arachidonic acid can be converted to a variety of biologically active end products. The first step is the release of arachidonic acid from membrane phospholipids, usually in response to stimulation of sensitive cells with such things as immune complexes, calcium ionophores, inflammatory stimuli and chemotactic peptides (49, 51). Release of arachidonic acid is the rate limiting step for all subsequent metabolism. Oxygen can then be inserted stereospecifically into unconjugated double bond systems (50) at positions 5, 12, and/or 15 of eicosanoic acids by the respective lipoxygenases. The products are hydroperoxy-eicosatetraenoic acids (HPETES). The 5-lipoxygenase produces 5-HPETE which is dehydrated yielding an epoxy (oxido) fatty acid (e.g. LTA\textsubscript{4}). LTA\textsubscript{4} is very unstable and can be hydrolyzed to LTB\textsubscript{4} or conjugated with glutathione to form LTC\textsubscript{4} by GSH-S-transferase, LTC\textsubscript{4} can then be converted to LTD\textsubscript{4} by gamma-glutamyl transpeptidase.
Figure 1. Oxygenation reactions involving arachidonic acid.
which may be converted to LTE₄ by dipeptidase (Fig. 2; 49, 51). Recent studies have shown LTE₄ can be further metabolized to N-acetyl LTE₄, LTE₄ sulfoxide, -hydroxy LTE₄, 6-trans LTB₄, LTF₄ and LTG₄ (55). Leukotriene B₄, LTC₄, LTD₄ and LTE₄ are biologically active. They are produced by and act primarily in host defense mechanisms, which include cells such as polymorphonuclear lymphocytes, mononuclear phagocytes and mast cells. They are also found in the central nervous system, and are being extensively studied in relation to allergy and inflammation. LTB₄ is a calcium ionophore and neutrophil chemotactant (49). Eosinophils generate LTC₄ and LTD₄ (19). Leukotrienes C₄, D₄, and E₄ are all sulfidopeptide metabolites. Together they comprise SRS-A, slow reactive substance A. The biological activities of these leukotrienes include: increased vascular permeability in the skin, stimulation of mucus secretion, constriction of the microvasculature and coronary arteries of both skin and lungs and effects on smooth muscle and other contractile cells. Leukotrienes released from the lung tissue of asthmatics after exposure to specific allergens appear to play a major pathophysiological role in immediate hypersensitivity reactions (49, 51).

The synthesis of leukotrienes requires 5-lipoxygenase activation. This enzyme catalyzes production of 5-HPETE from arachidonic acid by the dioxygenation of C-5 and then converts 5-HPETE to LTA₄. Results from human leukocyte
Figure 2. Conversion of LTA₄ to other peptidoleukotrienes.
5-lipoxygenases studies (49) suggest the enzyme is regulated by a complex, multicomponent system and requires activation by cofactors including Ca\(^{2+}\), phospholipid and ATP (19). These multiple cellular stimulatory components are unique among mammalian 5-lipoxygenases and much remains unknown about the enzyme's regulation and various stimulatory components.

The lipoxins are newly discovered oxygenated derivatives of arachidonic acid which are generated though a proposed epoxide intermediate. There are indications that both the 5- and 15-lipoxygenase pathways interact to form lipoxins in human leukocytes (50). The conversion of exogenous LTA\(_4\) to lipoxins, including LxA, in human platelets has also been reported (21). Lam's group has reported that phospholipase A\(_2\) isoenzyme generated LxB from endogenous arachidonic acid sources in porcine leukocytes (34). Two conjugated tetraene lipoxins, LxA (5,6,15L-trihydroxy-7,9,11,13-icosatetraenoic acid) and LxB (5D,14,15L-trihydroxy-6,8,10,12-icosatetraenoic acid) are biologically active but some of their isomers are not. The reported activities include dose-dependent suppression of natural killer cells without effects to target cell binding or cAMP levels, chemotaxis, and lung tissue contractions, all of which are distinct from those of prostaglandins, thromboxanes, or leukotrienes (46, 49). When submicromolar concentrations of LxA was added to neutrophils it stimulated
generation of superoxide anion and degranulation without substantial aggregation. Lipoxin A is as potent as LTB₄ with respect to generation of superoxide anion but two times less potent than either LTB₄ or the chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (fMLP) at eliciting degranulation (50).

Prostaglandins (PG), cyclooxygenase products, are produced in many cell types in response to stimuli (19). They act as local hormones that are quickly cleared from the system. The biosynthesis is a three step process: 1) cellular stimuli releases the C₂₀ precursor fatty acid, usually arachidonic acid, 2) the fatty acid is oxygenated to prostaglandin endoperoxide derivative PGH, 3) PGH is converted, depending on cell type, to the biologically active prostanoid forms (PGE₂, PGF₂, PGD₂, PGI₂ or thromboxane A₂) (51). The physiological effects of PGs include inflammation, clotting, osmotic gradients and smooth muscle contraction and involve nearly every system in the body.

All three general classes of arachidonic acid metabolites are involved in normal immune responses and pathological responses such as immediate hypersensitivity and inflammation (49). Arachidonic acid itself can stimulate the mobilization of stored intracellular Ca²⁺ and thus increase free Ca²⁺ in the cytosol. In polymorphonuclear lymphocytes (PMNs) this results in the
activation of a Ca\textsuperscript{2+}/phospholipid dependent protein kinase C, induction of PMN aggregation and O\textsubscript{2} radical production (52). Exogenous arachidonic acid partially inhibits ionophore stimulated LTB\textsubscript{4} production (22). The resulting balance between these responses may be any combination of 1) the nature of the cellular stimulation(s), 2) the distribution/availability of arachidonic acid in the cell's phospholipid pools, and 3) the enzymes available for subsequent metabolism of free arachidonic acid.

There are two broad classes of stimuli for release of arachidonic acid - physiological, or specific, and pathological, or nonspecific (51). When cultured cells, platelets or organs are radiolabeled with polyunsaturated fatty acids, physiological stimulation releases arachidonic acid but no other sn-2 fatty acids, whereas, pathological stimulation releases linoleate, olate, arachidonate and other fatty acids at the sn-2 position in proportion to the amount present. Physiological stimuli for arachidonic acid include hormones such as angiotensin II, bradykinin and epinephrine; proteases such as thrombin; and some antibody-antigen complexes. Responsive cells have specific receptors for these stimuli. Pathological stimuli, however, can affect any cell and include mechanical damage, ischemia, venoms such as mellitin, Ca\textsuperscript{2+} ionophores such as A23187 and tumor promoters such as phorbol esters.

There is essentially no free pool of arachidonic acid
within a resting/nonstimulated cell (29, 58). Arachidonic acid is almost exclusively esterified at the sn-2 position in a variety of phospholipids in the membrane. Arachidonate is not incorporated during de novo synthesis of glycerophospholipids, such as acylation of glycerol-3-phosphate or lysophosphatidic acid to give phosphatidic acid. Instead it is acylated at a later stage to a glycerolysophospholipid such as lysophosphocholine (29). Arachidonic acid is found in the lipids of the plasma membrane, endoplasmic reticulum, and possibly in the mitochondrion membrane (35). The majority of arachidonic acid is in phosphatidyl components (diacyl) which are the most metabolically active pool. The remaining small fraction is ether linked (alkylacyl or alkenylacyl) and is an inert or storage pool (18). Wilson et al. (58) showed that human platelets preferentially incorporate arachidonic acid and 8,11,14 eicosatrienoic acid over other free fatty acids. This is because platelets have two long chain acyl-CoA synthetases; one is active with a wide range of different fatty acids and the other, arachidonoyl-CoA synthetase, is specific for the polyunsaturated C\textsubscript{20} fatty acids. This specific enzyme has an optimum pH of 8, requires ATP, Mg\textsuperscript{2+}, CoA and arachidonic acid. It may control the level of free arachidonic acid, limit its metabolites in unstimulated cells and capture free extracellular arachidonic acid. When labeled, exogenous
arachidonic acid was added to platelets, free levels were kept low as the label was esterified into phospholipids without generation of oxygenated metabolites or much beta-oxidation of the added fatty acid (58). Non-arachidonic acid metabolite precursors (palmitic, stearic, oleic, and linoleic acids) had a relatively low rate of esterification into the phospholipids and were present in a significant amount in the free fatty acid pool.

When the fatty acid content of pancreatic islet phospholipids was determined (56), the relative abundance of phospholipids was phosphatidylcholine (PC) > phosphatidylethanolamine (PE) > phosphatidylinositol (PI) > phosphatidylserine (PS). Arachidonate constituted 17% of the total islet fatty acid content and PC contained 43% of the total arachidonate in the islets. When the islets were incubated with \[^{3}H\] arachidonic acid plus D-glucose the PC pool had the highest specific activity. When the islets were incubated with either 28mM glucose or 3mM glucose for 30 min, the total fatty acid content of PC in the cells treated with the higher glucose concentration was much lower than those treated with 3mM glucose. This suggests PC may be the major source of free arachidonic acid upon stimulation of the islets with D-glucose, which induces phospholipid hydrolysis.

A study using human umbilical vein endothelial cells demonstrated selective incorporation of specific fatty acids
into various phospholipids via deacylation-reacylation pathways (53). Arachidonic acid, eicosatrienoic acid and eicosapentaneoic acid showed a high rate of incorporation into the total phospholipid pool but other fatty acids such as docosahexaenoates and monohydroxyeicosatetraenoates showed a low rate of incorporation. The relative amount of eicosanoid precursor fatty acid incorporation was PC > PE > PI > PS but the specific activity of \(^{3}\)H-arachidonic acid in PI was twice that of any of the other fatty acids i.e. arachidonic acid was preferentially incorporated into PI.

The general scheme for arachidonic acid release in the cell begins when receptors are activated which stimulate \(Ca^{2+}\) mobilization. Calcium activates phospholipase C which leads to hydrolysis of phosphoinositides. One product of this hydrolysis is 1,2-diacylglycerol which in turn activates protein kinase C as well as releases a small amount of arachidonic acid. Protein kinase C (PKC) then activates phospholipase \(A_2\) which specifically cleaves arachidonic acid from the sn-2 position in the phospholipid pool(s) of the cell (Fig. 3; 4, 51). The level of arachidonic acid in the tissue will be determined by the relative role of hydrolysis and reesterification by acyl-transferases. The reesterification could be the rate limiting step in different tissues (29).

The concentration of intracellular calcium is a major (possibly the major) controlling factor for arachidonate
Figure 3. Model for the mobilization of arachidonic acid from PI and PC in response to physiological stimuli. GPC is glycerophosphocholine.
release, though there is no clear evidence of this in vivo (29). Evidence does exist showing that within 10 seconds of \( \text{Ca}^{2+} \) stimulation (by way of ionophore A23187) in rabbit PMNs and platelets, phospholipase C preferentially broke down polyphosphoinositides to inositol bisphosphate and inositol triphosphate (39). Similar results were obtained when the chemotactic peptide fMLP was used to stimulate rabbit PMNs. Purdon et al. (45) reported phospholipase C activated platelets preferentially hydrolyzed arachidonic acid containing species of 1,2-diacylPI. Inositol triphosphate (InsP\(_3\)) is thought to be an intracellular messenger responsible for additional \( \text{Ca}^{2+} \) mobilization. InsP\(_3\) biological activity correlates directly with specific and saturable cell membrane binding sites. Recently another potentially physiologically important inositol phosphate has been discovered - InsP\(_4\) - with a proposed function of regulating the amount of \( \text{Ca}^{2+} \) to which InsP\(_3\) has access. Membranes of HL-60 cells (see Cell Line) have InsP\(_4\) binding site with a 200 times higher affinity than for InsP\(_3\), though a definite intracellular receptor has yet to be established (8).

Phospholipase C generates 1,2-diacylglycerol (diglyceride) in addition to the inositolpolyphosphates (51). These 1,2-diacylglycerides activate protein kinase C (PKC) in vivo and in vitro (47). Actually PKC is activated by the simultaneous presence of phospholipid, a diglyceride
and Ca\(^{2+}\). The diglyceride-PKC interaction is highly specific whereas the phospholipid-PKC interaction is not as specific. The diglyceride increases the PKC affinity for Ca\(^{2+}\) but is short lived itself because of metabolism by phosphorylation and lipase-mediated hydrolysis. In unstimulated cells PKC was found in the cytoplasm. Upon stimulation the kinase was translocated to the membrane because of its binding with the diglyceride and was activated by interactions with phospholipid and Ca\(^{2+}\). At the membrane it phosphorylated its substrate(s) on serine or threonine residues.

One of the substrates for PKC is the phospholipase A\(_2\) (P-lipase A\(_2\)) inhibitory protein complex. Phosphorylation results in activation of P-lipase A\(_2\) (51). Stimulation of arachidonic acid liberation in most tissues is probably controlled by changes in phospholipase activity (29). In 1983, Ballou et al. (3) stated arachidonic acid released from membrane phospholipids after Ca\(^{2+}\) dependent platelet activation served as the sole substrate for endoperoxides and thromboxanes which regulate various platelet functions. There are two proposed pathways for rapid arachidonic acid release from stimulated platelets: 1) the activation of P-lipase A\(_2\) which hydrolyses arachidonic acid from sn-2 position of PC, PE, and to a lesser degree PS to generate free arachidonic acid and lysophospholipids; (PI seemed to be a poor substrate for P-lipase A\(_2\)) and 2) P-lipase C.
breaks down PI to 1,2-diacylglycerol which is further broken down by a specific lipase to release free arachidonic acid. Together these two pathways account for all free arachidonic acid. There are arguments that PC specific P-lipase A₂ leads to the majority of the free fatty acid (pathway 1) and note the diacylglycerol from PI by P-lipase C is rapidly converted to phosphatidic acid and not available for the diacylglycerol lipase pathway by P-lipase A₂. PC cleaved arachidonic acid rapidly leads to biologically active products.

Phospholipase A₂ selectivity in its release of arachidonic acid from thrombin-stimulated platelets has been shown (45). The primary membrane phospholipids cleaved were 1-acyl PC and to a lesser extent 1-acyl PE, while ether phospholipids became enriched with arachidonic acid. After thrombin stimulation of human platelets all 1,2-diacyl PC species arachidonic acid content decreased by approximately 50% and 1,2-diacyl PE by 20%. No changes were observed in non-arachidonic acid containing species. It was concluded that P-lipase A₂ was selective for membrane phospholipids with arachidonic acid and it may be that these phospholipids are compartmentalized within the platelet membrane proximal to the enzyme action site. Most enzymes involved in phospholipid biosynthesis and metabolism are in the endoplasmic reticulum with cytoplasmic access, but there have been reports of P-lipase A₂ hydrolysis/deacylation both
intracellularly and from the plasma and intracellular membranes.

Human neutrophils contain a Ca\(^{2+}\) dependent, cytosolic phospholipase with relative specificity for arachidonoyl PC that is optimally active at pH 8. Calcium ionophore stimulation leads to enhanced phospholipase activity on 1-o-alkyl-2-arachidonoylglycerophosphocholine (-GPC) and to a lesser extent 1-acyl-2-arachidonoyl-GPC (2). Bone marrow macrophage also exhibit an alkaline P-lipase A\(_2\) with selectivity for arachidonic acid (as compared to oleic or linoleic acids) from the sn-2 position of 1-acyl- and 1-alkyl-PC species upon stimulation.

Inflammatory stimuli induce P-lipase A\(_2\) in a mouse macrophage line to release arachidonic acid (36). A P-lipase A\(_2\) that hydrolyzes 1-o-hexadecyl-2-[\(^3\)H]arachidonoyl-GPC was identified in a cytosolic fraction. It showed Ca\(^{2+}\) dependence and optimum activity at pH 8. Preferred substrates were 1-acyl-2-arachidonoyl-GPC > 1-alky-2-arachidonoyl-GPC = 1-acyl-2-arachidonoyl-GPE >> 1-acyl-2-arachidonoyl-GPI. This enzyme also had specificity for sn-2 arachidonic acid as compared to PC with linoleic or oleic acid at the same position.

Recent observations agreed that arachidonic acid is released through P-lipase A\(_2\) and P-lipase C/diacylglycerol lipase pathways but went on to imply involvement of G protein(s) in arachidonic acid release. Fluoride, which
directly activates G proteins, was used on permeabilized neutrophils (and similarly on platelets and mast cells). The fluoride induction released arachidonic acid from the PI, PC and PE pools and seemed to be mediated through the two above mentioned pathways (42).

Different cells produce different end products from released arachidonic acid though they all may have the same, complete set of enzymes (28). Some cells produce both cyclooxygenase (PG) and lipoxygenase (LT) products from the released arachidonic acid. These metabolites of arachidonic acid can interact to modulate macrophage functions: PGE$_2$ reverses LTC$_4$ stimulation of macrophage lysosomal enzyme released and LTC$_4$ stimulates macrophage PGE$_2$ release (22). This indicated that leukotriene activation of macrophage may be self-regulating as PGE$_2$ inhibits cell activation. PGE$_2$ has been reported to inhibit rat neutrophil LTB$_4$ synthesis. Therefore it is possible PGE$_2$ prevents macrophage activation by inhibiting leukotriene production. The coordination of the synthesis of the products is unclear (49). Depending on the type of stimuli used, whether it is a membrane stimulant or can by phagocytosed, different metabolites are produced (28). M.L. Brown suggested in 1987 that specific pools of arachidonic acid may provide precursors for individual classes of eicosanoids (13).
CELL LINE: HL-60 cells were chosen as the tissue culture line to be used in this project. HL-60s are promyelocytic cells that were established in 1977 from the peripheral blood leukocytes of an acute promyelocytic leukemia (17, 23). They are homogenous and exist in an arrested yet pliant state of maturation, that of an immature myelomonocytic line. They have receptors for fMLP and insulin. The doubling time is from 20-24 hours and approximately 10% of a population will spontaneously differentiate into more mature myeloid forms (26). Phenotypic differentiation can be chemically induced. This change begins with an early event of G1/0 growth arrest (or precommitment) followed by a late event that controls the particular lineage differentiation. Induction also depends on an initial S phase-specific event (61).

A number of compounds stimulate the uncommitted HL-60 precursors to differentiate into monocyte/macrophage cells or mature granulocytes (27, 63). The monocyte/macrophage inducers include phorbol meristate acetate (PMA, which is also referred to as TPA, 12-0-tetradecanoyl-phorbol-13-acetate), 1,25-dihydroxyvitamin D₃, interferon gamma, tumor necrosis factor, sodium butyrate and actinomycin D. The granulocyte inducers include retinoic acid (RA), dimethylsulfoxide (DMSO), dimethyl formamide (DMF) and hypoxanthine. All these compounds act by arresting cell proliferation at the G0/1 phase followed by differentiation.
to the particular functional cell type (27, 60). In this study PMA and RA were used to differentiate the HL-60 cells.

Upon treatment of HL-60 cells with PMA there is a rapid cessation of DNA synthesis and cell proliferation. The cells become adherent and phagocytic, and lose azurophilic granules. There is an increase in levels of nicotinamide-adenine dinucleotidase, nonspecific esterase and acid phosphatase. The cells become cytocidal and cytostatic for tumor targets and produce surface antigens that react with anti-monocyte serum (54, 57). There is an increase in synthesis of specific genes like c-fos, c-fms and c-src and a decrease in others like c-myc and c-myb. Phosphorylation of certain nuclear proteins is also observed (32). Still other affects of PMA on HL-60 cells are increased polyamine levels, altered PC and triglyceride metabolism, decreased production of high molecular weight glycopeptides and generation of potent procoagulant activity. All of these are via specific receptors (57).

The PMA receptor copurifies with protein kinase C (PKC) suggesting the kinase may play a role in the differentiation process. However a study has shown that the effect of PMA is other than or in addition to simply activating PKC (33). As previously mentioned, PKC is a cytosolic enzyme until activated at which time it moves to the membrane. When PMNs were treated with PMA, there was a decrease in the number of $[^3]$H cytosolic PMA binding sites by a factor of three but an
increase of binding sites in the membrane by a factor of four (43). PMA stimulates a Ca\(^{2+}\) efflux in human platelets (44) and perhaps this is due to PKC actions on the plasma membrane Ca\(^{2+}\) pumps. Ebeling et al. (20) tested synthetic diacylglycerols (known activators of PKC) for inhibitory ability against PMA. C\(_4\)–C\(_{10}\) saturated sn-1,2-diacylglycerols were found to be potent activators of partially purified HL-60 PKC and competitive inhibitors of phorbol dibutyrate to phorbol diester receptors. When HL-60 cells were treated with either PMA or sn-1,2-dioctanoylglycerol identical phosphoprotein changes and morphological characteristics of macrophages were seen. This added to the evidence that PMA mimics the effects of diacylglyceride on PKC (38). Because nuclear membranes share many biochemical properties with plasma membranes such as phospholipid content and turnover, maybe PMA treatment associates the PKC to the nucleus. HL-60 nuclei have been shown to bind phorbol ester \textit{in vitro} and this binding was Ca\(^{2+}\) dependent. Compounds that activate PKC may increase nuclear binding of phorbol ester \textit{in situ} (32).

Undifferentiated HL-60 cells have a low metabolic rate of arachidonic acid and low P-lipase A\(_2\) activity. Unlike normal cells they incorporate a major portion of arachidonic and linoleic acids into ether lipids (storage fractions). However upon exposure to PMA fatty acid incorporation becomes similar to that of normal cells - it is incorporated
mainly into the active phospholipid fraction (18).

PMA is highly lipophilic and readily binds to plasma membranes which could alter methylation pathways and thus block cellular responses to certain stimuli. Membrane alterations associated with increased methylation of phospholipids include changes in fluidity, in the number of agonist binding sites, in activation of adenylate cyclase and in Ca\(^{2+}\) flux. Cassileth et al. (15) looked for alterations in PC synthesis by transmethylation and exogenous choline pathways in PMA treated HL-60 cells. Early changes (within the first 90 min of the 24-48 hr labeling period) showed that PC synthesized by methylation of PE with a 5-adenosyl-L-methionine intermediate was inhibited in a dose dependent fashion as compared to PC synthesis from endogenous choline which was enhanced and correlated inversely with the inhibition of the methylation pathway. Cabot et al. (14) found that PMA causes alterations in the amount of cellular triacylglycerols and ether-containing alkylacylglycerols and changes in phospholipid metabolism possibly implicating a role for lipids in the differentiation process. Alkylacylglycerol changes may be due to a general stimulus on neutral lipid synthesis or a consequence of PMA effects on alkylidihydroxyacetone phosphate synthase, acyl-CoA reductase and fatty alcohol:NAD oxidoreductase all ether lipid metabolizing enzymes. The alterations in the phospholipids
consisted of a marked increase of $^{32}$P incorporation into PC and PE, but the mechanism was unknown.

The mechanism of RA induction of differentiation of HL-60 cells is largely unknown, but it may be through plasma membrane signal(s) (62). One report showed that RA increased the activity of plasma membrane enzymes $\text{Na}^+\text{-K}^+\text{-ATPase}$ and adenylate cyclase (1). RA triggered an early increase in intracellular concentration of $\text{Na}^+$ and $\text{K}^+$ and increased the intracellular pH because of activation of a $\text{Na}^+\text{/H}^+$ exchange system. The $\text{Na}^+$ transport system stimulation led to the increase of intracellular $\text{Na}^+$ concentration and activation of $\text{Na}^+\text{-K}^+\text{-ATPase}$ which then increased the intracellular $\text{K}^+$ concentration.

HL-60 cells lack CRABP, a cellular binding protein that carries RA to the cell nucleus in some systems (9). This plus the fact that the carboxyl group on the terminal carbon of RA is critical for its biological activity in differentiation of HL-60s suggests the RA may act after it is activated with CoA-SH to become retinoyl-CoA. Another aspect of RA that may have a role in differentiation is its ability to reduce the expression of the c-myc oncogene present in HL-60 cells (9). This gene is not rearranged but highly amplified - expression is 10 times that in some other cell lines. RA reduces this expression by 80-90% during induction and differentiation.

RA changes the pattern of phosphoproteins in HL-60
cells. Abita et al. suggested this is probably because of a decrease in intracellular cAMP concentrations (1). Evidence for overall changes in cAMP binding proteins in studies with HL-60 cells, both undifferentiated and differentiated, is inconclusive (positive and negative changes) (11). Recently a study on the action of cAMP binding type II regulatory subunit, RII, of cAMP-dependent protein kinase on a DNA substrate has led to the suggestion that the RII may be the phosphoprotein substrate required for cAMP-directed transcriptional regulation. There is evidence for nuclear proteins involved in the regulation of differentiation-associated specific gene transcription. Briggs and his group studied RA differentiated and undifferentiated HL-60 cells. They found the quantity of the cAMP binding protein did not change upon differentiation but there was a second form of the subunit with an altered charge present after RA treatment. They concluded that the regulatory subunit of type II cAMP-dependent protein kinase may be involved in the nuclear functions associated with differentiation.

HL-60 cells and two HL-60 subclones (one resistant to RA and one resistant to DMSO) were tested for arachidonic acid uptake after differentiation. After treatment of all three cell types with both chemicals significant increases of arachidonic acid uptake were observed though the biological significance of this is unknown (24). Studies using $^{14}$C-arachidonic acid and Ca$^{2+}$ ionophore A23187,
compared the lipoxygenase products of undifferentiated HL-60 cells, DMSO and PMA differentiated cells. The study reported the precursor HL-60 cells were unable to produce LTB₄, but could generate limited LTC₄ and LTD₄. The DMSO induced granulocytes preferentially produced LTC₄ and LTD₄ where the PMA induced monocyte/macrophage cells preferentially generated LTB₄ (63).

THESIS PROPOSAL: The human promyelocytic leukemia cell line, HL-60, which can be differentiated with PMA to monocyte/macrophage cells or with retinoic acid to granulocytes were used as a model for studying any changes in the distribution or mobilization of arachidonic acid in the phospholipid pools as compared to each other and to undifferentiated cells. This cell line was chosen because upon differentiation the two different cell types preferentially produce different arachidonic acid metabolites. The questions to be addressed in this study are 1) Are there any differences in arachidonic acid distribution in the lipid pools of the different cell types? 2) Are there any differences in lipid composition in the different cell types? and 3) Are there any differences in the arachidonic acid turnover patterns in the various lipid pools in response to cell stimulation?
Tissue Culture:

1. Cells and Medium. HL-60 cells were purchased from American Type Culture Collection (ATCC). The cells were grown in Iscove's modified Dulbecco's media with L-glutamine and 25mM hepes buffer plus 10% heat inactivated fetal bovine serum (FBS). One liter of culture media contained 5 mls of an antibiotic mixture of penicillin (10,000 unit/ml) and streptomycin (10 mg/ml). The cells were maintained in a 5% CO₂ humidified atmosphere at 37°C. Cell counts and viability were determined with a hemocytometer and trypan blue exclusion, viability was always at least 85%.

2. Storage. To maintain a frozen stock supply, HL-60 cells were collected, centrifuged and the media/supernatant discarded. The cell pellet was resuspended in 2 ml of Iscove's media plus 20% FBS, cooled on ice for at least 15 minutes then mixed with an equal volume of chilled Iscove's media plus 10% DMSO. One ml aliquots were stored overnight at -70°C then transferred to liquid N₂ for permanent storage. To revive frozen cells, a 1 ml vial was thawed in a 37°C water bath for approximately 2 minutes and resuspended in 20-30 ml culture media to dilute the DMSO.
**Experimental Procedures:**

1. **Differentiation.**

   **Macrophage/monocyte-like cells:** HL-60 cells were differentiated to macrophage/monocyte-like cells with phorbol-12-myristate-13-acetate (PMA) (Sigma, St. Louis, Mo.). The PMA was resuspended in dimethylsulfoxide (DMSO). To induce differentiation, 100ng/ml of PMA was added to 2x10⁶ cells/ml and incubated two hours (48, 61). PMA treated cells presented many pseudopods and became very adherent to the tissue culture flask.

   **Granulocyte-like cells:** HL-60 cells were differentiated to granulocyte-like cells with retinoic acid (RA) (Sigma, St. Louis, Mo.) resuspended in ethanol to 1mM. To differentiate the cells, 1uM RA was added to 2x10⁶ cells/ml which were then incubated for 48 hours (1, 10, 60). A functional characteristic of normal peripheral blood granulocytes is the ability to reduce nitroblue tetrazolium (NBT). A sample of the RA treated cells was mixed with an equal volume of 0.2% NBT (Sigma) in Dulbecco's phosphate-buffered saline with PMA and incubated at 37°C for 25 min. A Wright-Giemsa stain was made to determine the number of cells with intracellular blue-black formazon deposits (17).

2. **Labeling.** Labeling experiments used either ³²PO₄ from ICN Radiochemicals or [³H]arachidonic acid (AA) from NEN Research. Cells were labeled by the addition of 0.3 to 0.5 uCi[³H]AA/sample. Phospholipid was labeled by adding 50
uCi of $^{32}$PO$_4$ to the entire cell population. Steady-state experiment labeling times were 16 hrs. Pulse-chase (turnover) experiments were as indicated in the results section.

3. Stimulation. Ionophore A23187 (Sigma) was resuspended in DMSO then diluted to 0.1mM in ethanol. Stimulation of the three cell types was accomplished using a concentration of 1uM ionophore.

Lipid Extraction and Analysis:

1. Extraction. Lipid was extracted by a modification of the Bligh and Dyer lipid method (5). One ml cell suspension samples were taken at appropriate times, and put on ice and frozen (-20°C) to stop further metabolism. Samples were then thawed and 0.2 ml were removed for future protein analysis if necessary. The remaining 0.8 ml were extracted by the modified Bligh and Dyer (5) method. To the 0.8 ml cell suspensions were added 3 ml chloroform : methanol : .01N HCl to give a 1:2:0.8 ratio of C:M:H$_2$O. The mixture was heated for 5 min at 68°C and allowed to cool to room temperature. The samples were centrifuged and the pellet resuspended in C:M:HCl to repeat the extraction procedure. The supernatant was mixed with 1 ml chloroform and 1 ml 0.3% NaCl, centrifuged and the lower (chloroform) layer containing the lipids was collected. The water and methanol phase was reextracted with 1.5 ml chloroform. All
chloroform extracts were pooled, evaporated to dryness and stored under N₂.

2. Analysis. Lipids were separated on SG81 silicic acid impregnated paper (59) or Silica gel LK6D thin layer chromatography (TLC) plates (31). The lipid extracts were resuspended in chloroform : methanol (2:1), 50µl of each sample evaporated and counted in a scintillation counter, and the remaining sample spotted on either paper or TLC plates. The solvent systems for the 2 dimension paper chromatography were 1st: chloroform : methanol : diisobutyl ketone : acetic acid : water (23:10:45:25:4 v/v/v/v/v), and 2nd: chloroform : methanol : diisobutyl ketone : pyridine : 0.5M ammonium chloride buffer (30:17.5:25:35:6 v/v/v/v/v). The phospholipids were separated on the TLC plates using a chloroform : ethanol : water : triethylamine (30:34:8:35) solvent system, the neutral lipids migrated with the solvent front. After separation the chromatographs were autoradiographed. If the experiment had been done using [³H]AA as a label, previously labeled ³²P-phospholipid was added to the spotted samples as markers, if the experiment label had been ³²P no additions were needed. After developing the film the separated lipids were identified against standards and cut or scraped into scintillation vials and counted on a Beckman LS 7500 scintillation counter.
Protein Assays:

Protein determinations were made using the Lowry method. A standard curve was obtained with bovine serum albumin (1ug/ul).
Chapter 3

RESULTS

Cell Differentiation

Undifferentiated HL-60 cells were stained with Wright-Giemsa stain and appeared as uniformly purple-colored cells. Retinoic acid (RA) treated cells had an obviously smaller and/or segmented, dark nucleus and lighter cytoplasmic areas. The following chart indicates the percentage of differentiation in each cell type:

<table>
<thead>
<tr>
<th></th>
<th>undiff.</th>
<th>diff.</th>
<th>% differentiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>untreated HL-60s</td>
<td>203</td>
<td>8</td>
<td>3.8</td>
</tr>
<tr>
<td>RA treated HL-60s</td>
<td>11</td>
<td>189</td>
<td>94.5</td>
</tr>
</tbody>
</table>

Retinoic acid treated cells (granulocytes) and undifferentiated HL-60 cells were added to 0.2% NBT in the presence or absence of 200 ng of PMA. Upon sitting overnight the entire pellet of the RA treated cells with PMA was dark indicating uptake of the NBT and incorporation as blue-black formazan deposits. The other three cell samples showed little to no darkening.

PMA treated cells were characteristically macrophage-like with pseudopodia production and a grainier cytoplasm as compared to undifferentiated HL-60 cells. After the period
of differentiation with PMA treatment the growth media was
decanted off of the adherent cells which were washed once
with Dulbecco's PBS before being scraped off for further
experimentation. With this procedure approximately 100% of
the cells used were assumed to be differentiated to
macrophage.

**Lipid Composition**

HL-60 cells were grown in the presence of $^{32}$PO$_4$ for 48
hours then divided into three portions: one untreated, one
portion treated with PMA and the third treated with RA.
Lipids were extracted, separated, quantified and counted as
described in Materials and Methods. The relative
phospholipid composition of all the cell types had similar
concentrations of the major lipids (Fig. 4).

**Uptake of Arachidonic acid in Lipid Pools after
Differentiation**

Control cells and HL-60 cells differentiated with PMA
or RA were labeled for 16 hours with $[^3]$H]arachidonic acid.
Lipids were extracted, separated, identified and counted as
above. The steady-state arachidonic acid distribution in
the neutral lipid and phospholipid pools of the three cell
types is shown in Figure 5. There were marked differences
in the distribution of arachidonic acid among the three cell
types after the labeling period. In the PMA differentiated
Figure 4. Comparison of Phospholipids in Differentiated and Undifferentiated HL-60 Cells. HL-60 cells were grown in the presence of $^{32}$P then differentiated with retinoic acid (granulocytes), phorbol-12-myristate-13-acetate (macrophages) or left undifferentiated. Lipids from the three cell types were extracted by the Bligh and Dyer method, separated by chromatography in two dimensions on SG81 silicic acid impregnated paper, and visualized by autoradiography. PC = phosphatidylcholine, PI = phosphatidylinositol, PS = phosphatidylserine, PE = phosphatidylethanolamine.
Figure 5. [\(^{3}\)H]Arachidonic Acid Distribution in Differentiated and Undifferentiated HL-60 Cells. HL-60 cells were differentiated with PMA or RA or left undifferentiated. The three cell types were labeled for 16 hrs then lipids extracted by the Bligh and Dyer method, separated by two dimensional chromatography and visualized by autoradiography.
(macrophage/monocyte) cells the neutral lipid pool contained the largest portion of arachidonic acid (40% of the total) whereas phosphatidylethanolamine (PE) and phosphatidylcholine (PC) contained 26% and 10% respectively. In contrast the major portion of the arachidonic acid in the RA treated (granulocyte) cells and undifferentiated cells was in the PE pool which contained 49% and 43% respectively. The neutral lipid accounted for 18% of the total \[^{3}\text{H}]\text{arachidonic acid} in the granulocyte type cells and less than 3% of the total label in the undifferentiated HL-60 cells.

**Uptake of Arachidonic acid in Lipid Pools of Differentiated Cells**

Total \[^{3}\text{H}]\text{arachidonic acid} uptake by differentiated and undifferentiated HL-60 cells was determined over an 8 hour period after addition of the label. There were similarities between the RA treated and undifferentiated cells, both of which differed from the PMA treated cells. The PMA differentiated cells showed a linear uptake throughout the 8 hour labeling period. The RA treated and undifferentiated cells showed a maximum uptake at 6 hours (Fig. 6). The macrophage/monocyte-like cells (Figure 7) showed a very rapid initial uptake of label in the neutral lipid fraction which then decreased from 60% to 20% of the total arachidonic acid added by the second hour and continued to
Figure 6. Uptake of $[^3$H]Arachidonic Acid by Differentiated and Undifferentiated HL-60 Cells. HL-60 cells were differentiated with either PMA or RA or left undifferentiated, then uptake was measured over an 8 hour interval following the addition of $[^3$H]arachidonic acid. Lipids were extracted using the Bligh and Dyer method, separated on LK6D silica gel thin layer chromatographs and visualized by autoradiography.
Figure 7. Uptake of [\(^{3}\text{H}\)]Arachidonic Acid in Lipid Pool of PMA Differentiated (Macrophage/monocyte-type) HL-60 Cells. Experimental procedure as in Figure 6. A. Data as counts per minute. B. Data as percent of recovered label.

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decrease to less than 10% over the remaining 8 hour test period. Coordinately there was a gradual rise in label in the phosphatidylserine (PS), phosphatidylinositol (PI) and PE pools. The arachidonic acid content of the PC pool initially decreased, corresponding to the rise in the neutral lipid, then rose to approximately 50% by the second hour and decreased to 41% by 8 hours. The RA treated and undifferentiated cells had continual loss of arachidonic acid from the PC pool for 8 hours and 6 hours respectively (Fig.s 8,9). This accompanied a continued uptake of arachidonic acid in the PE fraction and a more gradual increase in the PI fraction in both cases.

Turnover of Arachidonic acid during Differentiation

Cells were prelabeled with [³H]arachidonic acid, split and differentiated. Samples were taken over the time periods necessary for differentiation (2 hours for PMA treatment and 48 hours for RA treatment) to macrophage/monocyte and granulocyte-like cells as well as over 48 hours for the undifferentiated cells. The PMA treated samples showed no significant turnover in the lipid pools. The distribution of arachidonic acid in each pool was PE 35%, PC 30%, PI 13%, PS and neutral lipids 5% (Fig. 10). The RA differentiated and undifferentiated HL-60 cells both showed a loss of about one half (15%) of the arachidonic acid in the PC pools over the first 16 hours.
Figure 8. Uptake of $[^{3}H]$Arachidonic Acid in Lipid Pool of RA Differentiated (Granulocyte-type) HL-60 Cells. Experimental procedure as in Figure 6. A. Data as counts per minute. B. Data as percent of recovered label.

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Figure 9. Uptake of $[^3H]$Arachidonic Acid in Lipid Pool of Undifferentiated HL-60 Cells. Experimental procedure as in Figure 6. A. Data as counts per minute. B. Data as percent of recovered label.

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Figure 10. Turnover of $[^3]H$Arachidonic Acid During Differentiation of HL-60 Cells by PMA. The cells were prelabeled 4 hrs, washed, split and resuspended. 100ng/ml PMA was added to one third of the cells and 1 ml samples taken over the next 2 hrs. Lipids were extracted and processed as usual. A. Data as counts per minute. B. Data as percent of recovered label.
Figure 11. Turnover of $[^3H]$Arachidonic Acid During Differentiation of HL-60 Cells by RA. Experimental procedure as outlined in Figure 10 except 1uM RA was added to one third of the cells. A. Data as counts per minute. B. Data as percent of recovered label.
Figure 12. Turnover of [³H]Arachidonic Acid in Undifferentiated HL-60 Cells. Experimental procedure as in Figure 10 except the remaining one third of the cells were left undifferentiated. A. Data as counts per minute. B. Data as percent of recovered label.
of testing (Fig.s 11, 12). The PE pool however at least doubled its arachidonic acid content over 16 hours and PI had a 10% uptake of label. (In figures 11 and 12 sampling was continued through 48 hours but no further variations were observed after 16 hours.)

**Turnover of Lipids in Response to Stimulation**

Figures 13-18 represent the response of the major phospholipid pools of the three cell types upon stimulation with calcium ionophore A23187 as compared to unstimulated cells. The stimulated PMA treated cells showed no major changes in the phospholipid pools (Fig.s 13, 14). As seen in Figure 16, stimulation of the RA treated cells led to a 5% decrease of label in the PC pool between 15 and 30 minutes and was reversed (reincorporated) by 60 minutes. These changes inversely matched the changes that occurred in the PE pool. A similar turnover seemed to occur to a lesser extent upon stimulation of the undifferentiated cells (Figure 18).

**Turnover of Arachidonic acid in Response to Stimulation**

$[^3]H$ arachidonic acid-prelabeled differentiated and undifferentiated cells were stimulated with calcium ionophore and the major phospholipid pools were compared to those of unstimulated, control, cells (Fig.s 19-24). Twenty minutes after stimulation of the PMA treated cells a
Figure 13. Turnover of Phospholipid Fractions in PMA Treated HL-60 Cells. Cells were prelabeled with $^{32}$PO$_4$ for 24 hrs, split and one third of the population differentiated with 100ng/ml PMA for 2 hrs. The cells were washed, divided and one half left unstimulated. Lipids were extracted and processed as usual. A. Data as counts per minute. B. Data as percent of recovered label.
Figure 14. Turnover of Phospholipid Fractions in Stimulated PMA Treated HL-60 Cells. Cells were prelabeled, differentiated, washed and divided as in Figure 13 but the other half was stimulated with 1uM A23187. A. Data as counts per minute. B. Data as percent of recovered label.
Figure 15. Turnover of Phospholipid Fractions in RA Treated HL-60 Cells. Experimental procedures as in Figure 13 except 1μM RA was used to differentiate one third of the cells over 48 hrs. A. Data as counts per minute. B. Data as percent of recovered label.

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Figure 16. Turnover of Phospholipid Fractions in Stimulated RA Treated HL-60 Cells. Cells were prelabeled, differentiated, washed and divided as in Figure 15 but the other half was stimulated with A23187. A. Data as counts per minute. B. Data as percent of recovered label.
Figure 17. Turnover of Phospholipid Fractions in Undifferentiated HL-60 Cells. Experimental procedures as in Figure 13 except one third of the cells were left undifferentiated. A. Data as counts per minute. B. Data as percent of recovered label.
Figure 18. Turnover of Phospholipid Fractions in Stimulated Undifferentiated HL-60 Cells. Cells were prelabeled, differentiated, washed and divided as in Figure 17 but the other half was stimulated with A23187. A. Data as counts per minute. B. Data as percent of recovered label.

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turnover of label occurred between the PC and PE pools. The PE arachidonic acid content increased and the PC pool decreased. The change made PE the phospholipid pool with the highest arachidonic acid content (Figure 20). The neutral lipid pool changes, best seen in figures 20 C,D, showed a large increase in label upon stimulation. The maximum was reached 15 minutes after addition of ionophore and gradually declined throughout the remaining 60 minutes but never returned to the unstimulated level. The RA treated cells had an overall loss of arachidonic acid in the PC pool after stimulation with a significant increase in the neutral lipid pool (Fig. 22). The undifferentiated cells displayed only minor changes in the stimulated fraction. Fluctuations in PE began 10 minutes after stimulation and continued though 60 minutes when the pool reequilibrated to a content of 40% arachidonic acid (Fig. 24). The large patterns of change seen in the neutral lipid pool are not relevant because the pool contains less than 3% of the total arachidonic acid content of undifferentiated HL-60 cells.
Figure 19. \(^3\text{H}\)Arachidonic Acid Turnover in PMA Differentiated HL-60 Cells. Cells were prelabeled with \(^3\text{H}\)AA and treated as in Figure 13. A. Data as counts per minute. B. Data as percent of recovered label.
Figure 20. $[^3\text{H}]$Arachidonic Acid Turnover in PMA Differentiated HL-60 Cells upon Stimulation. Cells were prelabeled with $[^3\text{H}]$AA and treated as in Figure 14. A. Data as counts per minute. B. Data as percent of recovered label.
Figure 20. C. Data as percent of Time 0 for unstimulated cells. D. Data as percent of Time 0 for stimulated cells.

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Figure 21. $[^3]$H]Arachidonic Acid Turnover in RA Differentiated HL-60 Cells. Cells were prelabeled with $[^3]$H]AA and treated as in Figure 15. A. Data as counts per minute. B. Data as percent of recovered label.
Figure 22. $[^3]$HArachidonic Acid Turnover in RA Differentiated HL-60 Cells upon Stimulation. Cells were prelabeled with $[^3]$HAA and treated as in Figure 16. A. Data as counts per minute. B. Data as percent of recovered label.
Figure 22.  C. Data as percent of Time 0 for unstimulated cells.  D. Data as percent of Time 0 for stimulated cells.
Figure 23. $[^{3}H]$Arachidonic Acid Turnover in Undifferentiated HL-60 Cells. Cells were prelabeled with $[^{3}H]$AA and treated as in Figure 17. A. Data as counts per minute. B. Data as percent of recovered label.
Figure 24. \[^{3}\text{H}]\text{Arachidonic Acid Turnover in Undifferentiated HL-60 Cells upon Stimulation. Cells were prelabeled with} \[^{3}\text{H}]\text{AA and treated as in Figure 18. A. Data as counts per minute. B. Data as percent of recovered label.}

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Figure 24. C. Data as percent of Time 0 for unstimulated cells. D. Data as percent of Time 0 for stimulated cells.
Chapter 4

DISCUSSION

Arachidonic acid is an important eicosanoid, C\textsubscript{20} fatty acid, in animals. Through the arachidonic acid cascade it becomes oxygenated. This results in a number of biologically significant compounds (51). Different types of cells preferentially produce different metabolites of arachidonic acid (63). Why this occurs is unknown. There is a range of possible answers including: the type of cellular stimulation; the cell's sensitivity to the stimulation; arachidonic acid availability and the amount released; from which pool(s) arachidonic acid is released; the nature of the phospholipases activated; and/or the presence, distribution and activity of the specific enzymes of the arachidonate cascade.

The HL-60 cell line is a very good model for studying some of these possibilities. These cells are maintained as undifferentiated promyelocytic cells but upon stimulation will become macrophage/monocyte-like cells or granulocyte-like cells. By starting with a single kind of cell and differentiating it into one of two different types of cells, a number of intracellular changes can be examined. In this study the following aspects of the three cell types were determined: 1) Differences in total phospholipid pools, 2) Differences in arachidonic acid distribution within the phospholipid pools, and 3) Arachidonic acid mobilization.
from phospholipid pools.

It was found that there were no significant differences in the total phospholipid pools but there were marked differences in arachidonic acid distribution within the three cell types. Stimulation of the differentiated cell types resulted in turnover in the phosphatidylcholine (PC) pools. There is much evidence that PC is a/the major phospholipid pool and source of arachidonic acid not only in HL-60 cells (6, 7, 37, 40, 64) but other types of cells as well including pancreatic islets (56), endothelial cells (53), platelets (12, 45, 51), neutrophils (2), macrophage (2), monocytes (25) and erythrocytes (30).

Initially a comparison of total phospholipid was done on PMA and RA differentiated and undifferentiated HL-60 cells. All three cells were similar in major phospholipid distribution; PC being the most abundant, followed by PE then PI and PS which were approximately equal. When compared to undifferentiated cells, treatment with PMA caused the total phospholipid content to decrease in the PC pool but increase slightly in the PE, PI and PS pools. RA treatment resulted in a slight decrease in all but the PE pool which remained the same. This phospholipid distribution agrees with Naito et al. (40) and their studies on HL-60 cells. The general stability of the phospholipid pools helps to narrow the focus of possibilities that lead to differences between the cell types.
The steady-state pattern of labeled arachidonic acid distribution varied greatly in differentiated and undifferentiated cells. Distribution in the three cell types was as follows: macrophage/monocyte-like cells - neutral lipid > PE > PC > PI > PS; granulocyte-like cells - PE > neutral lipid > PC > PI > PS; and undifferentiated HL-60 cells - PE > PC > PI > PS > neutral lipid. These human macrophage/monocyte-type cells differ in arachidonic acid distribution from that reported for rabbit pulmonary alveolar macrophage. After overnight label with \[^{3}H\]arachidonic acid the rabbit label was dispersed in PC 26.7%, PE 22.8%, lyso(bis)PA 13.1% and PS + PI 9.2% (16). When HL-60 cells were treated with DMSO and differentiated into granulocyte-like cells arachidonic acid distribution was PE 67%, PC 24%, PI 4%, neutral lipid 2%, PS 1.7% and PA 1.2% (7). Except for the amount of arachidonic acid incorporated into the neutral lipid pool, the two granulocyte-like cells follow the same pattern. It is possible the different arachidonic acid metabolites produced by different cells results from the marked differences of distribution in the three cell types.

The uptake of arachidonic acid into differentiated and undifferentiated cells over an 8 hour period was determined. An initial uptake followed by a dramatic decrease was seen in the neutral lipid pool of the PMA treated cells. The PC pool appeared to take up most of the released arachidonic
acid as well as the PE and PI pools with relatively little change in the PS pool. The RA treated and undifferentiated cells were quite similar in that both showed a turnover between the PC and PE pools around 5 hours after addition of the label. The PI pool continued to gain label throughout the time course in both cell types. The RA differentiated and undifferentiated cells have the same general phospholipid pattern 8 hours after addition of \[^{3}H\]arachidonic acid as they did after an overnight labeling (16 hour) period. The PMA (macrophage/monocyte cells) differ in their arachidonic acid content in the PE and PC pools which are reversed between the two time periods. This may indicate the macrophage/monocyte cell types have a more dynamic arachidonic acid supply.

When uniformly prelabeled undifferentiated HL-60 cells were split and differentiated, the turnover of the arachidonic acid label was monitored throughout the differentiation period and showed yet another pattern of arachidonic acid distribution. The PMA differentiated cells showed no turnover in any of the pools and after 2 hours the arachidonic acid content was greatest in the PE pool and in decreasing amounts in the PC, PI, neutral lipid and PS pools.

There were changes seen in the RA treated cells. An initial decrease occurred in the PE pool which continued for 2 hours but then the arachidonic acid content of the pool
rose through the 16th hour when it leveled off and did not change for the remainder of the 48 hours. The PC pool lost radioactivity from time 0 to the 16 hour time point when it too remained unchanged. Phosphatidylinositol gradually gained label for 16 hours and then became inactive. A group studying undifferentiated HL-60 cells treated with DMSO reported a rapid transmethylation of PE into PC occurring maximally at 60 seconds after treatment (64). This was followed by a decrease in the PC pool and release of arachidonic acid. The results correspond to those seen in this study. Since these changes occurred before any morphological evidence of differentiation, it was suggested these early events may represent the generation of intracellular chemical signals of differentiation. Another slightly different interpretation suggests the changes in the arachidonic acid containing molecular species that occur during differentiation may play some part in preparing the cell for stimuli that could result in arachidonic acid release (40).

There were changes in the undifferentiated cells similar to those of the granulocyte-type cells. These changes were rather unexpected. The untreated cells suddenly showed turnover between the phospholipid pools. This observation suggests that the alterations which occurred primarily within the first 1.5 hours were, at least in part, instigated by the handling of the cells when they
were split and redistributed.

To determine the effect of calcium ionophore A23187 stimulation of the phospholipid pools of the three cell types, the cells were treated as when the total phospholipid composition was determined. The distribution of \(^{32}\text{PO}_4\) label within the lipid pools was the same for the two experiments (compare figures 13-18 with figure 4). Stimulation of the PMA treated cells caused no perturbations in any of the pools. However an apparent turnover occurred in the stimulated RA treated cells and to a lesser extent also in the stimulated undifferentiated cells, between the PC and PE pools. This may be a result of deacylation/reacylation between the different phospholipid pools. It was reversed within 1 hour in the granulocyte cells but not in the undifferentiated cells.

In contrast to the response in the phospholipid pools, stimulation with A23187 led to turnover in certain pools of arachidonic acid in all three cell types. The arachidonic acid distribution in the PMA treated unstimulated cells was PC > PE > PI > neutral lipid > PA ≈ PS. After stimulation there was a decrease of arachidonic acid in the PC and PI pools and an increase in the PE and neutral lipid pools. Unstimulated human \(^{3}\text{H}\)arachidonic acid prelabeled monocytes have been shown to have a time 0 arachidonic acid content in the PC pool of 36.9% and in the PE pool of 15.3% (25). Upon challenge with opsonized zymosan, 25% of the incorporated
label was released within 30 minutes and came from the PC and PI pools. Phosphatidylcholine and PI are also the pools from which arachidonic acid is released in stimulated rabbit PMNs (39). This reported data closely matches that obtained in this study. The percent of label at time 0 was 35.4% in the PC pool and 17.3% in the PI pool. Similar findings were reported for fMLP stimulated guinea pig alveolar macrophage which also lost arachidonic acid from the PC and PI pools with the PE pool remaining unchanged (37). The PE pool was not static but arachidonic acid was released from the diacyl PE fraction and taken up in the alkenyl-acyl PE fraction. In contrast stimulated rabbit alveolar macrophage only release arachidonic acid from PC pools (41).

Addition of A23187 calcium ionophore to the RA treated cells resulted in the release of arachidonic acid from the PC pool and an increase in the neutral lipid pool. Others have reported that both the PC and PE pools were the source of arachidonic acid release in DMSO differentiated HL-60 cells stimulated with A23187 (6) or with N-fNle-Lue-Phe-Nle-Tyr-Lys (7). But in both cases lipoxygenase product 5-HETE was esterified back into the phospholipid and neutral lipid pools within 5 minutes. In thrombin induced human platelets a decrease of arachidonate was also observed in the PC and PE pools and was accompanied by an increase in free and oxygenated arachidonate (12). Precursor arachidonic acid for 12-HETE and diHETE metabolites comes from the PC and PE
pools in rabbit, chicken, rat and dog erythrocytes as well (30). This data also showed increasing label in the neutral lipid pool beginning at 5 minutes and continuing throughout the test period. Whether this was reacylation of free arachidonic acid or esterification of arachidonic acid metabolites was not determined.

The undifferentiated HL-60 cells had no net change due to the stimulation. The decrease and subsequent increase of arachidonic acid in the PE fraction from 15 minutes through 60 minutes after stimulation may be a result of reesterification of either the freed arachidonic acid or certain metabolized products back into the pool. The apparent loss of arachidonic acid from the neutral lipid pool is irrelevant as there is less than 3% of the total arachidonic acid content of undifferentiated cells in that pool.

From this study it is evident that upon differentiation of the HL-60 cell line into two different types of cells, the distribution of arachidonic acid within the phospholipid pools is changed. Furthermore, stimulation of the undifferentiated and two differentiated cell types gives different arachidonic acid uptake and release patterns. However, the patterns of the undifferentiated and RA treated cells are quite similar to each other and both differ from the PMA treated cells. This correlates with the similarities and differences in the arachidonic acid
metabolites produced by each cell type. Undifferentiated and granulocyte-like cells produce LTC₄ and LTD₄ and PMA treated cells generate LTB₄. A possible explanation for this may be the variations in arachidonic acid distribution.
Chapter 5

SUMMARY

Differences in arachidonic acid uptake, distribution and release with stimulation were studied in an uncommitted promyelocytic cell line (HL-60) and the two cell types into which it can be differentiated - macrophage/monocyte-like cells and granulocyte-like cells. Analogous results were obtained between the undifferentiated and granulocytic cells which both produce the same types of arachidonic acid metabolites. In comparison dissimilar patterns were found in the macrophage/monocyte cell type which generates different metabolic end products from arachidonic acid.

1. There were no significant differences in phospholipid composition among the different cell types; PC accounted for 63% - 69% of the total label added, PE 9% - 16%, PS 4% - 5% and PI 2% - 7%.

2. There were marked differences in the distribution of arachidonic acid among the three different cell types. In PMA differentiated cells the neutral lipid contained 40% of the total, PE 26% and PC 10%. RA treated and undifferentiated cells contained the largest portion of arachidonic acid in the PE fraction with 49% and 43% respectively. Arachidonic acid composed 18% of the RA treated cells' neutral lipid pool and less than 3% of the undifferentiated cells' neutral lipid pool.

3. After differentiation granulocyte-like cells (RA
treated) had the most rapid initial uptake of arachidonic acid which peaked after 6 hours. The macrophage/monocyte-like cells (PMA treated) showed a slower and continued uptake throughout 8 hours. Uptake in the undifferentiated cells peaked at 6 hours.

4. Over the 8 hour $[^3H]$arachidonic acid uptake period tested after cell differentiation, the RA treated and undifferentiated cells exhibited a turnover of label between the PE and PC pools. The PMA treated cells had an early turnover between the neutral lipid pool (decrease) and the PC pool (increase).

5. During differentiation of $[^3H]$arachidonic acid prelabeled cells the PC pool lost radiolabel and the PE and PI pools gained counts in the RA treated and undifferentiated HL-60 cells. The phospholipid and neutral lipid pools of the PMA treated cells were unchanged throughout the differentiation period.

6. Stimulation of the three cell types with calcium ionophore generated no net change in the total phospholipid composition of any of the cell types.

7. Stimulation of the three cell types with calcium ionophore resulted in a different reorganization of arachidonic acid in the two differentiated cell types. The neutral lipid and PC pools differed the most between these cell types.


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