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# Metabolism of Arachidonate-containing Phospholipid Molecular Species in the Murine Macrophage-like Cell Line, P388d1

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# Metabolism of arachidonate-containing phospholipid molecular species in the murine macrophage-like cell line, P388D1

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# METABOLISM OF ARACHIDONATE-CONTAINING PHOSPHOLIPID MOLECULAR SPECIES IN THE MURINE MACROPHAGE-LIKE CELL LINE, P388D1

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In Partial Fulfillment

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Doctor of Philosophy in Biomedical Sciences

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by

Crystal Robbie Waites

May, 1991

APPROVAL

This is to certify that the Graduate Committee of

CRYSTAL ROBBIE WAITES

met on the

Thirtieth day of January, 1991

The committee read and examined her dissertation, supervised her defense of it in an oral examination, and decided to recommend that her study be submitted to the Graduate Council and the Associate Vice-President for Research and Dean of the Graduate School, in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biomedical Sciences.

Chair, Graduate Committee

Signed on behalf of the Graduate Council

Associate Vice-President for Research and Dean of the Graduate School

ABSTRACT

Metabolism of Arachidonate-Containing Phospholipid Molecular Species in the Murine Macrophage-Like Cell Line, P388D1

by

Crystal Robbie Waites

Glycerophospholipids of mammalian cells exist as chemically diverse structures with various fatty acids at the sn-1 and sn-2 positions. Arachidonic acid, a polyunsaturated fatty acid, which may be converted to biologically active eicosanoids such as prostaglandins, thromboxanes, and leukotrienes, is found predominantly in the sn-2 position of glycerophospholipids. The purpose of this study was to examine, at the level of the individual molecular species, the incorporation of arachidonate into phospholipids and its release from phospholipids during stimulation. In this way, the specificity of the enzymes controlling arachidonate metabolism could be examined in order to clarify the processes that control the metabolism of this precursor of potent biological mediators.

An investigation of the deacylation-reacylation mechanisms for the incorporation of arachidonic acid into the cellular phospholipids revealed that both the CoA-independent transacylation and CoA-dependent acylation mechanisms are active in the P388D1 macrophages. The CoA-independent transacylase preferentially acylated the alkyllysoglycero-phosphatidylcholine substrate with the polyunsaturates, arachidonate, and docosahexaenoate. The CoA-dependent pathways exhibited less selectively and acylated the alkyl-substrate with more saturated fatty acids. Supplementation of the P388D1 macrophages with the n-3 marine oil fatty acids, eicosapentaenoate and docosahexaenoate resulted in the enrichment of the cellular phospholipids with these polyunsaturates at the expense of arachidonate-containing molecular species. Using methodology, which permits the measure of both mass and specific radioactivity changes in the molecular species of phospholipids, it was determined that the arachidonate-containing species are preferentially degraded during stimulation with the calcium ionophore, A23187. Stimulation with calcium ionophore results in the activation of a calcium dependent phospholipase specific for the arachidonate-containing species.

Together, these results demonstrate that the incorporation and release of arachidonic acid is regulated by enzymes that bear distinct substrate specificities. The specificities of these enzymes can be directly related to the trafficking of arachidonate and its various esterified forms in cell phospholipids.

## DEDICATION

This is dedicated with love and deepest appreciation to my husband, Randy, my Mom, my Dad and my sister, Tammy. Their words of encouragement and their genuine belief in my ability made this possible. Thank you.

ACKNOWLEDGMENT

In the pursuit of a doctorate degree many obstacles are encountered. As each challenge or aggravation is met, one must decide between two paths: the road that leads to the degree or the road of defeat. It is oftentimes very difficult to see the light at the end of the tunnel, and convince oneself that the struggles, the personal confrontations with mentors, and the massive investment of time are worthwhile. More times than not, one is questioning one's capabilities and doubting one's potential.

Fortunately, for me and, hopefully, for others, there have been, and continue to be, friends and professors who unselfishly offer support and encouragement. Had I been given the opportunity to select a group of friends, I could never have chosen any as wonderful as the ones I have grown to love in the department of biochemistry. I know that I have been blessed to know so many kind people, and there is no question that I will miss everyone very much. To avoid omitting someone and possibly hurting that person's feelings, I will not list the people individually. I will say, however, that all you guys "graduate students, technicians, and secretaries", made my experience here bearable and oftentimes pleasurable, and for that I thank-you. To Dr. Lou Ernst-Fonberg, the one professor who encouraged me, who took the time from her busy schedule to speak with me, and for whom I hold the highest respect, I would like to express my appreciation. Thank-you for seeing something in me that I could not see, for expressing delight in my presentations, thesis, etc., and most importantly for being a friend.

To the members of my committee, Dr. Scott Champney, Dr. Arthur Hougland, Dr. Bill Mayberry, Dr. Lou Ernst-Fonberg, and Dr. Mitchell Robinson, I should like to communicate my appreciation. Thank-you for your time. To my major professor, Dr. Mitchell Robinson, I would like to express my thanks for the time taken to teach me lipid biochemistry.

Reality is that some of our paths may never cross again. I hope that life is good to each and every one of you. It is my prayer that God bless you and your families, always.

CONTENTS

LIST OF TABLES

TABLE Page

LIST OF FIGURES

## ABBREVIATIONS

| | |
|---|---|
| AA | Arachidonic acid |
| ATP | Adenosine 5'-triphosphate |
| BBOT | 2,5-bis-(5'-tert-butylbenzoxazolyl-[2']) thiophene |
| BSA | Bovine serum albumin |
| CoA | Coenzyme A |
| DG | Diradylglycerides |
| DMAP | 4-Dimethylaminopyridine |
| DMEM | Dulbecco's modified eagle's medium |
| DMSO | Dimethyl sulfoxide |
| Dpm | Disintegrations per minute |
| FBS | Fetal bovine serum |
| GroPCho | sn-Glycero-3-phosphocholine |
| GroPEtn | sn-Glycero-3-phosphoethanolamine |
| HEPES | N-[2-hydroxyethyl]piperazine-N'-[2-hydroxypropanesulfonic acid] |
| HETEs | Hydroxyeicosatetraenoic acids |
| HMG-CoA | 3-hydroxy-3-methylglutaryl-coenzyme A |
| HPLC | high pressure liquid chromatography |
| MEM | Modified eagle's medium |
| NL | Neutral lipid |
| PC | Phosphatidylcholine |
| PE | Phosphatidylethanolamine |
| PI | Phosphatidylinositol |
| PMA | Phorbol myristate acetate |
| PS | Phosphatidylserine |

| | |
|---|---|
| TLC | Thin-layer chromatography |
| TPA | Tetradecanoyl phorbol acetate |
| Tris | tris(hydroxymethyl) aminomethane |
| UV | Ultraviolet |

# CHAPTER 1

## Introduction

Glycerophospholipids of mammalian cells exist in chemically diverse structures with various fatty acids at the sn-1 and sn-2 positions. Arachidonic acid (AA), a polyunsaturated fatty acid which may be converted to biologically active eicosanoids such as prostaglandins, thromboxanes, and leukotrienes, is found predominantly in the sn-2 position of glycerophospholipids. Glycerophospholipids are an important source of arachidonate, which may be liberated by phospholipases in response to agonist stimulation (Nakagawa and Waku, 1989) and oxygenated via the cyclooxygenase pathway to prostaglandins or along the lipoxygenase pathway to leukotrienes and hydroxyeicosatetraenoic acids (HETEs). This transformation of AA into products of the lipoxygenase and cyclooxygenase pathways generates a family of lipid mediators; furthermore, these mediators evoke specific cellular and vascular responses associated with allergic and inflammatory reactions. As a result, the liberation of AA appears to be an important controlling step in the synthesis of these biologically active eicosanoids.

Since the level of AA in phospholipids affects the cells' ability to produce eicosanoids, the availability of AA, its liberation from esterification in the fatty acyl

chains of glycerophospholipids, and subsequent metabolism have been topics of numerous research studies. Initial research on the availability of free AA in the cytoplasm (Bills et al., 1977) and plasma (Hagenfeldt, 1975) revealed a disproportionately low amount in comparison to the level that is esterified. Free AA accounts for less than 0.1% of the total arachidonate present in rabbit alveolar macrophages in studies reported by Nakagawa and Waku (1989).

Work conducted by Lands and colleagues (1976) revealed that AA is not incorporated during de novo synthesis of glycerophospholipids, but through a deacylation and reacylation pathway. In this pathway, a phospholipid is converted to a lysophospholipid by cleavage of AA via a phospholipase $A_2$. Arachidonyl Coenzyme A (CoA) is formed by acyl CoA synthetase, and the arachidonate is then transferred into the sn-2 position of a lysophospholipid by the acyl CoA:lysophospholipid acyl-transferase.

Early reports stated that the low level of AA in the cytosol and plasma may be accounted for not only by high affinity of the lysophosphoglyceride acyltransferases for arachidonyl-CoA but also by the existence of separate acyltransferases for AA (Jezyk and Lands, 1968; Hasegawa-Sakai and Ohno, 1980). Chilton et al. (1983) reported selective acylation of alkyllysoglycero-phosphocholine (alkyllosyglycero-GroPCLo) and palmitoyllysoglycero-phosphocholine (palmitoyllyso-GroPCLo) with AA in human

neutrophils. The existence of a CoA-independent arachidonyl specific transacylation enzyme in rabbit alveolar macrophages (Robinson et al., 1985b; Sugiura et al., 1987) and human platelets (Kramer and Deykin, 1983; Kramer et al., 1984) has been reported. The operation of this pathway is demonstrated by the conversion of exogenous labelled lysophospholipid to phospholipid in the absence of exogenously supplied CoA.

Though the acyltransferases play a significant role in the availability of free AA for eicosanoid synthesis, the enzymes that hydrolyze AA from phospholipids are also of importance. Both Bills et al. (1977), studying fatty acid liberation by platelets aggregating in response to thrombin, and Hseuh et al. (1977), measuring fatty acid release from perfused heart or kidney stimulated by bradykinin, discovered that the release of acyl groups exhibited a marked specificity for AA in comparison to other fatty acids. This has been demonstrated in lymphocytes (Parker et al., 1979) and mouse peritoneal macrophages (Bonney et al., 1978; Scott et al., 1980). Capriotti et al. (1988) suggested that the most recently incorporated AA is the pool preferentially released when the cell is stimulated. This concept was supported by Furth and Laposata (1988) using EFD-1 (an essential fatty-acid-deficient mouse fibrosarcoma cell line) with findings that recently incorporated

arachidonate is preferentially released upon agonist stimulation.

The availability of arachidonate and the synthesis of eicosanoids may be affected by several factors. These include the synthesis and compartmentation of the acyltransferases and phospholipases, the degree of cellular activation, the extent of cellular immune response, etc. Changes in the fatty acid composition of glycerophospholipids in biomembranes are also closely associated with alterations in the physiological functions of various cells. For example, the level of AA may be affected by a simple change in the dietary regimen i.e., dietary enrichment with the N-3 marine oil fatty acids. Research conducted by Lokesh et al. (1989) with murine peritoneal macrophages demonstrated that dietary enrichment with the N-3 marine oil, docosahexaenoic acid, resulted in both decreased levels of arachidonate in cellular phospholipids and the suppression of eicosanoid synthesis. A reduction in the amount of arachidonate esterified in the alk-1-enylacyl-sn-glycero-3-phosphoethanolamine (alk-1-enylacyl-GroPEtn) was reported by Blank et al. (1989) following supplementation of the growth media of the murine macrophage-like cell line P388D1 with docosahexaenoate.

Macrophages are an important source of AA metabolites as evidenced by macrophages unusually high proportion of this fatty acid in their membranes. Comparative fatty acid

composition studies of phagocytes have shown that AA comprises nearly 20% of the total phospholipid of rabbit alveolar macrophages and less than 3% in guinea pig neutrophils (Mason et al., 1972). Analysis of resident peritoneal macrophages revealed that up to 25% of esterified fatty acid is AA (Scott et al., 1980). Macrophages release AA metabolites in response to a variety of stimuli such as phagocytosable zymosan particles, immune complex, calcium ionophores, and the phorbol esters. Various studies show that the source of arachidonate depends on the types of macrophages used and the stimulus employed (Nakagawa and Waku, 1989). The liberation of arachidonate from the sn-2 position of glycerophospholipids can occur through the activity of a phospholipase $A_2$. Such phospholipases have been characterized. In 1981, Wightman identified two phospholipase $A_2$ activities in murine macrophages (Wightman et al., 1981a). A phospholipase C was also characterized, which can liberate arachidonate by conversion of phosphatidylinositol to diacylglycerol. The diacylglycerol is then hydrolyzed by a diacylglycerol lipase to yield free AA (Wightman et al., 1981b). More recently, a membrane-bound phospholipase $A_2$ has been purified and characterized from P388D1, a macrophage-like cell line (Ulevitch et al., 1988).

The macrophage-like cell line P388D1, derived from a methylcholanthrene-induced lymphoid neoplasm (P388) of a

DBA/2 mouse (Dawe and Potter, 1975), was the cell line used in these investigations. Work conducted by Koren et al. (1975) demonstrated that the P388D1 cells possess characteristics typical for macrophages inclusing: adhesion to glass and plastic surfaces, mediation of antibody-dependent cell-mediated cytolysis, and expression of receptors for the Fc portion of IgG and for C3 on the cell surface. Moreover, the P388D1 failed to stain with fluorescein-conjugated rabbit anti-mouse Ig and fluorescein-conjugated rabbit anti-mouse brain serum, indicating the lack of surface Ig and T cell markers. Since P388D1 cells possess most of the characteristics of normal macrophages (Koren et al., 1975) and contain comparatively high amounts of AA, the P388D1 cells are well suited for studies on the release and incorporation of AA.

Phosphoglycerides may be divided into subclasses based on linkage at the sn-1 position. The diacyl subclass contains ester linkages at the sn-1 and sn-2 positions, while the alkyl and alkenyl subclasses contain the ether linkages 1-O-alkyl and 1-O-alk-1-enyl, respectively, at the sn-1 position. Analysis of the phosphoglycerides requires complete identification of the acyl or ether groups esterified to the sn-1 and sn-2 positions of the individual molecules. Recently developed methodology allowing quantification of molecular species provides information

important in understanding the arachidonate incorporation and release from cellular phosphoglycerides.

The evidence that a dietary regimen containing N-3 marine oil fatty acids may decrease the incidence of cardiovascular disease has prompted research into the metabolism of omega-3 polyunsaturated fatty acids. Arachidonate metabolites are involved in atherosclerotic plaque formation; therefore, the availability of the precursor, arachidonate, may represent a control point in this disease. Investigation of the effects of supplementation with the N-3 marine oil fatty acids, eicosapentaenoate and docosahexaenoate, may elucidate changes at the cellular level that may deter the pathological development of cardiovascular disease. The goal was to examine the effect of N-3 polyunsaturated fatty acid supplementation on arachidonate distribution in the cellular phospholipids. The consequences of supplementation on the distribution of other endogenous fatty acids in the cellular phosphoglycerides was investigated.

Diradylglycerides (DG) are intermediates in the biosynthesis and degradation of glycerolipids in eukaryotic cells and also function as intracellular second messengers (Bishop and Bell, 1988). DG second messengers activate protein kinase C, an enzyme crucial to cellular functions such as growth regulation. DG second messengers may be formed in response to extracellular stimuli such as hormones, growth factors, and neurotransmitters. The importance of DG in cellular activation and its potential role as a reservoir for AA prompted investigation into the

effects of N-3 supplementation on DG generation in P388D1 cells following stimulation with the phorbol ester, tetradecanoyl phorbol acetate (TPA).

# CHAPTER 2

## Materials and Methods

### Materials

Dimethyl sulfoxide (DMSO) and 4-dimethylaminopyridine (DMAP) were purchased from Aldrich. [1<3>-$^3$H]Glycerol (2.9 mCi/mmol) was purchased from Amersham. Silica gel H Uniplates were purchased from Analtech. The murine macrophage-like cell line, P388D1, was purchased from American Type Culture Collection. Bio-Rad's Protein Assay Dye Reagent was purchased from Bio-Rad. All solvents were high pressure liquid chromatography grade and purchased from Burdick and Jackson. Docosahexaenoic acid was purchased from Nu Check Prep, Inc. Adenosine 5'-triphosphate (ATP), benzoic anhydride, 2,5-bis-(5'-tert-butylbenzoxazolyl-[2']) thiophene (BBOT), bovine serum albumin (BSA), calcium ionophore (A23187), coenzyme A (CoA), 2',7'-dichlorofluorescein, Dulbecco's phosphate buffered saline, Dulbecco's modified eagle's medium (DMEM), eicosapentaenoic acid, fetal bovine serum, N-[2-hydroxyethyl]piperazine-N'-[2-hydroxypropanesulfonic acid] (HEPES), horse serum, L-α-phosphatidylcholine-oleoyl, L-α-phosphatidylcholine-from soybean, modified eagle's medium (MEM), penicillin, phospholipase C, RPMI-1640, streptomycin, tetradecanoyl phorbol acetate (TPA), and trypan blue dye were purchased from Sigma.

[5,6,8,9,11,12,14,15,-$^{3}$H(N)]arachidonic acid (100 mCi/mmol), [1,2,-$^{3}$H(N)]cholesterol (62 mCi/mmol), [2-palmitoyl-9,10-$^{3}$H(N)]phosphatidylcholine, L-α-dipalmitoyl (50 mCi/mmol), and L-1-[palmitoyl-1-$^{14}$C]lysopalmitoyl phosphatidylcholine (58.5 mCi/mmol) were purchased from New England Nuclear Research Products. [9,10-$^{3}$H]Hexadecyllyso-GPC was prepared biosynthetically by the method of Robinson et al. (1985b).

## Cell Culture

The P388D1 cells were cultured in RPMI-1640 medium supplemented with 25 mM HEPES, 10% fetal bovine serum (FBS), 100 units of penicillin/ml and 100 ug of streptomycin/ml (RPMI-complete), and maintained at 37°C in a humidified environment of 5% $CO_2$ in air. Cells were transferred every 4 to 5 days by scraping the cells free from the culture flask and diluting 1 to 3. P388D1 cells were routinely frozen in media containing 75% MEM, 15% FBS, and 10% DMSO and stored at -80°C. Cell viability was determined by trypan blue dye exclusion; cell number was determined by counting with a hemocytometer.

## Lipid Extraction

Lipid extraction was by the method of Bligh and Dyer (1959). For complete extraction of phosphatidylserine and phosphatidylinositol, the extraction was acidified using 1% acetic acid in the methanol fraction.

## Separation of Phospholipid Classes

The total lipid extract was evaporated to dryness under a stream of $N_2$ and taken up in chloroform/methanol (2:1) and banded onto silica gel H uniplates (20 x 20 cm). Phospholipid classes were separated by thin-layer chromatography (TLC) in a solvent system of chloroform/methanol/glacial acetic acid/water (50:25:8:3, v/v). For recovery of isolated phospholipid classes, developed plates were sprayed with 0.05% 2',7'-dichlorofluorescein in ethanol and viewed under UV light to locate the separated lipid fractions. Marked areas of the gel were scraped from the plate, and the lipids were then extracted by the method of Bligh and Dyer (1959). For determination of radioactivity, the lipid classes were visualized with $I_2$, scraped into counting vials and assayed in a liquid scintillation spectrometer.

## Preparation of Diradylglycerobenzoates

Phospholipids were hydrolyzed with 30 units phospholipase C Type XIII from Bacillus cereus for 3 h at room temperature according to the method of Mavis et al. (1972). The diradylglycerol products were extracted by the method of Bligh and Dyer (1959) and then converted to the benzoate derivatives as described by Blank et al. (1984) using 2 mg DMAP and 5 mg benzoic anhydride in 0.15 ml of benzene. Diradylglycerobenzoate derivatives were separated into the alkylacyl, alk-1-enylacyl, and diacyl subclasses by

TLC, using a solvent system of benzene/hexane/ether (50:45:5, v/v). The individual diradylglycerobenzoate subclasses were extracted from the gel with hexane/ethanol/water (1:1:1, v/v) and separated into molecular species by high pressure liquid chromatography (HPLC), monitored at absorbance of 230 nanometers utilizing a Beckman Ultrasphere C18 reverse-phase column (4.6 mm x 25 cm) at a flow rate of 1 ml/min (Blank et al., 1984). Fractions were collected from the HPLC directly into scintillation vials and assayed for radioactivity. Diacylglycerobenzoates were eluted with acetonitrile/isopropanol (70:30, v/v) at 35°C. Alk-1-enylacylglycerobenzoates were eluted with acetonitrile/isopropanol (65:35, v/v) at 35°C. The HPLC chromatograms were integrated by the computer software package, Maxima, purchased from Dynamic Solutions.

## Lipid Phosphorous Determination

The method of Rouser et al. (1966) was used to determine total phosphorous content with $Na_2HPO_4$ (1 mM) as a standard.

## Protein Determination

Protein was determined by the method of Bradford (1976) using the Bio-Rad assay kit with BSA as a standard.

## Membrane Preparation of P388D1 Cells

P388D1 cells were harvested, washed by centrifugation, and counted with a hemocytometer. Cells were suspended in Dulbecco's Phosphate Buffered Saline ($Ca^{+2}$, $Mg^{+2}$ free) and disrupted with a cell sonicator (four 1 s pulses) on ice. Nuclei and unbroken cells were removed by centrifugation (2500 g for 10 min), and the supernatant was centrifuged at 106,500 g for 60 min (Beckman L5-65 Ultracentrifuge with Ti 50 rotor). The pellet was resuspended in 20 mM tris(hydroxymethyl) aminomethane (Tris) (pH 7.1) to a protein concentration of 1 to 3 mg/ml. The membrane preparations were frozen in liquid nitrogen and stored at -80°C.

## Acyltransferase Assay

Incubations with P388D1 membranes contained 120 ug of membrane protein, 20 mM Tris (pH 7.4), [$^{3}$H]hexadecyllyso-GroPCLo (5 nmoles) or [$^{14}$C]palmitoyllyso-GroPCho (5 nmoles) and various combinations of cofactors including: CoA (0.1 mM), $MgCl_2$ (10 mM), ATP (10 mM), and $CaCl_2$ (10 mM). The total volume of the mixture was 0.4 ml. Reactions were initiated by the addition of the labelled lysophospholipid substrate. After incubation at 37°C for 15-30 min, the reactions were stopped by the addition of 2 ml of methanol. The internal standards oleoyllyso-sn-glycero-3-phosphocholine (GroPCho) (20 ug) and soy-GroPCho (20 ug) were added to provide sufficient lipid mass for

visualization by TLC. Lipids were extracted (Bligh and Dyer, 1959) from the incubation and separated by TLC, using the solvent system chloroform/methanol/glacial acetic acid/water (50:25:8:3, v/v). The zones corresponding to free fatty acid, lyso-PC and phosphatidylcholine (PC) were scraped and assayed for radioactivity.

Arachidonyl- and Palmitoyl- CoA Synthesis .

Acyl CoA-esters were synthesized from free acids as described by Okuyama et al. (1969). Palmitoyl and arachidonyl free fatty acids were converted to acyl chlorides by adding oxalyl chloride. This was repeated twice with approximately 0.7 ml oxalyl chloride, and the oily residue was dissolved in 0.5 ml of freshly distilled tetrahydrofuran. The acid chloride was added to a solution of CoA (5 mg) in 5 ml of tetrahydrofuran/water (7:3); the pH was maintained at 8 with 1 N NaOH, and the solution was vigorously stirred with a magnetic stir-bar in an Erlenmeyer flask. The pH was adjusted to approximately 4 with a few drops of $HClO_4$, and the tetrahydrofuran was evaporated under nitrogen. The volume was adjusted to 2 ml by adding $H_2O$ and 0.3 ml of 10% $HClO_4$, while cooling in an ice bath. The white precipitate was collected by centrifugation, rinsed twice with 5 ml of diethyl ether, and dissolved in 1 ml of $H_2O$. The pH was adjusted to 5.5 and the insoluble material was removed by centrifugation.

## Specificity of CoA-Dependent Acyltransferase for Acyllyso- vs Alkyllyso-GroPCho Acceptors

Incubations contained 60 ug of P388D1 membrane protein, 20 mM Tris (pH 7.4), and 5 nmoles of either [$^3$H]arachidonyl CoA or [$^3$H]palmitoyl CoA with either labelled alkyllyso- or acyllyso-GroPCho (5 nmoles) in a total volume of 0.4 ml. After incubation at 37°C for 10 min, the reactions were stopped by the addition of 2 ml methanol. The lipids were extracted and separated by TLC. Zones corresponding to phospholipids were scraped and assayed for radioactivity. Activity was calculated as follows:

$$\text{PC Product} = \frac{\text{original substrate (nmoles)} \times \% \text{ conversion to PC}}{\text{incubation time (min)} \times \text{total fraction (mg)}}$$

## Glycerol Labelling

Confluent cultures were incubated for 19 h in RPMI-complete and 5 uCi [$^3$H]glycerol/ml. The cells were harvested, and the lipids were extracted and separated on TLC as described previously. The diradylbenzoate derivatives of phosphatidylcholine and phosphatidyl-ethanolamine were then prepared (as described previously) and separated into subclasses by normal phase HPLC at an absorbance of 230 nm with isocratic elution at 1 ml/min using the following solvents: cyclohexane, 0.07% acetic/cyclohexane, 0.07% acetic, 5% ether (50:50 v/v).

## [$^3$H]Arachidonate Labelling and Stimulation of P388D1 Cells

P388D1 cells were scraped from culture dishes and transferred to a sterile glass scintillation vial. The cells were incubated for 30 min in 8 ml of RPMI containing 0.6 uCi/ml [$^3$H]arachidonate. The cells were then centrifuged (2500 g for 3 min) and resuspended in RPMI-complete. The cells were dispensed to culture vessels with RPMI-complete and were allowed to attach for 2 h. The monolayers were rinsed with DMEM and incubated for 0-60 min in 5 ml of DMEM containing BSA (1 mg/ml) with either no additions, DMSO (0.1%), or 10 uM A23187 in DMSO (final DMSO concentration: 0.1%). The reactions were stopped by the addition of methanol to the culture dishes. The lipids were harvested and extracted (Bligh and Dyer, 1959), separated by TLC and eluted by HPLC as described previously.

## Polyunsaturated Fatty Acid Supplementation of P388D1 Cells: Fatty Acid Supplementation

To increase the cellular content of polyunsaturated fatty acids, cells were grown in media containing 5% FBS supplemented with 5% horse serum since horse serum is more highly enriched with polyunsaturated fatty acids. All other conditions remained the same.

For specific enrichment of N-3 polyunsaturated fatty acids, cells were supplemented with eicosapentaenoic, docosahexaenoic acid, or a combination of both fatty acids.

A 1 ml solution of fatty acids in FBS (final fatty acid concentration of 0.8 uM) was added to 4 ml of RPMI-0 and dispensed to culture vessels containing approximately 2 x $10^7$ cells/vessel. After 24 h incubation, the supplemented cells and unsupplemented controls (maintained in RPMI-complete) were harvested in methanol, and the lipids were extracted for compositional studies (Blank et al., 1989).

TPA-Induced Diradylglycerol Generation

Confluent cultures were incubated for 16 h in RPMI-complete containing 0.5 uCi [$^3$H]myristate/ml to label phospholipids. After incubation, the cells were rinsed with DMEM-0 and incubated in DMEM/BSA (1 mg/ml) for 10 min with either DMSO or TPA (0.1 uM). The reactions were stopped by the addition of methanol, and the lipids were extracted by the method of Bligh and Dyer (1959). The lipids were then separated by TLC in a solvent system of hexane/ether (40:60, v/v). To determine the radioactivity of the diradylglycerols, the lipid classes were visualized with $I_2$, scraped into counting vials and assayed in a liquid scintillation spectrometer.

# CHAPTER 3

## Results

### The Incorporation of Arachidonate into Cell Phospholipids

#### Different Mechanisms for the Incorporation of Arachidonate into Phospholipids

The acylation extents of the CoA-dependent and CoA-independent mechanisms were compared in the P388D1 membranes by examining the extent of conversion of radiolabelled lysophospholipid substrates to phospholipids. Both alkyllyso- and acyllyso-GroPCho substrates were used. Membranes were incubated with the lysophospholipid substrate under conditions required for the activity of either the CoA-independent transacylation reaction (no cofactors), the CoA-dependent transacylation (CoA alone added) or the acyl-CoA dependent acylation (CoA, ATP, and $MgCl_2$). In incubations containing [$^3$H]hexadecyllyso-GroPCho the addition of CoA increased the formation of alkylacyl-GroPCho by 19% (Table 1). The increase in the extent of acylation with the addition of CoA indicated that a CoA-dependent transacylation, as well as a CoA-independent reaction is present. The addition of all cofactors required (CoA, ATP, and $MgCl_2$) for the incorporation of endogenous fatty acids into the alkyllyso-GroPCho substrate resulted in a 13% decrease in the extent of acylation when compared to the

Table 1. Acylation of Alkyllyso-GroPCho and Acyllyso-GroPCho by P388D1 Membranes. Incubations contained either 2.0 nmol of [$^3$H]hexadecyllyso-GroPCho or 1.6 nmol of [$^{14}$C]palmitoyllyso-GroPCho with 120 ug of membrane protein in 0.4 ml of 20 mM Tris buffer (pH 7.4) and, where included, $MgCl_2$ (10 mM), ATP (10 mM), and CoA (0.1 mM). Following incubation at 37°C for 15 min, the reactions were terminated, and the total lipids were extracted and analyzed by TLC as described in Methods.

| Substrate | [$^3$H]Hexadecyllyso-GroPCho | | | |
|---|---|---|---|---|
| Product | Alkylacyl-GroPCho | | Neutral Lipid | |
| Additions | Percent | | | |
| | Experiment | | Experiment | |
| | 1 | 2 | 1 | 2 |
| None | 28.2 | 19 | 0.5 | 1.1 |
| CoA | 29 | 25 | 0.7 | 1.5 |
| $MgCl_2$ | 15.2 | | 4.7 | |
| CoA, ATP and $MgCl_2$ | 27.1 | 17.2 | 1.8 | 3.5 |

| Substrate | [$^{14}$C]Palmitoyllyso-GroPCho | |
|---|---|---|
| Product | Diacyl-GroPCho | Neutral Lipid |
| Additions | Percent | |
| | Single Experiment | |
| None | 20.6 | 50.6 |
| CoA | 19.1 | 45.4 |
| $MgCl_2$ | ND* | ND* |
| CoA, ATP and $Mgcl_2$ | 47.7 | 20.8 |

*Not Determined

acylation extent in the absence of cofactors. This may have resulted from the inhibition of acylation by $MgCl_2$ in the mixture since $MgCl_2$ alone inhibited acylation. The acylation of [$^{14}$C]palmitoyllyso-GroPCho (1.6 nmol) showed no increase with the addition of CoA. This demonstrates that the CoA-dependent transacylation is more active with the alkyllyso-GroPCho substrate. Since both the alkyllyso- and acyllyso-GroPCho substrates were acylated in the absence of cofactors, there is transacylation (CoA-indep) activity present in the membranes.

The acylation product of [$^{14}$C]palmitoyllyso-GroPCho increased from 20.6% in the absence of cofactors to 47.7% with the inclusion of all cofactors. This increase in activity of the acyl:CoA acyltransferase was not observed in those incubations containing alkyllyso-GroPCho, indicating that the acyl:CoA acyltransferase selectively acylates the acyllyso-GroPCho substrate.

In the incubations with the alkyllyso-GroPCho substrate [$^{3}$H]hexadecyllyso-GroPCho, the neutral lipid (NL) fraction showed an increase in radioactive labelling with the inclusion of $MgCl_2$ in the incubation reaction. The neutral lipid accounted for less than 1.0% of the label under the conditions of the experiments outlined in Table 1 when $MgCl_2$ was not present and up to 4.7% when $MgCl_2$ was present. In those incubations containing the acyllyso-GroPCho substrate, [$^{14}$C]palmitoyllyso-GroPCho the quantity of radiolabel in the

neutral lipid fraction decreased from 50.6% observed in the absence of cofactors to 20.8% in the presence of all cofactors, while the amount of label in the PC increased. These results indicated that though the CoA-independent transacylation reaction is active toward both acyl- and alkyl-substrates, it is much more active toward the alkyllyso-GroPCho than the acyllyso-GroPCho. A much larger portion of the acyllyso-GroPCho was converted to NL; this could have reduced the apparent extent of acylation by lowering the amount of substrate available.

Selectivity of CoA-Dependent and CoA-Independent Acylation Reactions for Various Acyl Groups

The molecular species composition of the [$^3$H]hexadecylacyl-GroPCho formed by macrophage membranes in the presence of different cofactors was examined in order to assess the selectivity of the different acylation mechanisms for endogenous acyl groups. Examination of the molecular species composition of the [$^3$H]hexadecylacyl-GroPCho formed by macrophage membranes containing (A) no additions and (B) CoA (0.1 mM), ATP (10 mM), and $MgCl_2$ (10 mM) to determine the selectivity of the CoA-independent and CoA-dependent acylation reactions, respectively. The [$^3$H]hexadecylacyl-GroPCho formed from the acylation of [$^3$H]hexadecyllyso-GroPCho was analyzed by HPLC analysis of the alkylacyl-glycerobenzoate product. Figures 1A and 1B show the

radioactivity distribution in the products formed when [$^3$H]hexadecyllyso-GroPCho (2 nmol) was incubated with 120 ug of membrane protein for 15 min in the absence of cofactors (Figure 1A) and in the presence of CoA, ATP, and $MgCl_2$ (Figure 1B). Table 2 summarizes the percent incorporations. In the absence of cofactors, P388D1 membranes catalyzed the preferential acylation of [$^3$H]hexadecyllyso-GroPCho with arachidonate. The product, phosphatidylcholine, consisted predominantly of polyunsaturated species (74%) with the predominance of the 16:0-20:4[1] species (46.7%). The CoA-independent acylation reaction also exhibited a preference for the polyunsaturated acyl group docosahexaenoate (22:6) with 13.5% of this molecular species found in the newly formed PC (Table 2). With the addition of CoA, ATP, and $MgCl_2$, the products became less enriched in arachidonate (only 31.2% of the total labelled species formed) and demonstrated an increase in the incorporation of several other, more saturated, fatty acids. This CoA-dependent acylation of [$^3$H]hexadecyllyso-GroPCho resulted in increases in the 16:0-18:2 and the 16:0-18:1 radiolabelled molecular species (Table 2). These data suggest that in the P388D1 membranes, the CoA-independent pathway is highly

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[1] The acyl group denotation refers to length of the carbon chain and number of double bonds for the aliphatic groups and does not include those linkages contained in ether-linked acyl groups.

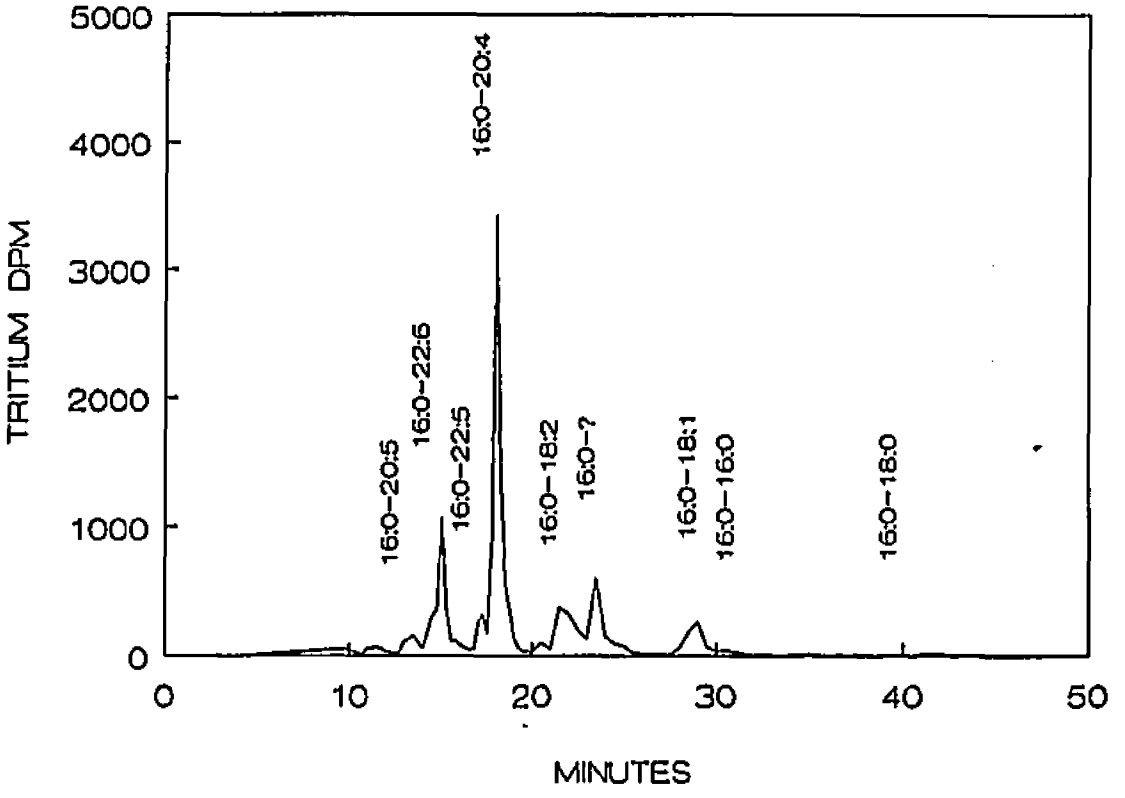


Figure 1A. Radioactivity Profile of [$^3$H]Hexadecylacyl-GroPCho Acylation Product Formed by the CoA-Independent Acylation Mechanism. Macrophages were disrupted by sonication, and total cell membranes were isolated by differential centrifugation. Membranes (120 ug protein) were incubated for 15 min with 2 nmoles of [$^3$H]hexadecyl-lyso-GroPCho in 0.4 ml of 20 mM Tris buffer (pH 7.4). Incubations contained no additions. The acylation product, [$^3$H]hexadecylacyl-GroPCho, was isolated and analyzed as the benzoate derivative as described in Methods to determine the distribution of acyl groups at the sn-2 position. Numbers above the peaks indicate the acyl groups contained within each individual chromatographic peak.

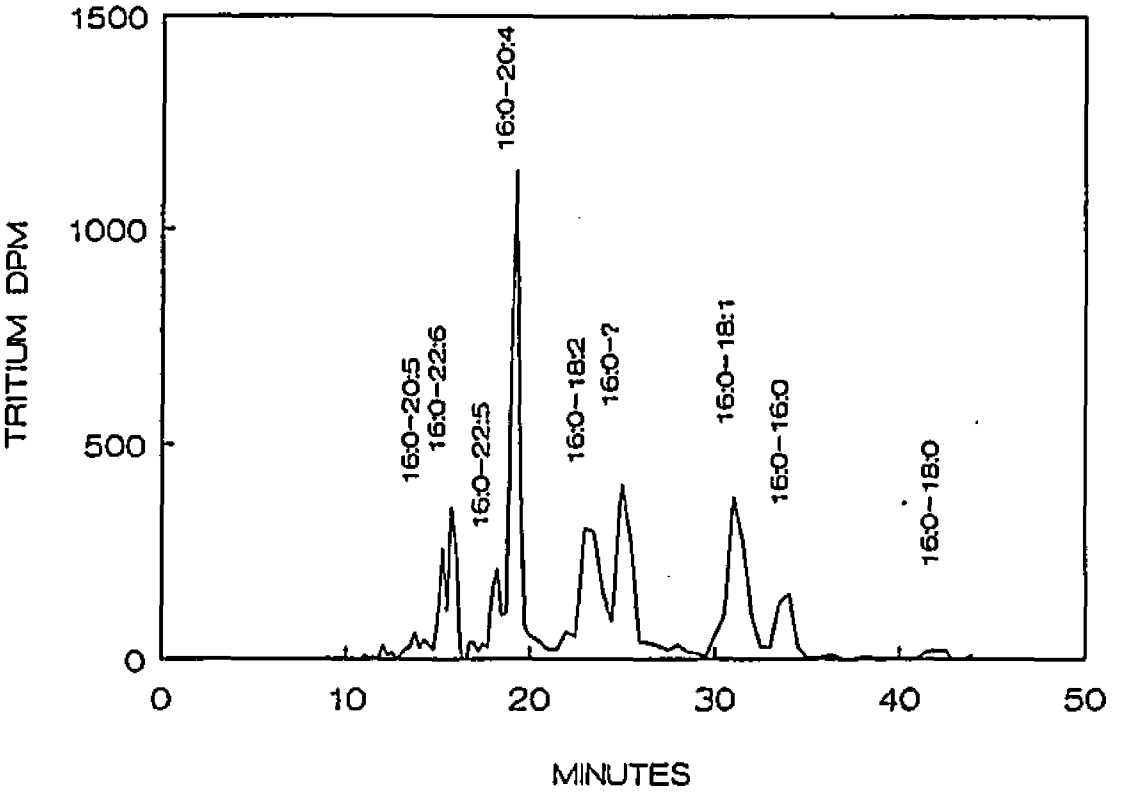


Figure 1B. Radioactivity Profile of [$^3$H]Hexadecylacyl-GroPCho Acylation Product Formed by the CoA-Dependent Acylation Mechanisms. Macrophages were disrupted by sonication and total cell membranes were isolated by differential centrifugation. Membranes (120 ug protein) were incubated for 15 min with 2 nmoles of [$^3$H]hexadecyllyso-GroPCho in 0.4 ml of 20 mM Tris buffer (pH 7.4). Incubations contained CoA (0.1 mM), ATP (10 mM) and $MgCl_2$ (10 mM). The acylation product, [$^3$H]hexadecylacyl-GroPCho, was isolated and analyzed as the benzoate derivative as described in Methods to determine the distribution of acyl groups at the sn-2 position. Numbers above the peaks indicate the acyl groups contained within each chromatographic peak.

Table 2. Distribution of Acyl Groups Incorporated into [$^3$H]Hexadecyllysophosphatidylcholine. Macrophages were disrupted by sonication, and total cell membranes were isolated by differential centrifugation. Membranes (120 ug protein) were incubated for 15 min with 2 nmoles of [$^3$H]hexadecyllyso-GroPCho in 0.4 ml of 20 mM Tris buffer (pH 7.4). Incubations contained (A) no additions or (B) CoA (0.1 mM), ATP (10 mM) and $MgCl_2$ (10 mM). The acylation product, PC, was isolated and analyzed as the benzoate derivative as described in Methods to determine the distribution of acyl groups at the sn-2 position. The data represent total tritium disintegrations per minute (Dpm) from chromatograms displayed in Figure 1 and is representative of several determinations.

Table 2. (Continued)

| Acyl Group† | Percent incorporated into [$^3$H]Hexadecyllyso-GroPCho† | |
|---|---|---|
| | No Additions | CoA, ATP and $MgCl_2$ |
| 20:5 | 3.2 | 5.3 |
| 22:6 | 13.5 | 8.7 |
| 22:5 | 4.5 | 6.2 |
| 20:4 | 46.7 | 31.2 |
| 18:2 | 6.1 | 10.6 |
| unidentified | 7.2 | 10.7 |
| 18:1 | 4.0 | 12.2 |
| 16:0 | 0.6 | 4.2 |
| 18:0 | ND* | ND* |
| Other | 14.2 | 10.9 |

*Not Detected

†Denotes aliphatic chain esterified in the sn-2 position of acylation product, [$^3$H]Hexadecylacyl-GroPCho.

†Values represent the percent of the total radioactivity contained in the individual aliphatic chains.

selective for the polyunsaturated acyl group arachidonate, while the CoA-dependent pathways are less selective and use several fatty acids as substrates.

Specificity of CoA-Dependent Acyltransferase for Acyllyso- vs Alkyllyso-GroPCho Acceptors

The CoA-dependent acylation was examined in more detail using exogenously added acyl-CoA substrate. By using radiolabelled acyl-CoA substrate, instead of labelled lysophospholipids, the preference of the acyltransferase for different lyso-GroPCho acceptors was determined. The specificity of acyl-CoA:lysophospholipid acyltransferase for acyl- vs alkyl-lysophospholipid was measured using two different acyl-CoA donor molecules. Table 3 shows the extent of formation of phosphatidylcholine when either 5 nmoles of (A) [$^3$H]palmitoyl-CoA or (B) [$^3$H]arachidonyl-CoA was incubated with P388D1 macrophage membranes in the presence of alkyllyso-GroPCho and acyllyso-GroPCho substrates. [$^3$H]Palmitoyl-CoA was incorporated into the acyllyso-GroPCho substrate, oleoyllyso-GroPCho at a extent that was three-fold greater than with the alkyllyso-GroPCho substrate, hexadecyllyso-GroPCho. These data indicate that acyllyso-GroPCho was the preferred substrate for the acyltransferase that utilizes [$^3$H]palmitoyl-CoA. Those incubations containing [$^3$H]arachidonyl-CoA with palmitoyllyso-GroPCho demonstrated a similar response.

TABLE 3. Specificity of CoA-Dependent Acyltransferase for Acyllyso- vs Alkyllyso-GroPCho Acceptors. Incubations contained 60 ug of P388D1 membrane protein, 20 mM Tris (pH 7.4) and 5 nmoles of either [$^3$H]Arachidonyl CoA or [$^3$H]Palmitoyl CoA with (A) no additions, (B) 5 nmoles palmitoyllyso-GroPCho, (C) 5 nmoles 1-oleoyllyso-GroPCho or (D) 5 nmoles 1-hexadecyllyso-GroPCho in a total volume of 0.4 ml. After incubation at 37°C for 10 min, the reactions were terminated and the total lipids were extracted and analyzed by TLC. The results are expressed as the means ± standard deviation for replicate determinations.

| Addition | Acylation Product Percent | |
|---|---|---|
| | with [$^3$H]Palmitoyl-CoA | |
| | Experiment | |
| | 1 | 2 |
| None | 5.3 | 5.0 |
| Oleoyllyso-GroPCho[1] | 29 | 23 |
| Hexadecyllyso-GroPCho[2] | 8.8 | 8.3 |
| | with [$^3$H]Arachidonyl-CoA | |
| | Experiment | |
| | 1 | 2 |
| None | 11.1 | 14 |
| Palmitoyllyso-GroPCho[+] | 52.4 | 53.2 |
| Hexadecyllyso-GroPCho[#] | 24.8 | 26.8 |

[+]Acyllyso-GroPCho Substrate

[#]Alkyllyso-GroPCho Substrate

A greater than two-fold increase in the formation of the acylation product was seen in those incubations containing the acyl-linked lyso-GroPCho substrate in comparison to the alkyllyso-GroPCho substrate. A comparison of the acylation of incubations containing [$^3$H]palmitoyl-CoA and [$^3$H]arachidonyl-CoA revealed that the arachidonyl-CoA was more actively incorporated into either substrate than the palmitoyl-CoA.

Effect of Membrane Lipid Composition on Acylation of Lyso-GroPCho in Intact Macrophages

In order to determine the effect of the fatty acid composition of the membrane on the specificities of the acylation reactions in intact macrophages, the acylation of [$^3$H]hexadecyllyso-GroPCho by macrophages grown in the presence and absence of polyunsaturated fatty acids was studied. Table 4 shows the effect of polyunsaturated supplementation on the incorporation of acyl groups into lysophosphatidylcholine of intact macrophages. Macrophages were grown to confluence in media containing 10% fetal calf serum (unsupplemented) or 5% fetal calf serum and 5% horse serum (supplemented) and were incubated for one hour in serum-free media containing [$^3$H]hexadecyllyso-GroPCho. Analysis of the product revealed that the acyl composition of macrophage lipids affects the distribution of acyl groups that are incorporated into lysophosphatidylcholine during

TABLE 4. Effect of Polyunsaturated Supplementation on the Distribution of Acyl Groups Incorporated into [$^3$H]Hexadecyllyso-GroPCho of Intact Macrophages. Macrophages were grown in media containing either 10% fetal calf serum (unsupplemented) or 5% fetal calf serum and 5% horse serum (supplemented). Cells were rinsed and incubated for one hour in serum-free media containing [$^3$H]hexadecyllyso-GroPCho. The acylation product, phosphatidylcholine, was isolated, converted to its diradylglycerobenzoate derivative, and analyzed on HPLC to determine the acyl composition at the sn-2 position. The data are representative of several determinations.

| Acyl Group† | Percent Incorporated into [$^3$H]Hexadecyllyso-GroPCho† | |
|---|---|---|
| sn-2 position | Unsupplemented | Supplemented |
| 20:5 | 1.7 | 2.6 |
| 22:6 | 4.2 | 3.1 |
| 22:5 | 4.9 | 5.2 |
| 20:4 | 18.3 | 34.1 |
| 18:2 | 21.9 | 30.4 |
| 18:1 | 26.3 | 8.5 |
| 16:0 | 4.2 | 2.4 |
| 18:0 | 0.5 | ND* |

*Not Detected

†Denotes aliphatic chain esterified at the sn-2 position of the acylation product, [$^3$H]Hexadecylacyl-GroPCho.

†Values represent the percent of the total radioactivity contained in the individual aliphatic chains.

acylation. HPLC analysis of the molecular species composition of phosphatidylcholine revealed that growth enrichment of the media with horse serum, a polyunsaturated fatty acid rich supplement, resulted in the increased acylation of the [$^3$H]hexadecyllyso-GroPCho with the polyunsaturates, arachidonate and linoleate (18:2). Supplementation resulted in an 85% and 36% increase in the incorporation of these acyl groups, respectively. A simultaneous enrichment with the polyunsaturates, eicosapentaenoate and docosapentaenoate, was also observed with horse serum supplementation. Mass analysis of phosphatidylcholine revealed a concurrent decrease in the saturated fatty acid 16:0 as well as a three-fold reduction in the monounsaturated fatty acid 18:1. Growth enrichment with polyunsaturated fatty acids determines the acyl groups incorporated into [$^3$H]hexadecyllyso-GroPCho, and is evidenced by an enhancement of the polyunsaturates in the acylation product. The reductions in the saturated fatty acids, 16:0 and 18:1, revealed that supplementation results in a redistribution at the molecular species level at the expense of these more saturated fatty acids.

Effect of Polyunsaturated Fatty Acid Supplementation on the CoA-Independent Acylation of [$^3$H]Hexadecyllyso-GroPCho

To elucidate the effect of polyunsaturated fatty acid supplementation on the selectivity of the CoA-independent

acylation reaction, it was necessary to examine this reaction in isolated membranes where the concentrations of cofactors could be varied. Membranes from macrophages grown in supplemented (5% horse serum and 5% fetal calf serum) and unsupplemented (10% fetal calf serum) media were prepared and the specificity of the CoA-independent acylation reaction was determined by incubation of these membranes with [$^3$H]hexadecyllyso-GroPCho in the absence of cofactors. Table 5 illustrates that a selectivity for arachidonate and docosahexaenoate was maintained irrespective of the media supplementation. These results indicated that the selectivity of the CoA-independent transacylation reaction for various acyl groups is not affected by the lipid composition of the membrane. Therefore, a CoA-independent reaction must be responsible for the higher incorporation of the polyunsaturates, arachidonate, docosapentaenoate, and eicosapentaenoate, into the cellular phospholipids of the P388D1 cells enriched in these fatty acids.

[$^3$H]Glycerol Labelling of Macrophages

The extent of synthesis de novo of the alk-1-enylacyl, alkylacyl and diacyl subclasses of PC and phosphatidylethanolamine (PE) of macrophages were determined by measuring the extent at which radiolabelled glycerol was incorporated into these fractions. Table 6 shows the subclass distribution of tritium in PC and PE from macrophages incubated for 19 h in media containing 10% fetal

TABLE 5. Effect of Polyunsaturated Supplementation on the CoA-Independent Acylation of [$^3$H]Hexadecyllyso-GroPCho. Membranes were prepared from P388D1 macrophages grown in media with a high (supplemented) or low (unsupplemented) content of polyunsaturated fatty acids. Membranes (120 ug protein) were incubated with [$^3$H]hexadecyllyso-GroPCho (2 nmol) for 15 min without cofactors. The acylation product, PC, was isolated and analyzed to identify and quantitate the acyl groups incorporated at the sn-2 position. The data are representative of two separate determinations.

| Acyl Group[+] | Percent Incorporated into [$^3$H]Hexadecyllyso-GroPCho[+] | |
|---|---|---|
| sn-2 position | Unsupplemented | Supplemented |
| 20:5 | 3.2 | 3.8 |
| 22:6 | 13.5 | 13.3 |
| 22:5 | 4.5 | 4.0 |
| 20:4 | 46.7 | 51.5 |
| 18:2 | 6.1 | 8.9 |
| 18:1 | 4.0 | 2.6 |
| 16:0 | 0.6 | 0.4 |
| 18:0 | 1.8 | ND* |

*Not Detected

[+]Denotes aliphatic chain esterified at the sn-2 position of the acylation product, [$^3$H]Hexadecylacyl-GroPCho.

[+]Values represent the percent of the total radioactivity associated with the individual aliphatic chains.

calf serum and 5 uCi [$^3$H]glycerol. The mass distribution of these subclasses, also shown in Table 6, was determined by normal-phase HPLC analysis of the diradylbenzoate derivatives of PC and PE (as described in Methods). Mass analysis revealed that the diacyl fraction is the predominant component of both phospholipids with the ether-linked subclass composing 47% and 25% of the PE and PC, respectively. In PC, the predominant form of ether-linked fraction is the alkylacyl subclass, while in PE, the alk-1-enylacyl fraction predominates. [$^3$H]Glycerol was incorporated mainly into the diacyl fraction of both phospholipid classes. The specific radioactivities of each subclass was calculated as a measure of the relative de novo synthetic extent. The high specific radioactivity of the diacyl subclass demonstrated that this fraction is synthesized de novo to a much higher extent than the ether-linked subclasses.

## Effect of N-3 Polyunsaturated Fatty Acid Supplementation on Cellular Phospholipids of P388D1 Macrophages

### Molecular Species Composition of Unsupplemented P388D1 Macrophages

As illustrated in Tables 7, 8, and 9, diacyl-GroPCho, diacyl-GroPEtn, and alk-1-enylacyl-GroPEtn differ considerably in their molecular species composition. The most predominant polyunsaturated group in diacyl-GroPCho

TABLE 6. Incorporation of [$^3$H]Glycerol into Subclasses of PC and PE of P388D1 Macrophages. P388D1 macrophages were incubated for 19 h in media containing 10% FBS and 5 uCi/ml [$^3$H]glycerol. The phospholipid classes, PC and PE, were isolated by TLC, converted to their benzoate derivatives, and separated into subclasses by normal phase HPLC. The mass amount of each subclass was determined by absorbance at 230 nm, and the radioactivity was assayed by scintillation counting of fractions eluted by HPLC. The results are representative of two separate determinations.

| Subclass | Percent Distribution+ Mass | Radioactivity | Specific Activity+ Dpm/nmol |
|---|---|---|---|
| | Phosphatidylcholine | | |
| Alk-1-enylacyl | 1.0 | 1.1 | 90 |
| Alkylacyl | 24.3 | 3.9 | 14 |
| Diacyl | 74.7 | 94.9 | 1102 |
| | Phosphatidylethanolamine | | |
| Alk-1-enylacyl | 41.1 | 4.3 | 42 |
| Alkylacyl | 6.1 | 0.6 | 40 |
| Diacyl | 52.8 | 95.1 | 714 |

+Values represent percents of the total mass and radioactivity recovered within each individual phospholipid class.

+Specific activity was calculated using the formula: disintegrations per minute ÷ total mass (nanomole).

appears to be 18:2, while diacyl-GroPEtn is more enriched with species containing more saturated acyl groups and is particularly enhanced with AA. The alk-1-enylacyl-GroPEtn, like the diacyl-GroPEtn, is also higher in the polyunsaturated fatty acid, AA. A predisposition to selected molecular species must accompany the various polar head groups. For example, while AA is mainly associated with 18:0 in diacyl-GroPEtn, it is mostly associated with 16:0 in diacyl-GroPCho. Similarly, the monoenoic group 18:1 is paired mainly with AA and 18:1 in alk-1-enyl-GroPEtn, while 18:1 is paired with 16:0 in diacyl-GroPCho. These results demonstrated that the pairing of acyl groups in each class is not random and that the nature of the polar head group has an effect on the composition of the acyl groups in individual molecules of phospholipids.

The selectivity must be further enhanced by the sn-1 linkage of the acyl group. The selectivity is demonstrated by differences in molecular species content of the various subclasses within a particular phospholipid class. For example, though both the alk-1-enylacyl-GroPEtn and diacyl-GroPEtn subclasses are enriched with AA, the acyl groups with which AA is predominantly paired varies among these two subclasses. AA is predominantly paired with 18:1 in the alk-1-enylacyl-GroPEtn and with 18:0 in the diacyl-GroPEtn. These differences indicate that the sn-1 linkage influences the acyl group distribution of a phospholipid molecule.

Table 7. Acyl Composition of Diacyl-GroPCho of P388D1 Macrophages. Diacyl-GroPCho was isolated and analyzed as described in Methods. Values represent percent in lipid class and are average weight % ± standard deviation. The data is representative of replicate determinations.

| Species[+] | Unsupplemented | Supplemented | Change |
|---|---|---|---|
| 18:1-20:5 | 0.5 ± 0.1 | 2.3 ± 0.02 | + 1.8 |
| 16:0-20:5<br>18:1-22:6 | 0.7 ± 0.1 | 9.9 ± 0.3 | + 9.2 |
| 16:0-22:6 | 1.2 ± 0.2 | 4.2 ± 0.9 | + 3.0 |
| 18:1-20:4 | 4.8 ± 0.4 | 5.4 ± 0.7 | + 0.6 |
| 16:0-20:4 | 6.3 ± 0.7 | 5.1 ± 0.2 | - 1.2 |
| 18:0-20:5 | ND* | 1.9 ± 0.2 | + 1.9 |
| 18:0-22:6 | 4.7 ± 0.5 | 2.9 ± 0.1 | - 1.8 |
| 18:1-18:2[+a] | 15.8 ± 1.7 | 11.7 ± 0.6 | - 4.1 |
| 16:0-18:2[+b] | 7.7 ± 0.5 | 7.4 ± 0.3 | - 0.3 |
| 18:0-20:4<br>16:0-20:3 | 3.5 ± 0.3 | 2.3 ± 0.1 | - 1.2 |
| 18:1-18:1 | 8.1 ± 1.1 | 3.5 ± 0.3 | - 4.6 |
| 16:0-18:1[+c] | 28.0 ± 1.8 | 21.6 ± 2.5 | - 6.4 |
| 16:0-16:0 | 5.5 ± 0.2 | 9.8 ± 0.7 | + 4.3 |
| 18:0-18:1<br>16:0-20:1 | 4.3 ± 1.0 | 3.8 ± 0.5 | - 0.5 |
| 16:0-18:0 | 1.4 ± 0.2 | 1.9 ± 0.3 | + 0.5 |
| Other | 7.5 | 6.3 | - 0.7 |

Table 7. (Continued)

*Not Detected

†Denotes the length of the carbon chain and the number of double bonds in the two aliphatic groups.

‡These contain additional species:

| a | b | c |
|---|---|---|
| 18:1-20:3 | 16:0-18:3 | 18:0-20:3 |
| 18:1-22:4 | 16:0-22:4 | 18:0-22:4 |

Table 8. Acyl Composition of Diacyl-GroPEtn of P388D1 Macrophages. Diacyl-GroPEtn was isolated and analyzed as described in Methods. Values represent percent in lipid class and are average weight % ± standard deviation. The data is representative of replicate determinations.

| Species[+] | Unsupplemented | Supplemented | Change |
|---|---|---|---|
| 18:1-20:5 | 0.3 ± 0.2 | 4.2 ± 0.5 | + 3.9 |
| 16:0-20:5<br>18:1-22:6 | 2.1 ± 0.4 | 12.3 ± 1.1 | + 10.2 |
| 16:0-22:6 | 2.3 ± 0.2 | 5.3 ± 1.2 | + 3.0 |
| 18:1-20:4 | 9.1 ± 0.7 | 7.7 ± 0.2 | - 1.4 |
| 16:0-20:4 | 9.1 ± 0.3 | 5.8 ± 0.4 | - 3.3 |
| 18:0-20:5 | 1.1 ± 0.4 | 15.0 ± 0.9 | + 13.9 |
| 18:0-22:6 | 4.7 ± 0.3 | 6.9 ± 1.2 | + 2.2 |
| 18:1-18:2[+a] | ND* | 2.4 ± 0.4 | + 2.4 |
| 16:0-18:2[+b] | 5.3 ± 0.5 | 6.7 ± 0.5 | + 1.4 |
| 18:0-20:4<br>16:0-20:3 | 23.3 ± 1.2 | 12.6 ± 0.6 | - 10.7 |
| 18:1-18:1 | 7.4 ± 0.6 | 2.4 ± 0.2 | - 5.0 |
| | 9.4 ± 0.6 | 7.3 ± 1.7 | - 2.1 |
| 16:0-16:0 | ND* | ND* | None |
| 18:0-18:1<br>16:0-20:1 | ND* | ND* | None |
| 16:0-18:0 | 3.2 ± 2.2 | 2.3 ± 0.9 | - 0.9 |
| Other | 22.3 | 9.1 | - 13.2 |

Table 8. (Continued)

*Not Detected

†Denotes the length of the carbon chain and the number of double bonds in the two aliphatic groups.

‡These contain additional species:

| a | b | c |
|---|---|---|
| 18:1-20:3 | 16:0-18:3 | 18:0-20:3 |
| 18:1-22:4 | 16:0-22:4 | 18:0-22:4 |

Table 9. Acyl Composition of Alk-1-enylacyl-GroPEtn of P388D1 Macrophages. Alk-1-enylacyl-GroPEtn was isolated and analyzed as described in Methods. Values represent percent in lipid class and are average weight % ± standard deviation. The data is representative of replicate determinations.

| Species[+] | Unsupplemented | Supplemented | Change |
|---|---|---|---|
| 18:1-20:5 | 2.7 ± 0.4 | 16.5 ± 4.8 | + 13.8 |
| 16:0-20:5<br>18:1-22:6 | 3.3 ± 0.5 | 5.2 ± 1.5 | + 1.9 |
| 16:0-22:6 | 3.7 ± 0.9 | 4.7 ± 1.7 | + 1.0 |
| 18:1-20:4 | 18.1 ± 3.2 | 10.4 ± 1.8 | - 7.7 |
| 16:0-20:4 | 1.0 ± 0.1 | 6.8 ± 0.5 | + 5.8 |
| 18:0-20:5 | 2.2 ± 0.3 | 2.1 ± 0.3 | None |
| 18:0-22:6 | 2.8 ± 0.1 | 4.9 ± 1.9 | + 2.1 |
| 18:1-18:2[+a] | 10.2 ± 3.3 | 4.1 ± 0.8 | - 6.1 |
| 16:0-18:2[+b] | ND* | 6.0 ± 1.0 | + 6.0 |
| 18:0-20:4<br>16:0-20:3 | 3.0 ± 0.1 | 1.2 ± 0.8 | - 1.8 |
| 18:1-18:1 | 11.2 ± 1.6 | 8.1 ± 1.6 | - 3.1 |
| Other | 41.8 | 30 | - 11.8 |

*Not Detected

[+]Denotes the length of the carbon chain and the number of double bonds in the two aliphatic groups (vinyl ether double bond in the alk-1-enylacyl subclass is not included).

[+]These contain additional species:

| a | b |
|---|---|
| 18:1-20:3 | 16:0-20:3 |
| 18:1-22:4 | 16:0-22:4 |

Effect of N-3 Fatty Acid Supplementation on the Phospholipid Class, Subclass, and Molecular Species Composition of P388D1 Macrophages

Studies were conducted to determine the effects of supplementation with the N-3 marine oil fatty acids, eicosapentaenoate and docosahexaenoate, on the distribution of phospholipid molecular species in P388D1 cells. P388D1 cells were incubated in media containing fetal calf serum (10%) with either no additions (unsupplemented) or with 0.8 uM eicosapentaenoic and 0.8 uM docosahexaenoic acids (supplemented) for 24 h. PC and PE were isolated and analyzed as their diradylglycerobenzoate derivatives. Tables 6, 7, and 8 illustrate the acyl group compositions of diacyl-GroPCho, diacyl-GroPEtn, and alk-1-enylacyl-GroPEtn from unsupplemented and supplemented (20:5 and 22:6) P388D1 macrophages, respectively. Figure 2A and B, HPLC chromatographic overlays of diacyl-GroPCho (Figure 2A) and figure 2B, diacyl-GroPEtn (Figure 2B), illustrate that supplementation with eicosapentaenoate and docosahexaenoate results in the enrichment of these fatty acids into the acyl groups of the phospholipid classes.

Effect of N-3 Supplementation on the Phospholipid Classes and Subclasses of the P388D1 Cells. Phosphorous determination revealed that supplementation of the P388D1 cells for 24 h with eicosapentaenoate and docosahexaenoate did not significantly alter the cellular content of the

phospholipid classes (Table 10). Normal-phase HPLC analysis of diradylglycerobenzoate derivatives of PC and PE revealed that supplementation with the N-3 marine oil fatty acids did not result in a change in the mass amounts of the diacyl, alkylacyl and alk-1-enylacyl subclasses of these phospholipids (Table 11). However, the data in Tables 7, 8 and 9 demonstrate that the acyl groups of the subclasses, diacyl-GroPCho, diacyl GroPEtn and alk-1-enylacyl-GroPEtn, became highly enriched with eicosapentaenoic and docosahexaenoic acids during supplementation. These results indicate that the effects of supplementation are manifested at the molecular species level. The enrichment with both eicosapentaenoic and docosahexaenoic acids resulted in a greater than two-fold enhancement of these acids into acyl groups of all subclasses studied. Because the overall mass content of PC and PE and the individual subclasses were unaffected by supplementation, the changes at the molecular species level were the result of a replacement of other molecular species with species containing docosahexaenoate and eicosapentaenoate.

Effect of N-3 Fatty Acid Supplementation on the Molecular Species Composition of P388D1 Macrophages. The newly incorporated polyunsaturates were coupled with the more saturated fatty acids: 16:0, 18:0 and 18:1. Supplementation of the cells, with both eicosapentaenoic and docosahexaenoic acids, caused a reduction in both the

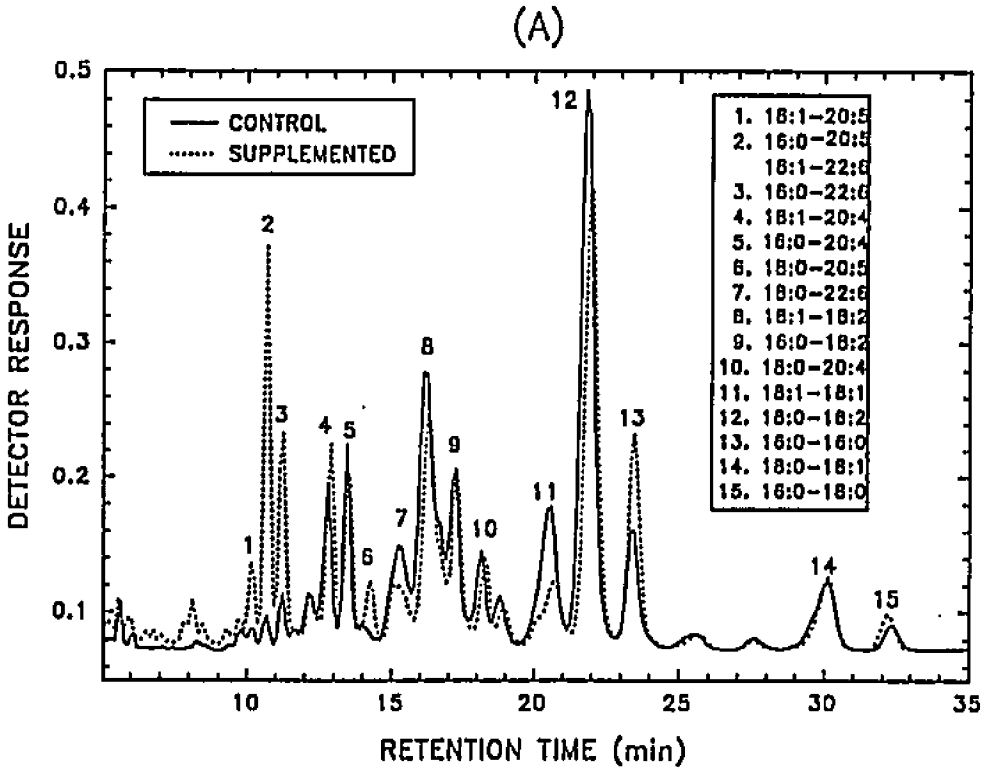


Figure 2A. HPLC Chromatograms of the Diradylglycerobenzoate Derivatives of Diacyl-GroPCho from Control and N-3 Supplemented P388D1 Macrophages. P388D1 macrophages were incubated in media containing fetal calf serum (10%) with either no additions (unsupplemented) or with 0.8 uM eicosapentaenoic and 0.8 uM docosahexaenoic acids (supplemented) for 24 h.

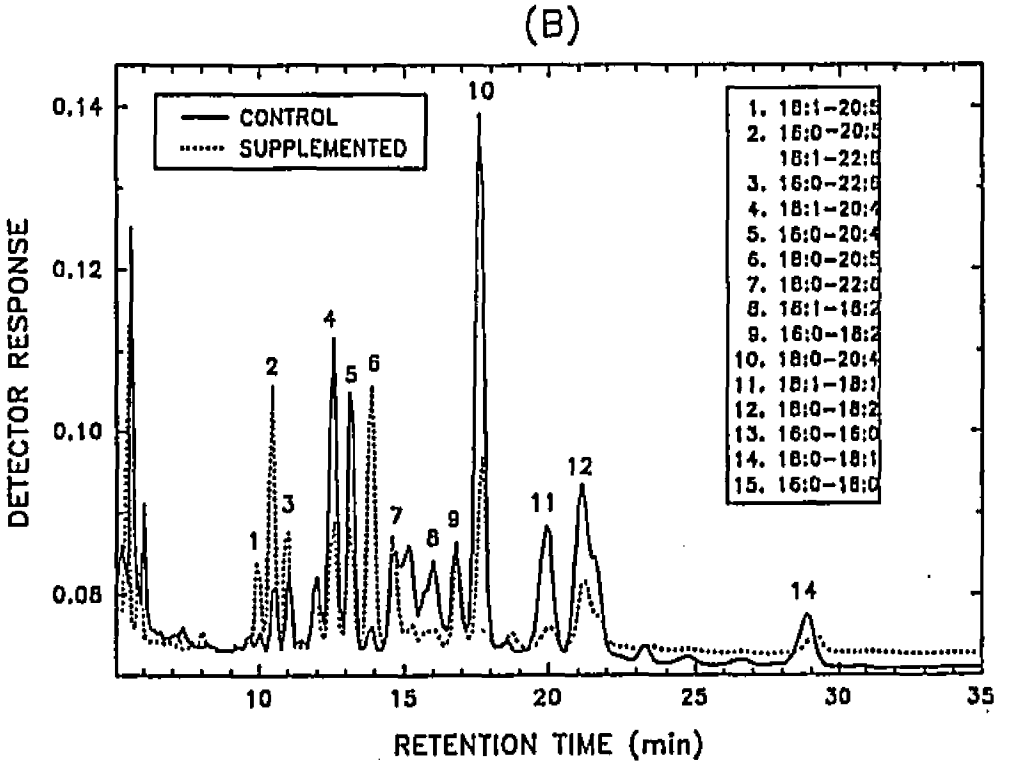


Figure 2B. HPLC Chromatograms of the Diradylglycerobenzoate Derivatives of Diacyl-GroPEtn from Control and N-3 Supplemented P388D1 Macrophages. P388D1 macrophages were incubated in media containing fetal calf serum (10%) with either no additions (unsupplemented) or with 0.8 uM eicosapentaenoic and 0.8 uM docosahexaenoic acids (supplemented) for 24 h.

Table 10. Effect of N-3 Supplementation on the Phospholipid Classes of the P388D1 Macrophages. Phospholipid classes were isolated and analyzed as described in Methods. Values represent percent in phospholipid classes. The data are representative of replicate determinations.

| | SUPPLEMENTATION | | | |
|---|---|---|---|---|
| | NONE | | 20:5 and 22:6 | |
| | (Percent of Total Phospholipids) | | | |
| | EXPERIMENT | | EXPERIMENT | |
| LIPID CLASS | 1 | 2 | 1 | 2 |
| PE | 30.4 | 26.7 | 31.3 | 27.7 |
| PS/PI | 13.1 | 12.5 | 15.5 | 13.1 |
| PC | 41.2 | 37.3 | 40.6 | 40.1 |

Table 11. Effect of N-3 Supplementation on the Phospholipid Subclasses of PC and PE of P388D1 Macrophages. The phospholipid subclasses were isolated and analyzed as described in Methods. Values represent percent in phospholipid classes. The data are representative of replicate determinations.

| SUBCLASS | SUPPLEMENTATION | |
|---|---|---|
| | NONE | 20:5 AND 22:6 |
| | (Percent of Total Class) | |
| | Phosphatidylethanolamine | |
| Alk-1-enylacyl | 32.2 ± 5.6 | 30.0 ± 1.6 |
| Alkylacyl | 5.2 ± 2.3 | 7.7 ± 2.2 |
| Diacyl | 57.5 ± 3.5 | 60.4 ± 1.6 |
| | Phosphatidylcholine | |
| Alk-1-enylacyl | 2.8 ± 1.8 | 1.6 ± 0.7 |
| Alkylacyl | 10.0 ± 2.5 | 8.4 ± 0.4 |
| Diacyl | 87.3 ± 4.1 | 90.2 ± 0.8 |

dioleoyl and the arachidonate-containing species in all of the subclasses analyzed. For example, supplementation with both polyunsaturates resulted in a 10% and 40% loss in the arachidonate-containing species of the diacyl-GroPCho and diacyl-GroPEtn, respectively (Tables 7 and 8). Both supplemented fatty acids were incorporated into the phospholipid classes at the expense of diolein (Tables 7, 8, and 9) in all subclasses studied: Alk-1-enylacyl-GroPEtn showed a 26% reduction in the dioleoyl species, while the diacyl-GroPCho and diacyl-GroPEtn showed reductions of 42% and 73%, respectively.

With supplementation, the diacyl-GroPCho exhibited a distinct reduction in the mass amounts of the two linoleic-paired species, with decreases from 15.8% to 11.7% in the 18:1-18:2 species and 28% to 21.6% in the 18:0-18:2 species. The diacyl-GroPEtn exhibited a loss in the 18:0-20:4 species during supplementation, resulting in a decrease from 23.3% to 12.6%. Alk-1-enylacyl-GroPEtn analysis displayed a major loss in its 18:1-containing species with reductions in the 18:1-20:4 species from 18.1% to 10.4% and in the 18:1-18:1 species from 11.2% to 8.1% during supplementation. These results indicate that enrichment with the N-3 marine oil fatty acids is coincident with redistributions in the molecular species of diacyl-GroPCho, diacyl-GroPEtn, and alk-1-enylacyl-GroPEtn.

Studies involving single fatty acid supplementation with either eicosapentaenoic or docosahexaenoic acid resulted in a noticeable enhancement of the individual polyunsaturates in the acyl groups of diacyl-GroPEtn with a variable response in the diacyl-GroPCho. Though supplementation with a single polyunsaturated fatty acid resulted in varying amounts of incorporation, it was apparent that enrichment with one fatty acid--i.e., eicosapentaenoic acid--did not decrease the content of the other--i.e., docosahexaenoic acid, in the cellular phospholipids studied (data not shown).

Effect of N-3 Fatty Acid Supplementation on TPA-Induced Diradylglycerol Generation. Studies were conducted to determine the effect of supplementation with the N-3 marine oil fatty acids, docosahexaenoate and eicosapentaenoate, on the generation of diradylglycerides. Following incubation of supplemented and unsupplemented P388D1 macrophages with [$^3$H]myristic acid for 16 h, the macrophages were incubated with either DMSO (0.1%) or TPA (0.1 mM) for 10 min. Isolation of the diradylglycerol fraction revealed that unsupplemented macrophages stimulated with TPA resulted in an increase of 27% in diradylglycerol (Table 12). However there was no significant effect of TPA observed on diradylglycerol generation in supplemented macrophages.

Table 12. Effect of N-3 Supplementation on TPA-Induced Diradylglyceride Generation. P388D1 macrophages were labelled with [$^3$H]myristate for 16 h, stimulated with TPA (0.1 mM) for 10 min. The diradylglycerides were then isolated, separated, and analyzed for radioactivity as described in Methods. Values are presented as mean percent ± standard deviation of five experiments performed in duplicate.

| | DIRADYLGLYCEROL (PERCENT OF TOTAL CELL RADIOACTIVITY) | |
|---|---|---|
| | - TPA | + TPA |
| Unsupplemented | 0.76 ± 0.04 | 1.05 ± 0.09 |
| Supplemented | 0.80 ± 0.05 | 0.81 ± 0.07 |

## Incorporation and Stimulated Release of Arachidonate from P388D1 Phospholipids

Studies were conducted to determine (1) the extent of incorporation of exogenous arachidonate into the phospholipid classes of the P388D1 macrophages and (2) the degree of selectivity of the phospholipases in the P388D1 macrophages for the arachidonate-containing species of phospholipids.

Specificity of Phospholipid Classes

Macrophages exposed to exogenous [$^3$H]AA actively incorporated it into their cellular phospholipids. At the end of a 2 hour incubation with [$^3$H]AA, 22.5% of the incorporated radioactive AA was present in phosphatidylcholine, while 38.4% was incorporated into phosphatidylethanolamine.

To determine whether the radioactivity of the cellular lipids was redistributed over time, the initial labelling with [$^3$H]AA was followed by a 24 h chase period. This chase period, which did not involve the addition of unlabelled AA, disclosed the trafficking of [$^3$H]AA among the phospholipids. During the 24 h chase period, PE showed an increase in radioactivity (43% to 57%), while the radioactivity in PC and NL decreased (Table 13).

To determine the release of [$^3$H]AA from cellular phosphoglycerides or the conversion of AA to neutral lipid products, AA-labelled P388D1 cells were incubated with ionophore A23187 (10 uM) or no stimulus for 60 min. Ionophore induced the release of labelled AA from all AA-containing phosphoglycerides (Table 14). Most of the [$^3$H]AA originated from the phosphatidylserine/phosphatidyl-inositol (PS/PI), while the remainder apparently originated from PE and PC, respectively. With ionophore stimulation, the content of radiolabel in the media increased from 4.3% ± 2.1

Table 13. Redistribution of Radiolabelled Arachidonic Acid in Macrophage Glycerolipids. Macrophages were labelled with [$^3$H]arachidonic acid and chased for either 2 or 24 h. The glycerolipids were then extracted and analyzed by TLC in the solvent system $CHCl_3$:MeOH:HAC:$H_2O$ (50:25:8:3,v/v). The radioactivity was determined by scintillation counting of zonal scrapings. The values are representations of two determinations ± standard deviation.

| Glycerolipid Class | Percent Distribution+ | |
|---|---|---|
| | 2 Hr. Chase | 24 Hr. Chase |
| Neutral Lipid | 7.5 ± 0.6 | 5.4 ± 2.0 |
| PE | 38.4 ± 2.3 | 57.7 ± 1.5 |
| PS/PI | 27.7 ± 1.0 | 26.1 ± 0.4 |
| PC | 22.5 ± 2.5 | 9.8 ± 1.1 |

+The amount of radioactivity in each glycerolipid is a percent of the total radioactivity recovered from all cellular lipids.

in the unstimulated to 30% ± 16.4 (mean ± standard deviation, n=3) in the stimulated macrophages.

Though the numbers are variable, a greater than 6-fold increase in radiolabel release was observed with ionophore stimulation. The NL showed a two-fold increase in radiolabel with the addition of ionophore. The liberation

Table 14. Distribution of Radiolabelled Arachidonic Acid in Macrophage Glycerolipids of Stimulated and Unstimulated P388D1 Macrophages. Macrophages, labelled with [$^3$H]arachidonic acid as described in Methods, were incubated with the $Ca^{+2}$ ionophore, A23187, for 60 min. The lipids were extracted, separated by TLC and the radioactivity was determined by scintillation counting of zonal scrapings. The mean from three experiments is presented with S.D. The percent change was determined by the difference in control and A23187 values.

| Glycerolipids | Treatment | Percent+ | Change |
|---|---|---|---|
| PC | Control | 20.7 ± 3.6 | |
| | A23187 | 20.5 ± 1.2 | -0.2 |
| PE | Control | 40.0 ± 3.3 | |
| | A23187 | 38.2 ± 4.1 | -1.8 |
| PS/PI | Control | 29.1 ± 2.6 | |
| | A23187 | 24.3 ± 2.0 | -4.8 |
| NL | Control | 7.5 ± 0.5 | |
| | A23187 | 14.8 ± 1.8 | +7.3 |

+The amount of radioactivity in each glycerolipid is a percent of the total radioactivity recovered from all cellular lipids.

of AA from phospholipid may have resulted from either direct release of AA or from the conversion of a particular phospholipid to a NL product such as diradylglycerol.

Specificity of Molecular Species

In order to determine the nature of the AA-containing molecular species that are degraded during stimulation of P388D1 macrophages, the phospholipids were isolated, converted to benzoate derivatives, and then analyzed by reverse-phase HPLC.

Analysis of the phospholipid subclasses diacyl-GroPCho, diacyl-GroPEtn and alk-1-enylacyl-GroPEtn (from stimulated and unstimulated macrophages) revealed a degradative specificity for the arachidonate-containing species in the ionophore treated macrophages. Figure 3, an HPLC chromatographic overlay of control and ionophore-stimulated diacyl-GroPEtn with a computer-generated subtraction chromatogram shown in the extreme bottom, illustrates this selective degradation of arachidonate-containing species. Incubations with the calcium ionophore, A23187 (10 uM), for 60 min resulted in the selective degradation of 18:1-20:4, 16:0-20:4 and 18:0-20:4. Similar results were obtained with diacyl-GroPCho and alk-1-enylacyl-GroPEtn.

Changes in Mass vs Specific Radioactivity

Two distinct calculations were necessary to quantitate the molecular species modifications in the phospholipid classes resulting from the addition of ionophore. The first analysis, the change in mass, was determined by the difference in percent total of the individual molecular

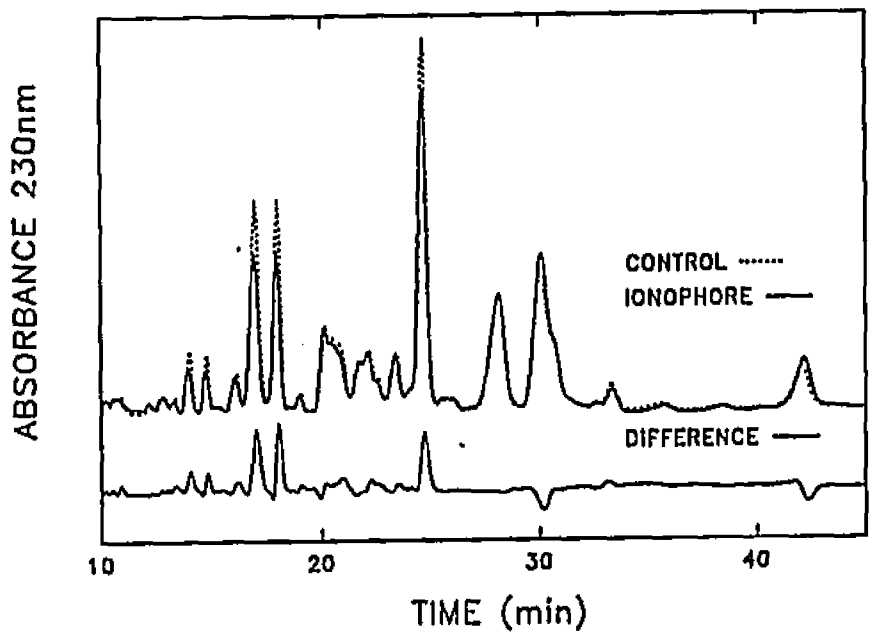


Figure 3. HPLC Chromatographic Overlay of Control and Ionophore-Stimulated Diacyl-GroPEtn. Macrophages, labelled with [$^3$H]arachidonic acid as described in Methods, were incubated with the $Ca^{+2}$ ionophore, A23187, for 60 min. Diacyl-GroPEtn was converted to its diradylglycerobenzoate derivative and analyzed on reverse-phase HPLC as described.

the phospholipid classes studied. The second analysis, the measurement of change in the specific radioactivity, was calculated using the formula:

disintegrations per minute ÷ nanomole content.

Table 15 presents the molecular species mass changes that occurred with the addition of the calcium ionophore, A23187, in the diacyl-GroPCho, diacyl GroPEtn and the alk-1-enylacyl-GroPEtn subclasses. When calcium ionophore was added, mass analysis revealed that the arachidonate-containing species were extensively degraded in these phospholipid subclasses. The species containing AA were more extensively degraded than species containing other acyl groups (data not shown). A calcium-dependent phospholipase selective for AA-containing species was active during ionophore stimulation of the P388D1 macrophages.

Analysis of the AA-containing species of diacyl-GroPCho, diacyl-GroPEtn and alk-1-enylacyl-GroPEtn demonstrated that treatment with calcium ionophore also resulted in a consistent decrease in the specific radioactivity of the AA-containing species. As depicted in Table 16, diacyl-GroPCho displayed the greatest loss in specific radioactivity of the arachidonate-containing species with a greater than 45% decrease following treatment with ionophore. Decreases in the specific radioactivity of the diacyl-GroPEtn and the alk-1-enylacyl-GroPEtn subclasses were also observed (Table 16).

Table 15. Effect of Calcium Ionophore on the Mass Distribution of Acyl Groups Paired with Arachidonate in Intact Macrophages. Macrophages were harvested and incubated in serum-free media containing 5 uCi [$^3$H]arachidonic acid at 37°C for 30 min. The cells were plated and allowed to attach for 2 h in supplemented media. The cells were rinsed with serum-free media and incubated in DMEM/BSA (1 mg/ml) with either DMSO (0.1%)(control) or 10 uM A23187 (stimulated) for 0-60 min. Diacyl-GroPCho, diacyl-GroPEtn, and alk-1-enylacyl-GroPEtn were isolated and analyzed as described in Methods. The data represent two separate determinations but are representative of several experiments. The amount of change was determined by the difference between control and ionophore stimulated values.

| Molecular Species+ | Percent of Total Subclass+ | | Amount of Change |
|---|---|---|---|
| | Control | Stimulated | |
| | Diacyl Phosphatidylethanolamine | | |
| 18:1-20:4 | 11.6 | 9.1 | -2.5 |
| 16:0-20:4 | 9.0 | 7.2 | -1.8 |
| 18:0-20:4 | 20.7 | 18.9 | -1.8 |
| | Alk-1-enylacyl Phosphatidylethanolamine | | |
| 18:1-20:4 | 16.7 | 15.3 | -1.4 |
| 16:0-20:4 | 15.6 | 14.0 | -1.6 |
| 18:0-20:4 | 8.5 | 7.8 | -0.7 |
| | Diacyl Phosphatidylcholine | | |
| 18:1-20:4 | 5.8 | 4.7 | -1.1 |
| 16:0-20:4 | 6.1 | 4.7 | -1.4 |
| 18:0-20:4 | 2.3 | 2.0 | -0.3 |

+Denotes the length of the carbon chain and the number of double bonds in the two aliphatic groups.

+Values represent the percent of radioactivity recovered with individual aliphatic groups within each subclass.

Table 16. Effect of Calcium Ionophore on the Specific Radioactivity of Acyl Groups Paired with Arachidonate in Intact Macrophages. Diacyl-GroPCho, diacyl-GroPEtn, and alk-1-enylacyl-GroPEtn were isolated and analyzed as described in Methods. The data represent two separate determinations but are representative of several experiments. The percent change was determined by the difference between control and A23187 stimulated values.

| ACYL GROUP+ | DPM/NMOLE+ | | PERCENT CHANGE |
|---|---|---|---|
| | Control | Stimulated | |
| | Diacyl Phosphatidylethanolamine | | |
| 18:1-20:4 | 14397 | 13339 | -7.4 |
| 16:0-20:4 | 15691 | 13531 | -13.8 |
| 18:0-20:4 | 9761 | 8417 | -13.8 |
| | Alk-1-enylacyl Phosphatidylethanolamine | | |
| 18:1-20:4 | 13725 | 10982 | -20.0 |
| 16:0-20:4 | 17324 | 15823 | -8.7 |
| 18:0-20:4 | 14199 | 13991 | -1.5 |
| | Diacyl Phosphatidylcholine | | |
| 18:1-20:4 | 12505 | 11495 | -8.1 |
| 16:0-20:4 | 17014 | 11248 | -33.9 |
| 18:0-20:4 | 11360 | 10887 | -4.2 |

+Denotes the length of the carbon chain and the number of double bonds in the two aliphatic groups.

+Specific Activity was calculated using the formula: disintegrations per minute ÷ total nanomole content.

# CHAPTER 4

# Discussion

## The Incorporation of Arachidonate into Cell Phospholipids: A Comparison of the Three Acylation Mechanisms

### Acylation of Alkyllyso-GroPCho vs Acyllyso-GroPCho

The CoA-independent transacylation pathway was found to be active toward both acyl and alkyl substrates in the P388D1 macrophages as demonstrated by the acylation of both compound types in the absence of added cofactors. The activity was much greater toward the alkyllyso-GroPCho than the acyllyso-GroPCho substrate. The higher activity toward the alkyllyso-GroPCho substrate indicates that the transacylase demonstrates a preference for ether-linked lysophospholipid substrates. However, the high content of radioactivity in the neutral lipid fraction in incubations with the acyllyso-GroPCho substrate may result from selective degradation of the acyllyso-GroPCho substrate or its acylation product, diacyl-GroPCho, masking the activity of the transacylase toward this substrate. Lysophospholipases may selectively cleave the acyl group from the sn-1 position of the acyllyso-GroPCho substrate. Therefore, the selective acylation of the alkyllyso-GroPCho may indicate either a selectivity for the acylation of the ether-linked substrate or a degradative selectivity for the acyllyso-GroPCho and/or diacyl-GroPCho.

In incubations containing the alkyllyso-GroPCho substrate, the addition of CoA alone increased the rate of acylation, demonstrating the presence of a CoA-dependent transacylation. The increased activity toward the alkyllyso-GroPCho substrate with the addition of CoA suggests that the acyltransferase may be acting in reverse and, therefore, functioning as a phospholipase and an acyltransferase by cleaving the acyl group of an endogenous phospholipid that is subsequently transferred to the radiolabelled lysophospholipid substrate.

The addition of CoA to incubations containing the acyllyso-GroPCho substrate did not increase the extent of acylation. Since there is no indication that the addition of CoA to the incubations eliminates the activity of the CoA-independent transacylase, the CoA-dependent transacylation mechanism must be specific for the alkyl form.

The addition of all cofactors (CoA, ATP, and $MgCl_2$) for the incorporation of endogenous fatty acids into the alkyllyso-GroPCho substrate resulted in a decreased rate of acylation when compared to the rate of acylation in the absence of cofactors. The decrease in hexadecylacyl-GroPCho product formation with the addition of CoA, ATP, and $MgCl_2$ suggests that the CoA and ATP-dependent pathway may be inhibited by a cofactor. Incubations with $MgCl_2$ alone resulted in decreased product formation and increased

radioactivity in the neutral lipid fraction. These data suggest that this cofactor may (1) inhibit transacylation of the alkyllyso-GroPCho substrate, (2) stimulate the lysophospholipase activity, and/or (3) stimulate phospholipase C activity in the P388D1 membranes. Inhibition of the transacylation results in decreased product formation, while the stimulation of the phospholipases results in the conversion of the alkyllyso-GroPCho to the neutral lipid products: free fatty acid, monoglyceride, or monoalkylglycerol. An increase in the neutral lipid product, identified as [$^3$H]hexadecylglycerol, was reported by Robinson et al. (1985b) in incubations of rabbit alveolar macrophages with [$^3$H]hexadecyllyso-GroPCho in the presence of $MgCl_2$; no inhibition of product formation was observed. Since our investigations, unlike those of Robinson et al. (1985b), did not reveal the identification of the neutral lipid product formed, further investigation of the effects of $MgCl_2$ are necessary.

The addition of all cofactors (CoA, ATP, and $MgCl_2$) stimulated the acylation rate of the acyllyso-GroPCho substrate. This indicates the acyl:CoA acyltransferase selectively acylates the acyllyso-GroPCho.

Selectivity of the Acylation Pathways for Acyl Groups

HPLC analysis was used to examine the alkylacyl glycerobenzoate derivatives of alkylacyl-GroPCho formed

during incubation of [$^3$H]hexadecyllyso-GroPCho with P388D1 membranes in the presence and absence of cofactors. This was used to determine the specificities of the CoA-independent and CoA-dependent acylation mechanisms in the acylation of the alkyllyso-GroPCho substrate. Several differences in the specificities of the CoA-independent and CoA-dependent pathways were observed. The CoA-independent pathway preferentially acylated the alkyllyso-GroPCho substrate with the polyunsaturates arachidonate (46.7%) and docosahexaenoate (13.5%). The CoA-dependent pathways exhibited less selectivity and acylated the alkyl-linked GroPCho substrate with several, more saturated, fatty acids i.e., palmitate, stearate and oleate (Table 2). The specificity of the CoA-independent transacylation pathway for arachidonic acid, in the acylation of [$^3$H]hexadecyllyso-GroPCho, has been reported for human polymorphonuclear leukocytes (Chilton et al., 1983), rabbit platelets (Malone et al., 1985), rat alveolar macrophages (Robinson and Snyder, 1985a) and rabbit alveolar macrophages (Robinson et al., 1985b; Sugiura et al., 1987). The studies by Robinson et al. (1985b) reported a highly selective acylation of alkyllyso- and acyllyso-GroPCho substrates with arachidonate; the choline glycerophospholipid products, formed from either precursor, contained up to 80% arachidonate.

Our investigation, the first to describe studies of this enzyme in a cultured cell line, demonstrates that the CoA-independent transacylation reaction selectively acylates the hexadecyllyso-GroPCho substrate with the $C_{20}$ polyunsaturate, arachidonate, and the $C_{22}$ polyunsaturate, docosahexaenoate. Similar results were obtained by Sugiura and co-workers (1987) who reported that 1-alkyllyso-GroPCho was rapidly acylated with arachidonate (greater than 50% in the acylation product), as well as the polyunsaturate docosahexaenoate.

Selectivity of the CoA-Dependent Pathway for Acyllyso- vs Alkyllyso-GroPCho Acceptors

The specificity of the acyl CoA:1-acyl-*sn*-glycero-3-phosphocholine acyltransferase for different acyl-CoAs was investigated by comparing the rates at which different radiolabelled CoA esters were incorporated into lysophospholipids of P388D1 membranes. The specificity of the reactions for alkyllyso- and acyllyso-GroPCho substrates was compared. The P388D1 membranes preferentially acylated the acyllyso-GroPCho substrate. The acylation extent for the acyllyso-GroPCho substrate, compared to that of the alkyllyso-GroPCho substrate, was three-fold higher when palmitoyl-CoA was a substrate and two-fold higher when the arachidonyl-CoA was the substrate (Table 3). The results may have been affected by the use of different acyllyso-

GroPCho substrates (palmitoyl vs oleoyl) in the two assays. These results indicate that the acyl CoA: 1-acyl-sn-glycero-phosphocholine acyltransferase of P388D1 membranes prefers acyl-linked substrates.

A comparison of the acylation rates for the two acyl CoAs, palmitoyl-CoA and arachidonyl-CoA, revealed that the arachidonyl-CoA was more actively incorporated into either substrate than the palmitoyl-CoA. These results indicate that either (1) the acyl CoA:1-acyl-*sn*-glycero-phosphocholine acyltransferase of P388D1 membranes prefers polyunsaturated acyl-CoAs, or (2) the acyl-CoA hydrolases within the cell more actively hydrolyze the palmitoyl-CoA. A high specificity of the acyl-CoA:acyltransferase for polyunsaturated fatty acids has previously been reported (Brandt and Lands, 1967). For example, the existence of a remodeling pathway for PC biosynthesis, catalyzed by an acyl-CoA:1-acyl-*sn*-glycero-3-phosphocholine acyltransferase selective for unsaturated fatty acyl-CoA esters, in human platelets was reported by McKean and coworkers (1982). Investigations with various cell types have shown that unsaturated fatty acids are incorporated into phospholipids by the deacylation-reacylation pathway with arachidonyl-CoA as the preferred fatty acyl donor.

Effect of Membrane Lipid Composition on Acylation Reaction Specificities

To examine the effect of the fatty acid composition of the membrane on the specificities of the acylation reactions in intact macrophages, the acylation of [$^3$H]hexadecyllyso-GroPCho by macrophages grown in the presence and absence of polyunsaturated fatty acids was studied. Macrophages were grown to confluence in media containing either 10% fetal calf serum (unsupplemented), or 5% FBS and 5% horse serum (supplemented), and then incubated with [$^3$H]hexadecyllyso-GroPCho for one hour. Table 4 shows that incubation of the supplemented macrophages with the [$^3$H]hexadecyllyso-GroPCho substrate resulted in the formation of a [$^3$H]hexadecylacyl-GroPCho product enriched with those polyunsaturates which predominate in horse serum: arachidonate, linoleate, docosapentaenoate and eicosapentaenoate.

To determine whether the selectivity of the CoA-independent transacylation for arachidonate and linoleate was maintained during supplementation with horse serum, membranes from supplemented and unsupplemented macrophages were prepared. The membranes were then incubated with [$^3$H]hexadecyllyso-GroPCho in the absence of cofactors. As shown in Table 5, horse serum supplementation had no effect on the selectivity of the CoA-independent transacylation for the polyunsaturates, arachidonate and linoleate. This indicates that the specificity of the CoA-independent

acylation reaction for various acyl groups is not affected by the lipid composition of the membrane.

Based on the results of these studies, it appears that the acyl:CoA acyltransferase preferentially acylates the acyllyso-GroPCho substrate, while the CoA-independent acyltransferase prefers the alkyllyso-GroPCho substrate. The CoA-independent transacylase exhibited selectivity for the polyunsaturates arachidonate and linoleate. These results, spurred speculation on the mechanisms involved in the incorporation of the horse serum polyunsaturates and the acylation of [$^3$H]hexadecyllyso-GroPCho. We propose that the horse serum polyunsaturates are incoporated into the membrane phospholipids by the acyl:CoA acyltransferase and/or the CoA-independent transacylase. Due to the substrate selectivities of these two systems, the acyl:CoA acyltransferase, most likely, acylates the acyllyso-GroPCho. These polyunsaturates may then be transferred from the diacyl-GroPCho into alkyllyso-GroPCho by the CoA-independent transacylase.

[$^3$H]Glycerol Labelling of Macrophages

The extent of synthesis de novo of the alk-1-enylacyl, alkylacyl and diacyl subclasses of PC and PE of macrophages were determined by measuring the extent to which radiolabelled glycerol was incorporated into these fractions. As shown in Table 6, the diacyl fraction is

synthesized de novo to a much higher extent than the ether-linked subclasses in both the PC and PE classes. The predominant ether-linked subclass in PC is alkylacyl while the predominate ether-linked subclass in PE is the alk-1-enylacyl subclass. The high proportion of alk-1-enylacyl-GroPEtn and alkylacyl-GroPCho is the prominent feature of the composition of the subclasses in macrophages (Nakagawa and Waku, 1989; Sugiura et al. 1983a). A high proportion of alkylacyl-GroPCho and alk-1-enylacyl-GroPetn have also been reported in bone-marrow derived blood cells such as neutrophils (Sugiura et al. 1982) and platelets (Sugiura et al. 1983b).

Molecular Species Composition of Unsupplemented P388D1 Macrophages

Analysis of diacyl-GroPCho, diacyl-GroPEtn and alk-1-enylacyl-GroPEtn in unsupplemented P388D1 cells revealed considerable differences in their molecular species composition (Tables 7, 8 and 9). Diacyl-GroPEtn is higher in the species containing polyunsaturated acyl groups and is particularly high in arachidonate. Linoleate (18:2) appears to be the most predominant polyunsaturated group in diacyl-PC.

A comparison of the acyl groups associated with individual unsaturated acyl groups in each lipid class revealed further distinctions among the phospholipid classes. For example, though arachidonate is primarily

associated with stearate in PE, it is mainly associated with palmitate in PC. Similarly, the monoenoic group oleate is paired predominantly with arachidonate and oleate in PE, while oleate is paired with palmitate in PC. These results demonstrate that the pairing of acyl groups in each class is not random and the nature of the polar headgroup affects the composition of the acyl groups in individual molecules of phospholipids.

The dissimilarity between the acyl group compositions of PC and PE may be explained by the differences in the selectivities of the acyl:CoA acyltransferase and the CoA-independent transacylase. The acyl:CoA acyltransferase is responsible for the incorporation of arachidonate into the acyllyso-GroPCho and perhaps, to a lesser extent, into the acyllyso-GroPEtn while the CoA-independent transacylase is involved in the incorporation of arachidonate into the ether-linked subclasses, alkylacyl-GroPCho and alk-1-enylacyl-GroPEtn. This proposal does not eliminate the possibility that an overlap of activities occurs. For example, the CoA-independent transacylase may transfer the polyunsaturated acyl groups from the diacyl-GroPCho into either the diacyl-GroPEtn, the alk-1-enylacyl-GroPEtn, or the alkyacyl-GroPCho.

<u>Effect of N-3 Fatty Acid Supplementation on the Phospholipid Class, Subclass and Molecular Species Composition of P388D1 Macrophages</u>

Effect of N-3 Supplementation on the Phospholipid Classes and Subclasses of the P388D1 Cells. To determine the effect of supplementation on the phospholipid classes and subclasses of the P388D1 macrophages, the macrophages were incubated for 24 h with eicosapentaenoate and docosahexaenoate. Table 10 shows that supplementation did not significantly alter the cellular content of the phospholipid classes. Table 11 shows that enrichment with the N-3 marine oil fatty acids did not result in a change in the mass amounts of the diacyl, alkylacyl and alk-1-enylacyl subclasses of PC and PE. These results indicate that the effects of supplementation are not manifested at the class or subclass levels. Since the total content of the subclasses did not change during supplementation, we conclude that as the 20:5 and 22:6 are incorporated, other acyl groups are depleted to the extent that no overall change is observed.

Effect of N-3 Fatty Acid Supplementation on the Molecular Species Composition of P388D1 Macrophages. The acyl groups of the subclasses diacyl-GroPCho, diacyl-GroPEtn and alk-1-enylacyl-GroPEtn, became highly enriched with eicosapentaenoate and docosahexaenoate during supplementation. Supplementation of the macrophages with eicosapentaenoate and docosahexaenoate resulted in a greater than 2-fold enrichment of these N-3 marine oil fatty acids into the cellular phospholipids. These polyunsaturates were

paired with the more saturated fatty acids: palmitate, stearate and oleate. A comparison of our studies with those of Blank et al. (1989) revealed similar results in the enrichment of the cellular phospholipids with the N-3 marine fatty acids. In agreement with our results, Blank et al. (1989) observed the greatest enrichment with the N-3 marine oil fatty acids in the alk-1-enylacyl-GroPEtn subclass. They also reported a loss of one-third of the eicosatetraenoate species of the alk-1-enylacyl-GroPEtn subclass during supplementation. We also observed a decrease in the amount of arachidonate in this ether-linked subclass. Additionally, we observed a loss of 20:4 in the diacyl-GroPEtn and the diacyl-GroPCho.

Supplementation with eicosapentaenoate and docosahexaenoate resulted in reductions of several molecular species of the various phospholipid classes. For example, the mass amounts of the dioleoyl and arachidonyl species were reduced during supplementation in the diacyl-GroPCho, diacyl-GroPEtn and the alk-1-enylacyl-GroPEtn classes. Blank et al. (1989) also reported that supplementation with eicosapentaenoate lowered the oleate content in PS/PI, diacyl-GroPEtn and alk-1-enylacyl-GroPEtn. HPLC analysis also revealed a reduction in the predominant molecular species of each phospholipid class. For example, linoleate, the predominate species of diacyl phosphatidylcholine, showed a reduction from 43.3% to 33.3% during

supplementation and oleate, the predominant species of alk-1-enylacyl-GroPEtn, was reduced from 29.3% to 18.5%. A redistribution in the phospholipid classes at the molecular species level was observed in the stearate-paired species. The molecular species 18:0-22:6 exhibited either no change or a decrease in abundance during supplementation in the different phospholipid classes.

These results indicate: (1) supplementation of the P388D1 macrophages with the polyunsaturates, docosahexaenoate and eicosapentaenoate, results in enrichment of the acyl groups of the cellular phospholipids with these fatty acids and (2) coincident with this enrichment with the N-3 marine oil fatty acids are redistributions in the molecular species of the cellular phospholipids, i.e., reductions in arachidonic acid. The enrichment of the cellular phospholipids with the *N*-3 marine oil fatty acids is consistent with reports in a variety of cell types including: rat platelets (Careaga-Houck and Sprecher, 1989), rainbow trout leucocytes (Pettitt and Rowley, 1990), human neutrophils (Prescott, 1984), and porcine pulmonary artery smooth muscle cells (Yerram and Spector, 1989). All authors reported enrichment with the N-3 fatty acids into the phospholipid classes of the various cell types. During this enrichment with the N-3 fatty acids, the authors also reported reductions in the abundance of arachidonic acid (Careaga-Houck and Sprecher, 1989;

Pettitt and Rowley, 1990; Yerram and Spector, 1989) or its metabolites (Prescott, 1984). In studies on the effect of a fish oil diet on the composition of phospholipids and eicosanoid production by rat platelets, Careaga-Houck and Sprecher (1989) observed decreases in the arachidonate-containing species of PC and PE during supplementation with the N-3 marine oil fatty acids; moreover, they reported reductions in the linoleate species of PC and the oleate species of PE during supplementation. These findings parallel our observations of the reductions in the less saturated fatty acids within the phospholipid classes.

Effect of N-3 Fatty Acid Supplementation on TPA-Induced Diradylglycerol Generation. Unsupplemented macrophages stimulated with TPA showed an increase in diradylglycerol generation, while no significant effect of TPA was observed on diradylglyceride generation in supplemented macrophages (Table 12). Diradylglycerides, serving as intermediates in the biosynthesis and degradation of glycerolipids and as activators of protein kinase C (Nishizuka et al. 1977), may be sources of arachidonate and its metabolites. Phosphatidylcholine may be degraded by phospholipases to diradylglycerol; the diradylglycerol may then be hydrolyzed by diradylglycerol lipases to free fatty acids and glycerol. Since arachidonate is predominantly esterified to the sn-2 position of phospholipids, the diradylglycerides function as a potential reservoir for arachidonic acid. The metabolites

of arachidonate have been implicated as contributors to atherosclerotic plaque formation in cardiovascular disease. Since diradylglycerides are a potential source of arachidonate, the abatement of TPA-induced diradylglyceride generation in supplemented cells diminishes the arachidonate content of the cell. This depletion in the potential source of arachidonate would then be manifested by decreased availability of arachidonate and its metabolites for pathological changes in the cell (i.e., atherosclerotic plaque formation). TPA is an analog of diradylglycerides and, when presented in the cell, mimics the activity of the diradylglycerides: TPA activates protein kinase C. Protein kinase C activates several target enzymes within the cell and results in heightened diradylglyceride production. One of the target enzymes, HMG-CoA reductase, catalyzes the reduction of HMG-CoA to mevalonate, the first committed step in the biosynthesis of cholesterol. Cholesterol has been implicated as another major contributor in the development of cardiovascular disease. The absence of effect by TPA on the DG generation in supplemented macrophages suggests that supplementation affects the responsiveness of the cell to activation. The absence of diradylglycerol generation in the presence of TPA indicates that the protein kinase C is not responsive to stimulation. This absence of protein kinase C response suggests a possible control point in the development of coronary heart disease. The *N*-3 marine oil

fatty acids may deter the development of coronary heart disease by limiting the level of protein kinase C in the supplemented cells.

## Stimulated Release of 20:4 from P388D1 Phospholipids

### Specificity of Phospholipid Classes

Macrophages actively incorporate exogenous arachidonic acid into cellular phospholipids. At the end of a 2 h incubation with [$^3$H]AA, the extent of [$^3$H]AA in cellular lipids was PE > PS+PI > PC > NL. To determine whether the radioactivity of the cellular lipids was redistributed over time, the initial labelling with [$^3$H]AA was followed by a 24 h chase period. Table 13 shows that during the 24 h chase period, the radioactivity in PE increased, while the radioactivity in PC and NL decreased. Based upon our data on the selectivity of the CoA-independent transacylase for alkyllyso-substrates, we propose that the CoA-independent transacylase is responsible for the transfer of AA from PC to PE during the chase period. Specifically, we believe the CoA-independent transacylase is transferring the AA from the diacyl PC into the alk-1-enylacyl-GroPEtn.

### Specificity for Arachidonate: Changes in Mass and Specific Radioactivity

Incubation of the P388D1 macrophages with the calcium ionophore A23187 resulted in a decrease in (1) the mass

distribution of acyl groups paired with AA in diacyl-GroPEtn, diacyl-GroPCho and alk-1-enylacyl-GroPEtn and in (2) the specific radioactivity of the AA-containing species of these cellular phospholipids (Tables 9 and 10). The AA-containing species were selectively degraded during stimulation with the ionophore. However, no distinct preference was observed among the species in which AA was paired with various sn-1 aliphatic groups: the 18:0-20:4, 18:1-20:4 and 16:0-20:4 groups were degraded to the same extent. The addition of the calcium ionophore, A23187, to the P388D1 cells results in the activation of a calcium dependent phospholipase with specificity toward AA-containing molecules. The fact that the AA-containing species were not preferentially degraded indicates that the phospholipase is not affected by the linkage at the sn-1 linkage of the molecule.

Capriotti et al. (1988) suggested that the most recently incorporated arachidonate is the pool preferentially released when the cell is stimulated. This concept was supported by Furth and Laposata (1988) using EFD-1 (an essential fatty acid deficient fibrosarcoma cell line) with findings that recently incorporated arachidonate is preferentially released upon agonist stimulation. By incubation of the cells for 24 h with [$^{3}$H]AA to label an endogenous pool and pulse labelling a newly incorporated pool with [$^{14}$C]AA and then stimulating with bradykinin, the

authors demonstrated that the "newly incorporated pool" is the source of the released arachidonate. Results indicate that several pools of arachidonate may exist in the P388D1 macrophages. The pool which is readily labelled is also preferentially stimulated and, therefore, readily releases the newly incorporated arachidonate. Our results show that a decrease in specific activity is manifested at the level of individual molecular species of arachidonate-containing phospholipids. Since specific activity is calculated using the formula: Dpm/nmol, a decrease would result from a loss in the amount of radioactivity present or from an increase in the mass amount of the particular molecule. We propose that several pools of arachidonate molecules exist in the cells; these pools may be located in close proximity to one another and as such, may serve as reservoirs for endogenous AA. During radiolabelling with [$^3$H]AA these molecular pools may contribute unlabelled AA. The replacement of radiolabelled AA with unlabelled, endogenous AA would result in a loss of specific activity. Moreover, an increase in the mass of the AA, caused by the existence of these unlabelled pools of AA and their contributions of AA would also result in a decrease in the specific activity. The proposal is, therefore, that the "pool" of esterified arachidonate that is selectively labelled and degraded does not correspond to a particular chemical form of arachidonate.

Investigations indicate that the deacylation-reacylation of arachidonate in the phospholipids of P388D1 involves a complex array of mechanisms. Nonetheless, several speculations may be drawn from the data. The specificity of the acyl:CoA acyltransferase indicates that arachidonate is initially incorporated into diacyl-GroPCho. Our investigation of the redistribution of arachidonate among the phospholipid classes revealed a transfer of arachidonate from diacyl-GroPCho to alk-1-enylacyl-GroPEtn. The specificity of the CoA-independent transacylation indicates that this pathway is responsible for the transfer of arachidonate from diacyl phospholipids to ether-linked subclasses i.e., the transfer of arachidonate from the diacyl subclass of PC to the alk-1-enylacyl subclass of PE. The deacylation reacylation of arachidonate in phospholipids of P388D1 cells is dependent upon the activities of several enzymes. The trafficking of arachidonate in the P388D1 phospholipids is, therefore, rather complex.

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