

EFFECTS OF ω 3-POLYUNSATURATED FATTY ACIDS ON THE
FORMATION OF *sn*-1,2-DIRADYLGLYCEROL, CYTOKINE SECRETION
AND THE REGULATION OF PROTEIN KINASE C ACTIVITY

By

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ω 3PUFA-induced changes in *sn*-1,2-DG, IL-6 and PKC activity

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PROTEIN KINASE C ACTIVITY

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ABSTRACT

The focus of my thesis was to investigate changes in the formation of second messenger *sn*-1,2-diacylglycerol (DG) and phospholipids (PL) in murine peritoneal macrophages *in vitro* and *in vivo* in response to varying concentrations of the ω 3-polyunsaturated fatty acids (ω 3PUFA), eicosapentaenoic (EPA, 20:5 ω 3) and docosahexaenoic (DHA, 22:6 ω 3) acids. My findings establish for the first time that the incorporation of EPA and DHA into DG and membrane PL occurs independently and dose-dependently, with EPA being incorporated to a greater extent than DHA.

It was of interest to investigate the functional correlations of the substitution of ω 3PUFA into DG and PL molecular species. DG is classically known as the physiological activator of protein kinase C (PKC). I investigated the effects on PKC activation by pure species of diacylglycerol (DAG) with oleoyl, arachidonoyl, eicosapentaenoyl or docosahexaenoyl at the *sn*-2 position. These results showed that DG species with DHA or EPA incorporated at the *sn*-2 position activate PKC. However, while DG with DHA at the *sn*-2 position activated PKC to a similar extent as did DG with AA at the *sn*-2 position (representative of physiological DG), DG with EPA at the *sn*-2 position activated PKC to a significantly lesser degree. To the best of my knowledge, this is the first demonstration in the literature that

the activation of PKC *in vitro* differs significantly among these molecular species of DAG.

The immunoregulatory cytokine, interleukin-6 (IL-6), is produced in response to tissue injury and inflammation and *in vitro* by LPS-stimulated macrophages. I investigated the correlation of ω 3PUFA substitution into DG and secretion of IL-6 by LPS-stimulated murine peritoneal macrophages. These results show for the first time that IL-6 secretion is attenuated by ω 3PUFA, and to a greater extent by DHA than by EPA.

I have obtained several novel findings that contribute to improved understanding of the underlying mechanisms by which ω 3PUFA function at the level of signal transduction. The ability to modify the fatty acid composition of the signalling molecule, DG, with ω 3PUFA can now be used for future research to further elucidate the functional ramifications of ω 3PUFA in cell signal transduction.

In Dedizione

Ai miei genitori, sorella e fratello

*O speculatore delle cose, non ti laldare di conoscere le cose
che ordinariamente per sè medesima la natura conduce, ma
rallegрати di conoscere il fine di quelle cose che son disegnate
dalla mente tua.*

Leonardo da Vinci

MS. G 47r

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The value of a person is not determined by their social status or by the number of letters behind their name but rather by their integrity and compassion for others. Students are rarely fortunate enough to encounter such people during the course of their graduate training, I however have been.

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LIST OF ABBREVIATIONS

AA	arachidonic acid (20:4 ω 6)
ACS	aqueous counting scintillant
ALA	α -linolenic acid (18:3 ω 3)
aPKC	atypical isoforms of protein kinase C
Ca ²⁺	calcium
COEE	corn oil ethyl ester
cPKC	classical isoforms of protein kinase C
DAG	<i>sn</i> -1,2-diacylglycerol
DAG kinase	<i>sn</i> -1,2-diacylglycerol kinase
DOG	<i>sn</i> -1,2-dioleoylglycerol
dFCS, dfcs	heat inactivated fetal calf serum
DG	<i>sn</i> -1,2-diradylglycerol
DHA	docosahexaenoic acid (22:6 ω 3)
DHEE	docosahexaenoic ethyl ester
DMPA	dimethylphosphatidic acid
DPA	docosapentaenoic acid (22:5 ω 3)
EPA	eicosapentaenoic acid (20:5 ω 3)
EPEE	eicosapentaenoic ethyl ester

HHBSS	Hepes-buffered hanks balanced salt solution
IP ₃	inositol 1,4,5-trisphosphate
IL-1	interleukin-1
IL-2	interleukin-2
IL-4	interleukin-4
IL-6	interleukin-6
LA	linoleic acid (18:2 ω 6)
LPS	lipopolysaccharide
LTB ₄	leukotriene B ₄
LTC ₄	leukotriene C ₄
LTC ₅	leukotriene C ₅
LTE ₅	leukotriene E ₅
2-ME	2-mercaptoethanol
MNC	mononuclear cells
mRNA	messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
NMR	nuclear magnetic resonance
nPKC	novel isoforms of protein kinase C
(n-3)EE	(n-3) ethyl ester concentrate
(n-3) PUFA	(n-3) polyunsaturated fatty acid
PA	phosphatidic acid

PAF	platelet activating factor (1- <i>O</i> -alkyl-2-acetyl- <i>sn</i> -glycero-3-phosphocholine)
PC	phosphatidylcholine
PC-AA	1-stearoyl-2-arachidonoyl- <i>sn</i> -glycero-3-phosphocholine
PC-DHA	1-stearoyl-2-docosahexaenoyl- <i>sn</i> -glycerol-3-phosphocholine
PE	phosphatidylethanolamine
PGE ₁	prostaglandin E ₁
PGE ₂	prostaglandin E ₂
PGI ₂	prostacyclin
PI	phosphatidylinositol
PIP	phosphoinositol-4-phosphate
PIP ₂	phosphoinositol-4, 5-bisphosphate
PKC	protein kinase C
PL	phospholipid or phospholipids
PMA	phorbol myristate acetate, 12- <i>O</i> -tetradecanoylphorbol-13-acetate
POPC	1-palmitoyl-2-oleoyl- <i>sn</i> -glycerol-3-phosphocholine
POPS	1-palmitoyl-2-oleoyl- <i>sn</i> -glycerol-3-phosphoserine
PUFA	polyunsaturated fatty acid
ω-3EE	ω-3 fatty acid ethyl ester concentrate
ω3PUFA	ω3-polyunsaturated fatty acid

PS	phosphatidylserine
RP-HPLC	reverse phase-high pressure liquid chromatography
SAG	1-stearoyl-2-arachidonoyl- <i>sn</i> -glycerol
SDG	1-stearoyl-2-docosahexaenoyl- <i>sn</i> -glycerol
SEG	1-stearoyl-2-eicosapentaenoyl- <i>sn</i> -glycerol
tlc, t.l.c., TLC	thin-layer chromatography
TNF- α	tumor necrosis factor- α
TxB ₂	thromboxane B ₂

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CHAPTER ONE

CHAPTER ONE: INTRODUCTION

1.1 ω 3-Polyunsaturated Fatty Acids

1.1.1 Historical Perspective

Current interest in the antiinflammatory, antithrombotic and antineoplastic effects of ω 3-polyunsaturated fatty acids (ω 3PUFA) was originally ignited by the work of Sinclair (1953) and later by Bang and Dyerberg (Bang et al 1971, Dyerberg and Bang, 1979). Sinclair (1953), while commissioned by the Canadian Government to study the diet of the Eskimos and Northern Indians, reported an almost total absence of cardiovascular diseases in the Eskimo population. Bang and Dyerberg (Bang et al 1971, Dyerberg and Bang, 1979) later studied a population of Greenland Inuit that reportedly had a lower incidence of coronary heart disease and compared them to a population of age- and sex-matched Danes. They found that the Inuit also had lower plasma levels of very low density lipoproteins, low density lipoproteins and triglycerides and elevated levels of high density lipoproteins, consistent with reduced risk of disease. Bang and Dyerberg attributed these differences between the Inuit and Danes to the differences in fatty acid composition of their respective diets. The diet of the Inuit was rich in

ω 3PUFA, including eicosapentaenoic acid (EPA, 20:5 ω 3) and docosahexaenoic acid (DHA, 22:6 ω 3) derived from marine organisms, whereas the diet of the Danes was rich in monounsaturated fatty acids and ω 6PUFA, including linoleic acid (LA, 18:2 ω 6) and to a lesser degree arachidonic acid (AA, 20:4 ω 6), obtained from meats and vegetables.

This pioneering work has opened the door to significant contributions being made to an understanding of how modifications in the fatty acid component of human dietary intake may influence the activity of certain diseases. Much research is still needed to elucidate the mechanisms by which ω 3PUFA contribute to amelioration of various diseases.

1.1.2 Clinical Significance of ω 3-Polyunsaturated Fatty Acids

Since the work of Bang and Dyerberg, the effects of ω 3PUFA have been studied clinically in patients with rheumatoid arthritis (Kremer et al 1985, Kremer et al 1987, Sperling et al 1987), atherosclerotic diseases (Lervang et al 1993, Lungershausen et al 1994), hyperlipidemia (Harris et al 1990, Schmidt et al 1993, Svaneborg et al 1994), ulcerative colitis (Grimminger et al 1993, Ross 1993), systemic lupus erythematosus (Das 1994), diabetes (Juhan et al 1982, Kamada et al 1986, Malasanos and Stacpoole 1991), transplantation (Maachi et al 1995), restenosis (O'Connor et al 1992) and colon cancer (Reddy et al 1991, Anti et al 1992, Anti et

al 1994).

Kremer and colleagues (1985) compared the clinical manifestations in patients with rheumatoid arthritis who took 1.8 g EPA daily for 12 weeks with those in control patients who took diets low in PUFA:saturated fat ratio. At the end of the 12 weeks, the EPA group showed significantly better improvements in outcomes, including morning stiffness, duration of fatigue, grip strength, number of tender joints and of swollen joints and others. During a follow-up period, the improvements in the experimental group deteriorated, reinforcing the evidence for a beneficial effect of EPA in rheumatoid arthritis.

Dietary supplementation with fish oil has been studied in patients with active ulcerative colitis (Stenson et al 1992). Daily supplementation with fish oil (EPA, 3.24 g and DHA, 2.16 g) for four months resulted in a reduction in prostaglandin E₂ (PGE₂) and leukotriene B₄ (LTB₄) measured in rectal dialysate, improvements in the histological index (mucin on the surface of the epithelium, erosion, superficial and deep inflammatory cell infiltrate, architectural distortion, fibrosis and loss of polarity) and a significant weight gain. Furthermore, in patients receiving simultaneous treatment with prednisone, the mean dose of prednisone decreased significantly from 12.9 g/d to 6.1 g/d compared to an increase from 10.4 g/d to 12.9 g/d in controls. The authors suggest that dietary supplementation with fish oil may be useful as an adjunct therapy in the treatment of patients with ulcerative colitis, particularly in those patients requiring long-term steroid therapy to control their symptoms.

Anti and colleagues (1992) determined the effect of dietary supplementation of ω 3PUFA in patients with sporadic adenomatous colorectal polyps. Patients took EPA (4.1 g/d) and DHA (3.6 g/d) for 12 weeks, after which mucosal proliferation was assessed with [3 H]thymidine autoradiography. After two weeks of therapy, the mean percentage of replicative S-phase cells in the upper colonic crypts was significantly reduced compared to baseline levels and remained reduced throughout the duration of the study. Fatty acid analyses of biopsies showed that EPA content was significantly increased at the expense of AA content. The authors suggest that effects of ω 3PUFA on colorectal adenomas may be related to their effects on the AA-eicosanoid pathway and that ω 3PUFA may be useful as an adjunct therapy in the reduction and/or prevention of colorectal cancer.

1.1.3 Functional Effects of ω 3-Polyunsaturated Fatty Acids

The clinical effects of ω 3PUFA on the amelioration of several diseases has been recognized and continues to be studied in ongoing clinical trials, but considerable research efforts also continue to be devoted to unravelling the mechanisms by which ω 3PUFA elicit their beneficial effects.

In the laboratory, some of the functional effects of ω 3PUFA examined to date include alterations in eicosanoid formation (Lokesh and Kinsella 1987, 1988b,

Lokesh et al 1988, Croft et al 1987, Chapkin et al 1991, Hardardottir et al 1992, Endres et al 1993, Lawrence and Sorrell 1994), lipidemia (Huang et al 1986, Al-Shurbaji et al 1991, Meydani et al 1993), macrophage function (Sugiura et al 1983, Dustin et al 1990, Calder et al 1990, Hubbard et al 1994), platelet function (Ahmed and Holub 1984, von Stacky et al 1985, Schick et al 1992, Bénistant et al 1993), erythrocyte function (Knapp et al 1994), neutrophil function (Chilton et al 1990, Sperling et al 1993, Hubbard et al 1994), and T lymphocyte function (Szamel et al 1989, Calder et al 1992, Fowler et al 1993, Shapiro et al 1993). The expression of adhesion molecules and surface proteins (De Caterina et al 1995, Hughes et al 1995, Jenski et al 1995), as well as the expression of a major histocompatibility antigen found on murine macrophages, Ia (Hubbard et al 1991) have been studied *in vitro*. Studies have also been conducted to determine the effect of ω 3PUFA on smooth muscle cell growth (Hui et al 1992, Shiina et al 1993), cytokine production (Billiar et al 1988, Endres et al 1989, Scales et al 1989, Hardardottir and Kinsella 1991, 1992, Whelan et al 1991, Calder and Newsholme 1992a, 1992b, Ertel et al 1993, Purasiri et al 1994, Somers and Erickson 1994), superoxide formation (Beckman and Crow 1993, Chaet et al 1994, Chen et al 1994), central nervous system growth and development (Anderson and Maude 1972, Tinoco 1982, Neuringer and Connor 1986, Neuringer et al 1986), gene regulation (Clarke and Jump 1994, Fernandes et al 1995) and kinase activity (Bell and Sargent 1987, Holian and Nelson 1992).

The most widely studied mechanism by which ω 3PUFA have been thought

to cause their antiinflammatory and antithrombotic effects is the replacement of cellular membrane AA by EPA and possibly DHA. The consequences of this replacement have been extensively investigated in the AA cascade, where the hydrolysis of AA by 5-lipoxygenase and prostaglandin endoperoxide synthase results in the formation of bioactive mediators named eicosanoids (leukotriene C₄ (LTC₄), leukotriene B₄ (LTB₄), thromboxane B₂ (TxB₂), prostacyclin (PGI₂) and PGE₂). The literature suggests that alterations in the ratio of bioactive AA-derived eicosanoids to bioactive or bionutral ω 3PUFA-derived eicosanoids may play a role in the amelioration of several of the clinical manifestations of the diseases described above (section 1.1.2).

Several additional mechanisms have also been examined, including alterations in membrane fluidity (Rabini et al 1993), postprandial lipidemia (Schmidt and Dyerberg 1994) and free radical formation (Fisher et al 1990, Chaet et al 1994).

1.1.4 Eicosapentaenoic Acid, Docosahexaenoic Acid and Other ω 3PUFA

The primary natural source of ω 3PUFA is unicellular marine algae. *Nannochloropsis* and *Isochrysis* are rich in EPA and DHA, respectively (Lands 1989). ω 3PUFA accumulate in higher marine organisms and ultimately in humans via the food chain. Linseed oil (Magrum and Johnston 1986) and blackcurrant seed

oil (Kunkel et al 1981) are sources of the shorter chain ω 3PUFA, α -linolenic acid (ALA, 18:3 ω 3).

The PUFA present in human tissues fall into four groups, namely ω 6, ω 7, ω 9 and ω 3. This designation refers to the location of the first double bond from the terminal methyl group. ω 6 and ω 3 fatty acids cannot be synthesized *de novo* from palmitic (16:0) or stearic (18:0) acids because mammals lack the enzymes required for Δ 12 and Δ 15 desaturation of stearic or oleic acids at carbons ω 6 and ω 3, respectively (Brenner 1974, Innis 1993). Therefore, linoleic (18:2 ω 6) or γ -linolenic (18:3 ω 6) and probably α -linolenic (18:3 ω 3) acids are essential fatty acids required by mammals and must be obtained from the diet. The ω 6 essential fatty acids can be acted upon enzymatically by elongases and desaturases to form ω 6PUFA, for example AA. The same enzymes appear to have less affinity for ALA (Figure 1) (Innis 1991, Sardesai 1992), and very small amounts of EPA and DHA can be made (Sardesai 1992) (with the possible exception of DHA in the central nervous system (Neuringer and Connor 1986)).

The unique structural characteristics of EPA and DHA are perhaps pivotal in the pharmacological and physiological mode of action of these ω 3PUFA (Figure 2). EPA and DHA are fatty acid chains of 20 and 22 carbons, respectively. EPA is identical to AA except for a fifth double bond at carbon 17. DHA has a sixth double bond at carbon 4.

There has been controversy since the 1930's over whether ALA is an essential nutrient required for mammalian growth and development (Burr and

Burr 1930). Relatively high concentrations of DHA have been discovered in the central nervous system (Anderson and Maude 1972, Tinoco 1982, Neuringer and Connor 1986). ω 3PUFA are required for proper skin development and function (Bjerve et al 1987), testicular growth and sperm maturity (Ayala et al 1972, Salem et al 1986) and development of the retina and visual function (Anderson and Maude 1972, Neuringer and Connor 1986, Neuringer et al 1986). Neurological disorders directly associated with DHA deficiency in brain and other tissues are Menkes' Disease (Menkes et al 1962, O' Brian and Sampson 1966), multiple sclerosis (Gerstl et al 1965, Bernsohn and Stephanides 1967), infantile and juvenile forms of ceroid-lipofuscinosis (Svennerholm et al 1975) and a syndrome associated with ω 6-rich but ω 3-poor baby formula (Holman et al 1982, Bozian and Moussavian 1982). The essentiality of ω 3PUFA in infant and adult human diet is now recognized. Because premature, very low birth weight and full term babies all undergo rapid brain growth and neurological development, the formulation of milk supplements is recommended to include the same proportions of saturates, monoenes, dienes, ω 6PUFA and ω 3 PUFA as are found in human breast milk (Bjerve et al 1993).

Several reports describe the pharmacological similarities and differences between the longer chain ω 3PUFA (EPA and DHA) and the shorter chain ω 3PUFA, ALA (Kunkel et al 1981, Chapkin and Carmichael 1990, Garg et al 1990, Whelan et al 1991, Chan et al 1993, Hubbard et al 1994). As detailed above, ALA is the precursor of EPA and DHA in certain algae, but this conversion is not

quantitatively measurable in humans. Therefore, ALA potentially elicits different effects in humans from EPA and DHA. Consideration has been given to the role of ALA in inflammatory, thrombotic and neoplastic diseases. Since both ALA and linoleic acid (LA, 18:2 ω 6) are desaturated and elongated by the same enzymes (Figure 1), substrate competition may occur between them.

Garg and colleagues (1990) investigated the inhibitory effect of linseed oil (as a source of ALA) and fish oil (as a source of EPA and DHA) on the rate limiting enzyme in AA biosynthesis, Δ 6-desaturase, from the hepatic microsomal membrane of weaning rats. Both sources of ω 3PUFA inhibited the conversion of linoleic to γ -linolenic acid and reduced plasma and tissue levels of AA. Formation of 4- and 5-series leukotrienes and of PGE₂ was determined in peritoneal macrophages obtained from mice fed a series of concentrations of linseed oil or fish oil concentrate, to supply dietary ω 3/ ω 6 ratios of 0.2, 0.4 and 1.0 (Whelan et al 1991). In the linseed oil diet group, there was a significant accumulation of DHA in liver and macrophage PL. 4-series leukotrienes and PGE₂ levels were reduced in the linseed oil and fish oil diet groups with the highest ω 3/ ω 6 ratios. Leukotriene E₅ formation was found in all fish oil diet groups as well as in the linseed oil group with the highest ω 3/ ω 6 ratio. These results suggest that ALA is preferentially metabolized to DHA and that ALA, like DHA, is capable of contributing to the formation of 5-series leukotrienes.

The cytolytic capacity of macrophages, tumor necrosis factor- α (TNF- α) secretion and eicosanoid production have been compared among mice fed diets

containing linseed oil, fish (menhaden) oil or safflower oil (containing principally 18:2 ω 6 but no appreciable amount of ω 3PUFA) (Hubbard et al 1994). Significantly less LTC₄ was produced by macrophages from mice fed linseed or menhaden oil compared to macrophages from mice in the safflower oil group. Macrophages taken from the latter mice did not produce LTC₄, unlike macrophages in the other groups. Overall, the effects of linseed oil on the production of eicosanoids from macrophages were not as pronounced as those of menhaden oil. Functionally, on stimulation with lipopolysaccharide (LPS) or LPS and interferon- γ (IFN- γ), macrophages from mice fed linseed oil had a reduced cytolytic capacity, as measured by TNF- α production, nitric oxide production and cytolysis against P815 mastocytoma cells, compared to macrophages from mice fed menhaden or safflower oil. However, tumoricidal capacity was similar among the diet groups with the exception of macrophages stimulated with LPS for 24 hr, where tumoricidal capacity was significantly elevated compared to linseed and safflower oil groups. These results suggest that the effects of ALA on eicosanoid formation are similar to those of the larger chain ω 3PUFA, but the effects of ALA on tumoricidal capacity remains similar to that of ω 6PUFA. Moreover, the beneficial clinical effects of ω 3PUFA may be specific for EPA and DHA.

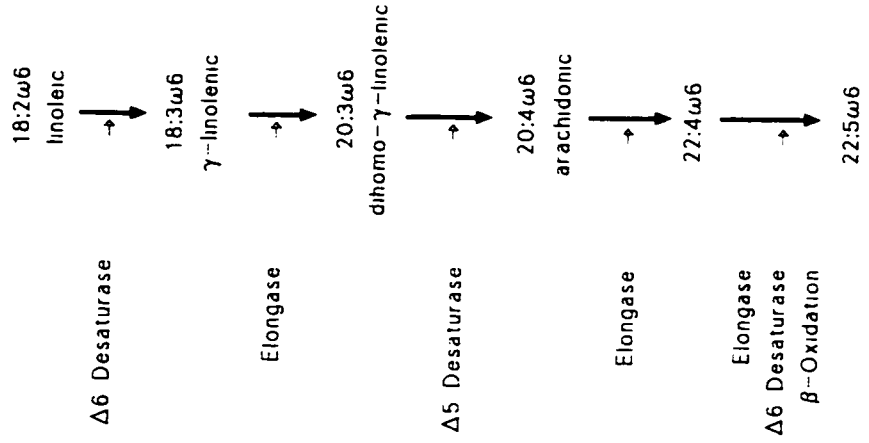
The effects of diets containing blackcurrant seed oil (principally rich in ALA, 18:3 ω 3) or corn oil (containing principally ω 6PUFA, but no appreciable amount of ω 3PUFA) on diacyl, alkyacyl and alkenylacyl subclasses of murine peritoneal macrophage phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were

Figure 1. Enzymatic Conversion Of Essential and Nonessential Fatty Acids.*

The ω 6 and ω 3 families of essential fatty acids and the ω 9, ω 7 and saturated families of nonessential fatty acids are enzymatically converted from short chain fatty acids to long chain fatty acids by a series of desaturases, elongases and β -oxidation. For the essential fatty acid families, enzymatic conversion commences with Δ 6 desaturase, whereas for the nonessential fatty acids, enzymatic conversion commences with Δ 9 desaturase. Small arrows indicate enzymatic step.

*modified from Lipids (1996) 31: S272.

ESSENTIAL FATTY ACIDS ω 6 Family



NONESSENTIAL FATTY ACIDS Saturated Family ω 7 Family

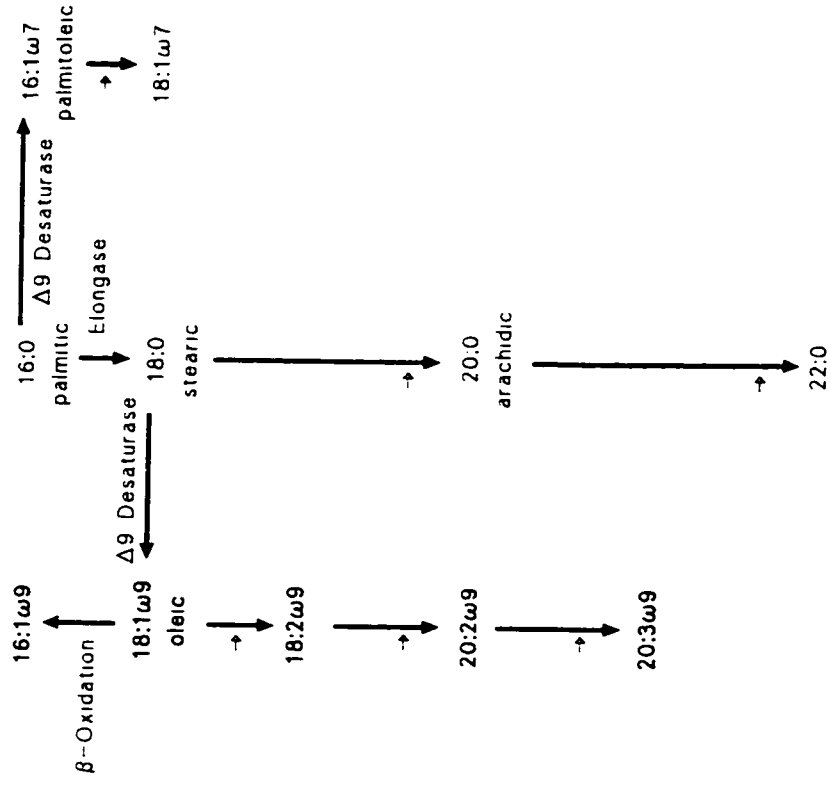
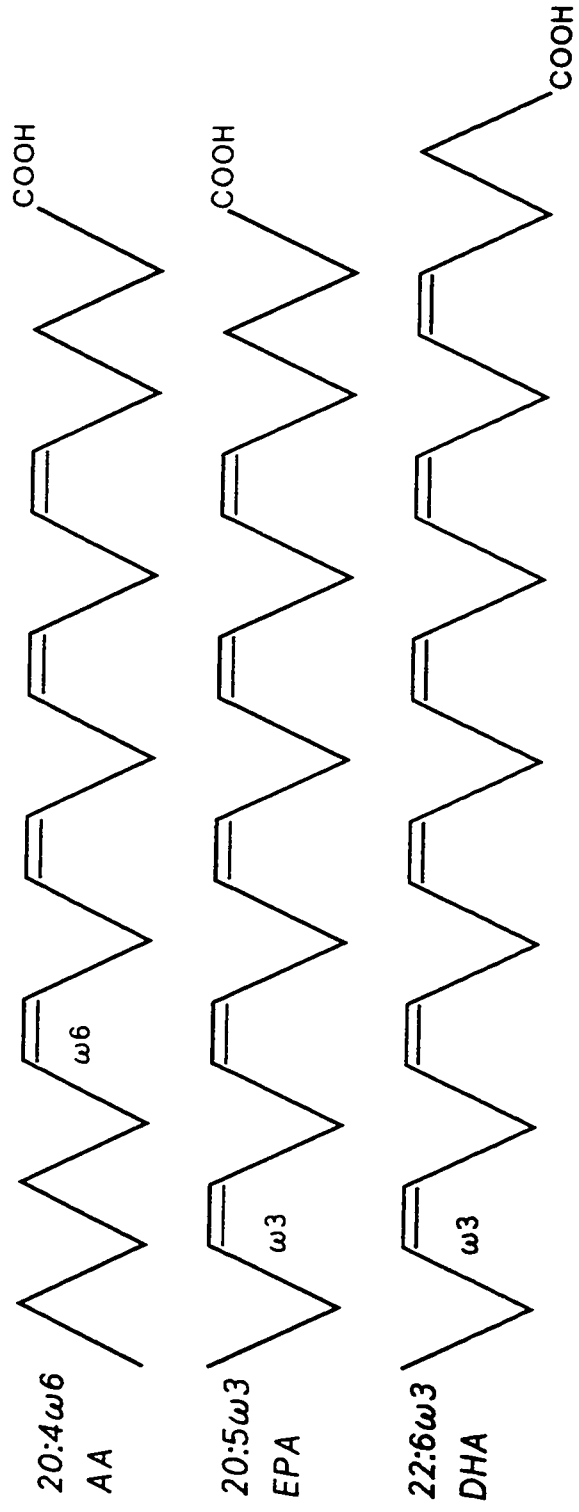


Figure 2. The Structure of Polyunsaturated Fatty Acids.

The identification of a fatty acid (x:y&z) is given by the number of carbons in the fatty acid chain (x) followed by the number of double bonds (y) and the location of the first one from the terminal methyl carbon (z). Arachidonic acid (AA, 20:4&6) contains 20 carbons with 4 double bonds, eicosapentaenoic acid (EPA, 20:5&3) contains 20 carbons with 5 double bonds and docosahexaenoic acid (DHA, 22:6&3) contains 22 carbons with 6 double bonds.



studied in mice fed diets for four weeks (Chapkin and Charmichael 1990). PC and PE subclass composition showed significant increases in macrophage membrane docosapentaenoic acid (DPA, 22:5 ω 3) and DHA in the blackcurrant compared to corn oil groups. PE subclass content of AA was significantly reduced and replaced by 20:3 ω 6 and 22:5 ω 3, whereas in PC, AA replacement by ω 3PUFA derived from 18:3 ω 3 precursor did not occur.

Clearly EPA, DHA and ALA share similarities to some degree in their effect on altering eicosanoid production and membrane PL fatty acid composition, but the mechanisms by which they alter disease activity may be entirely distinct.

1.2 Membrane Phospholipids

The cell membrane has a highly heterogeneous composition, consisting principally of several classes, subclasses and molecular species of PL, cholesterol and diverse proteins. The distribution of amphipathic lipids throughout the membrane, or those lipids composed of both a polar group, such as phosphate, amine or alcohol, and a non-polar group, is defined by the physiological and pharmacological state of the cell.

In aqueous conditions, PL organize into a lipid membrane bilayer structure in which the polar portion of each molecule is oriented towards the aqueous phase and the nonpolar portion of each molecule is oriented inwards towards the

membrane, as first proposed by Danielli and Davson (1935). This model was later redefined as a fluid mosaic structure where globular protein molecules are variously distributed within, across and associated with the phospholipid bilayer (Singer and Nicolson 1972).

Membrane PL are composed of three side chains linked to a glycerol backbone. In general, two hydrophobic fatty acid radyl side chains are linked at positions *sn*-1 and *sn*-2 and a phosphate-linked alcohol group occupies position *sn*-3. At the *sn*-1 position of the PL in many cell types, including murine peritoneal macrophages, saturated fatty acids (principally 16:0 and 18:0) constitute as much as 98% of the substituents (Blank et al 1994, Sugiura et al 1983), whereas the *sn*-2 position is almost exclusively occupied by polyunsaturated fatty acids (two or more double bonds) in ester (acyl) linkage (Akoh and Chapkin 1990, Chapkin et al 1988 and 1991, Sugiura et al 1983). At the *sn*-3 position, the alcohols linked to the phosphate group are principally choline, serine, ethanolamine, glycerol or inositol.

PL are grouped by classes, which are defined by the moiety at *sn*-3, so that the principal PL classes are PC, phosphatidylserine (PS), PE and phosphatidylinositol (PI), respectively. These PL classes appear to serve different functions, which have only been partially defined to date. Depending on the cell type, zwitterionic PC forms approximately 30-45 mol% of the total membrane PL and functions to maintain the structural integrity of the membrane as well as to participate in receptor-mediated phospholipase formation of second messengers (Sugiura et al 1983). Zwitterionic PE forms approximately 25-30 mol% of the total

membrane PL and is involved in maintaining the structural integrity of the membrane along with PC (Sugiura et al 1983). Furthermore, PE can be converted to PC. Anionic PS forms approximately 10-20 mol% of the membrane PL and is necessary for the activation of several isoforms of protein kinase C (Sugiura et al 1983). Anionic PI constitutes only approximately 3-5 mol% of the total membrane PL but is highly metabolically active and involved in signal transduction, initially via phosphorylation to PIP and PIP₂ (Hofmann and Majerus, Sugiura et al 1983, 1982, Connolly et al 1986).

PL classes are further subdivided into subclasses based on the nature of the linkage at the *sn*-1 position. 1-acyl, 1-alkyl and 1-alk-1'-enyl linkages occur in different proportions in each PL class. For example, PI consists almost entirely of 1-acyl subclass while PC and PE contain significant amounts of 1-alkyl and 1-alkenyl subclasses, respectively.

Even PL subclasses are not single compounds but families of molecular species. These species are defined by the specific pair of fatty acids that are linked at *sn*-1 and *sn*-2. For example, PI in many cell types (Sugiura et al 1983, Akoh and Chapkin 1990) consists to a large extent of the 18:0-20:4 species, while other PL classes are more heterogeneous. Little is known about the biological significance of the different molecular species, but it is interesting that PI and PC, which are involved in cascades of intracellular signal transduction (see below) are relatively enriched in AA at the *sn*-2 position.

1.2.1 Membrane Fluidity

Within a PL molecule there are gradients of increasing fluidity from the glycerol backbone outward. The fluidity of a membrane PL is dependent on two components, the fatty acid side chains and the phosphate-linked alcohol group. The fluidity of each fatty acid moiety increases with increasing distance from carbon 1 (C1). For example, in a 20-carbon fatty acid, the fluidity of the C1 - C5 portion is less than the fluidity between C10 - C20. Similarly, there is a gradient of fluidity between the glycerol backbone and the phosphate-linked alcohol group at *sn*-3, with greater fluidity as the distance from the glycerol backbone increases. Membrane fluidity can be modified by several factors, such as fatty acid chain length and number of double bonds (Erand 1985, Slater et al 1994). Changes in PL fluidity may lead to changes in important regulatory events in cell signalling because of conformational changes and accessibility of membrane protein, enzymes and ion channels.

1.2.2 Arachidonic Acid In Membrane Phospholipids

The replacement of AA by EPA and DHA at the *sn*-2 position of membrane PL can be induced in a number of cell types including macrophages (Chapkin and Carmichael 1990, Marignani and Sebaldt 1995), neutrophils (Chilton et al 1993),

splenocytes (Huang et al 1986), platelets (Galloway et al 1985, von Schacky et al 1985), adipocytes (Raclot and Groscolas 1994) and hepatocytes (Huang et al 1986, Chapkin et al 1992).

In mammalian studies, there is competition between ω 3PUFA and AA for the esterase responsible for fatty acid incorporation into cellular membrane PL (Ahmed and Holub 1984, Maehr et al 1994). In unstimulated macrophages, ω 3PUFA compete with free AA for acylation into membrane PL but do not interfere with reacylation of phospholipase A₂ (PLA₂)-hydrolyzed AA after stimulation of macrophages with PMA, even though reacylation of PLA₂-hydrolysed EPA or DHA may occur (Lokesh and Kinsella 1994). It is possible that these and other enzymes are selective towards AA (Galli et al 1993, Lokesh and Kinsella 1994, Walsh et al 1994), so that phospholipase-catalyzed hydrolysis of EPA and DHA from the *sn*-2 position of membrane PL may differ from that of AA. Furthermore, ω 3PUFA are more avidly retained in membrane PL due to their slower rate of turnover that results from reduced mitochondrial β -oxidation rates, reduced mobilization rates and elevated acylation/reacylation rates (Galli et al 1993, Raclot and Groscolas 1994).

1.2.3 Membrane Lipids in Signal Transduction

1.2.3.1 Diradylglycerol

Receptor-mediated G protein-coupled phospholipase hydrolysis of PL results in the formation of second messengers that participate in cellular activation and differentiation (Figure 3). The second messengers, *sn*-1,2-DG and inositol 1,4,5-trisphosphate (IP_3), derived by PLC-catalyzed hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2), are involved in the activation of several isoforms of protein kinase C (PKC) and the mobilization of intracellular calcium (Ca^{2+}), respectively (Exton 1988, Löffelholz 1989, Stabel and Parker 1991, Dennis et al 1992). DG formation can occur from precursors other than PI, for example, the hydrolysis of PC (Uhing et al 1989, Stabel and Parker 1991) or may occur indirectly, via a two-step process beginning with phospholipase D (PLD)-catalyzed hydrolysis of PC to phosphatidic acid (PA) and free choline (Exton 1988, Löffelholz 1989, Uhing et al 1989, Stabel and Parker 1991, Dennis et al 1992). PA is hydrolyzed to DG by phosphatidic acid phosphohydrolase (Dennis et al 1991).

1.2.3.2 Diradylglycerol Molecular Species

Like each PL class, DG is a family of molecular species defined by the pair of fatty acid substituents at the *sn*-1 and *sn*-2 positions (Figure 4). There are

approximately 30 relatively common molecular species of DG found in mammalian cells. A variety of cell types form DG biphasically in response to stimulation by various agonists (Honeycutt and Niedel 1986, Dougherty et al 1989, Sebaldt et al 1992). After stimulation of murine peritoneal macrophages either by a receptor-mediated G protein-coupled mechanism with platelet-activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, PAF) or by elevation of intracellular calcium with the divalent cation ionophore, ionomycin, the molecular species profile of early-phase DG resembles that of basal DG and that of PI, while the profile of late-phase DG more closely resembles that of PC (Sebaldt et al 1992).

Most analytical methods of lipid analysis that separate PL from neutral lipids are not adequate to resolve molecular species of PL or DG from one another. At most, thin layer chromatography (TLC) band broadening or reverse phase-high pressure liquid chromatography (RP-HPLC) peak smearing is seen to occur. Specific methods have been developed to resolve molecular species based on their differences in chain length, double bond count and *sn*-1 linkage. Argentation TLC was pioneered by Renkonen and resolves a derivative of DG into bands corresponding to the total number of double bonds in the two fatty acid residues (Renkonen 1968, Marignani and Sebaldt 1995). Reverse-phase HPLC of a specialized derivative of DG, dimethyl-phosphatidic acid, is an alternative method that fractionates species into a different pattern (Kennerly 1986, Sebaldt et al 1992).

Hui et al (1992) have studied the effect of altering the cell membrane PL of vascular smooth muscle cells *in vitro* by the addition of EPA, linoleic acid (18:2 ω 6)

or AA to culture media on the formation of DG by arginine vasopressin stimulation. EPA treatment reduced but linoleic acid increased the proportion of AA in membrane PL. Complete abolition of DG formation was reported to occur in the EPA group.

Dietary supplementation with ω 3PUFA has proven to be an effective *in vivo* method to alter total DG mass in unstimulated and concanavalin A (ConA)-stimulated splenocytes from mice fed diets containing purified EPA ethyl esters or DHA ethyl esters for three weeks. Both EPA and DHA were incorporated into splenocyte DG, but total DG mass was reduced more in the EPA ethyl ester group (Fowler et al 1993b).

Alteration of the fatty acid moieties of membrane PL either *in vitro* or *in vivo* provide an opportunity to study both the structural and functional effects of ω 3PUFA. By controlling the ratio of ω 3PUFA to other, more common fatty acid constituents of membrane PL, a better understanding of the cellular and molecular mechanisms by which ω 3PUFA alter disease activity may eventually be achieved.

Figure 3. Receptor-Mediated Signal Transduction.

The membrane phospholipid molecule consists of three components: R1, the first fatty acid located in the *sn*-1 position, R2, the second fatty acid located in the *sn*-2 position and P-X, the alcohol head group located in the *sn*-3 position. Lipase activity produces a series of second messengers. Cytosolic phospholipase A₂ (cPLA₂) activity liberates the fatty acid at the *sn*-2 position, mainly AA which is the precursor for eicosanoids, prostaglandins (PG), thromboxanes (Tx) and leukotrienes (LT). Phospholipase C_β (PLC_β) activity hydrolyses the phosphodiester bond between the phosphorylated alcohol group and the glycerol back bone, forming diradylglycerol (DG). Phospholipase D (PLD) activity hydrolyzes the bond between the alcohol group and the phosphate group, liberating phosphatidic acid (PA). AA, DG and PA are known activators of protein kinase C (PKC).

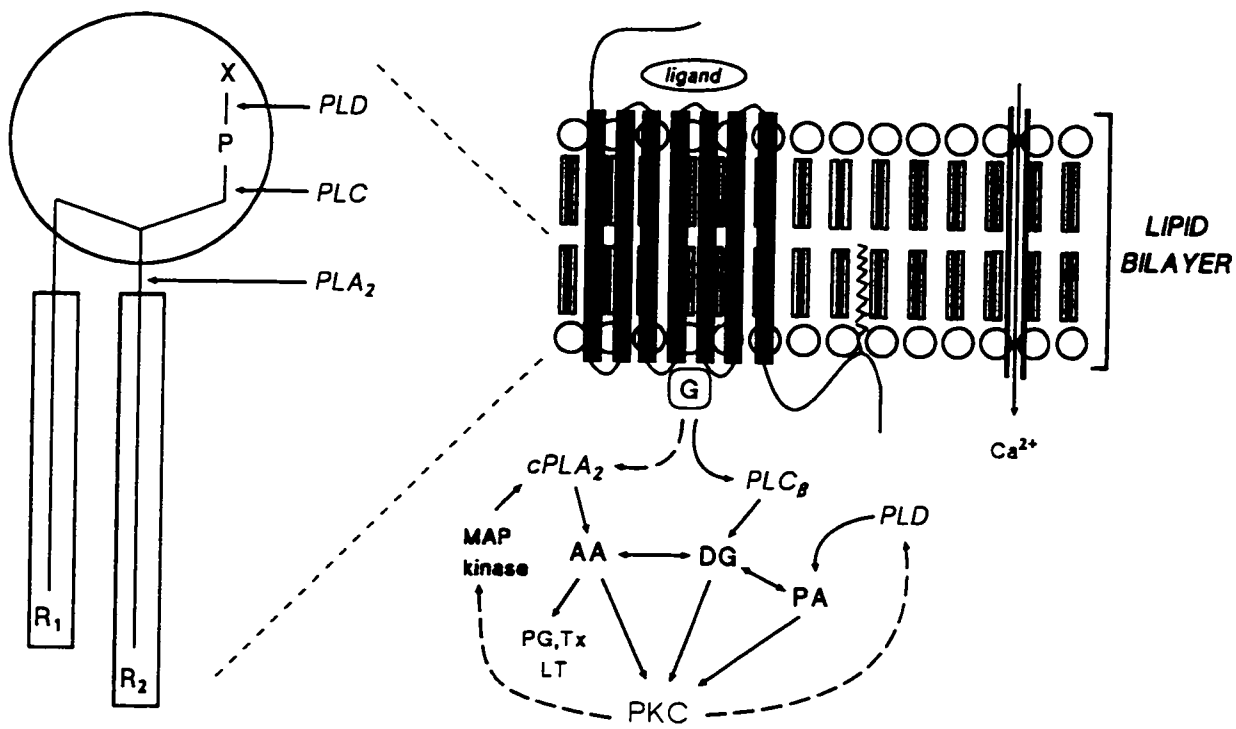
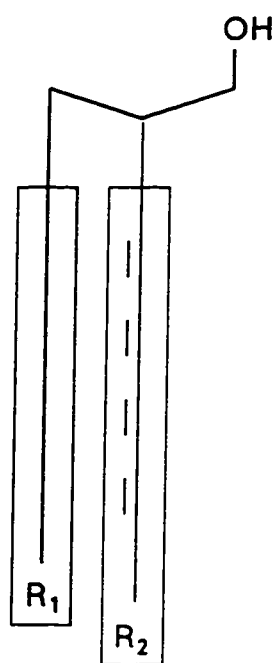
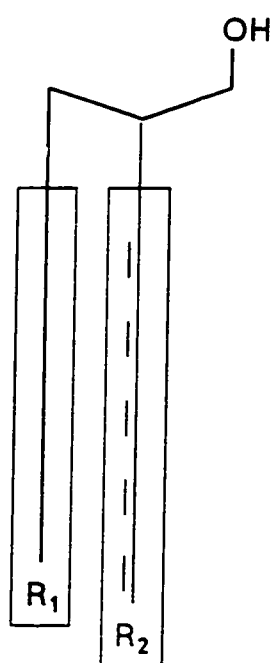


Figure 4. Diradylglycerol Molecular Species.

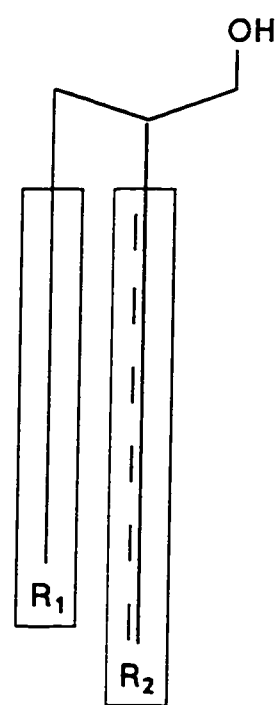
The diradylglycerol molecular species 18:0-20:4 ω 6 (1-stearoyl-2-archidonoyl-*sn*-glycerol, SAG), 18:0-20:5 ω 3 (1-stearoyl-2-eicosapentaenoyl-*sn*-glycerol, SEG) or 18:0-22:6 ω 3 (1-stearoyl-2-docosahexaenoyl-*sn*-glycerol, SDG). Small lines within the *sn*-2 substituted fatty acid represent double bonds.



$18:0-20:4\omega 6$



$18:0-20:5\omega 3$



$18:0-22:6\omega 3$

1.2.3.3 Protein Kinase C

The function of DG is to activate PKC, a principal enzyme involved in the transduction of a large number of signals committed to cellular growth and differentiation (Inoue et al 1977). PKC is a family of serine/threonine-specific protein kinases first discovered by Nishizuka and coworkers (Inoue et al 1977). There are currently twelve known members of the PKC family, which are subdivided into three groups based on their biochemical and structural properties. All isoforms share a similar requirement for activation, namely their insertion into membrane regions high in anionic PS (Burns et al 1990, Senisterra and Epand 1993, Newton 1995). The originally characterized isoforms are the conventional or classical isoforms (cPKC) α , β I, β II and γ , which are grouped together because of their calcium dependence and a requirement for anionic lipids, DG or phorbol esters for activation (Castagna et al 1982, Nishizuka 1986, Bell and Burns 1991). The novel isoforms (nPKC) δ , ϵ , η , θ and μ , are calcium-independent and are activated by DG or phorbol esters (Leibersperger et al 1990, Liyanage et al 1992, Schaap and Parker 1990, Koide et al 1992, Johannes et al 1994, Nishizuka 1995). The atypical isoforms (aPKC) ζ , λ and ι , are calcium-independent, insensitive to DG or phorbol esters and can be activated by free fatty acids (Nishizuka 1995).

1.2.3.4 The Structure of Protein Kinase C

The amino acid structure of PKC derived from cloned cDNA (Figure 5) has four conserved (C1-C4) and five variable (V1-V5) regions with a C-terminal catalytic domain (45 kDa) and an N-terminal regulatory domain (20-40 kDa) (Newton 1995). Separating the regulatory domain from the catalytic domain is the hinge region (V3). At the N-terminus, there is a pseudosubstrate region that is removed by proteolysis for the enzyme to become activated. The pseudosubstrate is immediately followed by the C1 region, which is rich in cysteine and is required for DG/phorbol ester binding site. The C2 region includes recognition binding sites for calcium and acidic lipids. The C3 region contains the ATP binding motif, and the final conserved region, C4, contains both the phosphate transfer region and the substrate binding site. Both C3 and C4 are highly conserved between cPKC, nPKC and aPKC (Stabel and Parker 1991, Newton 1995).

1.2.3.5 Diacylglycerol Activation of Protein Kinase C

DG, one of the first known second messengers to activate PKC (Nishizuka 1988), functions by increasing the affinity of PKC for the membrane (Stabel and Parker 1991, Mosior and Epand 1993). Activation of PKC is highly specific for *sn*-1,2-DG, as both *sn*-1,3-DG and *sn*-2,3-DG are incapable of activating PKC (Young

and Rando 1984, Nomura et al 1986). Temporal and structural changes in the formation of DG could potentially alter the enzymatic activity of PKC.

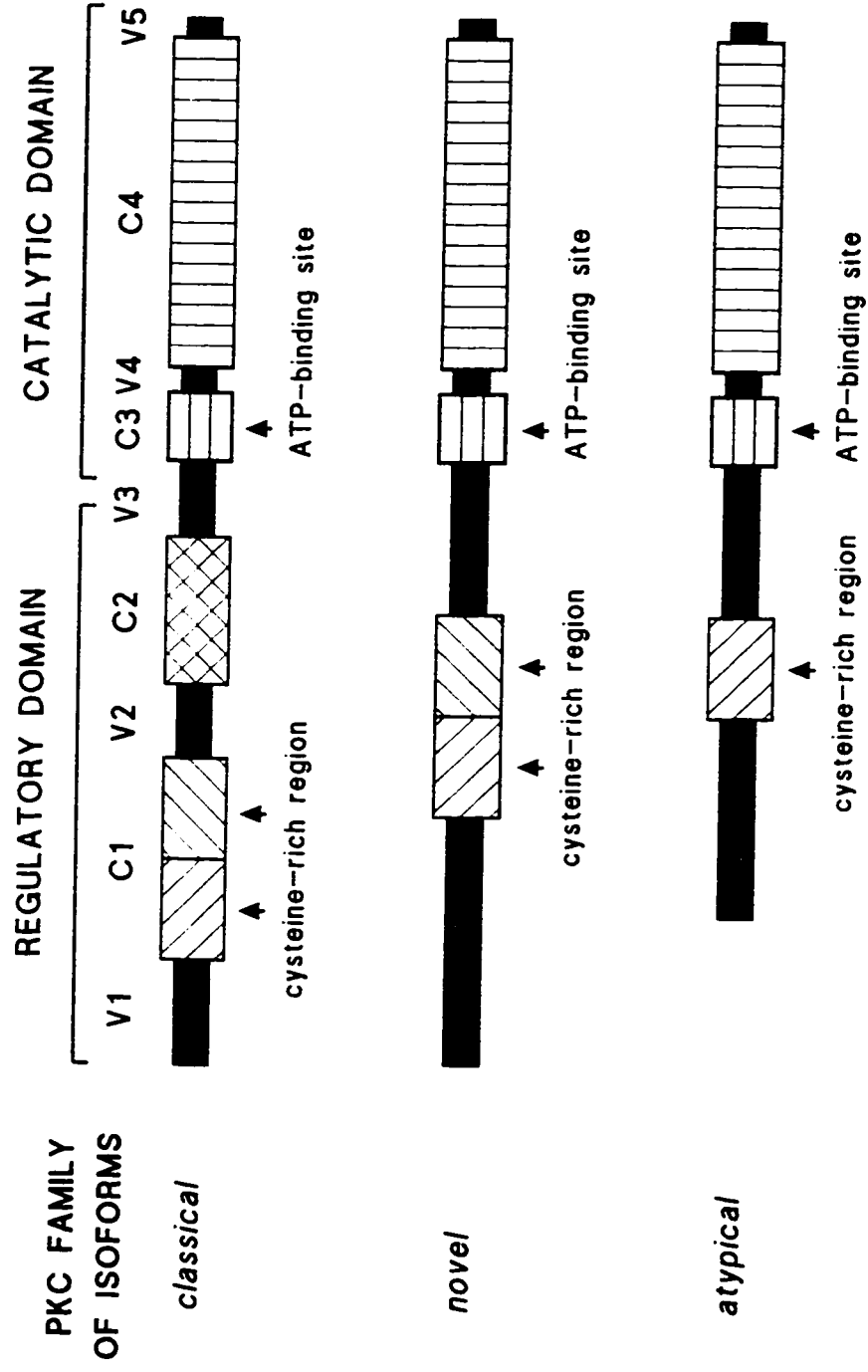
Several researchers have studied the effects of free fatty acids on PKC activation *in vitro*. Both saturated and *trans*-unsaturated free fatty acids are incapable of activating cPKC while *cis*-unsaturated fatty acids such as oleic, linoleic, AA, ALA, EPA, DHA, dotriacontatetraenoic (32:4 ω 6) and tetratriacontahexaenoic (34:6 ω 3) acids greatly enhance the phosphorylating capacity of cPKC *in vitro* (Szamel et al 1989, Lester et al 1991, Shinomura et al 1991, Hardy et al 1994).

These studies provide partial evidence that free fatty acids, including those of the ω 3-type, have the potential to modulate the PKC signalling pathway. This may be of particular relevance for studies where the consequences of cell membrane PL incorporation of ω 3PUFA on DG formation and the subsequent alterations in PKC activity by structurally modified DG are explored.

Figure 5. Structure of Protein Kinase C Isoform Families*.

The classical family of PKC consists of α , β I, β II and γ isoforms. The novel family consists of δ , ϵ , η , θ and μ isoforms. The atypical family consists of ζ , λ and ι isoforms. In the classical family there are four conserved regions (C1 - C4) and five variable regions (V1 - V5) and the C2 region is associated with calcium binding. The other families have variations of these regions. The catalytic domain is conserved in all isoforms.

*modified from J. Biol Chem. 270: 28495.



1.3 Mononuclear Phagocytes

Mammalian mononuclear phagocytes arise from bone marrow myeloid progenitor cells (Cotran et al 1989), travel through the circulatory system as monocytes and migrate to reside as tissue macrophages (histiocytes) into tissue compartments such as the peritoneal cavity (peritoneal macrophages), the liver (Kupffer cells), the skin (Langerhans cells), the brain (microglial cells), the kidney (mesangial cells), the pleural cavity (alveolar macrophages) and sites of inflammation (Adams 1979, Cotran et al 1989).

The ubiquitous nature of macrophages is perhaps one of the most characteristic attributes of this multifunctional cell, that is, macrophages can both wreak havoc or can take on a curative role. Which role is played at any time depends on the nature of the injury and the conditions or milieu surrounding the affected tissue or organ system.

1.3.1 Macrophages

Macrophages are characterized by their chemotactic and phagocytic competence at several levels of host defense. Chemotaxis, derived from the Greek word *chemeia* or "alchemy" plus *taxis*, or "arrangement", is the movement towards or away from a chemical stimulus. Phagocytosis, derived from the Greek word

phagein, or "to eat", is the process by which macrophages ingest and dispose of foreign materials (Cotran et al 1989). Macrophages have specific surface receptors that are necessary for host defense against microorganisms, antitumorigenic actions through cytotoxic mechanisms, T-cell mediated immune responses and secretion of a myriad of inflammatory mediators (Alexander 1976, Currie 1976, Karnovsky and Lasdin 1978, North 1978, Adams 1979, Koerner et al 1987, Gosset et al 1992, Glaser et al 1993, Sperber 1994, Hughes et al 1995).

The pathology of chronic inflammation is determined by the nature of the pathogenic insult, the extent of tissue injury and the immune response mounted by the host. The histology of chronic inflammation is characterized by numerous macrophages, lymphocytes, eosinophils and plasma cells at the site of inflammation (Cotran et al 1989). Macrophages play a key role in the elimination of the pathogenic agent from the site of injury as well as in restoring the integrity of the damaged tissue to baseline functional capacity. Since macrophages are not constitutively activated, their migration to sites of injury is governed by chemoattractants released from the area of inflammation. IFN- γ and LPS are chemoattractants released from injured cells and from invading bacteria, respectively (Introna et al 1986).

1.3.2 Stimulation of Macrophages

Under controlled experimental conditions, cell surface receptor-mediated G protein-coupled PL hydrolysis in macrophages forms second messengers, IP_3 and DG, that are involved in calcium mobilization and PKC activation (section 3.3). These latter events can be reproduced under experimental conditions in biological systems through the use of physiological and non-physiological agonists.

1.3.2.1 Platelet Activating Factor

Biological activation of leukocytes, monocytes and macrophages through a receptor-mediated G protein-coupled mechanism with 1-ether linked PL, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (PAF), results in the formation of inositol phosphates (Camussi et al 1981, Bachelet et al 1986, Prescott et al 1990). PAF is not constitutively found in cells, but its production can be induced through *de novo* synthesis from acetyl-CoA or through remodeling of membrane lyso-PAF upon stimulation. Physiological activation of this receptor-mediated event can be replicated in murine peritoneal macrophages by the addition of PAF to culture media (Prpic et al 1988). Macrophages incubated with PAF for up to 120 s formed several inositol phosphates as well as elevated levels of DG (Sebaldt et al 1992) and intracellular calcium (Prpic et al 1988), both essential components in the activation of PKC.

1.3.2.2 Tumor Promoting Phorbol Ester

In response to cellular mitogens such as the tumor-promoting phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate (PMA, also referred to as TPA) (Castagna et al 1982, Sebaldt et al 1992) and the divalent cation ionophore, ionomycin (Sebaldt et al 1992), post-receptor-mediated events occur which lead to the formation of DG, and these events can be studied *in vitro*.

Nishizuka and colleagues (Kikkawa et al 1989, Nakamura and Nishizuka 1994) suggested that the activation of PKC by phorbol esters may be involved in late-phase production of DG by PC-specific PLC or PLD hydrolysis of PC (section 1.2.3). The experimental use of phorbol esters mimics a sustained action of DAG whereas under physiological conditions, DAG acts only transiently. Moreover, the experimental use of PMA allows for the quantification of DG derived from PLD hydrolysis of PC (Daniel et al 1986). Castagna and colleagues (1982) were the first to show that PMA could replace DAG as an activator of calcium-dependent PKC *in vitro*. They showed that PMA significantly increased the affinity of PKC for Ca^{2+} and PL, suggesting that the phorbol ester and DAG binding sites on PKC are one and the same.

1.3.2.3 Calcium Ionophore

Intracellular Ca^{2+} signalling is partially regulated by IP_3 that functions to mobilize Ca^{2+} from internal stores (Berridge and Irvine 1989). One of the physiological functions of calcium is to control the binding affinity of cPKC (section 1.2.3.3) for membrane PL (Mosior and Epand 1994). Intracellular Ca^{2+} concentrations can be increased by mobilizing extracellular Ca^{2+} with the Ca^{2+} ionophore, ionomycin. The effects of Ca^{2+} on formation of DG molecular species, for example, can be determined *in vitro* by mimicking the effects of IP_3 with the use of Ca^{2+} ionophores. Sebaldt and colleagues (1992) have shown that ionomycin stimulates the formation of DG in murine peritoneal macrophages. Similarly, several other researchers have described increases in DAG formation with the use of ionomycin in canine epithelial cells (Peterson and Walter 1992), bovine parathyroid cells (Shoback et al 1988) and human fibroblasts (Blitterswijk et al 1991). Furthermore, ionomycin has been found to activate PC-specific PLC through a PKC-dependent pathway in murine peritoneal macrophages (Uhing et al 1989).

1.3.2.4 Lipopolysaccharide

LPS is a member of the endotoxin family of toxins derived from the cell walls of gram-negative and some gram-positive bacteria (Morrison and Ulevitch 1978). The mechanism by which LPS activates macrophages is not entirely understood. However, it is known that the activation of macrophages requires the binding of LPS to an LPS-binding protein that circulates in serum (Wright et al 1990). This complex then binds to CD14, a phosphoinositol-glycan-linked membrane protein (Morrison and Ryan 1987, Tobias et al 1993). Cytokines such as IL-1 and TNF- α are capable of enhancing expression of CD14 while interleukin-4 (IL-4) attenuates expression (Ziegler-Heitbrock and Ulevitch 1993). CD11 and CD18 are additional cell surface binding proteins suggested to be involved in LPS stimulation (Wright et al 1990).

1.3.2.5 Cytokines

The immune system is the primary source of cytokines, proteins that act both as mediators of disease as well as mediators responsible for defense against disease (Mizel 1989). For this reason, the expression and regulation of cytokines are tightly controlled (Adams 1989). In general, individual cytokines can be secreted from a variety of cells and are pleiotropic, capable of eliciting different responses in different cells and tissues.

1.3.2.6 Interleukin-6

Interleukin-6 (IL-6), a multipotent cytokine with a molecular mass ranging between 19-32 kDa and previously known as β 2-interferon, B-cell stimulating factor 2 and hepatocyte stimulating factor, is produced by a number of cell types including macrophages/monocytes (Aarden et al 1987), fibroblasts (Weissenbach et al 1980), endothelial cells (Corbel and Melchers 1984), mast cells (Plaut et al 1989), T and B lymphocytes (Hirano et al 1988) and rat peritoneal mast cells (Nordan 1986, Leal-Berumen et al 1995). IL-6 is a potent mediator in the production of acute phase proteins from hepatocytes in response to tissue injury or inflammation (Heinrich et al 1990). IL-6 has been isolated from a variety of tumor cell lines and human carcinomas (Erroi et al 1989, Evans et al 1991, 1992, Utsumi et al 1990, Strassman et al 1992).

The multiple molecular mechanisms underlying the synthesis and expression of IL-6 can be invoked by cytokines such as interleukin-1 (IL-1) (Shalaby et al 1989) and TNF- α (Van Damme et al 1987). IL-6 expression also occurs in response to viral infections (Sehgal et al 1988), AA-derived eicosanoids prostaglandin E₁ (PGE₁) and PGE₂ (Leal-Berumen et al 1995), LPS (Nordan 1986) and the priming of cells with IFN- γ followed by TNF- α (Shalaby et al 1989).

CHAPTER TWO

CHAPTER TWO: OBJECTIVES

2.0 OBJECTIVES

The general objective of this research was to investigate changes in the formation of diacylglycerol (DG) molecular species and in interleukin-6 (IL-6) secretion in murine peritoneal macrophages as a result of the incorporation of ω 3-polyunsaturated fatty acids (ω 3PUFA) into membrane phospholipids. The effects on protein kinase C (PKC) activation by pure species of diacylglycerol with specific PUFA at the *sn*-2 position were also investigated.

Specific Objective I

The initial specific objective was to determine whether dietary supplementation with concentrates of ethyl esters of mixed ω 3PUFA, purified eicosapentaenoic acid and/or purified docosahexaenoic acid at various concentrations alters the formation of DG in murine peritoneal macrophages *in vivo* (Chapters Three, Four and Five).

Specific Objective II

The specific objective was to develop an *in vitro* model that could reproduce the dietary-induced alterations in DG and PL molecular species observed *in vivo*. (Chapter Six).

Specific Objective III

The specific objective was to demonstrate a correlation between interleukin-6 (IL-6) secretion in murine peritoneal macrophages and the incorporation of eicosapentaenoic and docosahexaenoic acids into DG molecular species by *in vitro* (Chapter Seven).

Specific Objective IV

The specific objective was to compare the activation of PKC *in vitro* by purified molecular species of DAG with different PUFA at the *sn*-2 position (Chapter Eight).

CHAPTER THREE

PUBLISHED MANUSCRIPT

Marignani, P. A. and Sebaldt, R. J. (1995) Formation Of Second Messenger Diacylglycerol In Murine Peritoneal Macrophages Is Altered After In Vivo (n-3) Polyunsaturated Fatty Acid Supplementation. *Journal of Nutrition* 125:3030-3040.

PREAMBLE

The purpose of these experiments was to address the hypothesis that ω 3PUFA have biological functions in addition to the known suppression of formation of AA-derived eicosanoids. I did this by determining the effects of prolonged dietary supplementation with ω 3PUFA concentrate *in vivo* on the formation of molecular species of DG and membrane PL in murine peritoneal macrophages.



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10 August 1996.

The Journal of Nutrition
Dr. Willard J. Visek
University of Illinois, College of Medicine
190 Medical Sciences Building
306 South Mathews, Urbana, Illinois USA 61801

Dear Dr. Willard J. Visek

I am completing a PhD thesis at McMaster University entitled *The Effects Of n-3 Polyunsaturated Fatty Acids On The Formation Of n-1,3-Diacylglycerol, Cytokine Secretion And On The Regulation Protein Kinase C Activity*. I would like your permission to reprint the following journal article in my thesis.

Mariignani PA and Sebaldt RJ. (1995) Formation of second messenger diacylglycerol in murine peritoneal macrophages is altered after in vivo (n-3) polyunsaturated fatty acid supplementation. *J. Nutr.* 125: 3030-3040.

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3.1 ABSTRACT

The consequences of macrophage membrane incorporation of (n-3) polyunsaturated fatty acids ((n-3)PUFA) on diradylglycerol (DG) formation and phospholipase-mediated signal transduction in vivo remain largely unexplored. Three- to four-week old C57BL/6 mice were randomly assigned to diets in which the 10% lipid component of the purified diet was either corn oil ethyl ester (control) or (n-3) ethyl ester ((n-3)PUFA-enriched). After four weeks, thioglycollate-elicited peritoneal macrophages were harvested and assayed for: (i) total DG mass, (ii) DG molecular species fraction analyses by argentation TLC and (iii) separation of phospholipid classes and analyses of their molecular species fractions. We found that basal DG mass and the increase in DG mass after stimulation with platelet activating factor or phorbol myristate acetate were similar in control and (n-3)PUFA-enriched diet groups, whereas ionomycin-stimulated DG formation was less in the (n-3)PUFA-enriched diet group. Analyses of DG molecular species fractions showed that the proportions of species with five and six double bonds were markedly greater in the (n-3)PUFA-enriched diet group compared to the control diet group. The molecular species fractions of all phospholipid classes including phosphatidylinositol showed substantial incorporation of (n-3)PUFA. These results demonstrate that dietary enrichment with (n-3)PUFA induces marked changes in DG metabolism in murine peritoneal macrophages. These changes may contribute to the mechanisms of antiinflammatory, antithrombotic and antineoplastic actions associated with dietary (n-3)PUFA supplementation.

3.2 INTRODUCTION

The antiinflammatory, antithrombotic and antineoplastic effects of dietary supplementation with (n-3)polyunsaturated fatty acids ((n-3)PUFA)³ (eicosapentaenoic acid (EPA, [20:5n-3]) and docosahexaenoic acid (DHA, [22:6n-3])) have been widely studied for several years. A large body of research that provides insight into potential mechanisms of (n-3)PUFA action has been generated (Chan et al. 1993, Hargreaves and Clandinin 1987, Huang et al. 1986, Kremer et al. 1985, Whelan et al. 1991). A mechanism that is capable of satisfactorily explaining the effects of (n-3)PUFA on cell growth and differentiation has yet to be discovered.

The hypothesis most commonly used to explain many of the actions of (n-3)PUFA is that these highly unsaturated fatty acids reduce cellular membrane content of arachidonate (AA, [20:4n-6]) (Galloway et al. 1985, Lokesh and Kinsella 1987). A great deal of the literature has focused on the consequent attenuation of formation of eicosanoids derived from the hydrolysis of AA by lipoxygenases and cyclooxygenase (leukotriene C₄, leukotriene B₄, thromboxane B₂, prostacyclin and prostaglandin E₂) (Croft et al. 1987, Needleman et al. 1979, Whelan et al. 1991). However, other actions of (n-3)PUFA have been observed whose mechanisms are less well understood. These include the suppression of cytokine formation (tumor necrosis factor- α , interleukin-1 and interleukin-6) (Endres et al. 1989, Hardardottir and Kinsella 1992), modulation of macrophage adhesion and phagocytosis and lymphocyte interleukin-2-mediated proliferation (Calder and Newsholme 1992),

inhibition of natural killer cell activity (Yaqoob et al. 1994), changes in platelet function (Ahmed and Holub 1984, Bénistant et al. 1993) and antitumorigenic effects (Hardardottir and Kinsella 1992, Hubbard et al. 1994).

Recently, we have shown that the biosynthesis of diradylglycerol (DG), the physiological activator of protein kinase C (PKC) in murine peritoneal macrophages, is substantially altered after in vitro supplementation of culture media with EPA or DHA (Sebaldt and Marignani 1995). Total DG mass was significantly increased by EPA and even more potently by DHA in both basal and stimulated macrophages. Furthermore, the distribution of molecular species was altered and reflected substantial incorporation of EPA or DHA, largely at the expense of AA. These results reveal that (n-3)PUFA, in addition to their effects on eicosanoid biosynthesis, have the potential to significantly alter a second messenger of intracellular signal transduction, and this may account, at least in part, for some of their less well understood actions.

We report the results of investigations into alterations in total DG mass and DG molecular species composition as well as in membrane phospholipids that are induced in vivo in peritoneal macrophages of C57BL/6 mice after 4 wk of dietary enrichment with (n-3)PUFA.

3.3 MATERIALS AND METHODS

3.3.1 Materials

All solvents were from Caledon (Georgetown, ON, Canada). *sn*-1,2-diacylglycerol kinase (DAG kinase), ionomycin and phorbol myristate acetate (PMA) were from Calbiochem (La Jolla, CA). Diethylene triamine pentaacetic acid (DETAPAC), imidazole, ATP, DL-dithiothreitol (DTT) and phospholipase C (#7147) were from Sigma (St. Louis, MO). Diazald was from Aldrich (Milwaukee, WI). RPMI 1640 media without L-glutamine, fetal calf serum, penicillin, streptomycin and Hanks balanced salt solution were from Gibco (Grand Island, NY). L-glutamine was purchased from Fisher (Unionville, ON, Canada). Culture dishes were from Corning (Corning, NY) and Falcon (Franklin Lakes, NJ). [γ - 32 P]ATP and [γ - 32 P]H₃PO₄ were from Dupont-NEN (Markham, ON, Canada). Platelet activating factor (PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) and dioleoylglycerol standards were purchased from Avanti Polar Lipids (Alabaster, AL). ACS was from Amersham (Oakville, ON, Canada). The (n-3) ethyl ester concentrate ((n-3)EE) and corn oil ethyl ester concentrate (COEE) were graciously supplied by the NIH/NOAA Fish Oil Test Materials Program (Charleston, SC). Basal mix without 10% oil (TD89047) was purchased from Harlan Teklad Diets (Madison, WI). Its composition was such that addition of 10% lipid reconstituted a complete purified diet that contained casein, 20%, DL-methionine, 0.3%, sucrose, 45%, corn starch, 15%, cellulose, 5%, mineral mix (AIN-76), 3.5%, vitamin mix (AIN-76A), 1%, choline bitartrate, 0.2%.

3.3.2 *Animal Care and Diets*

Pathogen-free 3-4 week old C57BL/6 mice (Charles River Laboratory, NY) were acclimated for 24 h in microisolator cages with access to pre-irradiated rodent laboratory chow (PicoLab, Purina Mills, Richmond, IN) and were given free access to water. Mice were randomly allocated to cages (4 - 5/cage) and individually weighed. Mice were weighed weekly to monitor growth. Cages were assigned to either control or (n-3)PUFA-enriched diet groups (see below). Mice were fed for 4 wk, and diets and feeding jars were changed daily. The diets were prepared by reconstituting basal mix with 10% lipid. COEE contained principally [18:2n-6] (498mg/g), [18:1n-9] (221 mg/g) and [16:0] (98 mg/g), with only 1.0% (n-3) fatty acids ([18:3n-3] (8.6 mg/g)). In (n-3)EE, the (n-3)PUFA content was 85% of total fatty acids, or 778 mg/g, principally EPA (416 mg/g), DHA (237 mg/g) and [18:4n-3] (87 mg/g). To match the increased degree of fatty acid unsaturation in (n-3)PUFA-enriched diets (Shapiro et al. 1994), the vitamin E concentration was tripled from the value of 50 mg/kg used in the control diets by the addition of DL- α -tocopheryl acetate. Feeding jars and all utensils required to prepare the weekly diets were autoclaved prior to use. The diets were prepared in weekly batches under sterile conditions and were stored under nitrogen at -20°C . All animal procedures were approved by the University's Animal Research Ethics Board.

3.3.3 *Macrophage Culture and Stimulation*

Mice were injected intraperitoneally with 1 mL brewer's thioglycollate (DIFCO, Detroit, MI). After three days, elicited macrophages were harvested by peritoneal lavage (80-90% macrophages), plated and enriched for adherent cells by incubation for 2 h (>98% macrophages) as described (Adams 1979). The macrophage monolayers were washed three times with Hanks balanced salt solution containing 10 mmol/L Hepes, pH 7.4 (HHBSS). The washed macrophage monolayers (15×10^6 per 16 cm^2 well and 2×10^6 per 9.62 cm^2 well) were cultured for an additional 16 h in RPMI 1640 media containing 2 mmol/L L-glutamine, 125 units/mL penicillin and 6 mg/L streptomycin at 37°C in a 5% CO_2 /100% H_2O atmosphere. Based on previous work in our laboratory, culture media were not supplemented with decomplexed fetal calf serum to minimize ex vivo loss of incorporated (n-3)PUFA (Marignani and Sebaldt, in preparation). To those wells destined for phospholipid analyses, $3.7 \times 10^7 \text{ Bq/L}$ [^{32}P] H_3PO_4 was added to label the phospholipids. After incubation, the macrophage monolayers were washed three times with HHBSS, left in 2.0 mL HHBSS and placed in a water bath at 37°C . To these wells, 400 μL of one of the following was added (final concentration shown): ionomycin (10 $\mu\text{mol/L}$), PMA (0.1 mg/L), PAF (0.1 $\mu\text{mol/L}$) or HHBSS control, as described (Sebaldt et al. 1990). The timed 10-min stimulations were stopped by the addition of 2 mL methanol. DG mass was quantitated in wells containing 2×10^6 macrophages. Molecular species analyses of DG and phospholipids were performed in wells containing 15×10^6 macrophages. All conditions were repeated

in duplicate or triplicate wells for each experiment.

3.3.4 *Quantitative Analysis of Diradylglycerol*

After termination of stimulation, wells with 2×10^6 macrophages were scraped into 13 x 75 mm screw-top glass tubes, and DG was extracted into chloroform, using methanol and 1.0 mol/L NaCl in the aqueous phase, by the Bligh-Dyer procedure as described (Sebaldt et al. 1992). DG was derivatized to phosphatidic acid (PA) in the presence of DAG kinase and 1.85×10^9 Bq/L [γ - 32 P]ATP and separated from ceramides by TLC. As described (Sebaldt et al. 1992), Silica Gel 60 plates (Analtech, Newark, DE) were preconditioned in acetone and heat-activated immediately prior to use. [32 P]PA spots were visualized (Kodak film, XAR-5) and scraped into scintillation vials to which 10 mL ACS scintillant was added. Radioactivity was quantitated on a Packard 1900CA liquid scintillation counter. DG mass (pmol) was calculated by reference to the radioactivity counted for 1 nmol of [γ - 32 P]ATP.

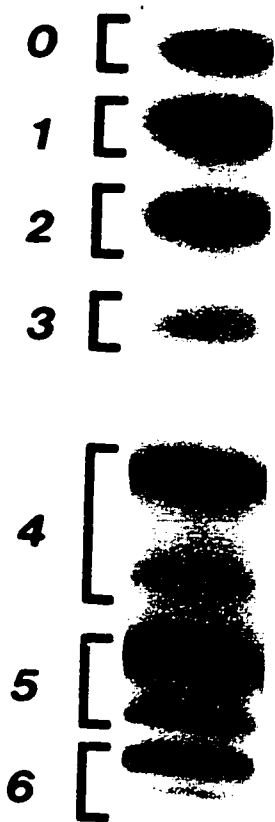
3.3.5 *Molecular Species Fractionation of Diradylglycerol*

Wells with 15×10^6 macrophages were scraped, extracted and derivatized to [32 P]PA as described above except that [γ - 32 P]ATP was used at 1.11×10^{10} Bq/L. After TLC, [32 P]PA spots were scraped into 13 x 75 mm screw-top glass tubes, extracted from the silica and further derivatized to [32 P]dimethylphosphatidic acid by dissolving in 50 μ L chloroform, adding 50 μ L of 0.15 mol/L ethereal

diazomethane and incubating (sealed with Parafilm) at 20°C for 10 min (Kennerly 1986, Sebaldt et al. 1992). Samples were fractionated into bands corresponding to the total double bond content (0 - 6) in the two fatty acid residues by argentation TLC, as previously described (Kennerly 1986, Sebaldt et al. 1992). Plastic-backed TLC plates (Macherey-Nagel, Düren, Germany) were heat-activated at 65°C for 20 min, 90°C for 20 min and 105°C for 40 min. To improve resolution of the highly unsaturated fatty acid species (4 - 6 double bonds), the first mobile phase was modified from that used previously (Sebaldt et al. 1992) to chloroform:methanol (180:20, v/v) and run up to 10 cm, and the second mobile phase was chloroform:methanol (197:3, v/v). Fractions corresponding to 0 - 3 double bonds were visualized as single bands, while fractions corresponding to 4 - 6 double bonds were each visualized as doublets by autoradiography (Fig. 1) as previously described (Kennerly 1986). Bands were individually cut out and quantified by liquid scintillation. The mol/100 mol of total DG sample contained in each fraction was calculated as the percentage of its cpm relative to the total cpm of the sample's 7 fractions. We verified that chemically pure *sn*-1-palmitoyl- and stearoyl-species with *sn*-2 AA, EPA and DHA that were synthesized in our laboratory migrated as expected in the principal bands of the doublets labelled 4, 5 and 6, respectively. We also verified that in natural DG, the contributions of the minor bands of fractions 4, 5 and 6 were 16-28% of the respective total fraction.

Figure 1. Fractionation of diradylglycerol molecular species by argentation TLC.

Sample autoradiogram of diradylglycerol molecular species separated by the total number of double bonds present in the two fatty acid residues. Labels (0 - 6) indicate the total number of double bonds in the fatty acid side chains for each fraction. Excellent separation of all 7 fractions was achieved.



origin →

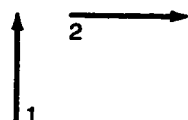
3.3.6 *Separation and Molecular Species Fractionation of Phospholipids*

Unstimulated wells with 15×10^6 macrophages were washed three times with HHBSS to remove $[^{32}\text{P}]\text{H}_3\text{PO}_4$. Phospholipids were extracted into chloroform, using methanol and 0.1 mol/L HCl in the aqueous phase, by the Bligh-Dyer method as described (Sebaldt et al. 1992). Phospholipid classes were separated by two-dimensional TLC on 5% MgAc-treated, heat-activated Silica Gel HLF plates (Analtech) (Sebaldt et al. 1992). The first mobile phase was composed of chloroform: methanol: ammonium hydroxide (130:50:8, v/v/v), and run up for 2 h. The plates were rotated 90° and the second mobile phase, composed of n-butanol:glacial acetic acid:water (150:25:25, v/v/v), was run up for 4 h. Phospholipids were visualized by autoradiography, and spots corresponding to phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI) (Fig. 2) were scraped into 13 x 75 mm screw-top glass tubes.

3.3.7 *Multilinear Regression Analysis: Apparent Metabolic Origin of Diacylglycerol*

To estimate the apparent metabolic origin of DG in (n-3)PUFA-enriched and control diet groups, we performed multilinear regression analyses on the results of molecular species fractionations using Fig.P version 6.0c software (Biosoft, Cambridge, UK). Compositions of PAF-, PMA- and ionomycin-stimulated DG were compared to those of basal DG, PC, PI, PS and PE as previously described (Sebaldt et al. 1992).

Figure 2. Separation of phospholipid classes by two-dimensional TLC.
Sample autoradiogram of phospholipids separated by class. PC: phosphatidylcholine, PE: phosphatidylethanolamine, PS: phosphatidylserine, PI: phosphatidylinositol. Excellent separation was achieved, including PI from PS.



PC



PE



PI



PS



origin →

3.3.8 Nitrogen Environment

To minimize the likelihood of oxidation, N₂ was used extensively throughout the preparation of the diets, during the handling of the dietary lipid concentrates and throughout sample preparation and analyses. The lipid concentrates were infused with N₂ and sealed under a blanket of N₂ and stored at -70°C. Peroxide value determinations were periodically performed on both COEE and (n-3)EE to monitor for any undesirable increases in peroxides (AOCS 1973). All solvents were bubbled with N₂ immediately prior to use. All samples were spotted onto TLC plates under a blanket of N₂. Sample tubes were sealed with Parafilm under N₂ prior to centrifuging or vortexing. For overnight storage, all samples were dried under a stream of N₂ and sealed with Parafilm and kept at -20°C.

3.3.9 Statistical Analyses

Results are expressed as mean $\pm$ SEM. Student's t-tests were performed using the Fig.P computer program. One-way and two-way analyses of variance followed by post hoc comparisons with Duncan's multiple range test at significance levels between $p < 0.05$ to $p < < 0.0001$ were used to test for significant differences in the (n-3)PUFA-induced changes in proportions of each of the molecular species fractions between diet groups, among cell stimulation conditions and among phospholipid classes.

3.4 RESULTS

3.4.1 *Mouse Growth*

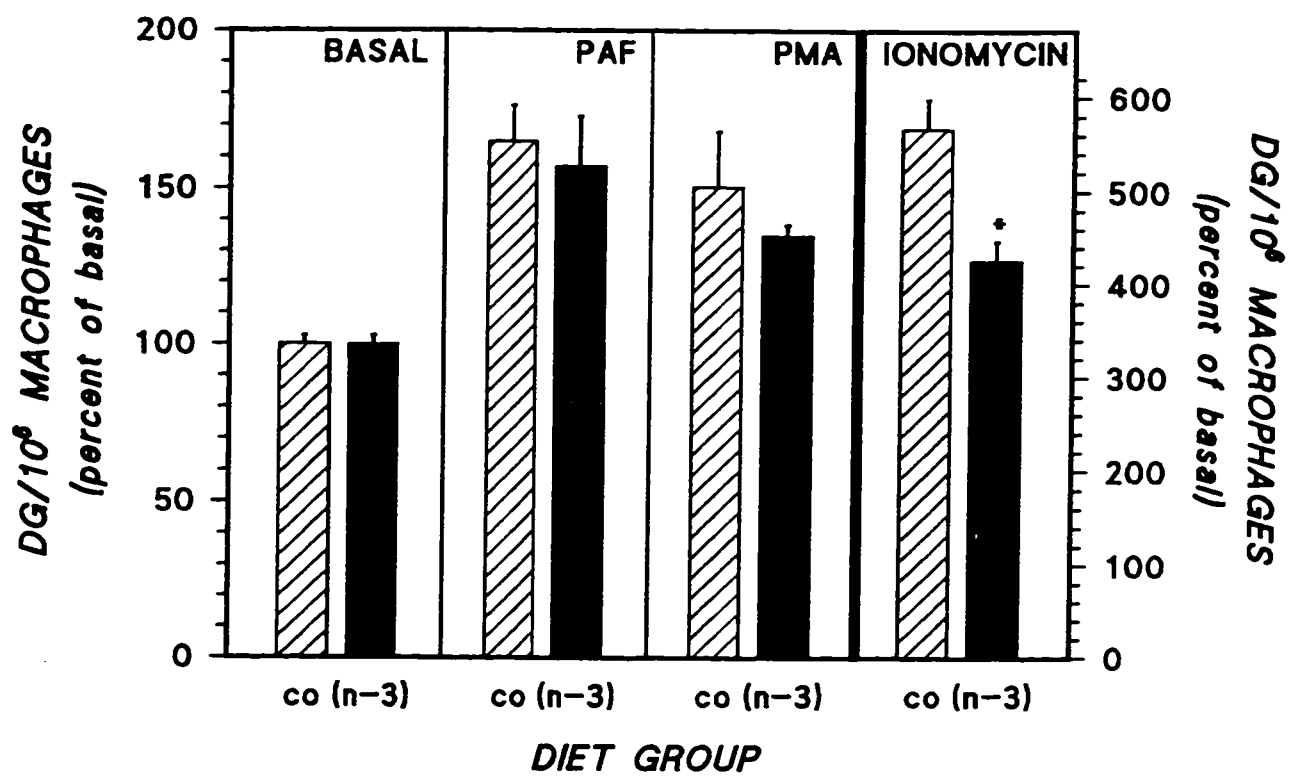
Mice weighed approximately 12 g at the start of the study and approximately 24 g after 4 wk in both the control and (n-3)PUFA-enriched diet groups. There was no statistically significant difference between the groups' weights either at baseline or at any of the weekly weighings.

3.4.2 *Quantitative Analyses of Diacylglycerol*

Previous observations in our laboratory have shown that the loss of membrane-incorporated (n-3)PUFA that occurs *ex vivo* over a 16 h incubation period is less than 5% (Marignani and Sebaldt, in preparation). Macrophages were either left unstimulated (n=18) or stimulated with PAF (0.1 $\mu\text{mol/L}$) (n=6), PMA (0.1 mg/L) (n=6) or ionomycin (10 $\mu\text{mol/L}$) (n=6) for 10 min, and total DG mass was quantitated by *E. coli* DAG kinase assay. Basal DG mass was not significantly different between control and (n-3)PUFA-enriched diet groups (50 - 100 $\text{pmol}/10^6$ macrophages) (Fig. 3). DG mass after PAF stimulation increased to $165 \pm 11\%$ ($p < 0.001$) and $157 \pm 16\%$ ($p < 0.005$) of basal, respectively, but these increases did not differ significantly between diet groups. DG mass after PMA stimulation increased similarly, to $150 \pm 18\%$ ($p < 0.05$) and $135 \pm 3\%$ ($p < 0.002$) of basal, respectively. However, after stimulation with ionomycin, DG mass increased greatly, to $567 \pm 32\%$ ($p < 0.0001$) and $426 \pm 21\%$ ($p < 0.0001$) of basal, respectively.

Figure 3. Quantitative analysis of diradylglycerol (DG).

Macrophages were left unstimulated (basal) or were exposed to platelet activating factor (PAF) ($0.1\mu\text{mol/L}$), phorbol myristate acetate (PMA) (0.1 mg/L) or ionomycin ($10\text{ }\mu\text{mol/L}$) for 10 min. Total DG mass under basal conditions was similar in control (co) and (n-3)polyunsaturated fatty acid-enriched (n-3) diet groups ($50 - 100\text{ pmol}/10^6$ macrophages) ($n=18$) and is expressed as 100%. Results for agonist-stimulated DG are expressed as percent of respective basal in each diet group, mean $\pm$ SEM ($n=6$), with results for ionomycin-stimulated DG shown on the larger scale at right. Level of statistical significance for differences between diet groups: *: $p<0.005$.



Ionomycin-stimulated DG mass was significantly lower in the (n-3)PUFA-enriched diet group compared to control diet ($p < 0.005$).

3.4.3 *Molecular Species Fractionations of Dirdylglycerol*

Fig. 4 shows DG molecular species fractions as percentages of total DG for each argentation TLC band (0 to 6 double bonds). In general, within either diet group, the proportions of the molecular species fractions did not differ significantly between basal DG and DG after PAF, PMA or ionomycin stimulation. One exception to this was the fraction of DG with six double bonds in the (n-3)PUFA-enriched diet group, where the proportion in PAF-stimulated DG was 18.6 ± 0.8 mol/100 mol compared to that in basal (10.9 ± 1.0 mol/100 mol, $p < 0.0001$).

Between the diet groups, differences in proportions of DG molecular species fractions were striking. The proportions of DG that contained highly unsaturated fatty acids (i.e. the sum of DG fractions four to six) were greater ($p < 0.05$) in the (n-3)PUFA-enriched diet group (56-64 mol/100 mol of total DG) than in the control diet group (46-51 mol/100 mol) (PUFA index (4+5+6), Table 1). The ratio of fractions five plus six to fraction four, which is an estimate of the (n-3):(n-6) PUFA content ratio, was markedly greater ($p < 0.05$) in the (n-3)PUFA-enriched diet group (2.22-5.54) than in the control diet group (0.22-0.26) (PUFA index (5+6)/4, Table 1). In further detail, the proportional content of species with five (Fig. 4F) and six (Fig. 4G) double bonds was 497-584% and 289-437% of the respective values in the control diet group. The prominent increase in these highly unsaturated species

Figure 4. Molecular species fractionation of diradylglycerol (DG).

Macrophages were prepared and stimulated as in Fig. 3. Results of molecular species fractionations for basal (n=18) and agonist-stimulated DG (n=6 each) are shown as a proportional content (mol/100 mol of total DG) of each molecular species fraction obtained as shown in Fig. 1, from fully saturated species (panel A) to species with six double bonds (panel G), mean $\pm$ SEM. Levels of statistical significance for differences between (n-3)polyunsaturated fatty acid-enriched diet group (n-3) and control diet group (co): *: $p < 0.01$, **: $p < 0.001$, ***: $p < 0.0001$.

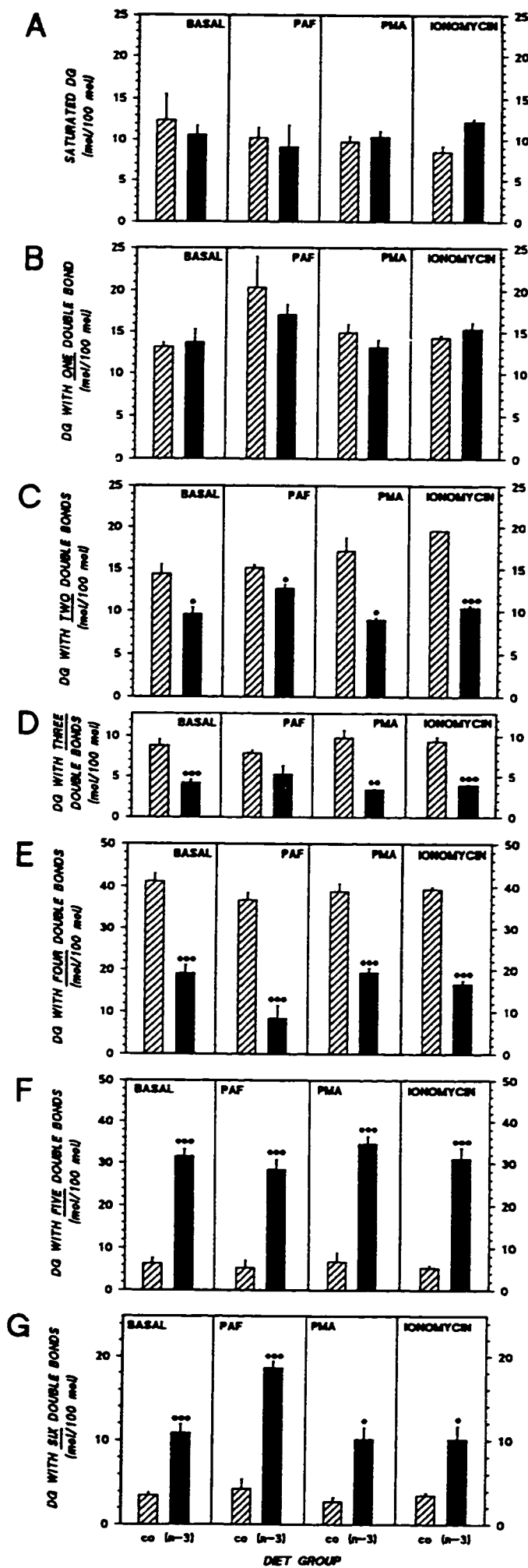


TABLE 1
Polyunsaturated fatty acid (PUFA) indices of diradylglycerol molecular species in
peritoneal macrophages from mice fed control or (n-3)PUFA-enriched diets¹

PUFA Index ²	BASAL		PAF		PMA		IONOMYCIN	
	control	n-3	control	n-3	control	n-3	control	n-3
4+5+6	51.2± 2.0	65.6± 3.8	46.5± 4.3	55.7± 4.8	48.4± 0.8	64.1± 0.5	48.3± 0.6	58.0± 0.9
(5+6)/4	0.24± 0.03	2.26± 0.43	0.26± 0.07	8.42± 2.78 ³	0.25± 0.08	2.33± 0.13	0.22± 0.02	2.47± 0.18

¹For each experimental condition (basal, platelet activating factor (PAF), phorbol myristate acetate (PMA) and ionomycin stimulation), the sum of diradylglycerol (DG) fractions with 4, 5 and 6 double bonds (4+5+6, mol/100 mol) was calculated to determine an index of highly unsaturated fatty acid content. The ratio (5+6)/4 was calculated as an index of the (n-3):(n-6) content ratio. Results are expressed as mean ± SEM for basal (n=18) and agonist-stimulated (n=6, each).

²For each index and for each condition of cell stimulation, the difference between control and (n-3)PUFA-enriched diet groups was significant at $p<0.05$.

³In the (n-3)PUFA-enriched diet group, the (5+6)/4 index was significantly greater for PAF-stimulation than for the other conditions ($p<0.05$).

TABLE 2

Molecular species fractions of diradylglycerol (DG): comparison among cell stimulants of the ratios ((n-3) polyunsaturated fatty acid-enriched to control) of their proportional content in total DG¹

FRACTION ² (no. of double bonds)	DIFFERENCES ³ (at $p < 0.05$)
two	PAF < PMA PAF < IONOMYCIN
three	PAF < PMA PAF < IONOMYCIN
four	PAF < BASAL BASAL < PAF
five	IONOMYCIN > BASAL
six	PAF > IONOMYCIN

¹In each experimental condition (basal, (n=18), platelet activating factor (PAF, n=6), phorbol myristate acetate (PMA, n=6) and ionomycin (n=6) stimulation), for each molecular species fraction (2 - 6) of DG, the ratio of its proportion of total DG in the (n-3)polyunsaturated fatty acid-enriched diet groups (mol/100 mol) to its proportion of total DG in the control diet group (mol/100 mol) was calculated.

²Fractions 2 - 4 were less and fractions 5 - 6 were greater in the (n-3)polyunsaturated fatty acid-enriched diet group, relative to control.

³For each molecular species fraction (2 - 6), one-way analysis of variance was used to test for differences among the ratios determined for basal and PAF-, PMA- and ionomycin-stimulated conditions.

was at the expense of species with two (Fig. 4C), three (Fig. 4D) and especially four (Fig. 4E) double bonds, whose proportional content was reduced to 52-84%, 34-84% and 23-50% of respective values in the control diet group. All differences between diet groups were significant ($p < 0.001$ to $p < < 0.0001$), with the sole exception of the proportion of DG with three double bonds after PAF stimulation. The proportion of saturated DG molecular species (Fig. 4A) was similar in both dietary groups at 10-13 mol/100 mol of total DG as was the fraction with one double bond (13-20 mol/100 mol, Fig. 4B).

We also examined whether the extent of (n-3)PUFA-induced differences in DG molecular species composition varied with the nature of cell stimulation. We compared the ratios between diet groups of the proportions of molecular species with two to six double bonds for the four conditions of basal and PAF-, PMA- and ionomycin-stimulated (Table 2). We found that the proportional reductions in the fractions of DG with two and three double bonds were less after PAF than after PMA and ionomycin stimulation ($p < 0.05$). However, the proportional reductions ($p < 0.05$) in the fractions of DG with four double bonds and augmentations ($p < 0.05$) in those with five and six double bonds were generally similar among stimulants.

3.4.4 *Molecular Species Fractionations of Phospholipids*

Murine peritoneal macrophages were prepared as described in Materials and Methods. Membrane phospholipids PC, PI, PS and PE were extracted,

separated (Fig. 2), hydrolyzed to DG and then analyzed by argentation TLC for molecular species fractions (Fig. 5). The proportions of phospholipid molecular species fractions differed markedly between diet groups. The proportions of phospholipids that contained highly unsaturated fatty acids (i.e. the sum of DG fractions four to six) were greater ($p < 0.05$) in the (n-3)PUFA-enriched diet group (45-77 mol/100 mol of total phospholipid) than in the control diet group (33-65 mol/100 mol) (PUFA index (4+5+6), Table 3). An estimate of the (n-3):(n-6) PUFA content ratio was substantially greater ($p < 0.05$) in the (n-3)PUFA-enriched diet group (1.69-4.74) than in the control diet group (0.13-0.33) (PUFA index (5+6)/4, Table 3). In further detail, the proportional content of species with five (Fig. 5F) and six (Fig. 5G) double bonds was 497-1212% and 218-377% of the respective values in the control diet group. The large increase in these highly unsaturated species was at the expense of species with two (Fig. 5C), three (Fig. 5D) and especially four (Fig. 5E) double bonds, whose proportional content was reduced to 53-78%, 43-73% and 28-49% of respective values in the control diet group. Differences between the diet groups were significant at $p < 0.05$ to $p < < 0.0001$.

We also examined whether the extent of these profound (n-3)PUFA-induced changes in phospholipid molecular species composition varied among phospholipid classes. We determined the ratios between diet groups of the proportions of molecular species fractions two to six, comparing the four phospholipids PC, PI, PS and PE (Table 4). We found that the proportional reductions in the fractions of phospholipid with two and three double bonds were

Figure 5. Molecular species fractionation of phospholipid (PL) classes.

Macrophages were prepared as in Fig. 4 but left unstimulated, and PL classes were separated as shown in Fig.2. Results of molecular species fractionations for phosphatidylcholine (PC, n=12), phosphatidylinositol (PI, n=8), phosphatidylserine (PS, n=8) and phosphatidylethanolamine (PE, n=10) are shown as a proportional content (mol/100 mol of PL) of each molecular species fraction obtained as shown in Fig. 1, from fully saturated species (panel A) to species with six double bonds (panel G), mean $\pm$ SEM. Levels of statistical significance for differences between (n-3)polyunsaturated fatty acid-enriched diet groups (n-3) and control diet group (co): *: $p < 0.05$, **: $p < 0.005$, ***: $p < 0.0001$.

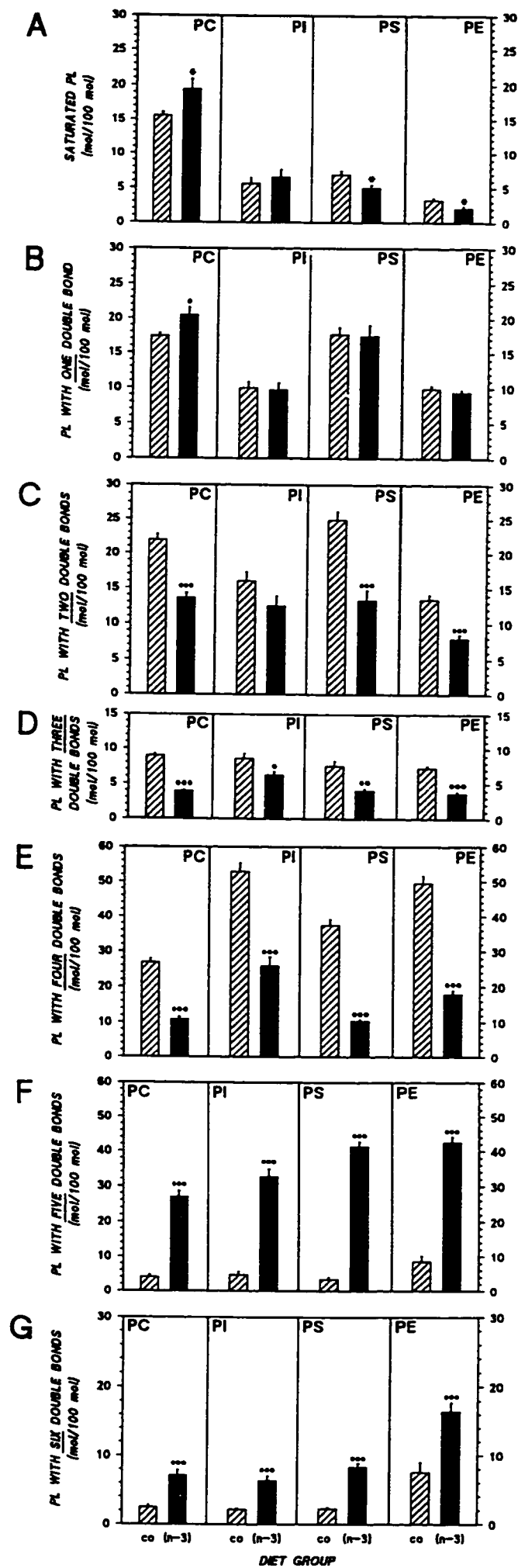


TABLE 3
Polyunsaturated fatty acid (PUFA) indices of phospholipid molecular species in
molecular species in peritoneal macrophages from mice fed control or (n-3)PUFA-enriched diets^{1,2}

PUFA Index	Phosphatidylcholine control	Phosphatidylcholine n-3	Phosphatidylinositol control	Phosphatidylinositol n-3	Phosphatidylserine control	Phosphatidylserine n-3	Phosphatidylethanolamine control	Phosphatidylethanolamine n-3
4+5+6	33.4±0.8	45.2±1.9	59.7±2.5	65.0±2.8	43.1±1.1	60.2±0.7	65.6±1.4	77.1±2.7
(5+6)/4	0.25±0.04	3.26±0.29	0.13±0.02	1.64±0.23 ³	0.15±0.03	4.81±0.26 ³	0.34±0.06	3.34±0.19

¹For each phospholipid (phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS) and phosphatidylethanolamine (PE)), the sum of phospholipid fractions with 4, 5 and 6 double bonds (4+5+6, mol/100 mol) was calculated to determine an index of highly unsaturated fatty acid content. The ratio (5+6)/4 was calculated as a index of (n-3):(n-6) content ratio. Results are expressed as mean ± SEM for PC, (n=12), PI, (n=8), PS, (n=8) and PE, (n=10).

²For each index and for each phospholipid, the difference between control and (n-3)PUFA-enriched diet group was significant at p<0.05.

³In the (n-3)PUFA-enriched diet group, the (5+6)/4 index was significantly less for PI than for the other phospholipids (p<0.05) and significantly greater for PS than for the other phospholipids (p<0.05).

TABLE 4

Molecular species fractions of phospholipid classes: comparison among phospholipid classes of the ratios ((n-3) polyunsaturated fatty acid-enriched to control) of their proportional content in each phospholipid class¹

FRACTION ² (no. of double bonds)	DIFFERENCES ³ (at $p < 0.05$)
two	PI < PS PI < PE
three	PI < PC PI < PS PI < PE PI < PC
four	PI < PS PI < PE PC < PE
five	PS > PI PS > PC PS > PE PI > PE PC > PE
six	PS > PC PS > PE PI > PE PC > PE

¹In each phospholipid class (phosphatidylcholine (PC, $n=12$), phosphatidylinositol (PI, $n=8$), phosphatidylserine (PS, $n=8$) and phosphatidylethanolamine (PE, $n=10$)), for each molecular species fraction (2 - 6), the ratio of its proportion of the phospholipid in the (n-3)polyunsaturated fatty acid-enriched diet groups (mol/100 mol) to its proportion of the phospholipid in the control diet group (mol/100 mol) was calculated.

²Fractions 2 - 4 were less and fractions 5 - 6 were greater in the (n-3)polyunsaturated fatty acid-enriched diet group, relative to control.

³For each molecular species fraction (2 - 6), one-way analysis of variance was used to test for differences among the ratios determined for each phospholipid class ($p < 0.05$).

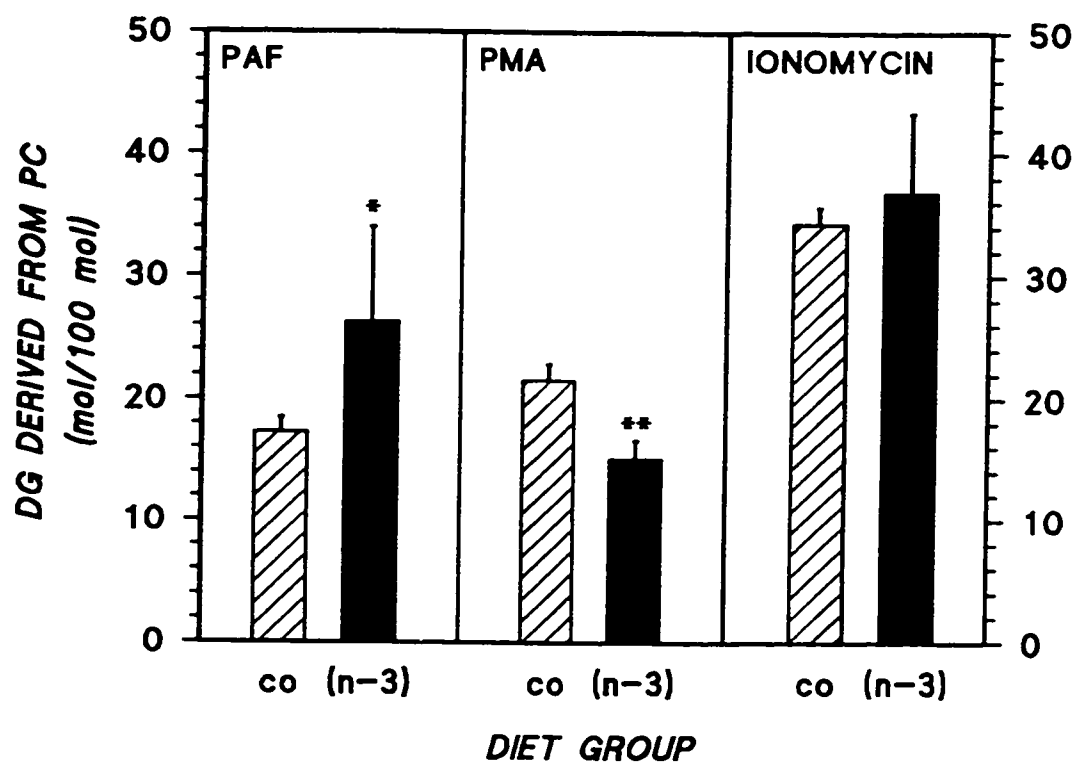
less in PI ($p < 0.05$) than in the other phospholipids, and the proportional augmentations in fractions with five and six double bonds were generally less in PE ($p < 0.05$) and greater in PS ($p < 0.05$) than in the other phospholipids.

3.4.5 *Apparent Metabolic Origin of Diradylglycerol*

The extent of apparent metabolic origin from PC hydrolysis of total DG samples after 10 min stimulation was determined by multilinear regression analyses of their fractional composition profiles (Fig. 6). PC hydrolysis appeared to account for 17-21 mol/100 mol of DG after PAF or PMA stimulation in the control diet group, whereas in the (n-3)PUFA-enriched diet group the fraction was 26 mol/100 mol after PAF stimulation ($p < 0.02$) and 15 mol/100 mol after PMA stimulation ($p < 0.05$). After ionomycin stimulation, 34 mol/100 mol of DG appeared to derive from PC hydrolysis, and there was no difference between the diet groups.

Figure 6. Multilinear regression analysis of the apparent metabolic origin of diradylglycerol (DG).

Multilinear regression analysis was performed on the fractional composition profiles of stimulated DG samples shown in Fig. 4 to estimate the contribution of phosphatidylcholine (PC) hydrolysis to their formation, expressed as a percent of total DG. Results are shown for DG after stimulation with platelet activating factor (PAF), phorbol myristate acetate (PMA) and ionomycin. Levels of statistical significance for differences between (n-3)polyunsaturated fatty acid-enriched diet group (n-3) and control diet group (co): *: $p < 0.02$, **: $p < 0.05$.



3.5 DISCUSSION

In this paper, we demonstrate for the first time that altering the membrane fatty acid composition of murine peritoneal macrophages by prolonged dietary supplementation with (n-3)PUFA *in vivo* results in the formation of second messenger DG that reflects the (n-3)PUFA-enriched molecular composition of the parent phospholipid(s) from which it is formed. In particular, we find that there is a massive enrichment in molecular species with five and six double bonds, reflecting EPA and DHA incorporation at the *sn*-2 position.

Several animal and human studies have shown that (n-3)PUFA compete with AA for the esterase responsible for fatty acid incorporation into cellular membrane phospholipids (Ahmed and Holub 1984, Maehr et al. 1994). Recently, Lokesh and Kinsella (1994) have demonstrated that (n-3)PUFA compete with free AA for acylation into membrane phospholipids of unstimulated cells. However, they do not interfere with reacylation of phospholipase A₂-hydrolyzed AA upon stimulation of macrophages with PMA, even though reacylation of phospholipase A₂-hydrolyzed EPA or DHA may occur. As a result of this and of other possible enzyme selectivities towards AA (Galli et al. 1993, Lokesh and Kinsella 1994, Walsh et al. 1994), the availability of phospholipids with EPA and DHA in the *sn*-2 position to undergo hydrolysis to DG by Phospholipase C may differ from those with AA. We have previously shown that the biosynthesis of DG in murine peritoneal macrophages is significantly increased after *in vitro* supplementation of

culture media by EPA and even more potently by DHA in both basal and stimulated macrophages (Sebaldt and Marignani 1995). The distribution of molecular species is substantially altered and reflects extensive incorporation of EPA or DHA, largely at the expense of AA.

It has been shown (Galli et al. 1993, Raclot and Groscolas 1994) that highly unsaturated PUFA with 20 or more carbons turn over more slowly and are more avidly retained than other fatty acids in cell membrane phospholipids because of reduced mitochondrial β -oxidation rates, reduced mobilization rates and elevated acylation/reacylation rates of PUFA. This may explain the greater content of highly unsaturated fatty acids that we found (PUFA index (4+5+6), Tables 1 and 3). Our results, which are obtained using a 4 wk mouse feeding schedule, are therefore likely to reflect those of a physiological steady-state.

Our results show that enrichment of diets with (n-3)PUFA did not cause a significant attenuation of total DG mass formed after stimulation with the physiological agonist, PAF, or the PKC activating phorbol ester, PMA. Thus, "physiological" DG formation appears to be regulated in such a way that DG mass is maintained at predetermined levels regardless of the details of DG molecular species composition. However, ionomycin-stimulated DG mass was significantly reduced in (n-3)PUFA-enriched diet groups relative to control. Thus, "total capacity" for DG formation may be reduced by (n-3)PUFA-induced changes in other calcium-dependent signal transduction pathways, which ultimately reduce the hydrolyzing ability of phospholipase C and/or phospholipase D to form DG

and/or enhance the divalent cation ionophore-stimulated DG degradation pathway (Roldan and Murase 1994, Walsh et al. 1994).

Despite the fact that the total DG mass in the (n-3)PUFA-enriched diet group was generally similar to that in the control diet group, DG molecular species fractionation studies provided clear evidence that the individual molecular species that comprise DG in basal and in stimulated cells are very different between the diet groups. Argentation TLC allows for the separation of DG molecular species by the total number of double bonds within the two fatty acid side chains. The *sn*-1 position in the phospholipids of many cell types, including murine peritoneal macrophages, is occupied (> 98%) by saturated fatty acids (16:0 and 18:0) (Blank et al. 1994, Sugiura et al. 1983), and the *sn*-2 position is occupied almost exclusively by unsaturated fatty acids (one or more double bonds) (Akoh and Chapkin 1990, Chapkin et al. 1988 and 1991, Sugiura et al. 1983). Therefore, argentation TLC (Fig. 1) readily provides excellent separation of DG molecular species that contain AA, EPA and DHA (four, five and six double bonds, respectively). (n-3)PUFA-induced changes in DG molecular species composition were generally similar for the different cell stimulants, with the exception of PAF (Table 2). This may be due, in part, to differences among the stimulants in functional pools of phospholipids that are involved in DG biosynthesis and/or in molecular species selectivities of DG metabolizing pathways.

Complete separation by two-dimensional TLC of membrane phospholipids (Fig. 2) permitted the characterization of the four individual phospholipids, PC, PI,

PS and PE (Fig. 5). It was expected from earlier work (Chapkin et al. 1988 and 1991, Chapkin and Carmichael 1990, Chilton et al. 1993) that the incorporation of EPA and DHA into macrophage membrane phospholipids would occur at the expense of *sn*-2-esterified AA in phospholipids PC, PS and PE, while PI would be found resistant to (n-3)PUFA incorporation. Our results suggest that there is substantial replacement of AA even in PI. Even the high PUFA content we report for PI is underestimated, while the apparent preservation of fractions with two and three double bonds (Table 4) is an overestimate, because the DAG kinase that we use to enzymatically convert DG to PA contains traces of bacterial membrane DG. In our laboratory, blank tubes treated with the enzyme do not generate any measurable DG with four or more double bonds, but do show reproducible quantities in the fractions corresponding to zero to two double bonds and traces with three. The absolute amounts correspond closely to those we generally measured in our samples of PI, the least abundant of the four major phospholipids. Despite the extensive incorporation of (n-3)PUFA into PI, the degree of AA preservation was significantly higher, both absolutely (Fig. 5) and relatively (Table 4), than in the other phospholipid classes. This suggests that enzymatic specificities may exist that selectively preserve the content of AA in PI yet do not select against EPA and DHA incorporation.

The proportional content of PE with 5 and 6 double bonds (Fig. 5E and 5F) is five-fold and two-fold greater, respectively, in the (n-3)PUFA-enriched diet group compared to control. Murine peritoneal macrophage PE consists principally of

plasmalogens (alk-1'-enylacyl subclass), with lesser contributions of diacyl and alkylacyl subclasses. Since the dominant *sn*-2-esterified fatty acid in plasmalogens is AA (Akoh and Chapkin 1990, Blank et al. 1994, Chapkin and Carmichael 1990, Sugiura et al. 1983), our results imply significant incorporation of (n-3)PUFA into the plasmalogen subclass of PE. This substitution could potentially affect membrane structural and metabolic functions of PE such as its conversion to PC.

The proportional content of PS with 5 and 6 double bonds (Fig. 5E and 5F) is twelve-fold and four-fold greater, respectively, in the (n-3)PUFA-enriched diet group relative to control. Since PS is a cofactor in PKC activation (Nishizuka 1988), it can be speculated that PS enrichment with (n-3)PUFA may alter PKC function.

The hydrolysis by phospholipase C and/or phospholipase D of PC, rather than of PI, provides the more sustained elevation of DG that may be necessary for effective signal transduction (Billah 1993, Exton 1990, Sebaldt et al. 1992). We estimated the apparent metabolic origin from PC of the sustained phase of DG formation after 10 min stimulation with PAF, PMA and ionomycin (Fig. 6). As previously discussed (Sebaldt et al. 1992), multilinear regression analysis presumes that any sample of stimulated DG consists of a mixture of pre-existing basal DG and DG derived from the hydrolysis of PC, PI, PS and/or PE. The distinct molecular species profiles of each of these sources permits the calculation of an estimate of their proportional contributions to the stimulated DG samples. Our results suggest that PC hydrolysis contributes substantially to the total DG formed.

Limitations of this form of analysis include the assumption that there is no significant preferential degradation of certain DG molecular species and that each parent phospholipid is hydrolyzed from a uniform pool (Sebaldt et al. 1992). Nevertheless, we find it intriguing that the (n-3)PUFA-enriched diet treatment may differentially affect the contribution of PC hydrolysis to DG formation, relative to control, depending on which cell stimulant is used.

Our results demonstrate novel effects of (n-3)PUFA beyond the better-known changes in eicosanoid formation and cytokine production. The profound changes associated with diet-induced (n-3)PUFA-enrichment of peritoneal macrophage membrane phospholipids result in a reduced "maximal capacity" for stimulated DG formation and the biosynthesis of DG that is enriched in highly unsaturated (n-3)PUFA. These changes may have significant implications for the normal second messenger function of DG, namely the activation of PKC early in the receptor-mediated signal transduction cascade of many important intercellular agonists. The formation of (n-3)PUFA-enriched DG may play a significant role in the diverse outcomes associated with (n-3)PUFA administration in vivo.

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CHAPTER FOUR

PUBLISHED MANUSCRIPT

Marignani, P. A. and Sebaldt, R. J. (1996) The Formation of Diradylglycerol Molecular Species In Murine Peritoneal Macrophages Varies Dose-Dependently With Dietary Content of Purified Eicosapentaenoic And Docosahexaenoic Ethyl Esters. *Journal of Nutrition* 126: 2738-2745.

PREAMBLE

The purpose of these experiments was to address the hypothesis that the physicochemical differences between the two principal ω 3PUFA components of fish oil, EPA and DHA, result in preferential incorporation of one over the other. I did this by determining the effect of purified diets containing either EPEE or DHEE or a mixture of both on the incorporation of EPA and DHA into DG and membrane PL.



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Dear Dr. Willard J. Visek

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Marignani PA and Sebaldt EJ. (1996) The Formation of Diacylglycerol Molecular Species In Murine Peritoneal Macrophages Varies Dose-Dependently With Dietary Content of Purified Eicosapentaenoic And Docosahexaenoic Ethyl Esters. *J. Nutr.* 000: 00-00. (Ms. No. 96-3287)

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4.1 ABSTRACT

Substantial effects of dietary fish oil-derived fatty acid ethyl esters on the metabolism of diradylglycerol (DG) have recently been described. We undertook to isolate the separate effects of (n-3)eicosapentaenoic acid (EPA) and (n-3)docosahexaenoic acid (DHA) on DG metabolism. For three weeks, male C57BL/6 mice were fed one of six purified diets, where the lipid component was either 3 g/100 g corn oil ethyl ester (COEE) (control diet) or 1 g/100 g COEE plus 2 g/100 g of one of EPA ethyl ester (EPEE), DHA ethyl ester (DHEE) or an EPEE:DHEE mixture. Peritoneal macrophages were analyzed for DG content and for molecular species distributions of DG and of phospholipid classes. We found that the degree of incorporation of EPA and of DHA into DG in macrophages is dose-dependent on the dietary concentration of EPEE and DHEE, under basal conditions and after stimulation with platelet activating factor, phorbol myristate acetate and ionomycin. Incorporation of EPA and DHA into phospholipids is also significant and dose-dependent in each phospholipid class. For both DG and phospholipid molecular species, the incorporation of EPA in the *sn*-2 position is considerably greater than that of species with DHA under conditions of equimolar dietary content. These results demonstrate that (a) incorporation of EPA and of DHA into DG are independent and dose-dependent on dietary content, (b) EPA is incorporated with greater affinity than DHA, and (c) these effects on DG metabolism appear to result from corresponding effects on parent membrane phospholipids. Physiologically and therapeutically relevant differences may exist between EPA and DHA.

4.2 INTRODUCTION

In the present study, experimental diets with purified eicosapentaenoic ethyl ester (EPEE), docosahexaenoic ethyl ester (DHEE) or mixtures thereof as their lipid component were used to determine whether there exists a difference between EPA and DHA in the changes in DG metabolism that result from their incorporation into membrane phospholipids in murine peritoneal macrophages. We also investigated whether incorporation of EPA or of DHA into DG is dose-dependent and whether the extent of incorporation of individual (n-3)PUFA into membrane phospholipids, from which DG is derived, differs between EPA and DHA. Finally, this study determined whether replacement of resident fatty acids in the *sn*-2 position of membrane phospholipids by EPA or DHA causes different changes in total mass of DG.

Recent *in vivo* findings show that when mice are fed diets that contain 10 g/100 g of either corn oil ethyl ester or mixed (n-3)PUFA ethyl ester, the latter group shows substantial enrichment of DG molecular species with five and six double bonds, reflecting EPA and DHA incorporation at the *sn*-2 position in peritoneal macrophage phospholipids (Marignani and Sebaldt 1995, Marignani and Sebaldt 1996). Earlier *in vitro* studies (Sebaldt and Marignani 1995) have shown that the (n-3) polyunsaturated fatty acids [(n-3)PUFA], eicosapentaenoic [EPA, 20:5(n-3)] and docosahexaenoic acid [DHA, 22:6(n-3)], when added to macrophage culture media, alter the formation of DG molecular species to substantially

differing extents under equimolar conditions. Those results indicated that (n-3)PUFA, in addition to altering eicosanoid biosynthesis (Croft et al. 1987, DeCaterina et al. 1995, Hubbard et al. 1994, Whelan et al. 1991), also have the potential to significantly modify a critical second messenger of intracellular signal transduction. This may account, at least in part, for some of the less well understood actions of (n-3)PUFA (Chan et al. 1993, Huang et al. 1986, Hubbard et al. 1994, Pascale et al. 1993).

4.3 METHODS AND MATERIALS

4.3.1 *Materials*

sn-1,2-diacylglycerol kinase, ionomycin and phorbol myristate acetate (PMA) were from Calbiochem (La Jolla, CA). Diethylene triamine pentaacetic acid (DETAPAC), imidazole, ATP, DL-dithiothreitol (DTT) and phospholipase C (#7147) were from Sigma (St. Louis, MO). Diazald was from Aldrich (Milwaukee, WI). RPMI 1640 media without L-glutamine, heat-deactivated fetal calf serum (dFCS), penicillin, streptomycin and Hanks balanced salt solution (containing calcium and magnesium) were from Gibco (Grand Island, NY). L-glutamine was from Fisher (Unionville, ON, Canada). Culture dishes were from Corning (Corning, NY) and Falcon (Franklin Lakes, NJ). [γ - 32 P]ATP and [γ - 32 P]H₃PO₄ were from Dupont-NEN (Markham, ON, Canada). 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (PAF) and dioleoylglycerol standards were from Avanti Polar Lipids (Alabaster, AL). Aqueous Counting Scintillant (ACS) was from Amersham (Oakville, ON, Canada).

4.3.2 *Animal care and diets*

Pathogen-free 3-4 week old male C57BL/6 mice (Charles River Laboratory, Montreal, PQ) were acclimated for 24 h in microisolator cages with access to standard nonpurified diet and water. Mice were randomly allocated to cages (4 - 5/cage), individually weighed and monitored for growth weekly. Mice were fed

purified diets for 3 wk. Diets were prepared by reconstituting basal mix (TD89047; Harlan Teklad Diets, Madison, WI) with lipid. The composition of basal mix was such that addition of 3 g lipid to 97 g basal mix reconstituted a complete purified diet that contained casein, 21.5%, DL-methionine, 0.32%, sucrose, 48.4%, corn starch, 16.1%, cellulose, 5.4%, mineral mix (AIN-76), 3.8%, vitamin mix (AIN-76A), 1.1%, choline bitartrate, 0.22%. For the control diet (A), basal mix was reconstituted with 3 g corn oil ethyl ester (COEE)/100 g diet. For the experimental diets (B - F), basal mix was reconstituted with 2 g (n-3)PUFA ethyl ester and 1 g COEE/100 g diet, where the former was either: (B) 0 g eicosapentaenoic ethyl ester (EPEE) and 2 g docosahexaenoic ethyl ester (DHEE); (C) 0.5 g EPEE and 1.5 g DHEE; (D) 1 g EPEE and 1 g DHEE; (E) 1.5 g EPEE and 0.5 g DHEE; or (F) 2 g EPEE and 0 g DHEE. Table 1 summarizes the lipid composition (3 g/100 g diet) of each diet. COEE contained primarily [18:2(n-6)] (498mg/g), [18:1(n-9)] (221 mg/g), [16:0] (98 mg/g) and [18:3(n-3)] (8.6 mg/g). EPEE contained predominantly [20:5(n-3)] (905 mg/g) and [22:6(n-3)] (2.3 mg/g). DHEE contained principally [22:6(n-3)] (935 mg/g) and [20:4(n-3)] (7.6 mg/g). All experimental diets contained COEE as a source of essential fatty acids and DL- α -tocopheryl acetate (150 mg/kg) to prevent the increased susceptibility of the (n-3)PUFA to oxidation due to the increased degree of fatty acid unsaturation (Marignani and Sebaldt 1995). Diets and feeding jars were changed daily. All animal procedures were approved by the McMaster University Animal Research Ethics Board.

TABLE 1

Composition of lipid portion of control and experimental diets¹

Diet	Corn oil ethyl esters	Eicosapentaenoic ethyl ester	Docosahexaenoic ethyl ester
<i>g/100 g diet</i>			
A ²	3.0	0.0	0.0
B	1.0	0.0	2.0
C	1.0	0.5	1.5
D	1.0	1.0	1.0
E	1.0	1.5	0.5
F	1.0	2.0	0.0

¹total dietary lipid=3.0 g/100 g²control diet

4.3.3 *Macrophage Stimulation*

After receiving purified diets for three weeks, mice were injected intraperitoneally with 1 mL brewer's thioglycollate (DIFCO, Detroit, MI). After 3 d, elicited macrophages were harvested by peritoneal lavage (80-90% macrophages), plated and enriched for adherent cells by incubation for 2 h (>98% macrophages) as described (Adams 1979). The macrophage monolayers were washed three times with Hanks balanced salt solution containing 10 mmol/L Hepes, pH 7.4 (HHBSS). The washed macrophage monolayers (15×10^6 per 16 cm² well and 2×10^6 per 9.62 cm² well) were cultured for an additional 16 h in RPMI 1640 containing 2 mmol/L L-glutamine, 1.25×10^5 U/L penicillin and 6 mg/L streptomycin at 37 °C in a 5% CO₂/100% H₂O atmosphere (Marignani and Sebaldt 1996). In order to visualize the location of individual phospholipids on the two-dimensional TLC plates, 37 MBq/L of [³²P]H₃PO₄ was added to those wells destined for phospholipid analyses. After incubation, the macrophage monolayers were washed three times with HHBSS, left in 2.0 mL HHBSS and placed in a water bath at 37 °C. Macrophages were stimulated for 10 min by the addition of 400 µL of one of the following (final concentrations shown): PAF (0.1 µmol/L), ionomycin (10 µmol/L), PMA (0.1 mg/L) or HHBSS (unstimulated control), as described (Marignani and Sebaldt 1995). All conditions were repeated in duplicate or triplicate wells for each experiment.

4.3.4 *Total Diradylglycerol Mass*

After termination of stimulation by the addition of 2 mL methanol, wells with 2×10^6 macrophages were scraped into 13x75 mm screw-top glass tubes, and DG was extracted into chloroform using methanol and 1.0 mol/L NaCl in the aqueous phase by the Bligh-Dyer (1959) procedure as described (Sebaldt et al. 1992). DG was derivatized to phosphatidic acid (PA) in the presence of DAG kinase and 1.85 GBq/L [32 P]ATP, separated from ceramides by TLC and quantitated as previously described (Marignani and Sebaldt 1995).

4.3.5 *Determination of Diradylglycerol Molecular Species*

After termination of stimulation by the addition of 2 mL methanol, macrophages were scraped, DG was extracted and derivatized to [32 P]PA as described above except that [32 P]ATP was used at 11.1 GBq/L. After TLC, [32 P]PA spots were scraped into 13x75 mm screw-top glass tubes, extracted from the silica and further derivatized to [32 P]dimethylphosphatidic acid (DMPA) and fractionated into bands corresponding to the total double bond content (0-6) in the two fatty acid residues by argentation TLC, as previously described (Marignani and Sebaldt 1995).

4.3.6 *Determination of Phospholipid Molecular Species*

Macrophages were washed three times with HHBSS to remove excess [32 P]H₃PO₄. Phospholipids were extracted into chloroform, using methanol and 0.1

mol/L HCl in the aqueous phase, by the Bligh-Dyer (1959) method as described (Sebaldt et al. 1992). Phospholipid classes phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS) and phosphatidylethanolamine (PE) were separated by two dimensional TLC on 5% Mg-acetate-treated, heat-activated Silica Gel HLF plates (Analtech, Newark, DE) and extracted, converted successively to DG, [32 P]PA and DMPA, and analyzed as described (Marignani and Sebaldt 1995).

4.3.7 *Nitrogen Environment*

The oxidation of PUFA was minimized by the extensive use of N₂ throughout the preparation of the diets and throughout sample preparation and analyses. The ethyl esters were infused with N₂ and sealed under a blanket of N₂ and stored at -70°C. Peroxide value determinations were periodically performed on all lipid ethyl esters to monitor any undesirable increases in peroxides (American Oil Chemists' Society 1973). Solvents were bubbled with N₂ immediately prior to use. Sample tubes were sealed with Parafilm under N₂ prior to centrifuging or vortexing. For overnight storage, all samples were dried under a stream of N₂, sealed with Parafilm and kept at -20°C.

4.3.8 *Statistical Analyses*

Results are expressed as mean $\pm$ SEM. For results shown in Fig. 3, statistically significant differences between diets were determined when p values

were less than 0.05 by Student's two-tailed t-test calculated using Fig.P version 6.0c software (Biosoft, Cambridge, UK). For results shown in Fig. 1 and 2, the relationship between dose (dietary EPEE or DHEE) and mol/100 mol product (proportion in total sample of corresponding molecular species fraction) was examined by both linear and quadratic regression analyses for each molecular species fraction. These were performed using Fig. P version 6.0c. In order to determine whether quadratic regression analyses significantly improved the goodness of fit over linear regression, F values for linear and quadratic regressions were calculated as shown below (Kelman and Whiting 1980) and were compared at $p=0.05$.

$$\frac{(SS_{\text{linear}} - SS_{\text{quadratic}}) / (df_{\text{linear}} - df_{\text{quadratic}})}{SS_{\text{quadratic}} / df_{\text{quadratic}}}$$

We found that quadratic regression analyses significantly improved the goodness of fit over that obtained with linear regression for the dose-response curves of molecular species fractions with five double bonds, but not for those with four or six.

4.4 RESULTS

4.4.1 *Animal Growth*

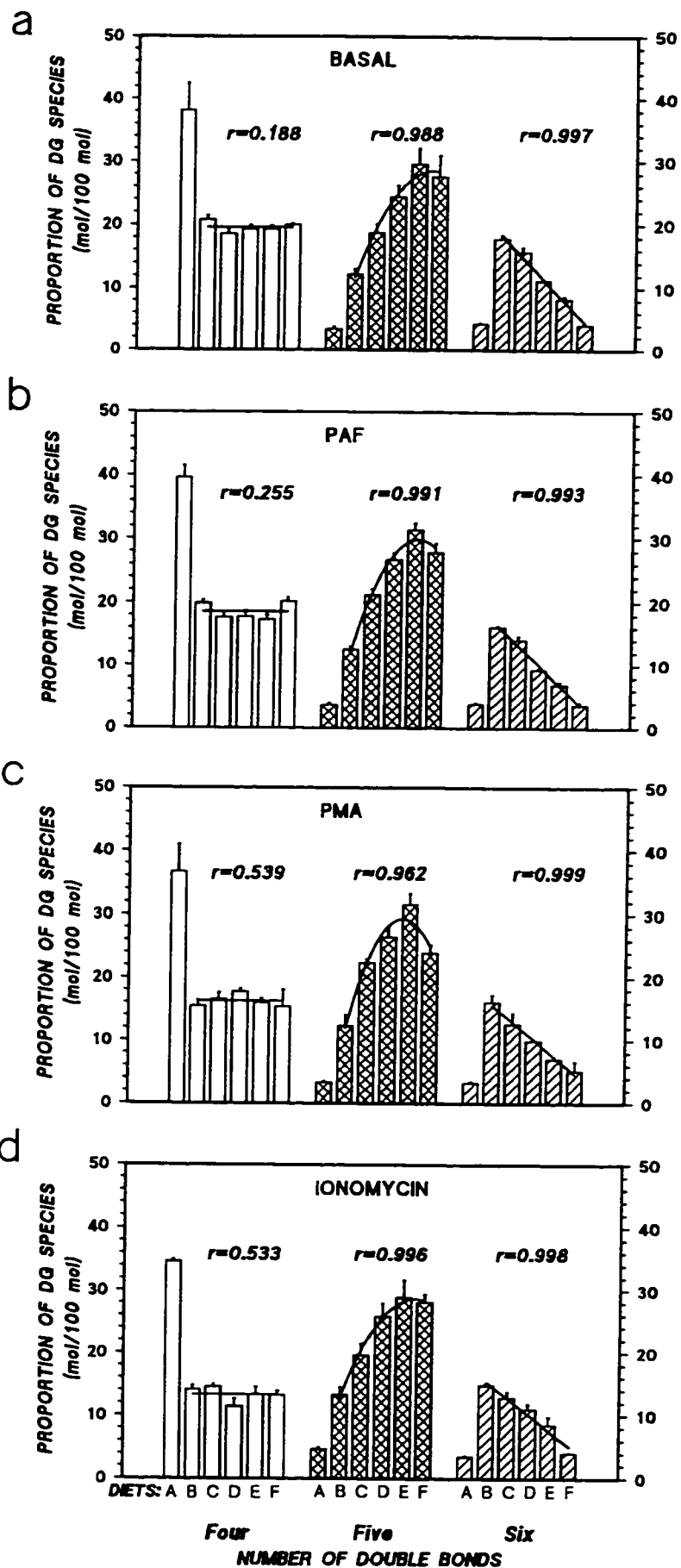
No difference in weight was found between diet groups on day 0, when mice weighed approximately 12 g. Overall, mice gained approximately 10 g after three weeks of consuming experimental diets. Weight gain did not differ significantly among diet groups.

4.4.2 *Diradylglycerol Molecular Species*

The results shown in Fig. 1 are the first demonstration that a direct dose-response relationship exists between the dietary concentration of EPEE or DHEE (ranging from 0 to 2 g/100 g diet) and the proportion of DG molecular species with 5 or 6 double bonds, respectively (Fig. 1, diets B-F). This relationship was confirmed by regression analyses. Goodness of fit by linear regression analyses for the proportions of molecular species with four and six double bonds was not significantly improved by quadratic regression, while quadratic regression analyses provided a significantly better fit (by F test, as described in Methods) for the proportions of molecular species with five double bonds. Regression analyses were performed for the results under basal and all stimulation conditions. The shapes of the dose-response curves (Fig. 1 a-d) were similar among (a) basal DG and DG formed after (b) PAF, (c) PMA or (d) ionomycin stimulation. Therefore, only the results for basal DG (Fig. 1a) will be described in further detail, as follows.

FIGURE. 1 Diradylglycerol molecular species analyses from basal and stimulated peritoneal macrophages from mice fed purified diets.

Macrophages were prepared and left unstimulated or stimulated for 10 min with 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (PAF), 0.1 $\mu\text{mol/L}$; phorbol myristate acetate (PMA), 0.1 mg/L or ionomycin, 10 $\mu\text{mol/L}$. Results for basal and stimulated DG are shown for molecular species fractions with four (left), five (middle) and six (right) double bonds and are expressed as mol/100 mol of total diradylglycerol (DG), mean $\pm$ SEM (n=4). Control (A) and experimental (B - F) diets are defined in Table 1. Correlation coefficients are shown for quadratic regressions for fraction five and for linear regressions for fractions four and six.



The proportion of basal DG molecular species with four double bonds (Fig. 1a, left) was 38 mol/100 mol in macrophages from mice fed the control diet (A) and 19-20 mol/100 mol in macrophages from mice fed the experimental diets (B-F). However, among the experimental diets, there was no relationship between diet and the proportion of basal DG with four double bonds. The incorporation of EPA (five double bonds) was 3 mol/100 mol in macrophages from mice fed the control diet (A) and 12-30 mol/100 mol in macrophages from mice fed the experimental diets (B-F) (Fig. 1a, middle), while incorporation of DHA (six double bonds) was 4 mol/100 mol in macrophages from mice fed the control diet (A) and 15 mol/100 mol in macrophages from mice fed the experimental diets (F-B) (Fig. 1a, right). The proportion of DG with five and six double bonds clearly varied directly with EPA and DHA concentration in the diet, respectively, as described above. In all cases, the regression coefficients were significantly different from zero ($p < 0.05$).

4.4.3 *Phospholipid Molecular Species*

The results shown in Fig. 2 demonstrate a dose-response relationship between the dietary concentration of EPEE or DHEE (ranging from 0 to 2 g/100 g diet) and the proportion of molecular species with 5 or 6 double bonds, respectively, in macrophages from mice fed experimental diets (Fig. 2, diets B-F). This relationship was confirmed by both linear and quadratic regression analyses and is similar to the results for DG. Goodness of fit by linear regression analyses for the proportions of molecular species with four and six double bonds was not

significantly improved by quadratic regression, while quadratic regression analyses provided a significantly better fit (by the F test - see Methods) for the proportion of molecular species with five double bonds. Regression analyses were performed for all phospholipid classes. Even though the proportions of phospholipid molecular species differed among classes, the shapes of the dose-dependency curves were similar (Fig. 2 a-d). Therefore, only the results for PC will be described in further detail as follows.

The proportion of PC molecular species with four double bonds (Fig. 2a, left) was 29 mol/100 mol in macrophages from mice fed the control diet (A) and 8-10 mol/100 mol in macrophages from mice fed the experimental diets (B-F). However, among the experimental diets, no relationship was found between diet and the proportion of PC with four double bonds ($p > 0.05$). The incorporation of EPA was 3 mol/100 mol in macrophages from mice fed the control diet (A) and 9-22 mol/100 mol in macrophages from mice fed the experimental diets (B-F) (Fig. 2a, middle), while incorporation of DHA was 2 mol/100 mol in macrophages from mice fed the control diet (A) and 3-9 mol/100 mol in macrophages from mice fed the experimental diets (B-F) (Fig. 2a, right). In all cases, regression coefficients were significantly different from zero ($p < 0.05$).

FIGURE. 2 Phospholipid molecular species analyses from peritoneal macrophages from mice fed purified diets.

Macrophage preparation and phospholipid (PL) separation and analyses are described in Methods. Results are shown for molecular species fractions with four (left), five (middle) and six (right) double bonds and are expressed as mol/100 mol of total phospholipid, mean $\pm$ SEM (n=4). Control (A) and experimental (B - F) diets are defined in Table 1. Correlation coefficients are shown for quadratic regressions for fraction five and for linear regressions for fractions four and six.

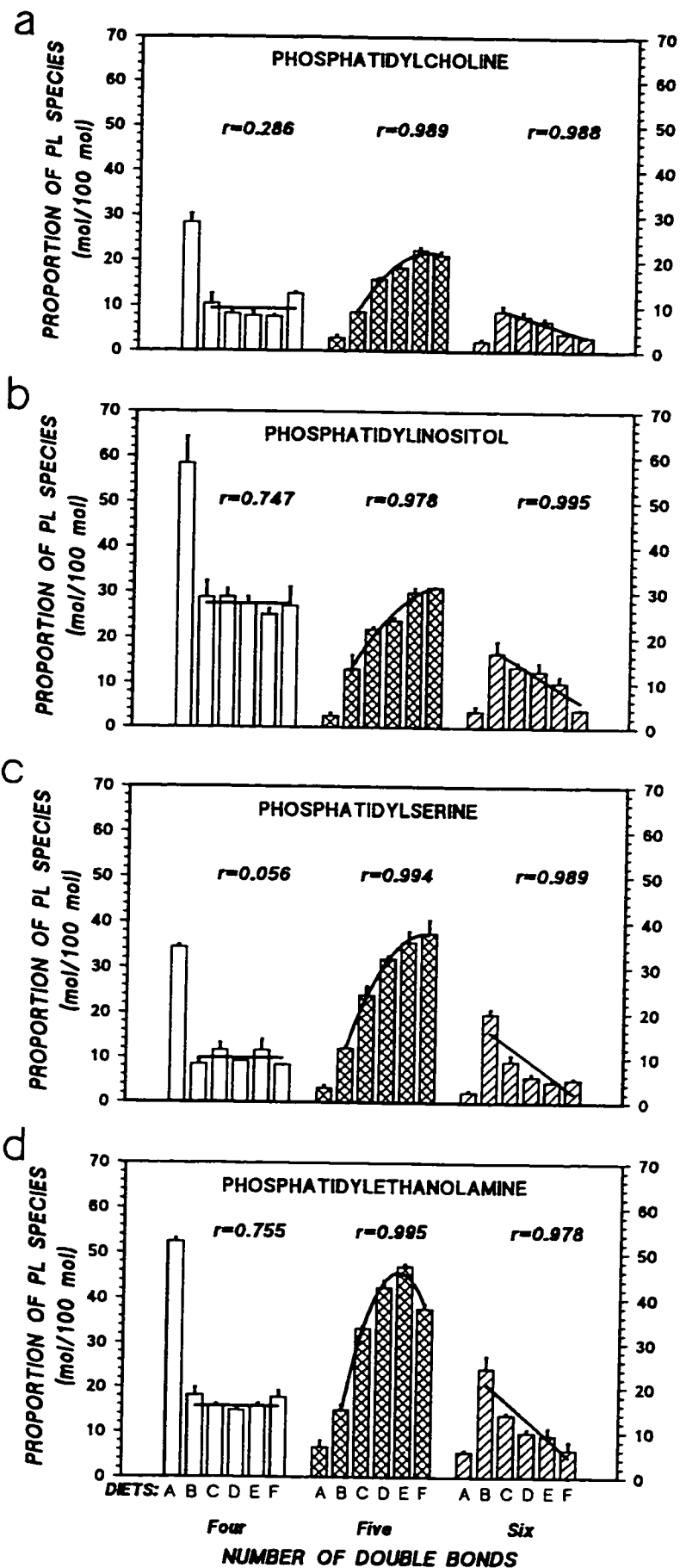
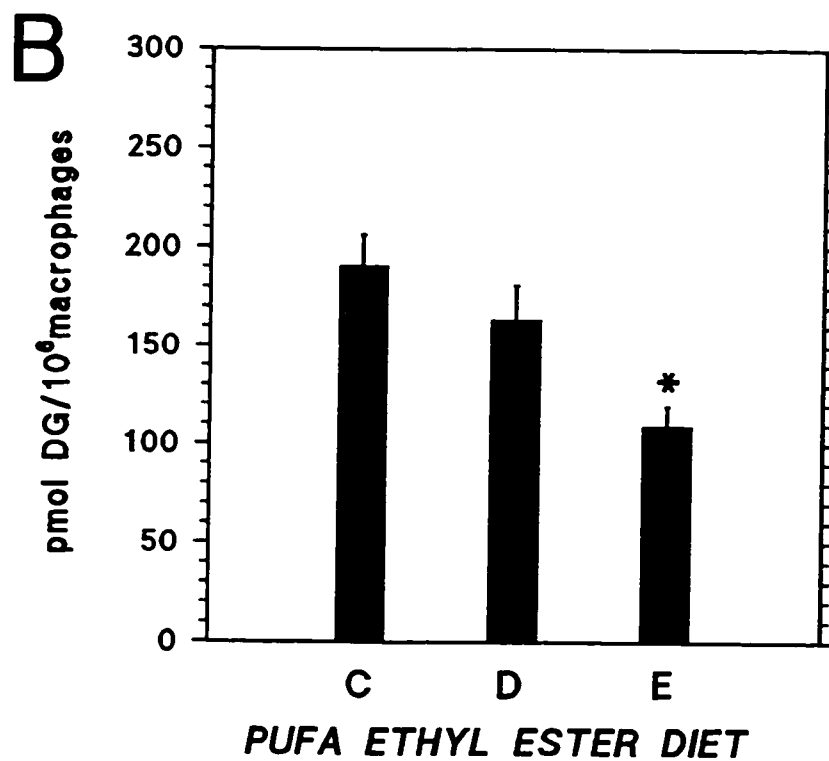
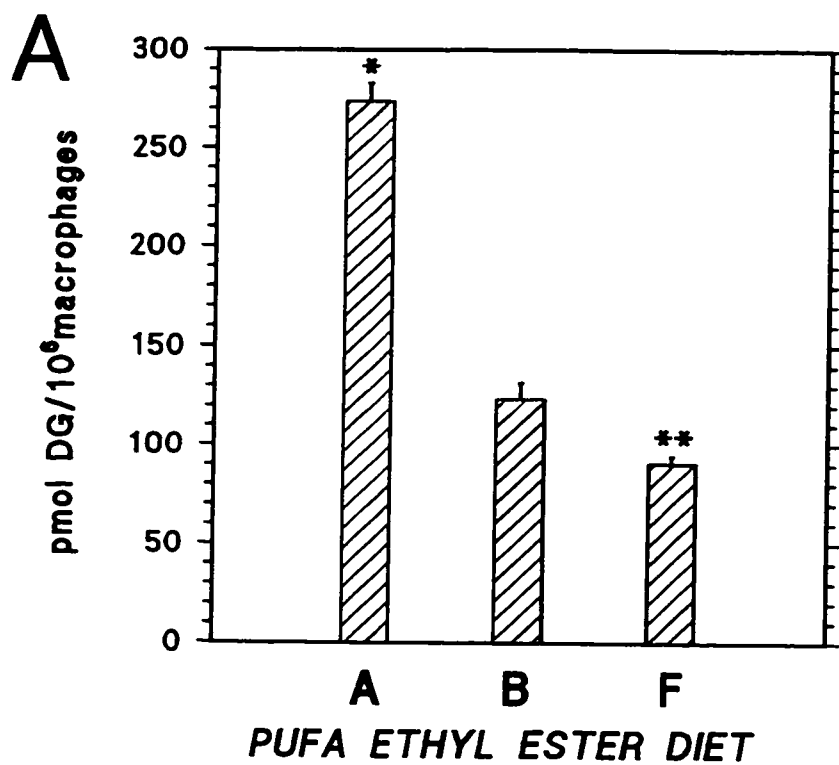


FIGURE. 3 Total basal diradylglycerol mass in unstimulated peritoneal macrophages from mice fed purified diets.

Results are expressed as pmol/ 10^6 macrophages, mean $\pm$ SEM (n=6). a. Comparison of effects of EPEE, DHEE and COEE on total diradylglycerol (DG) mass. Total dietary lipid concentration was held constant at 3 g/100 g, including at least 1 g/100 g COEE. The remaining 2 g/100 g lipid was COEE (A), EPEE (F) or DHEE (B). b. Dose-effect relationship between EPEE and DHEE and total DG mass. Total dietary lipid content was held constant at 3 g/100 g, including 1 g/100 g COEE. The remaining 2 g/100 g lipid was (n-3)PUFA ethyl ester (EPEE and DHEE), and the EPEE:DHEE varied from 0.5:1.5 (C) to 1:1 (D) to 1.5:0.5 (E). Statistically significant differences were determined when p values were less than 0.05. DG mass in diet groups A and E is significantly different from others (*, $p < 0.05$). DG mass in diet group F is significantly different from others (**, $p < 0.05$).



4.4.4 *Total diradylglycerol mass*

Total DG mass was quantitated in unstimulated macrophages by *E. coli* DAG kinase assay. In the control diet group (A) (Fig. 3a), total DG mass was 274 pmol/ 10^6 macrophages, but in experimental diet groups B and F, total DG mass was significantly lower, 123 and 90 pmol/ 10^6 macrophages, respectively ($p < 0.05$). Furthermore, the total DG mass in diet group F (EPEE without DHEE) was significantly lower than that in diet group B (DHEE without EPEE) ($p < 0.05$).

This difference between EPEE and DHEE was examined in further detail by dose-response studies (diet groups C-E) (Fig. 3b). Total DG mass was progressively lower, from 178 to 109 pmol/ 10^6 macrophages, in diet groups C to E (increasing EPEE and decreasing DHEE). Total DG mass in diet group E was significantly lower ($p < 0.05$) than that in diet groups C and D.

We also quantitated total DG mass after stimulation of macrophages with PAF, PMA or ionomycin in all diet groups. In the current study, the greater amounts of total DG mass observed after stimulation were comparable to previously reported changes (Marignani and Sebaldt 1995). Unlike the case for basal DG mass described above, we did not find significant differences among the diet groups in total DG mass after stimulation.

4.5 DISCUSSION

In this study we show for the first time a direct correlation between the amount of longterm diet-supplied (n-3) ethyl ester, either EPEE or DHEE, and the extent of replacement of resident *sn*-2 fatty acids by EPA or DHA in both membrane phospholipids and in DG. Our results explicitly show significant dose-dependent increases in the proportions of both phospholipid and DG molecular species that have *sn*-2 esterified fatty acids with 5 and 6 double bonds, even when essential fatty acids are present. We provide clear evidence that fatty acids with 5 double bonds are retained with greater affinity than those with 6 in macrophage membrane PC, PS, PE and PI. Presumably it is as a result of this that the proportion of DG formed with 5 double bonds at the *sn*-2 position is correspondingly greater than that with 6 when equimolar amounts of EPA and DHA ethyl esters are present in the diet. We have shown this to be true for both basal and stimulated DG. We have also analyzed our DG and phospholipid samples (unpublished data not shown) by a reverse-phase HPLC method described previously (Sebaldt et al. 1992) which resolves DG species in a different pattern than argentation TLC. These analyses show that the increases in fatty acids with 5 and 6 double bonds correspond to peaks comigrating with the diacyl subclass of *sn*-1-palmitoyl and *sn*-1-stearoyl DG with *sn*-2-EPA and *sn*-2-DHA, respectively. These results support the view that the physiological effects of EPA and DHA are potentially distinct. The magnitude of the differences between EPA

and DHA warrants further investigation.

Overall, the molecular species profiles of DG with four, five and six double bonds under basal as well as PAF-, PMA- and ionomycin-stimulated conditions were similar. This may suggest that, regardless of the stimulant introduced to macrophages in culture, the accessibility of phospholipid-incorporated PUFA as a group to hydrolyzing phospholipases remains tightly regulated (Galli et al. 1993, Lokesh and Kinsella 1994, Walsh et al. 1994).

DG molecular species formed in the various experimental diet groups were significantly different from one another and from those in the control diet groups, indicating that even a modest change in the PUFA component in the diet has a significant impact on DG composition. Common to the experimental diets was 1 g/100 g COEE as a source of essential fatty acid. Interestingly, molecular species with four double bonds remained stable regardless of the composition (EPA versus DHA) of the (n-3)PUFA component in the diet (Fig. 1) while the proportions of DG molecular species fractions five (containing EPA) and six (containing DHA) reflected the dietary lipid composition. Specifically, the mol/100 mol of fraction five changed as much as 10 mol/100 mol for each dietary increment change of 0.5 g/100 g EPEE, compared to a 2-5 mol/100 mol change in the proportion of fraction six for each dietary increment of 0.5 g/100 g DHEE. These results likely reflect corresponding changes in membrane phospholipids (see below) but may also suggest that phospholipase activity towards phospholipid-incorporated EPA is greater than that for incorporated DHA. The DHA moiety may impose steric

hindrance that does not permit access by and subsequent action of phospholipases. Because these differences between EPA and DHA are also observed *in vitro* (Sebaldt and Marignani 1995), they are unlikely to reflect differences in their intestinal absorption.

Molecular species in each phospholipid class (Fig. 2) parallel the DG molecular species findings (Fig. 1) in that the degree of EPA incorporation into membrane phospholipids was greater than that of DHA incorporation. This was true for all phospholipid classes analyzed, suggesting that the affinity for incorporation into membrane phospholipids of murine peritoneal macrophages is greater towards EPA than DHA. These results are consistent with our earlier studies (Marignani and Sebaldt 1995) where we reported that the proportion of phospholipid molecular species with five double bonds was disproportionately greater than that with six, given the ratio of species present in the diet in those studies.

Quantification of DG revealed that the decrease in basal total DG mass paralleled the decrease in the DHEE component and increase in the EPEE component of the diet (Fig. 3b). Diet B (DHEE without EPEE) reduced DG mass to 45% of control while diet F (EPEE without DHEE) reduced DG mass to 33% of control, and the reductions are significantly different. This suggests that the incorporation of (n-3)PUFA into membrane phospholipids attenuates basal levels of total DG mass in murine peritoneal macrophages. Furthermore, EPA more readily attenuates the formation of basal DG than does DHA.

The incorporation of EPA or DHA at the *sn*-2 position of DG and phospholipid molecular species is largely at the expense of AA incorporation (Marignani and Sebaldt 1995). The substitution of AA at the *sn*-2 position by EPA and/or DHA has been associated with numerous pharmacological and physiological changes, particularly in inflammatory mediating cells (Needleman et al. 1979, Whelan et al. 1991, Huang et al. 1986; Croft et al. 1987, Chan et al. 1993, Chilton et al. 1993, Fowler et al. 1993a) and more recently in neoplastic diseases (Hardardottir and Kinsella 1992; Hubbard et al. 1994). At equimolar dietary concentration (diet D), EPA incorporation into DG is approximately 2.5 times greater than DHA incorporation, for basal DG as well as for PAF, PMA and ionomycin stimulated DG (Fig. 1). Moreover, the incorporation of EPA into PE and PS is approximately 4.5 times greater than that of DHA and the incorporation of EPA into PC and PI is approximately 2.5 times greater than that of DHA (Fig.2). The substitution of AA by EPA and DHA in PS and PE could potentially affect membrane structure and metabolic function, such as PE conversion to PC and signalling pathways where enzyme activity is dependent upon anionic PS.

Other investigators have reported related interesting effects of (n-3)PUFA ethyl esters. Both phospholipid and DG molecular species consist of diacyl, alkyacyl and alkenylacyl subclasses. Work by Chapkin and colleagues has shown that a large proportion of PL-incorporated EPA and DHA is substituted into the diacyl subclass followed by lesser amounts into alkylacyl and alkenylacyl subclasses (Akoh and Chapkin 1990, Chapkin and Carmichael 1990, Chapkin et al.

1992, Fowler et al. 1993b). Furthermore, total DG mass in unstimulated and concanavalin A-stimulated murine splenocytes from mice fed diets with purified EPA ethyl esters was lower than that found in mice fed diets with purified DHA ethyl esters (Fowler et al. 1993b). Our results for total DG mass and for molecular species distributions of DG and phospholipids extend these and our own earlier findings (Marignani and Sebaldt 1995) and also demonstrate a dose-response relationship. In addition, we have very recently examined (a) macrophage interleukin-6 production in tissue culture studies and (b) protein kinase C (PKC) activation in lipid vesicle studies, where we have found pronounced and differential effects of EPA and DHA (unpublished data).

The implications of dietary supplementation with EPA, DHA or mixtures of these (n-3)PUFA in terms of the resulting altered formation of DG in signal transduction are not fully understood. The incorporation of (n-3)PUFA into membrane phospholipids and the eventual formation of DG in both reduced total mass and with EPA or DHA esterified at the *sn*-2 position may be of significance in a number of receptor-mediated signal transduction pathways. Thus far, the only well-described physiological function for DG is to participate in the activation of PKC (Nishizuka 1986). Potentially, lesser amounts and structurally modified *sn*-1,2-DG may alter the activation of PKC and affect the regulation of signalling pathways that control cell growth and differentiation.

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CHAPTER FIVE

PUBLISHED MANUSCRIPT

Marignani, P. A. and Sebaldt, R. J. (1996b) ω -3 Polyunsaturated Fatty Acid-Induced Changes In The Molecular Species Composition of Diradylglycerol In Murine Peritoneal Macrophages Remain Stable During Incubation *Ex Vivo*. *Lipids* 31:771-776.

PREAMBLE

The purpose of these experiments was to determine the hypothesis that incubation of macrophages *ex vivo* after several weeks of ω 3PUFA dietary supplementation *in vivo*, particularly when dfcs is included in the culture media, causes a diminution of the extent of ω 3PUFA incorporation into DG and PL. I did this by comparing the extent of EPA and DHA incorporation into DG after both standard (14h) and abbreviated (2h) incubations *ex vivo*, in both presence and absence of dfcs.

**AOCS
PRESS**

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August 14, 1996

Paola Marignani
McMaster University
Faculty of Health Sciences
Medical Sciences, Room 4N55S
Hamilton, Ontario
Canada L8N 3Z5

Dear Paola Marignani:

With this letter we grant your request to include the following material to be included in your thesis:

Marignani, P.A., R. J. Sebaldt, "Polyunsaturated Fatty Acid-Induced Changes In The Molecular Species Composition of Diradylglycerol In Murine Peritoneal Macrophages Remain Stable During Incubation Ex Vivo, *LIPIDS* 31:771-776.

Please acknowledge the original source, publisher, author(s) and date.

Sincerely,



Catherine Sexton
Publications Department

Enclosures

5.1 ABSTRACT

Three- to four-week-old C57BL/6 mice were maintained for four weeks on diets in which the 10% lipid component was the ethyl esters of either corn oil or ω -3 polyunsaturated fatty acids (ω -3 PUFA). Incubation of macrophages *ex vivo* for 14 hours, a normal incubation time used for macrophage studies, in the absence of fetal calf serum did not diminish the extent of incorporation of 20:5 ω -3 (EPA) and 22:6 ω -3 (DHA) moieties into membrane phospholipids and into diradylglycerol (DG) relative to that after a very abbreviated incubation time. We conclude that studies to examine the effects of dietary ω -3 PUFA on DG formation and related physiological effects in macrophages can be performed after a normal *ex vivo* incubation of at least 14 hours without experiencing a significant loss of incorporated ω -3 PUFA.

5.2 INTRODUCTION

Humans are incapable of *de novo* synthesis of ω -3 polyunsaturated fatty acids (ω -3 PUFA) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Incorporation of these PUFA into cell membrane lipids reflects their dietary intake (1-3). However, functional studies on lipid second messengers conducted *ex vivo* after manipulation of dietary PUFA content are potentially problematic because a rapid loss of the diet-induced changes in fatty acid composition might occur during the customary prolonged (12-16 h) incubation of adhered macrophage monolayers *ex vivo* (4-8). A number of physiological functions of macrophages require 8 - 16 h incubation *ex vivo* to reach stable basal levels (4), but the stability of phospholipid (PL) and diradylglycerol (DG) molecular species compositions over this prolonged interval is unknown. In the present study, we investigated to what extent previously described dietary ω -3 PUFA-induced changes in PL and DG molecular species in murine peritoneal macrophages (3) remain stable after a customary prolonged incubation period *ex vivo* relative to a much more abbreviated incubation. Our results define a basis of validity on which functional studies of the effects of dietary ω -3 PUFA may be performed *ex vivo*.

5.3 MATERIALS AND METHODS

5.3.1 *Animal care and diets.*

Ethyl ester concentrates were graciously supplied by the NIH/NOAA Fish Oil Test Materials Program (Charleston, SC). Pathogen-free 3-4 week old C57BL/6 mice (4 - 5/cage) (Charles River Laboratory, Montreal, PQ) were assigned to either corn oil (COEE) or ω -3 fatty acid ethyl ester (ω -3EE) diet groups for four weeks as previously described (3) and allowed access to food and water *ad libitum*. Mice were individually weighed on day 0 and weekly thereafter to monitor growth.

5.3.2 *Macrophage culture and stimulation.*

Mice were injected intraperitoneally with 1 ml brewer's thioglycollate (DIFCO, Detroit, MI). Macrophages were harvested and washed as described (3). For quantitative analyses of total DG mass, macrophage monolayers ($2 \times 10^6/9.62 \text{ cm}^2$ well) were adhered for 2 h, washed and cultured for an additional 2 h (abbreviated incubation) or 14 h (standard incubation). For DG and PL molecular species analyses, macrophage monolayers ($15 \times 10^6/16 \text{ cm}^2$ well) were similarly adhered and incubated. The culture medium was RPMI 1640 with (+dfcs) or without (-dfcs) 10% heat-deactivated fetal calf serum, as indicated. Macrophage monolayers were then washed with Hanks balanced salt solution containing 10 mmol/L Hepes, pH 7.4 (HHBSS), and were stimulated for 10 minutes with either PAF (0.1 μM), ionomycin (10 μM), PMA (0.1 $\mu\text{g/ml}$) or HHBSS control, as

described (3). All conditions were repeated in duplicate or triplicate wells for each experiment.

5.3.3 *Lipid analyses*

Lipids were extracted by the Bligh-Dyer method (9). For total DG mass, DG was derivatized to phosphatidic acid, purified by t.l.c. and quantitated as described (3). For DG molecular species composition, DG was derivatized to dimethylphosphatidic acid and separated into molecular species fractions by argentation thin-layer chromatography (t.l.c.) as described (3). PL classes were separated and hydrolyzed to DG, then further analysed like DG for molecular species fractions.

5.3.4 *Statistical analysis*

Results are expressed as mean $\pm$ SEM or as mean and range, as indicated. Statistical significance of differences between experimental conditions was tested by Student's two-tailed t-test using the Fig.P computer program (version 6.0c, Biosoft, Cambridge, UK).

5.4 RESULTS

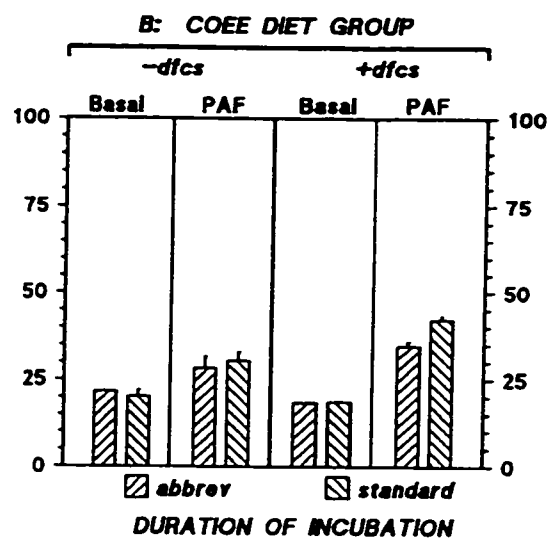
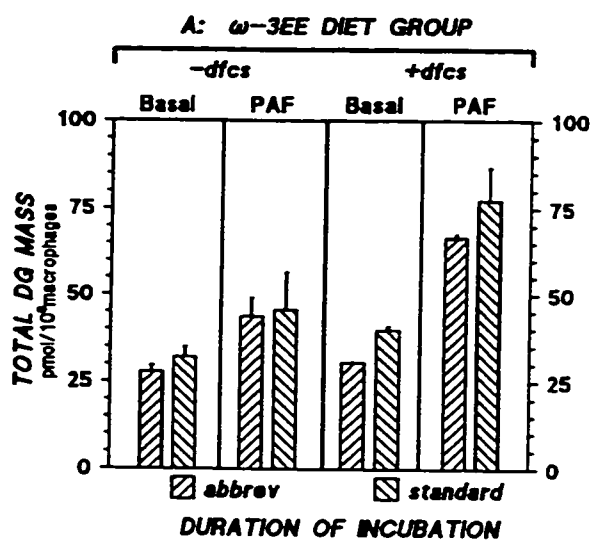
5.4.1 *Animal growth*

Mice gained approximately 12 g after four weeks in both the ω -3EE and COEE diet groups. Between diet groups, weights were not statistically significant at baseline or at weekly weighings.

5.4.2 *The effects of incubation time, with and without fetal calf serum, on the total mass of diradylglycerol*

Mice were fed either ω -3EE or COEE diets for four weeks, after which peritoneal macrophages were elicited, harvested, adhered for 2 h and incubated *ex vivo* for an abbreviated (2 h) or a standard (14 h) duration, in either +dfcs or -dfcs medium. Macrophages were then left unstimulated to determine total basal DG mass or stimulated with PAF (0.1 μ M) for 10 min. In the ω -3EE diet group (Figure 1A), basal DG mass was lower after abbreviated than after standard incubation time in both -dfcs medium (27.7 ± 1.7 pmol/ 10^6 macrophages compared to 31.9 ± 2.9 , n.s.) and in +dfcs medium (30.5 ± 0.1 pmol/ 10^6 macrophages compared to 39.8 ± 0.9 , $p < 0.05$). Similarly, after macrophage stimulation with PAF (Figure 1A), DG mass was lower after abbreviated than after standard incubation time in both -dfcs medium (43.7 ± 5.4 pmol/ 10^6 macrophages compared to 45.6 ± 10.8 , n.s.) and in +dfcs medium (66.5 ± 1.1 pmol/ 10^6 macrophages

Figure 1. The effects of incubation time in the presence and absence of dfcs on the total mass of diradylglycerol in murine peritoneal macrophages. Mice were fed diets containing either A. ω -3 fatty acid ethyl ester (ω -3EE) or B. corn oil ethyl ester (COEE) for four weeks. Macrophages were harvested, adhered for 2 hours, incubated for an additional 2 (abbrev) or 14 (standard) hours in either the absence (-dfcs) or the presence (+dfcs) of fetal calf serum in the culture media, and assayed for DG mass either under basal conditions or after 10 min PAF stimulation, as described in Methods. Results are expressed as pmol DG/ 10^6 macrophages, mean $\pm$ SEM (n=4).

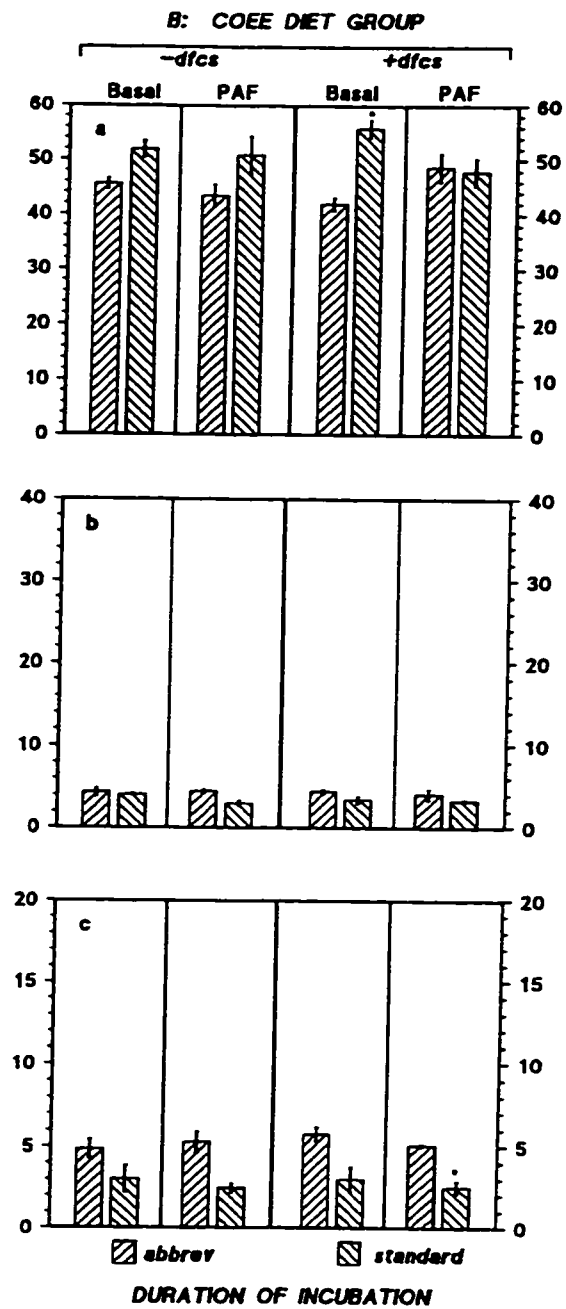
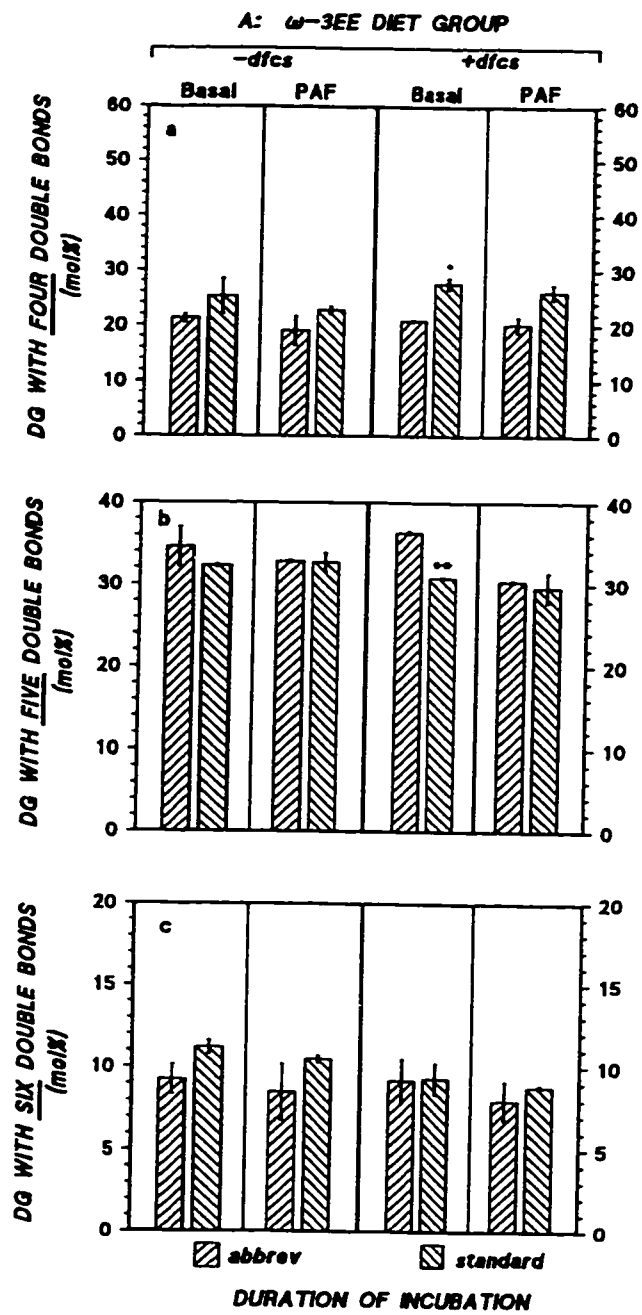


compared to 77.3 ± 8.1 , $p < 0.05$). However, in the COEE diet group (Figure 1B), both basal and PAF-stimulated DG mass were similar after abbreviated and standard incubation times, in both -dfcs and +dfcs media.

5.4.3 *The effects of incubation time, with and without fetal calf serum, on the molecular species composition of diradylglycerol*

Macrophages were prepared, incubated and stimulated as above. Under both basal and PAF-stimulated conditions, our results (Figure 2) generally show that the proportions of molecular species of DG after the standard incubation time did not differ significantly from the proportions present after only the abbreviated incubation time. Exceptions to this were seen *only* in +dfcs medium as follows: (a) In the ω -3EE diet group (Figure 2A), the fraction of basal DG with five double bonds (containing EPA) was 36.1 - 36.5 mol% after abbreviated incubation in +dfcs medium but was lower by about 17% (30.7 - 30.9 mol%) after standard incubation (Figure 2Ab). This difference was associated with a reciprocal change in DG species with 4 double bonds (containing AA), whose proportion was 20.7 - 21.0 mol% after abbreviated incubation but was 19% higher (26.6 - 28.7 mol%) after standard incubation (Figure 2Aa). (b) In the COEE diet group (Figure 2B), the fraction of basal DG with four double bonds (containing AA) was 40.7 - 43.1 mol% after abbreviated incubation in +dfcs medium but was 25% higher (54.2 - 57.4 mol%) after standard incubation (Figure 2Ba). This difference was associated with a reciprocal change in the fraction of DG with six double bonds (containing DHA),

Figure 2. The effects of incubation time in the presence and absence of dfcs on the molecular species composition of diradylglycerol in murine peritoneal macrophages. Mice were fed diets containing either A. ω -3 fatty acid ethyl ester (ω -3EE) or B. corn oil ethyl ester (COEE) for four weeks. Macrophages were prepared as in Figure 1 and assayed for DG molecular species composition, as described in Methods. Results for basal and for PAF-stimulated DG after 2 (abbrev) or 14 (standard) hours of incubation without (-dfcs) or with (+dfcs) fetal calf serum are shown for molecular species fractions with (a) four, (b) five, and (c) six double bonds. Results are expressed as mol% of total DG, mean and range (n=2).



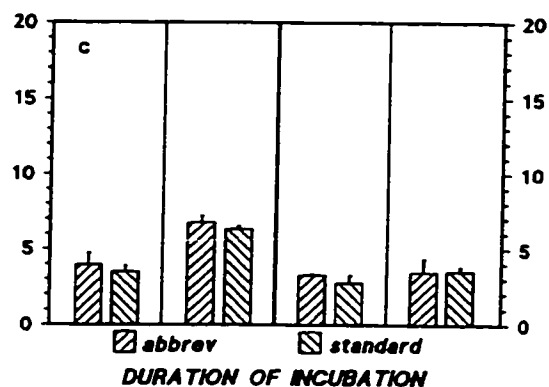
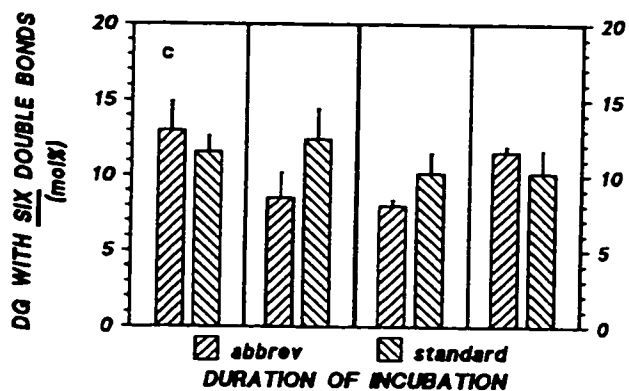
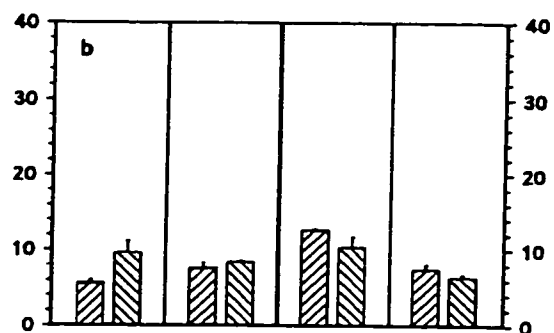
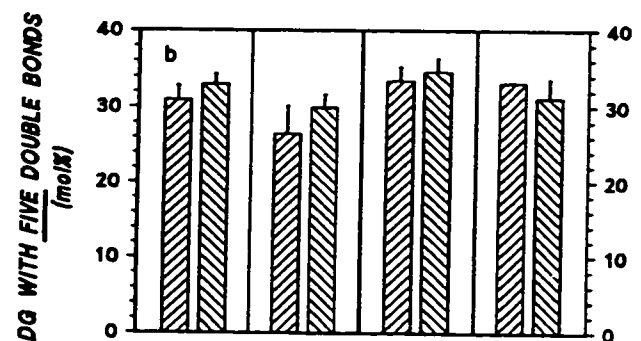
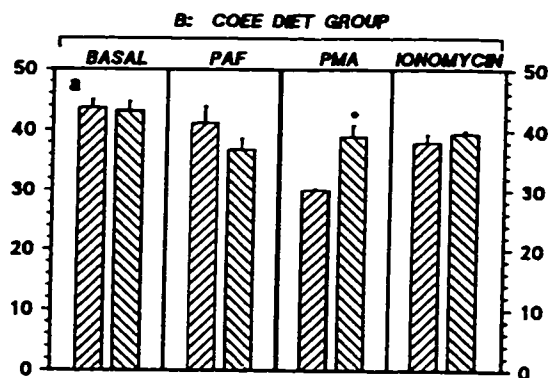
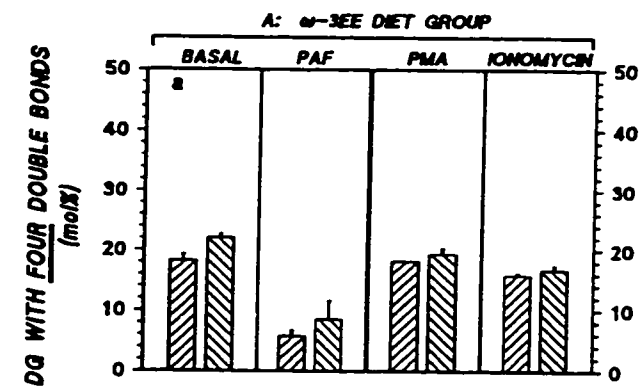
which was 5.3 - 6.2 mol% after abbreviated incubation but was lower by approximately 50% (2.3 - 3.8 mol%) after standard incubation time (Figure 2Bc). In summary, significant differences in the molecular species composition of DG were not found between abbreviated and standard incubation times unless dfcs was present in the culture media.

The above results confirmed our concerns that supplementation of culture media with dfcs may alter the molecular species composition of DG that results from a diet with a controlled lipid content. The remaining experiments were therefore conducted in -dfcs medium. Our results (Figure 3) show that within each diet group and for each macrophage stimulus, the distinctive proportions of DG molecular species with four, five and six double bonds measured after standard incubation were not significantly different from those after abbreviated incubation. An isolated exception to this is the results with PMA stimulation in the COEE diet group (Figure 3Ba), where the mol% of species with four double bonds after abbreviated incubation (30 mol%) was lower than after standard incubation (40 mol%), the difference being due to a greater fraction of species with lesser degrees of unsaturation.

5.4.4 The effects of incubation time on the molecular species composition of phospholipid classes

Since DG is formed from membrane PL, and the above results show a stability of DG molecular species composition over a period of standard incubation

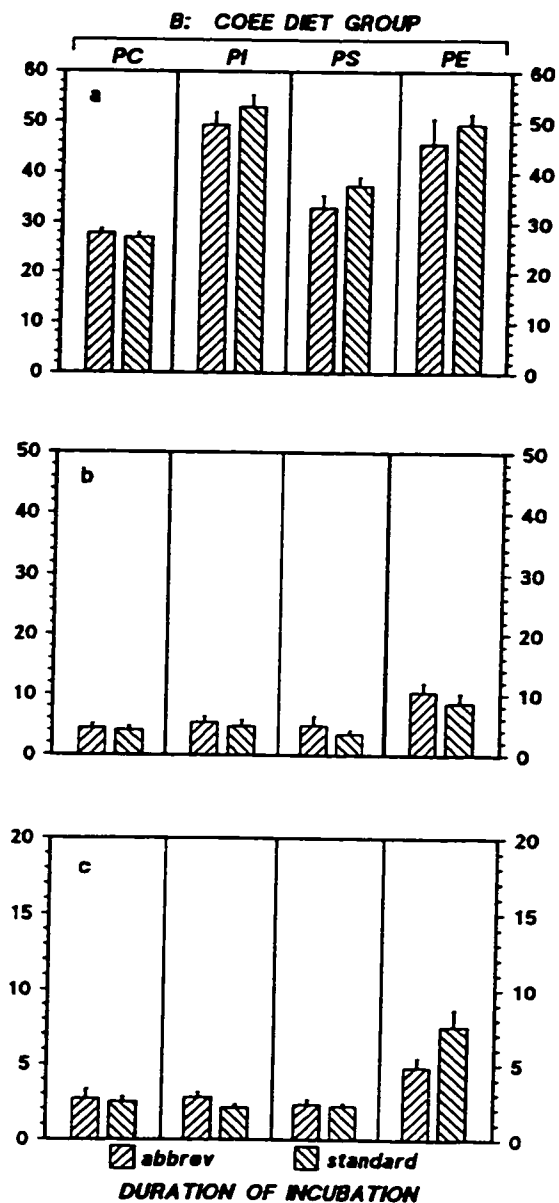
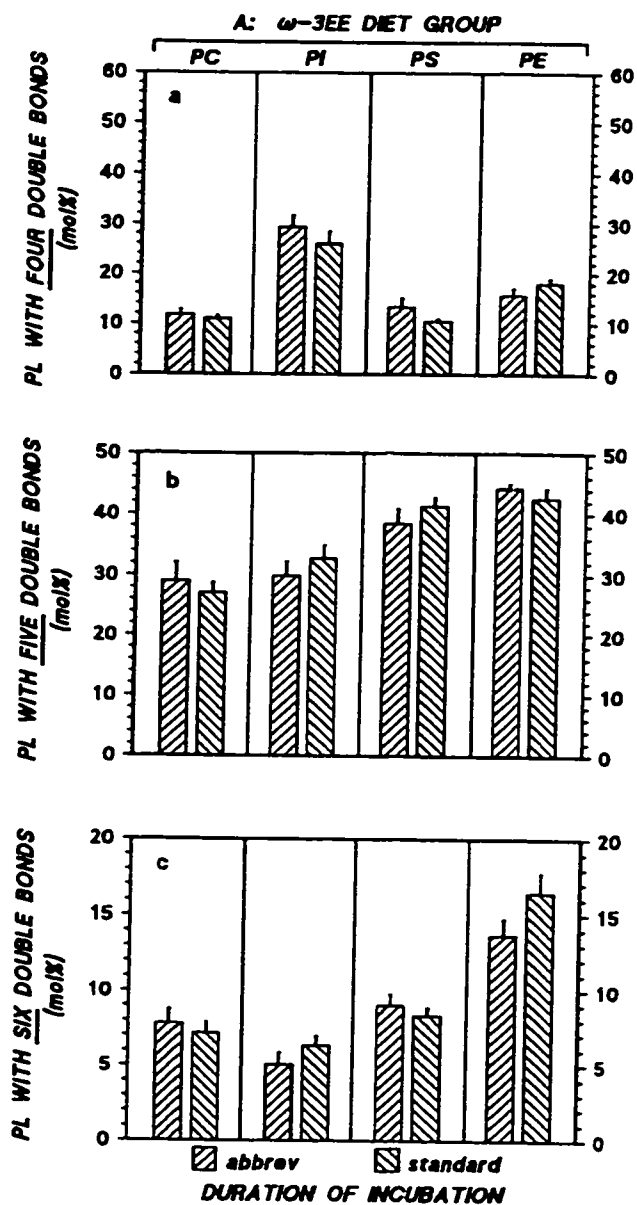
Figure 3. The effects of incubation time on the molecular species composition of diradylglycerol in variously stimulated murine peritoneal macrophages. Mice were fed diets containing A. ω -3 fatty acid ethyl ester (ω -3EE) or B. corn oil ethyl ester (COEE) for four weeks. Macrophages were harvested, adhered for 2 h, incubated in media without dfcs for an additional 2 (abbrev) or 14 (standard) hours and stimulated as described in Methods. Molecular species compositions of diradylglycerol (DG) were analyzed as described in Methods. Results are shown for basal (n=18) and agonist-stimulated conditions (PAF, 0.1 μ M; PMA, 0.1 μ g/ml and ionomycin, 10 μ M; n=6 each) for species with (a) four, (b) five, and (c) six double bonds. Results are expressed as mol% of total DG, mean $\pm$ SEM. *: p<0.02 relative to 2-h incubation.



ex vivo, we wished to determine whether a corresponding stability of the dietary ω -3 PUFA-induced changes in membrane PL could also be demonstrated.

Murine peritoneal macrophages were prepared after four weeks of either ω -3EE or COEE diets, as described in Methods. After 2 h adherence and additional incubation *ex vivo* without dfcs for either an abbreviated (2h) or a standard (14h) duration, membrane PL classes PC (n=12), PI (n=8), PS (n=8) and PE (n=10) were extracted, separated and analyzed for molecular species fractions (Figure 4). Characteristic differences between the PL classes in their molecular species profiles are as previously described (3). For each PL class, however, the characteristic species distribution found after abbreviated incubation remained entirely preserved after standard incubation. No discernible trend over time was observed within any PL class in any molecular species fraction, in either the ω -3EE (Figure 3A) or the COEE (Figure 3B) diet group. These results show that dietary ω -3 PUFA-induced enrichment of macrophage membrane PL with EPA and DHA does not significantly degrade over the standard *ex vivo* incubation time.

Figure 4. The effects of incubation time on the molecular species composition of phospholipid classes in murine peritoneal macrophages. Mice were fed diets containing A. ω -3 fatty acid ethyl ester (ω -3EE) or B. corn oil ethyl ester (COEE) for four weeks. Macrophages were prepared as in Figure 3 and incubated in media without dfcs for 2 (abbrev) or 14 (standard) hours. Phospholipid (PL) classes were separated and their molecular species compositions were analyzed as described in Methods. Results are shown for phosphatidylcholine (PC; n=12), phosphatidylinositol (PI; n=8), phosphatidylserine (PS; n=8) and phosphatidylethanolamine (PE; n=10) for species with (a) four, (b) five and (c) six double bonds. Results are expressed as mol% of each PL, mean $\pm$ SEM.



5.5 DISCUSSION

It is a significant concern that cell membrane lipid modifications achieved by dietary changes *in vivo* might quickly be lost during incubation of cells in *ex vivo* experiments. In the current study, we characterized the effects of incubation time *ex vivo* on molecular species distributions of DG and PL.

We found that the total DG mass in macrophages in the ω -3EE diet group tended to be greater after standard than after abbreviated incubation when dfcs was present in the culture media. It was stable under most other conditions (Figure 1A). However, many other normal physiological functions of macrophages equilibrate only after several hours of incubation *ex vivo* (4). Our results (Figure 1) suggest that total DG mass is similarly not yet stable after abbreviated incubation (2 h), particularly if dfcs is present in the culture media. For this reason, it is often important that macrophage monolayers not be studied *ex vivo* immediately after achieving adherence.

Molecular species of DG with EPA at the *sn*-2 position were diminished after standard incubation *ex vivo* in +dfcs medium, relative to abbreviated incubation, but this diminution was abolished in -dfcs medium (Figure 2Ab). Furthermore, under both basal conditions and after stimulation of macrophages with either the physiological agonist, PAF, the tumor promoting phorbol ester, PMA, or the divalent cation ionophore, ionomycin, we found that the proportions of DG molecular species studied in -dfcs medium were not different if the duration

of *ex vivo* incubation was abbreviated to 2 h or was a standard 14 h (Figure 3). Molecular species profiles of the PL classes PC, PI, PS, and PE were similarly stable over the duration of the standard incubation (Figure 4), in other words, there was no evidence of degradation of the lipid modifications that had previously been achieved *in vivo*. This PL stability presumably underlies the stability of the DG molecular species profiles discussed above.

We conclude that, at least under the conditions reported in this study, lipid modifications of macrophage membranes that are achieved *in vivo* after four weeks of dietary enrichment with ω -3 PUFA are not lost *ex vivo* to any significant degree over the standard 14 h incubation time. These observations validate the use of *ex vivo* studies of macrophages to assess functional implications of dietary ω -3 PUFA enrichment at the cellular level.

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CHAPTER SIX

PUBLISHED MANUSCRIPT

Sebaldt, R. J. and Marignani, P. A. Diacylglycerol (DG) formation is altered by n-3 highly unsaturated fatty acids with marked differences between eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids: A novel mechanism of action of n-3 fatty acids in signal transduction. In: *The 3rd International Conference On Eicosanoids and Other Bioactive Lipids in Cancer, Inflammation and Radiation Injury*. pp 931-939. Eds: S. Nigam, K.V. Honn, L.J. Marnett. Kluwer Academic Publishers, Boston, 1995.

PREAMBLE

The purpose of these experiments was to address the hypothesis that the modifications in DG molecular species observed *in vivo* can be replicated *in vitro* in normal cultured murine peritoneal macrophages by the addition of DHA and EPA to culture media. I did this by developing an *in vitro* system that could reproduce the changes in DG and PL molecular species composition observed *in vivo*.

6.1 INTRODUCTION

After binding to their specific cell surface receptors, many hormones and other agonists cause phospholipase-mediated turnover of membrane phosphoinositide and/or phosphatidylcholine (PC) as their initial signal transducing event. The resulting cleavage products include the important second messenger, diacylglycerol and other diacylglycerols (DG), which activates protein kinase C (PKC), a central cell regulatory enzyme. Alterations in PKC activation may be associated with development of pathological states including chronic inflammation and neoplastic transformation. It is therefore reasonable to hypothesize that perturbations in agonist-induced DG formation, particularly if present over prolonged periods, would potentially exert marked effects on cell activation and function.

Like the membrane phospholipids from which it is derived, DG second messenger is a family of molecular species that differ in the combination, permutation and linkage of the fatty acid residues at the *sn*-1 and *sn*-2 positions. Therefore, biological perturbations of DG formation may be reflected not only in changes in total mass of DG but also in changes in its composition, or distribution of molecular species. The potential biological relevance of the latter is suggested by evidence that certain molecular species, particularly those with highly unsaturated fatty acids such as arachidonate (AA or 20:4(n-6)) at the *sn*-2 position, may play unique cell regulatory roles.

We recently hypothesized that long-chain n-3 polyunsaturated fatty acids (PUFA), particularly the fish oil-derived eicosapentaenoic and docosahexaenoic acids (EPA or 20:5(n-3) and DHA or 22:6(n-3)) may have a unique and therefore virtually unexplored ability to alter agonist-induced DG formation. EPA differs from AA by the presence of an additional double bond at the n-3 position. When administered *in vitro* or *in vivo*, n-3 PUFA are incorporated and partially replace n-6 fatty acids, particularly AA, in cell membrane and other phospholipids. The resulting changes in the formation of prostaglandins, leukotrienes and other eicosanoid mediators have been investigated in considerable detail, as have the often marked differences in the biological activities of the AA- vs. the EPA-derived analogs. However, as noted by Galli (1), n-3 fatty acids have the potential to affect a large variety of other cell mediators in addition. Hui et al (2) have recently described reductions in vasopressin-stimulated DG mass in vascular smooth muscle cells after preincubation with 30 μ M EPA. That such additional, eicosanoid-independent effects may have biological significance is suggested by the results of studies of fish oils conducted over the past two decades, a number of which have described physiological or clinical effects that are not readily explained solely on the basis of altered eicosanoid formation.

In this report, we summarize and extend our recently reported findings (3) of substantially altered DG formation, both basal and agonist-induced, in murine peritoneal macrophages exposed to EPA or DHA *in vitro*. In particular, we report our findings of (i) dose-dependent increases in total DG mass and (ii) dose-

dependent incorporation of n-3 PUFA into DG with attendant changes of DG molecular species distributions. We further report dose-dependent changes in membrane phospholipid molecular species distributions that correlate with the above. Finally, we report that substantial differences exist between EPA and DHA with respect to the magnitude of some but not other induced changes in DG formation.

To our knowledge, our results represent the first description of n-3 PUFA-induced alterations in DG metabolism that defines dose-dependent quantitative and qualitative changes and reports differences in some of these effects between EPA and DHA.

6.2 MATERIALS AND METHODS

Murine peritoneal macrophages were obtained from 5-7 week old C57BL mice housed and fed in aseptic conditions, 3 days after intraperitoneal injection of 1 ml of sterile thioglycollate solution. Approximately 20 million cells per mouse, >90% macrophages, were obtained and washed, plated and adhered for 2 h to flat-bottomed plastic culture dish wells, rinsed free of nonadherent cells and further incubated for 16 h in standard tissue culture conditions, as previously described (4). The RPMI 1640-based culture media contained 10% FCS and were supplemented with indicated final concentrations of EPA or DHA, ranging from 0-100 μ M, added in EtOH to a uniform final EtOH concentration of 0.1% (v/v). Cultures were then rinsed, left in HBSS at 37° C, and stimulated with the receptor-binding, calcium-mobilizing macrophage agonist, platelet activating factor (PAF) or control for 10 min, as previously described (4).

The timed stimulations were terminated by addition of MeOH, reaction mixtures were scraped into glass tubes and lipid was extracted by the Bligh-Dyer CHCl_3 :MeOH procedure using 1 M NaCl in the aqueous phase (4). DG was radiophosphorylated using *E. coli* DG kinase and [32 P]ATP by the method of Preiss (5) and separated from labelled sphingosine products by TLC.

For DG mass quantitation in samples from designated culture wells, liquid scintillation counting and normalization to a dioleoylglycerol standard was used. Results were expressed in pmol DG per million cells (mean of triplicate wells).

For DG molecular species analysis of samples from designated culture wells, radiophosphorylated DG was extracted, further derivatized to [^{32}P]dimethylphosphatidic acid, and analyzed by Ag-TLC with liquid scintillation counting of separated bands as previously described (6). Briefly, this separates DG molecular species into bands corresponding to the number of double bonds present in their fatty acid moieties (7). Since over 95% of DG molecular species in the murine peritoneal macrophage system contain a saturated fatty acid at the *sn*-1 position (8), DG species with AA, EPA or DHA at the *sn*-2 position will separate by Ag-TLC into bands 4, 5 and 6, respectively, which we have confirmed to occur using synthesized pure DG species. For each sample of DG, results for each TLC band 0-6 (i.e. fraction of DG molecular species) were expressed as a percent of the total DG sample (mean of duplicate wells), and the results for bands 4, 5 and 6 are shown in the Figs below.

For phospholipid molecular species analysis, cell cultures in separate wells were incubated and supplemented as described above and additionally supplemented with 5 μCi [^{32}P] H_3PO_4 . After 16 h, these wells were left unstimulated and extracted as above but using 0.1 N HCl in the aqueous Bligh-Dyer phase. Phospholipids were well separated by two-dimensional TLC into PC, PE, PS and PI classes (9), visualized by autoradiography, extracted and cleaved to DG by *B. cereus* phospholipase C as described (6), then phosphorylated and analyzed for molecular species distributions as described above for DG. Given below are the results for PC, the principal phospholipid source of DG in macrophages that is

formed after 10 min of PAF stimulation (6).

Results are expressed as means $\pm$ SEM. Each complete experiment was performed in duplicate. All data were stored, normalized, combined where appropriate, statistically analyzed and presented graphically using the scientific software package, Fig.P for Windows (Biosoft).

6.3 RESULTS

6.3.1 *Effect of EPA and DHA on Basal Mass of DG*

Macrophages were incubated for 16 h in the presence of 0, 25, 50, 75 or 100 μ M EPA (Fig.1) or DHA (Fig.2). Total DG mass present under basal conditions and in the absence of PUFA supplementation (pmol per million cells) is shown as the leftmost bar in each Fig. as 100%. The next 4 bars in each Fig. show that the presence of n-3 PUFA for 16 h in the culture medium dose-dependently increases the cellular mass of basal DG. In particular, at 50 and 100 μ M supplementation, EPA causes increases of 34% and 72% in basal DG mass over unsupplemented basal control, while DHA causes increases of 120% and 213%. We conclude that both n-3 PUFA are effective, but DHA is approximately three times more potent than EPA in elevating DG mass in basal macrophages, on a basis of molar concentration of PUFA in the culture medium.

6.3.2 *Effect of EPA and DHA on PAF-Stimulated Mass of DG*

Previous work has established that the concentration of PAF we used is saturating (maximal) in our system (10). In Figs. 1 and 2, the sixth bar compared to the first shows the increase in DG mass (to 165% $\pm$ 41%) that results from cell

Figure 1. Diradylglycerol Mass: Effect of Eicosapentaenoic Acid.

Macrophages were prepared and incubated for 16 h in media containing eicosapentaenoic acid (EPA) at 0, 25, 50, 75 or 100 μ M. After incubation, macrophages were either left unstimulated or were stimulated for 10 min with 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (PAF), 0.1 μ mol/L. Dose-effect relationship between EPA and total DG mass were analyzed as described in the Materials and Methods. Results are expressed as mean $\pm$ SEM (n=3).

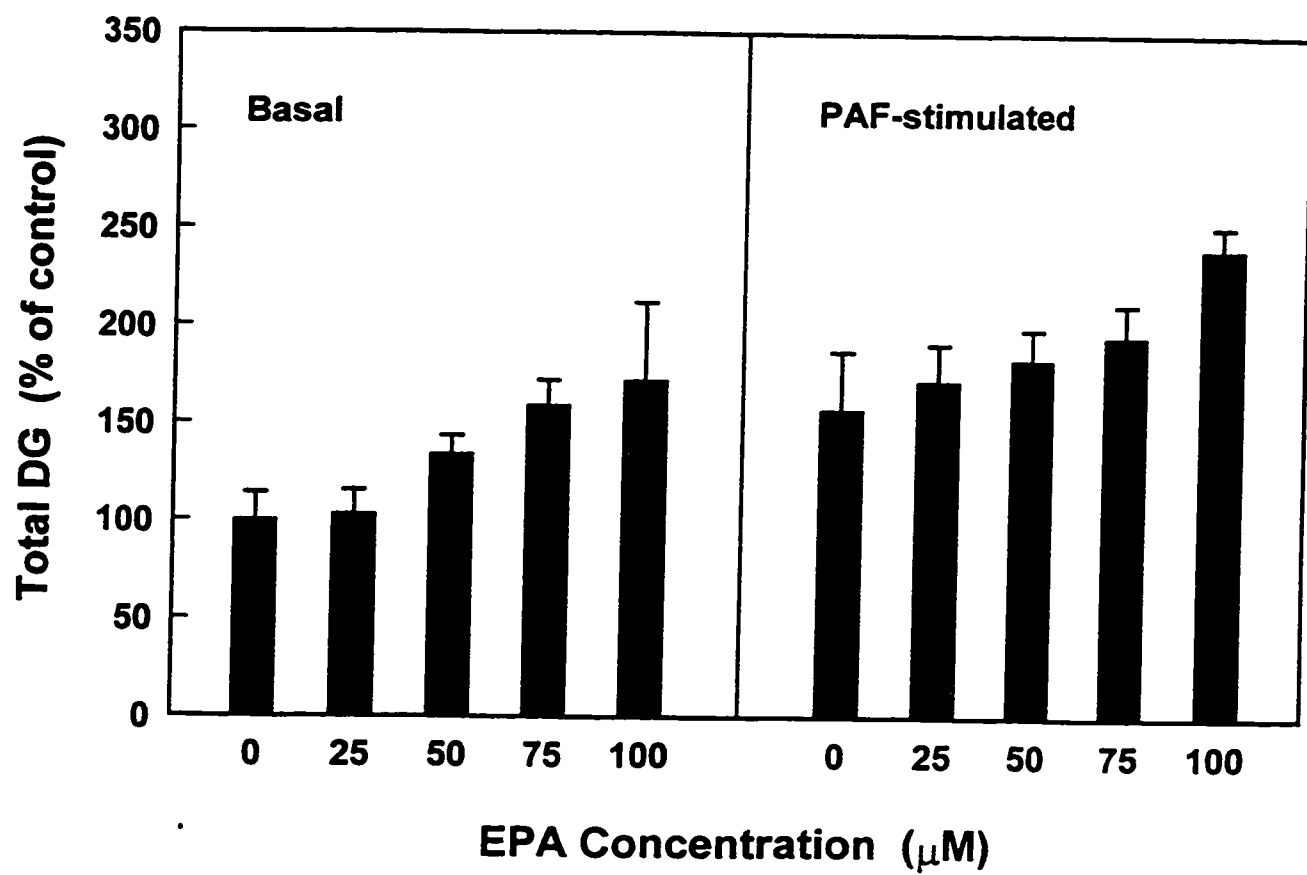
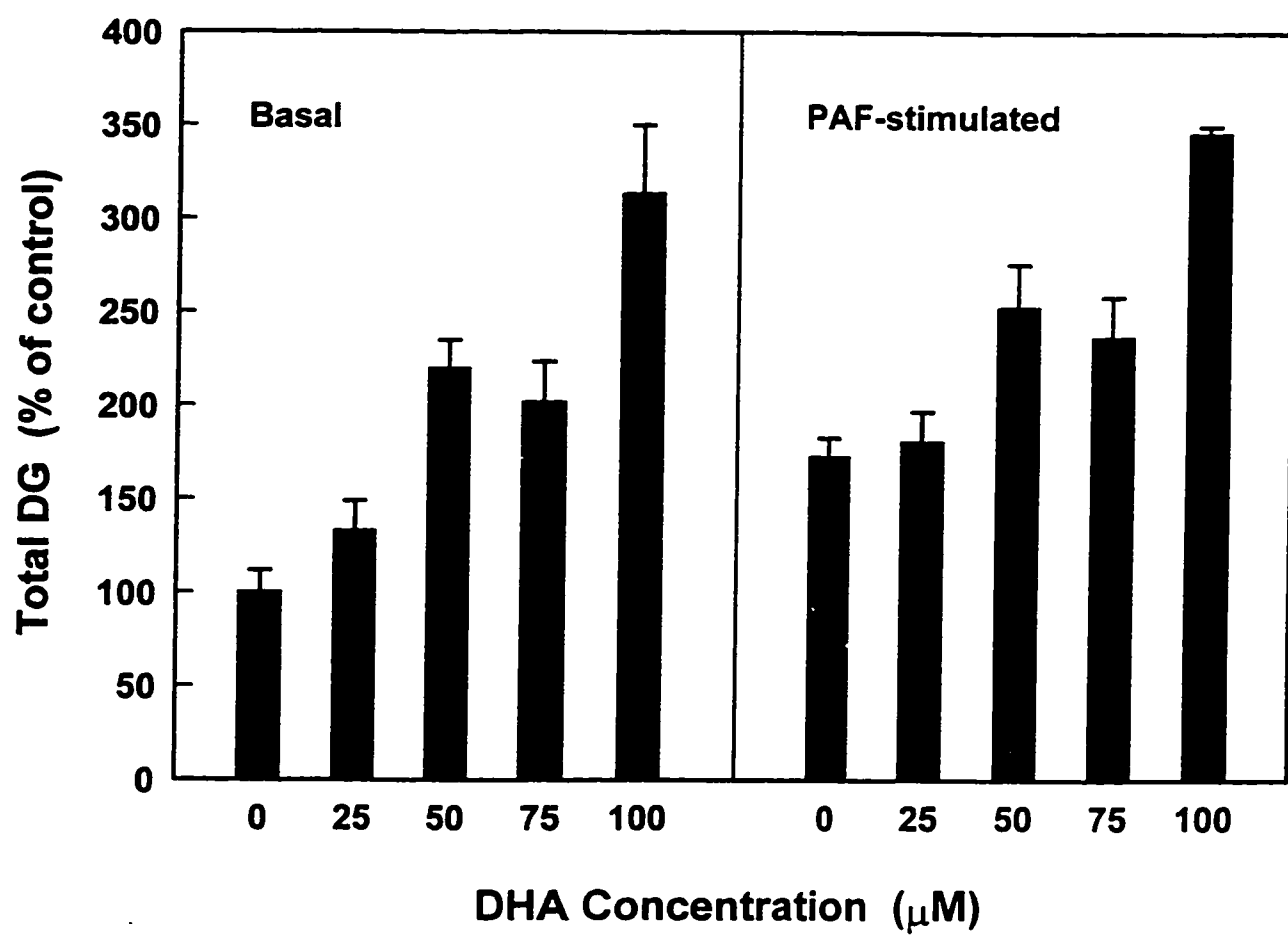


Figure 2. Diradylglycerol Mass: Effect of Docosahexaenoic Acid.

Macrophages were prepared and incubated for 16 h in media containing docosahexaenoic acid (DHA) at 0, 25, 50, 75 or 100 μ M. After incubation, macrophages were either left unstimulated or were stimulated for 10 min with 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (PAF), 0.1 μ mol/L. Dose-effect relationship between DHA and total DG mass were analyzed as described in the Materials and Methods. Results are expressed as mean $\pm$ SEM (n=3).



stimulation by PAF for 10 min in the absence of PUFA supplementation. The next 4 bars in each Fig. show that the presence of n-3 PUFA for 16 h in the culture medium dose-dependently potentiates PAF-stimulated DG formation to supra-maximal levels. In particular, at 50 and 100 μ M supplementation, EPA causes increases of 15% and 47% in PAF-stimulated DG mass above unsupplemented PAF-stimulated control, while DHA causes increases of 46% and 100%. We conclude that both n-3 PUFA are effective, but DHA is approximately two to three times more potent than EPA in potentiating DG formation in PAF-stimulated macrophages.

6.3.3 *Effect of EPA and DHA on Molecular Species Composition of Basal DG*

Macrophages were incubated for 16 h in the presence of 0, 25, 50, 75 or 100 μ M EPA (Fig.3) or DHA (Fig.4). Each Fig. shows 3 clusters of bars that represent, respectively, the percent of total DG whose species have 4, 5 and 6 double bonds. The leftmost bars of each of the 3 clusters shows the distribution of species that have 4, 5, and 6 double bonds under basal conditions and in the absence of PUFA supplementation.

The next 4 bars in each cluster in each Fig. show that the presence of n-3 PUFA for 16 h in the culture medium dose-dependently alters the proportions of DG molecular species that have 4, 5 and 6 double bonds in basal DG. In particular,

Figure 3. Diradylglycerol Molecular Species: Effect of Eicosapentaenoic Acid. Macrophages were prepared and incubated in eicosapentaenoic acid (EPA) as described in Figure 1. Results for basal DG are shown for molecular species fractions with four (#4, left), five (# 5, middle) and six (#6, right) double bonds and are expressed as percent of total diradylglycerol (DG), mean $\pm$ SEM (n=4).

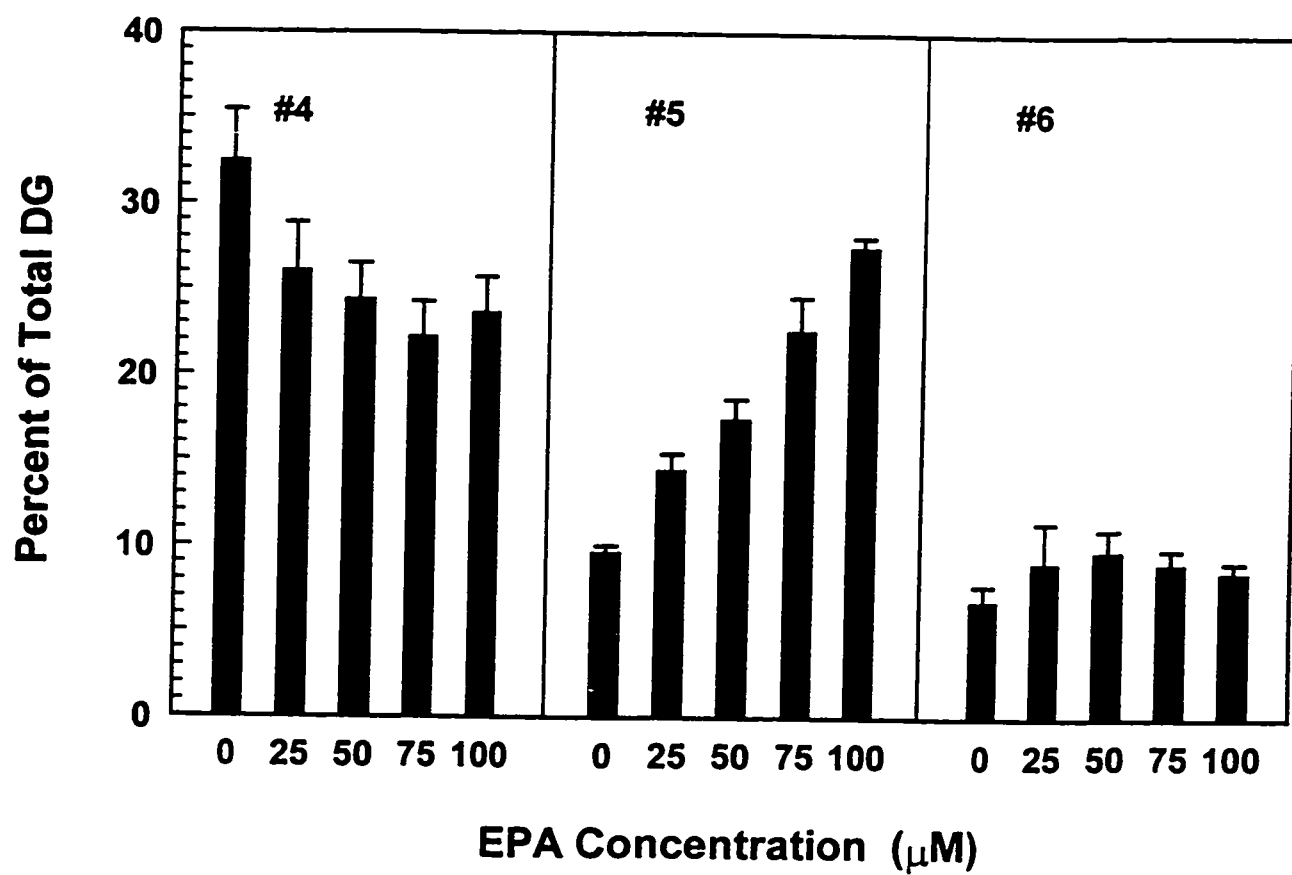
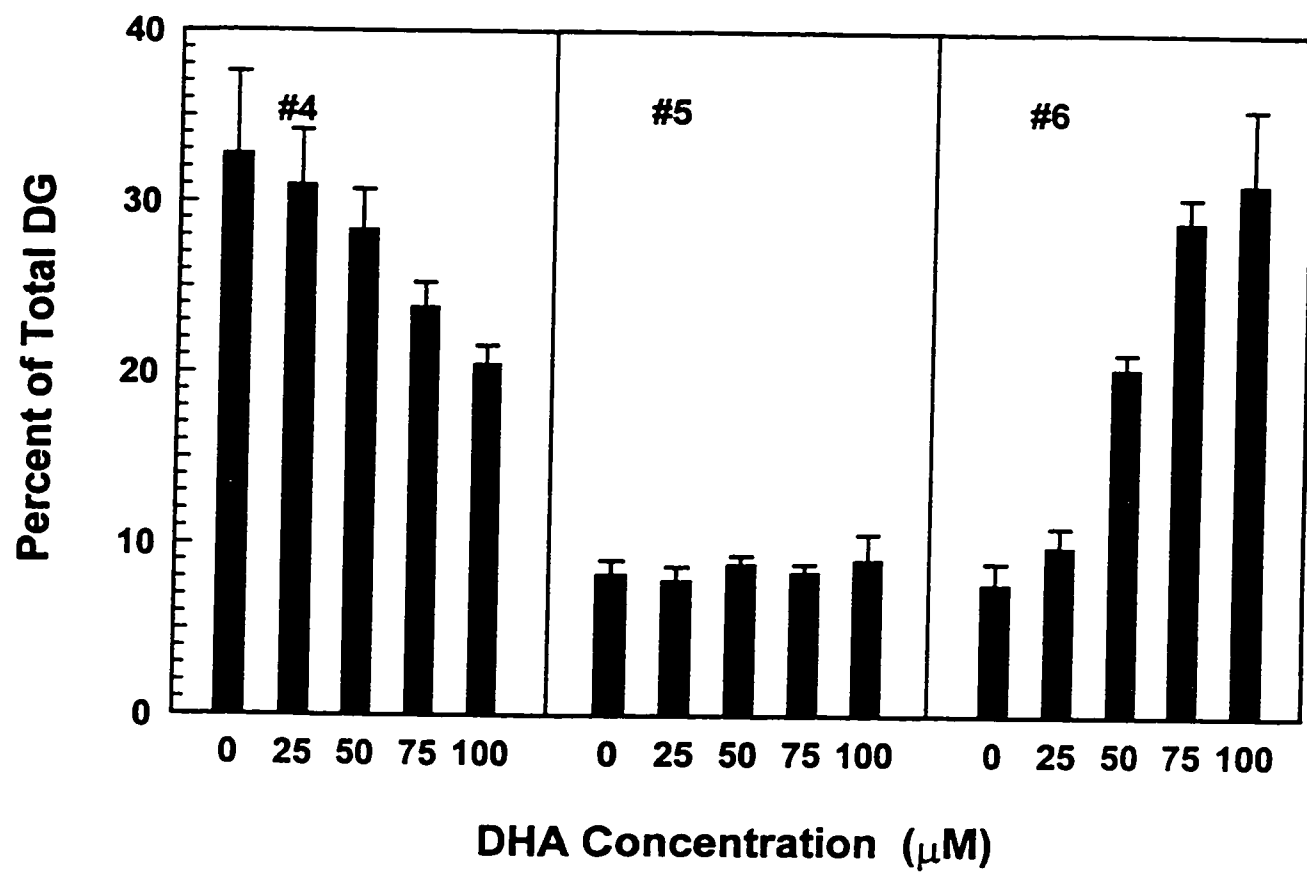


Figure 4. Diradylglycerol Molecular Species: Effect of Docosahexaenoic Acid. Macrophages were prepared and incubated in docosahexaenoic acid (DHA) as described in Figure 1. Results for basal DG are shown for molecular species fractions with four (#4, left), five (# 5, middle) and six (#6, right) double bonds and are expressed as percent of total diradylglycerol (DG), mean $\pm$ SEM (n=4).



particular, at 50 and 100 μM , EPA causes DG species with 5 double bonds to increase from 9.6% of total basal DG in the absence of PUFA supplementation to 17% and 28%, while DHA causes species with 6 double bonds to increase by a similar degree, from 7.6% of total to 20% and 31%. Both n-3 PUFA also reduce the proportion of DG species that have 4 double bonds, from 32% of total basal DG in the absence of PUFA supplementation to 24-28% and 20-24% at 50 and 100 μM , respectively. There are also dose-dependent decreases in the content of more saturated DG species (Ag-TLC bands 0-3, results not shown). Incidentally, our results also demonstrate the occurrence of only a minor degree, at most, of conversion of EPA to DHA and no retroconversion of DHA to EPA. We conclude that EPA and DHA are very substantially incorporated into the molecular species of basal DG in a dose-dependent manner, that EPA and DHA show no major differences in their degree of incorporation (per unit concentration in the culture medium) into DG molecular species, and that at least half of this incorporation is at the expense of arachidonate while the remainder is at the expense of more saturated molecular species.

6.3.4 Effect of EPA and DHA on Molecular Species Composition of PAF-Stimulated DG

Results for the molecular species distributions of PAF-stimulated DG in response to increasing concentrations of EPA or DHA supplementation are quantitatively extremely similar to and qualitatively identical to the above results for basal DG and are not shown.

6.3.5 Effect of EPA and DHA on Molecular Species Composition of Basal Phosphatidylcholine

Hydrolysis of cell membrane PC accounts for the majority of DG formed "late" after macrophage stimulation by agonists such as PAF (6). We wished to show that the compositional changes in DG described above could be accounted for by corresponding changes in cell membrane PC. Incorporation of EPA and DHA into PC have repeatedly been demonstrated previously, but to our knowledge no dose-response has heretofore been characterized.

Results for the molecular species distributions of PC under basal conditions in response to increasing concentrations of EPA or DHA supplementation are shown in Figs 5 and 6 using the same format as in the preceding two Figs for DG. We conclude that the molecular species distribution in membrane PC under basal

conditions has a significantly lower content of species with 4 double bonds than does basal DG in the absence of PUFA supplementation, as we have previously described (6), that EPA and DHA are dose-dependently incorporated into PC molecular species to a substantial and similar degree that closely mirrors the incorporation into basal and PAF-stimulated DG, and that this increase is less at the expense of arachidonate and more at the expense of more saturated species than is the case for DG. The latter observation is likely due to the fact that a portion of the total DG after PAF stimulation is contributed by pre-existing basal DG and/or by phosphoinositide hydrolysis.

Figure 5. Phosphatidylcholine Molecular Species: Effect of Eicosapentaenoic Acid.

Multilinear regression analysis was performed on the fractional composition profiles of stimulated DG samples shown in Figure 3 to estimate the contribution of phosphatidylcholine (PC) hydrolysis to their formation, expressed as a percent of total PC. Macrophages were prepared and incubated in eicosapentaenoic acid (EPA) as described in Figure 1. Results for basal DG are shown for molecular species fractions with four (#4, left), five (# 5, middle) and six (#6, right) double bonds and are expressed as percent of total diradylglycerol (DG), mean $\pm$ SEM (n=4).

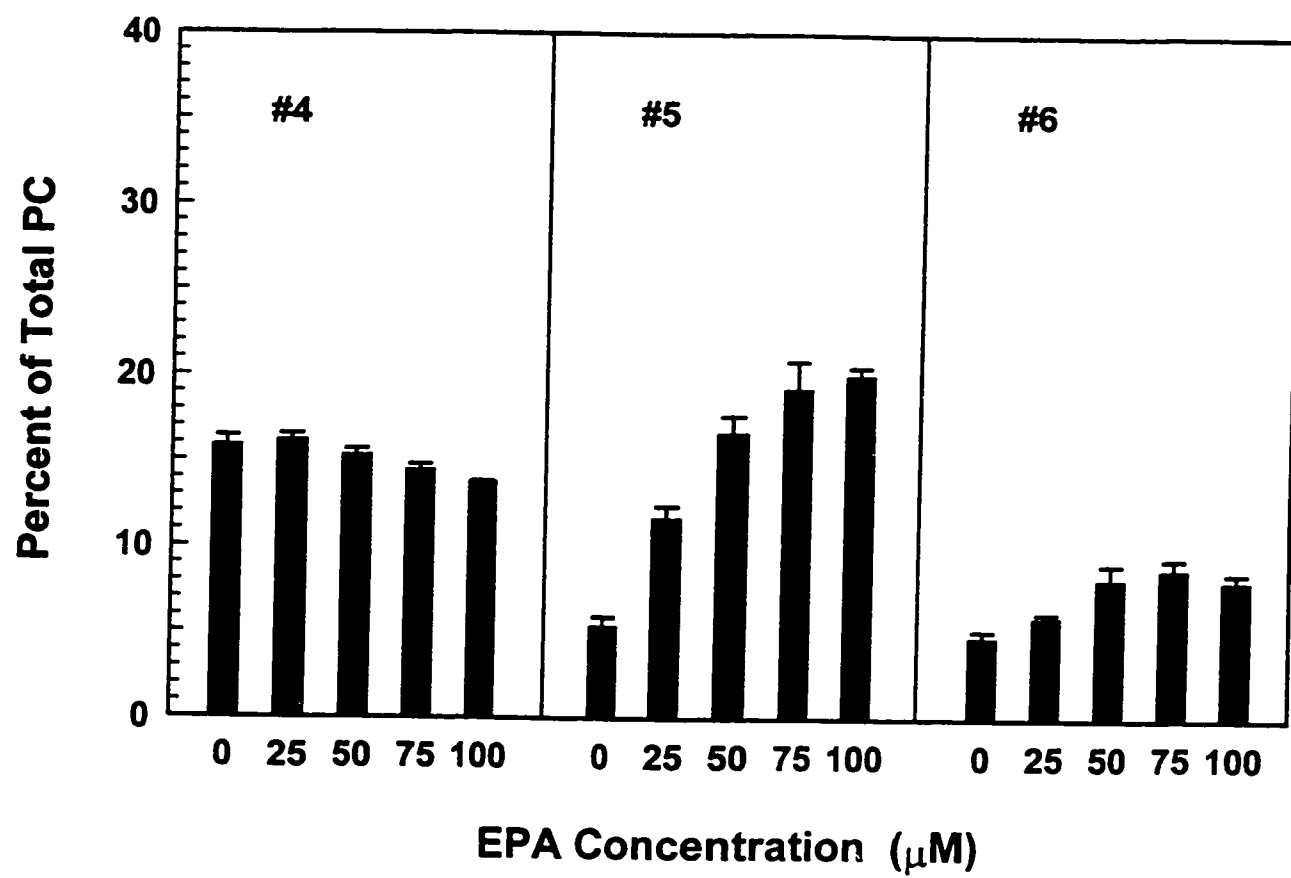
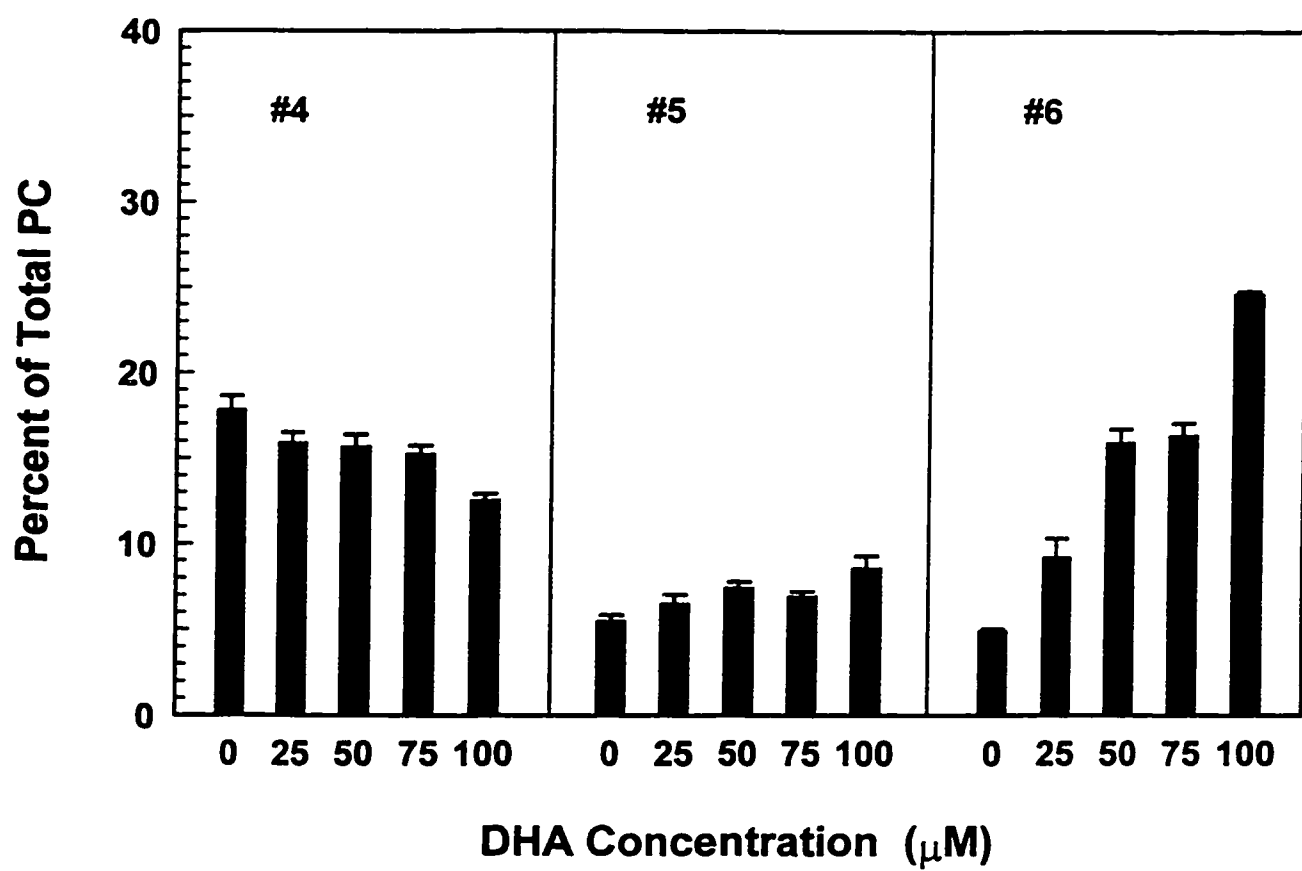


Figure 6. Phosphatidylcholine Molecular Species: Effect of Docosahexaenoic Acid.

Multilinear regression analysis was performed on the fractional composition profiles of stimulated DG samples shown in Figure 4 to estimate the contribution of phosphatidylcholine (PC) hydrolysis to their formation, expressed as a percent of total PC. Macrophages were prepared and incubated in docosahexaenoic acid (DHA) as described in Figure 1. Results for basal DG are shown for molecular species fractions with four (#4, left), five (# 5, middle) and six (#6, right) double bonds and are expressed as percent of total diradylglycerol (DG), mean $\pm$ SEM (n=4).



6.4 DISCUSSION

N-3 PUFA induce a series of biochemical and physiological effects *in vitro*, including altered formation of bioactive eicosanoids. Furthermore, in the form of fish oils or, as more recently studied, purified n-3 ethyl ester preparations, n-3 PUFA have clinical effects of potential eventual clinical utility in a variety of thrombotic, inflammatory, neoplastic and metabolic diseases. Many of these clinical effects have been ascribed to well-studied alterations in eicosanoid formation. Others are not readily explained on that basis, however.

DG is a crucial intracellular mediator of early events in the receptor-mediated signal transduction cascade of a wide variety of hormones and other cell agonists. Our results show for the first time that n-3 PUFA can affect the cellular biosynthesis of DG both quantitatively and qualitatively and do so in a virtually linear dose-dependent fashion over most of the 0-100 μM concentration range. We have further shown that EPA and DHA are incorporated to a very similar degree (mol% incorporated per molar concentration in the culture medium) into both basal and PAF-stimulated DG molecular species as well as into PC molecular species. At the same time, these results demonstrate a substantial difference between EPA and DHA in the magnitudes of their potentiation of total DG mass under both basal and PAF-stimulated conditions.

Our findings represent significant, controllable and differential alterations in the formation of DG, a centrally important cell activating second messenger,

that are induced by EPA and DHA. Prolonged dietary regimens that result in tissue lipid enrichment in n-3 PUFA *in vivo* may cause their biological and clinical effects, at least in part, by the mechanism of altering DG biosynthesis and the signal transduction pathways that involve the phospholipase/DG/PKC system.

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CHAPTER SEVEN

PUBLISHED MANUSCRIPT

Marignani, P. A. and Sebaldt, R. J. (1996) Interleukin-6 Secretion From Murine Peritoneal Macrophages Correlates With Changes In Incorporation of Eicosapentaenoic and Docosahexaenoic Acid Into Diradylglycerol. *J. Immunol.* *submitted*

PREAMBLE

The purpose of these experiments was to address the hypothesis that membrane incorporation of ω 3PUFA alter the secretion of the immunoregulatory cytokine IL-6 in murine peritoneal macrophages. I did this by determining whether correlation exists *in vitro* between IL-6 secretion in macrophages and ω 3PUFA incorporation into DG molecular species.

7.1 ABSTRACT

The effects of membrane incorporation of ω 3 polyunsaturated fatty acids (ω 3PUFA) on the secretion of the immunoregulatory cytokine, interleukin-6, and on the formation of the second messenger, diacylglycerol (DG), were investigated in murine peritoneal macrophages. Macrophage monolayers were incubated for 14h in control media or in media supplemented with 50 or 100 μ M of eicosapentaenoic (EPA, 20:5 ω 3) or docosahexaenoic (DHA, 22:6 ω 3) acids. After incubation, macrophage monolayers were stimulated for an additional 0, 2, 4, 8, or 12 h with 10 ng/ml lipopolysaccharide (LPS). We found that LPS-stimulated macrophages enriched with ω 3PUFA produced significantly less measurable interleukin-6 (IL-6), compared to control macrophages. This was true for both ω 3PUFA, but DHA was more potent in attenuating IL-6 production than was EPA. IL-6 secretion highly correlated with the effect of incorporation of ω 3PUFA into DG. Argentation thin-layer chromatography (TLC) analyses of DG molecular species profiles showed that the proportion of EPA and DHA incorporation at the *sn*-2 position increased as a function of dose and at the expense of arachidonate. Furthermore, reverse-phase-high pressure liquid chromatography (RP-HPLC) analysis showed that the predominant DG molecular species was 18:0-20:5 ω 3 in macrophages incubated in EPA-supplemented media, whereas in DHA-supplemented media, the predominant species was 16:0-22:6 ω 3. These results are the first to characterize simultaneous changes in IL-6 secretion and DG molecular

species composition in LPS-stimulated macrophages. Our findings warrant further investigation into how ω 3PUFA-containing molecular species of DG regulate potent mediators of inflammation.

7.2 INTRODUCTION

For years, researchers have been interested in the pharmacological actions and physiological implications of replacement of the fatty acid moieties of membrane phospholipids by ω 3 polyunsaturated fatty acids (ω 3PUFA). Early research focused on the anti-inflammatory and anti-thrombotic effects of eicosapentaenoic acid (EPA, [20:5 ω 3]) and docosahexaenoic acid (DHA, [22:6 ω 3]) on mediators of inflammation in macrophages, neutrophils and platelets (Chan et al. 1993, Huang et al. 1986, Whelan et al. 1991). ω 3PUFA have been reported to ameliorate various disease processes through the reduction of the membrane phospholipid content of the fatty acid arachidonate (AA, [20:4 ω 6]) (Galloway et al. 1985, Lokesh and Kinsella 1987). In so doing, the amounts of AA-derived eicosanoids such as leukotriene C₄ (LTC₄), leukotriene B₄ (LTB₄) thromboxane B₂ (TxB₂), prostacyclin (PGI₂) and prostaglandin E₂ (PGE₂) are attenuated while ω 3PUFA-derived eicosanoids are formed in greater amounts (Croft et al. 1987, Lokesh et al. 1988, Hardardottir et al. 1992).

Other actions of ω 3PUFA have been observed, including an eicosanoid-independent increase in macrophage production of nitric oxide (Chaet et al. 1994), modulation of macrophage adhesion and phagocytosis (Calder et al. 1990) and cytokine-mediated lymphocyte proliferation (Calder et al. 1992, Calder and Newsholme 1992), inhibition of natural killer cell activity (Yaqoob et al. 1994), inhibition of neutrophil migration (Ferrantes et al. 1994) and neutrophil superoxide

generation (Chen et al. 1994), changes in platelet function (Ahmed and Holub 1984, Benistant et al. 1993, Chan et al. 1993, von Schacky et al. 1985), modulation of calcium (Ca^{2+}) channels and cytosolic Ca^{2+} (Pepe et al. 1994), LTB_4 receptor down-regulation (Lawrence and Sorrell, 1994), amelioration of systemic lupus erythematosus (Das 1994, Robinson et al. 1993), reduction of postprandial hyperlipidemia (Chamberlain and Bolton 1987, Endres et al. 1989, Schmidt et al. 1993, Svaneborg et al. 1994), reduction of various cardiovascular risk factors (Huang et al. 1986, Lungershausen et al. 1994, Schmidt et al. 1993, Semplicini and Valle 1994), suppression of vascular smooth muscle proliferation (Shiina et al. 1993) and antitumorigenic effects (Anti et al. 1992, Deschner et al. 1990, Hardardottir and Kinsella 1992, Hubbard et al. 1994, Madhavi and Das 1994, Pascale et al. 1993, Rose et al. 1994, Welsh et al. 1993).

Previously, we have shown that the biosynthesis of diradylglycerol (DG) in murine peritoneal macrophages is substantially altered after *in vitro* supplementation of culture media with EPA or DHA (Sebaldt and Marignani 1995). More recently, we demonstrated for the first time that altering the membrane fatty acid composition of murine peritoneal macrophages by prolonged dietary supplementation with ω 3PUFA *in vivo* results in an impressive enrichment in molecular species with five and six double bonds, reflecting EPA and DHA incorporation at the *sn*-2 position, largely at the expense of arachidonic acid (AA, 20:4 ω 6) with four double bonds (Marignani and Sebaldt 1995, 1996a, 1996b).

The incorporation of ω 3PUFA into membrane phospholipids and the

formation of DG with EPA or DHA esterified at the *sn*-2 position may be of significance in a number of receptor-mediated signal transduction pathways, including cytokine production. Conceivably, structural modifications made to *sn*-1,2-DG may alter the activation of kinases and eventually affect signalling pathways that are pivotal to cell growth and differentiation (Marignani et al. 1996). The actions of ω 3PUFA in inflammatory or thrombotic conditions such as rheumatoid arthritis and coronary heart disease have been intensively studied at the level of eicosanoid production (Galloway et al. 1985, Huang et al. 1986, Croft et al. 1987, Lokesh and Kinsella 1987, Sperling et al. 1987, Lokesh et al. 1988, Whelan et al. 1991, Hardardottir et al. 1992, Chan et al. 1993). In this study, we were interested in determining the effect of ω 3PUFA on the production of IL-6, a multipotent cytokine produced by a number of inflammatory mediating cells such as macrophages, fibroblasts and mast cells (Leal-Berumen, 1994; Heinrich et al. 1990). We show the correlation between ω 3PUFA and the secretion of IL-6 and DG molecular species composition in murine peritoneal macrophages after LPS stimulation.

7.3 MATERIALS AND METHODS

7.3.1 *Materials*

sn-1,2-diacylglycerol kinase (DAG kinase) was from Calbiochem (La Jolla, CA). Phospholipase C (#7147), LPS *Escherichia coli* serotype 055-B5 (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) were from Sigma (St. Louis, MO). RPMI 1640 media, dfcs, penicillin, streptomycin and HBSS were from Gibco BRL (Grand Island, NY). L-glutamine was purchased from Fisher (Unionville, ON, Canada). Culture dishes and microtiter plates were from Corning (Corning, NY) and Falcon (Franklin Lakes, NJ). [γ - 32 P]ATP was from Dupont-NEN (Markham, ON, Canada). Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were from Biomol (Plymouth Meeting, PA).

7.3.2 *Animal Care*

Pathogen-free 4-5 week old C57BL/6 mice (Charles River Laboratory, Montreal, PQ) were acclimated for 72 h in microisolator cages with access to pre-irradiated rodent laboratory chow (PicoLab, Purina Mills, Richmond, IN) and water. All animal procedures were approved by the University's Animal Research Ethics Board.

7.3.3 *Macrophage Culture and Stimulation*

Mice were injected with 1 mL brewer's thioglycollate (i. p.) (DIFCO, Detroit, MI). After three days, elicited macrophages were harvested by peritoneal lavage (80-90%

macrophages), plated (15×10^6 per 16 cm^2 well) and enriched for adherent cells by incubation for 2 h (>98% macrophages) as described (Adams 1979). Macrophage monolayers were washed three times with HBSS containing 10 mM Hepes, pH 7.4 (HHBSS), and were cultured at 37°C in a 5% CO_2 /100% H_2O atmosphere for an additional 14h in either control media (RPMI 1640 media with 2 mM L-glutamine, 125 units/ml penicillin, 6 mg/L streptomycin and 10% heat deactivated fetal calf serum (dfcs)) or similar media supplemented with 50 or 100 μM of EPA or DHA.

After incubation, macrophage monolayers were washed three times with HHBSS, fresh identical media was added and macrophages were then stimulated for an additional 0, 2, 4, 8, or 12 h with 10 ng/ml LPS. DG molecular species analyses were performed by argentation TLC and RP-HPLC (Marignani and Sebaldt 1995, Sebaldt et al. 1992). For each time point, media was removed and frozen at -70°C for IL-6 bioassay.

7.3.4 *Interleukin-6 Bioassay*

Aarden et al. (1987) previously described the measurement of IL-6 bioactivity using the B9 hybridoma proliferation assay. B9 cells were grown in RPMI 1640 media supplemented with 1% Pen-Strep, 5% dfcs, 2-mercaptoethanol (2-ME) and a supernatant source of IL-6. B9 cells were cultured (2.5×10^3 /well) for 72 h in the absence or presence of IL-6-containing samples, after which 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) (0.5 mg/ml), 10 μl /well, was added to each sample and incubated for 4h at 37°C . 10% Triton-HCl, 50 μl /well, was added to

samples followed by an additional 18-20 h incubation at 25°C in the dark. For each bioassay, duplicate samples and standards were assayed in triplicate. Absorbance was measured at 540 nm. IL-6 content was determined by comparing sample absorbency readings with a standard IL-6 curve and expressed as ng IL-6/ml. 1 unit is equivalent to the amount of sample required to induce a half-maximal response. Standard curves were constructed in the presence and absence of EPA and DHA. Furthermore, in the absence of macrophages, all media were assessed for B9 proliferative capability for each time point under basal as well as LPS-stimulated conditions.

7.3.5 *Molecular Species Fractionation of Diradylglycerol by Argentation TLC*

After each time point, macrophages were scraped, DG was extracted by the Bligh-Dyer procedure (1959), derivatized to [^{32}P]phosphatidic acid (PA) with DAG kinase and 300 $\mu\text{Ci/ml}$ [$\gamma\text{-}^{32}\text{P}$]ATP and separated from ceramides by TLC. After TLC, [^{32}P]PA spots were scraped into 13 x 75 mm screw-top glass tubes, extracted from the silica, derivatized to [^{32}P]dimethylphosphatidic acid (DMPA) and fractionated into bands corresponding to the total double bond content (0-6) in the two fatty acid residues by argentation TLC, as previously described (Marignani and Sebaldt 1995).

7.3.6 *Molecular Species Fractionation of Diacylglycerol by Reverse Phase-HPLC*

DMPA samples were analysed by RP-HPLC (Ultrasphere 4.6 x 150 mm, 5 μ m C₁₈ column, Beckman) to yield twelve distinct peaks. The mobile phase was HPLC grade methanol:water (98.75:1.25, v/v) at a flow rate of 1.0 ml/min. Radioactive effluent was directly monitored by an in-line β -radiation detector (0.5 ml cell, Berthold). Total cpm were measured in successive 5s time windows (Sebaldt et al. 1992). Radiochromatograms were digitally reconstructed and smoothed without peak distortion by the Savitzky and Golay (1964) moving window least-squares technique, and peaks were expressed as a percent of the radiochromatogram total (Fig.P program version 6.0c, Biosoft, Cambridge, UK). The locations of molecular species standards have been identified in our laboratory and are in agreement with those determined by Kennerly (1986). Peak 0; C_{16:0-20:5} (1-palmitoyl-2-eicosapentaenoyl) and peak 3; C_{18:0-20:5} (1-stearoyl-2-eicosapentaenoyl). Peak 1; C_{16:0-22:6} (1-palmitoyl-2-docosahexaenoyl) and peak 5; C_{18:0-22:6} (1-stearoyl-2-docosahexaenoyl). Peak 2; C_{16:0-20:4} (1-palmitoyl-2-arachidonoyl) and peak 6; C_{18:0-20:4} (1-stearoyl-2-arachidonoyl). Results are expressed as mean $\pm$ SEM. The relationship between IL-6 secretion and ω 3PUFA incorporation and retention was examined by linear regression analyses using Fig.P version 6.0c.

7.3.7 *Determination of Viability*

The viability of peritoneal macrophages (2×10^6 /well) cultured in ω 3PUFA-supplemented media or control media was determined by standard Trypan Blue

exclusion procedure after a 14 h incubation followed by LPS stimulation for 8 h.

7.3.8 *Nitrogen Environment*

To minimize the likelihood of PUFA oxidation, nitrogen was used extensively throughout sample preparation and analyses. EPA and DHA stocks were infused with nitrogen and sealed under a blanket of nitrogen and stored at -70°C. Peroxide value determinations were periodically performed on the ω 3PUFA to monitor for any undesirable increases in peroxides (AOCS 1973). All solvents were bubbled with nitrogen immediately prior to use. All samples were spotted onto TLC plates under a blanket of nitrogen. Sample tubes were sealed under nitrogen prior to centrifuging or vortexing. For overnight storage, all samples were dried under a stream of nitrogen, sealed and kept at -20°C.

7.4 RESULTS

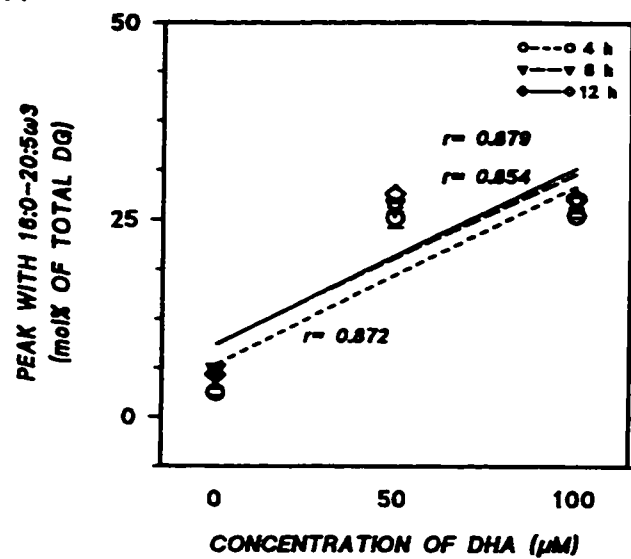
7.4.1 *Interleukin-6 Secretion In Macrophages Correlates with ω 3PUFA Incorporation Into Diacylglycerol.*

The changes in secretion of IL-6 in LPS-stimulated macrophages that are associated with the incorporation of EPA or DHA into DG were analyzed. Secretion of IL-6 (Figure 1) decreased as a function of increased incorporation into DG of EPA (mol% of total). Figure 1A shows the inverse relationship between IL-6 secretion and EPA incorporation into the argentation TLC band that contains 20:5 ω 3 that occurs 4, 8 and 12 hr after LPS stimulation of macrophages, with correlation coefficients of 0.902, 0.989, and 0.992, respectively. Figure 1B shows the inverse relationship between IL-6 secretion and EPA incorporation into the RP-HPLC peak that contains 18:0-20:5 ω 3, with correlation coefficients of 0.897, 0.999 and 0.995, respectively.

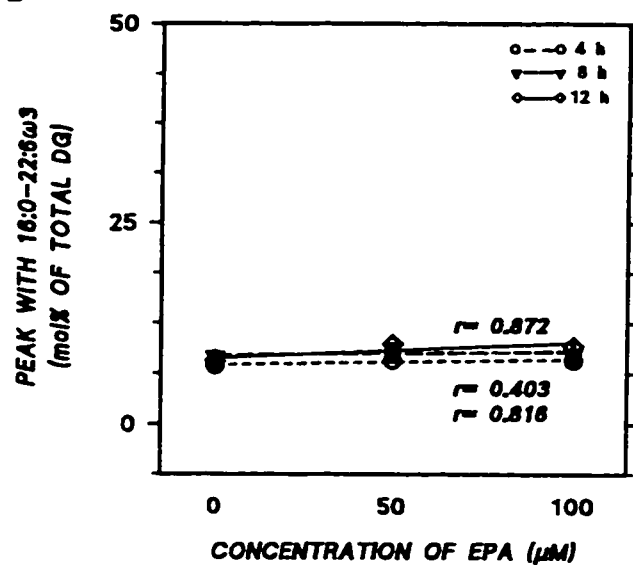
Secretion of IL-6 (Figure 2) also decreased as a function of increased incorporation into DG of DHA (mol% of total). Figure 2A shows the inverse relationship between IL-6 secretion and DHA incorporation into the argentation band that contains 22:6 ω 3 that occurs 4, 8 and 12 hr after LPS stimulation of macrophages, with correlation coefficients of 0.961, 0.990, and 0.987, respectively. Figure 2B shows the inverse relationship between IL-6 secretion and DHA incorporation into the RP-HPLC peak that contains 16:0-22:6 ω 3, with correlation coefficients of 0.891, 0.969 and 0.975, respectively.

FIGURE 1. Correlation Between Interleukin-6 Secretion and EPA Incorporation Into Diradylglycerol. Macrophages were incubated for 14 h in media supplemented with 0, 50 or 100 μ M EPA, prepared and stimulated for an additional 0, 2, 4, 8 or 12 h with LPS (10 ng/ml). Regression analyses was performed to determine the relationship between IL-6 secretion (ng/ml) and EPA incorporation into DG (mol% of total DG) for A. Argentation TLC and B. RP-HPLC.

A



B



C

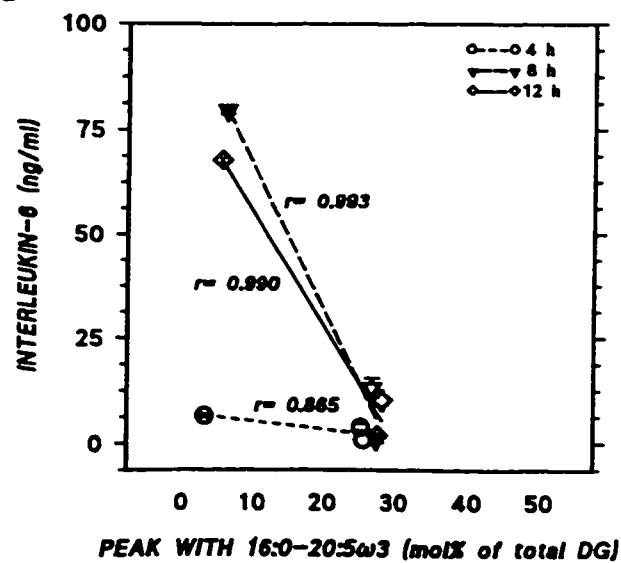
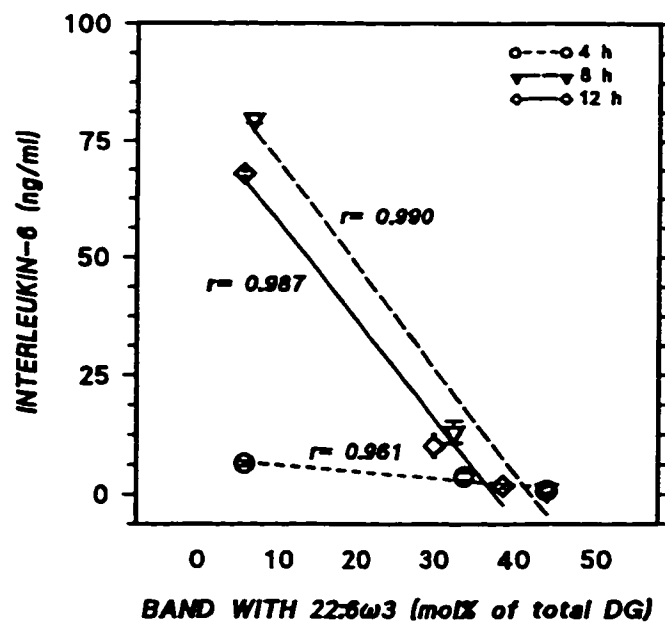
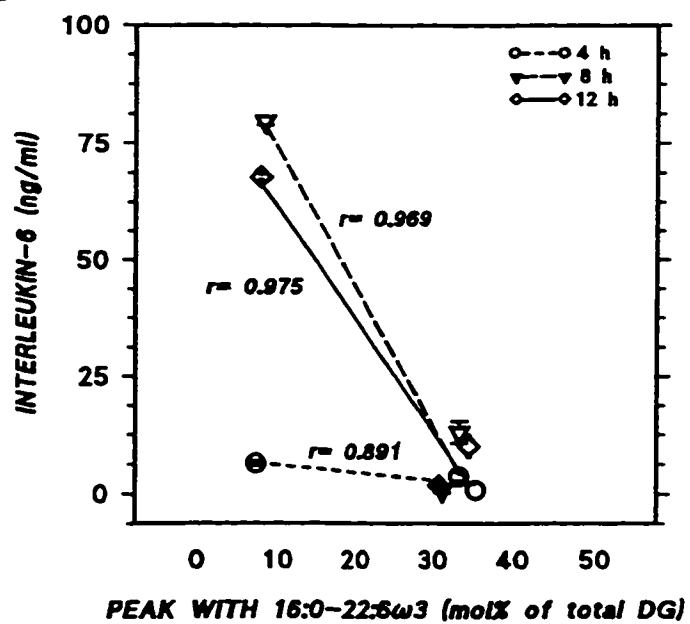


FIGURE 2. Correlation Between Interleukin-6 Secretion and DHA Incorporation Into Diradylglycerol. Macrophages were incubated for 14 h in media supplemented with 0, 50 or 100 μ M DHA, prepared and stimulated for an additional 0, 2, 4, 8 or 12 h with LPS (10 ng/ml). Regression analyses was performed to determine the relationship between IL-6 secretion (ng/ml) and DHA incorporation into DG (mol% of total DG) for A. Argentation TLC and B. RP-HPLC.

A



B



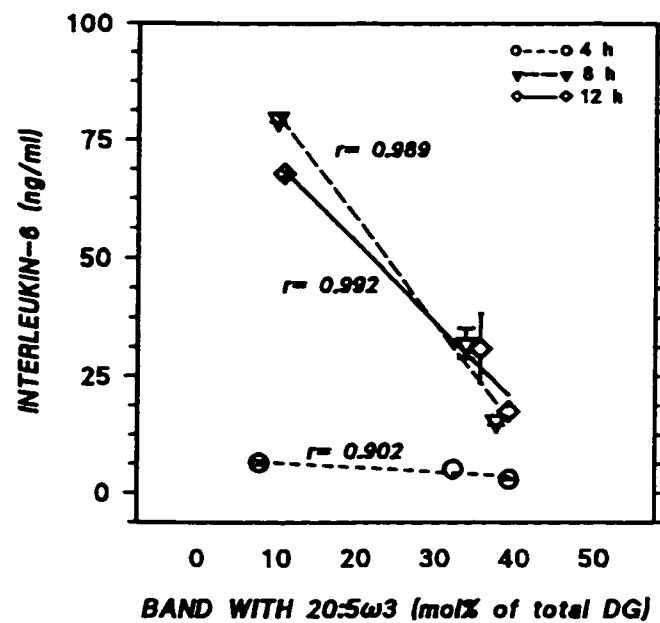
Conversion of EPA to DHA was not observed in LPS-stimulated macrophages incubated in EPA-containing media (Figure 3A). However, retroconversion of DHA to EPA did appear to occur (Figure 3B): there was increasing formation of 16:0-20:2 ω 3 species of DG with increasing concentration of DHA in the culture media. Figure 3C shows the correlation between IL-6 secretion and content of this species in DG after 4, 8 and 12 h incubation with DHA, with correlative coefficients of $r=0.865$, 0.993 and 0.990 , respectively. Interestingly, the 18:0-20:5 ω 3 species of DG that appeared in EPA-supplemented media did not increase with increasing DHA in the media.

7.4.2 *Interleukin-6 Secretion In Lipopolysaccharide Stimulated Macrophages*

Macrophages stimulated with 10 ng/ml LPS (Figure 4) resulted in a time-dependent release of IL-6 with detectable levels beginning at 2 h (ranging between 0.05 - 0.40 ng/ml) and increasing thereafter. At 8 h, macrophages incubated in control media produced the greatest concentration of IL-6, 76 ng/ml. Supplementation of media with EPA, 50 and 100 μ M, resulted in a 1.5- and 5-fold reduction in IL-6 concentration, respectively, compared to control media. Supplementation of media with 50 μ M DHA caused a 5- fold reduction in IL-6 concentration, compared to control, while an even more profound 30-fold reduction occurred with a DHA concentration of 100 μ M.

FIGURE 3. Retroconversion of DHA to EPA and Correlation to Interleukin-6 Secretion. Macrophages were incubated for 14 h in media supplemented with A. 0, 50 and 100 μ M EPA or B and C. 0, 50 and 100 μ M DHA, prepared and stimulated with LPS (10 ng/ml) for an additional 0, 2, 4, 8 and 12 h. A. Regression analyses was performed to determine the relationship between DHA concentration and formation of 16:0-20:5 ω 3. B. EPA concentration and conversion of 16:0-20:5 ω 3 to 16:0-22:6 ω 3. and C. IL-6 secretion (ng/ml) and formation of 16:0-20:5 ω 3.

A



B

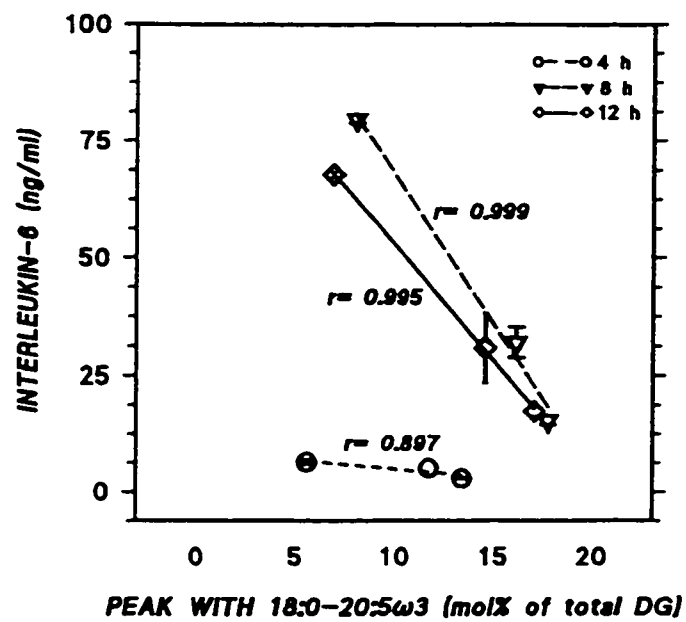
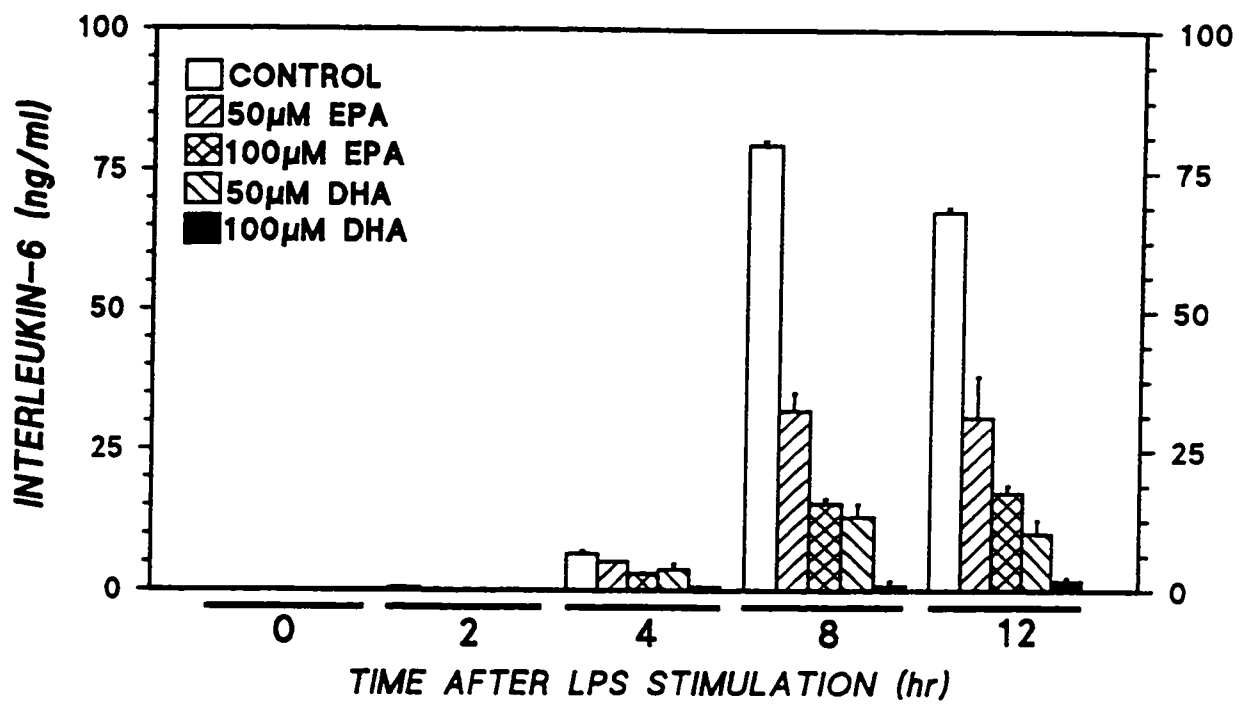


FIGURE 4. IL-6 Secretion From LPS-Stimulated Murine Peritoneal Macrophages. Macrophages were incubated for 14 h in 0, 50 and 100 μ M EPA and DHA, prepared and stimulated for an additional 0, 2, 4, 8 and 12 h with LPS (10 ng/ml). Supernatants were assessed for IL-6 secretion and expressed as mean values $\pm$ SEM (n=6).



Control experiments were conducted to assess whether ω 3PUFA-supplemented media alone induced proliferative activity in B9 cells. Half-maximal response did not differ significantly among the six different ω 3PUFA-supplemented media (Figure 5), suggesting that the sensitivity of the B9 assay is not compromised by the addition of ω 3PUFA. Experiments were also conducted to assess whether ω 3PUFA-supplemented media in the absence of macrophages could stimulate B9 proliferation under either unstimulated or LPS-stimulated conditions. We found that for each time point, ω 3PUFA-supplemented media in the absence of macrophages did not induce B9 proliferation (IL-6 levels were below the sensitivity of the B9 assay, data not shown).

Basal concentrations of IL-6 measured with unstimulated macrophages were detectable only after 4 h incubation but were well below 0.25 ng/ml (data not shown).

7.4.3 *Diradylglycerol Molecular Species Fractionation*

7.4.3.1 *EPA Supplementation*

Murine peritoneal macrophages were prepared as described in Materials and Methods. DG molecular species were analyzed by argentation TLC (Marignani and Sebaldt 1995) and RP-HPLC (Sebaldt et al. 1992). The proportions of DG molecular species fractions with 20:4 ω 6, 20:5 ω 3 or 22:6 ω 3 in the *sn*-2 position differed among conditions, reflecting the fatty acid component and concentration in the supplemented media. When macrophages were incubated

for 14 h in control media or media supplemented with 50 or 100 μ M EPA followed by 0-12 h LPS stimulation (Figure 6A), argentation TLC band 5 (Figure 6Ab), which includes DG species with EPA, contained the greatest proportion of DG molecular species. This proportion increased from <10 mol% (control media) to 20 - 35 mol% (50 μ M EPA) and to 29 - 39 mol% (100 μ M EPA). The fraction of species with EPA remained stable after the initial two hours of LPS stimulation. The early increase in species with EPA was predominantly at the expense of 20:4 ω 6 (Figure 6Aa) whose the proportion continued to decrease as a function of time as well as a function of increasing dose of EPA. The proportion of DG molecular species with DHA at the *sn*-2 position (Figure 6Ac) remained stable after the initial 2 h after LPS stimulation.

HPLC fractionation of DG molecular species (Figure 6B) from LPS-stimulated macrophages incubated in media supplemented with 50 or 100 μ M EPA showed that the predominant species of EPA-containing DG was 18:0-20:5 ω 3 (10 - 18 mol%) (Figure 6Bb), with some 16:0-20:5 ω 3 (5 - 8 mol%) (Figure 6Be). Both DHA-containing species 18:0-22:6 ω 3 and 16:0-22:6 ω 3 (Figure 6Bc,f) remained low at < 10 mol%. Decreases in 18:0-20:4 ω 6 coincided with the increase in DG 18:0-20:5 ω 3 while the proportion of 16:0-20:4 ω 6 remained relatively stable over time at < 18 mol% (Figure 6Bd).

7.4.3.2 DHA Supplementation

When macrophages were incubated for 14 h in control media or media

supplemented with 50 or 100 μM DHA followed by 0 - 12 h LPS stimulation (Figure 7A), argentation TLC band 6 (Figure 7Ac), which includes DG species with DHA, contained the greatest proportion of DG molecular species. This proportion increased from < 5 mol% (control media) to 20-40 mol% (50 μM DHA) and to 29 - 43 mol% (100 μM DHA). The fraction of species with DHA remained stable after the initial two hours of stimulation with LPS. The early increase in species with DHA was predominantly at the expense of 20:4 ω 6 (Figure 7Aa), whose proportion continued to decrease as a function of time as well as a function of increasing dose of DHA. The proportion of DG molecular species with EPA at the *sn*-2 position (Figure 7Ab) remained stable over time after LPS stimulation. However, at 50 μM DHA, DG molecular species with 20:5 ω 3 at the *sn*-2 position was elevated between 25 - 30 mol% after 4 hours LPS stimulation. This was not observed at 100 μM DHA where the proportion of DG molecular species with 20:5 ω 3 at the *sn*-2 position remained relatively low and stable at > 12 mol%.

HPLC fractionation of DG molecular species (Figure 7B) from LPS-stimulated macrophages incubated in media supplemented with 50 or 100 μM DHA showed that the predominant species of DHA-containing was 16:0-22:6 ω 3 (5 - 35 mol%) (Figure 7Bf). Interestingly, a significant fraction of the EPA-containing species 16:0-20:5 ω 3 (13 - 28 mol%) appeared over time (Figure 7Be). Decreases in DG molecular species 18:0-20:4 ω 6 coincided with the increase in 16:0-22:6 ω 3, while the proportion of 16:0-20:4 ω 6 remained relatively stable and low over time, < 10 mol% (Figure 7Bd).

FIGURE 5. ω 3PUFA Effect On IL-6 Standard Curves. The assessment of ω 3PUFA-supplemented media on the proliferation of B9 cells in the IL-6 standard curve. Results are shown for mean values $\pm$ SEM (n=6).

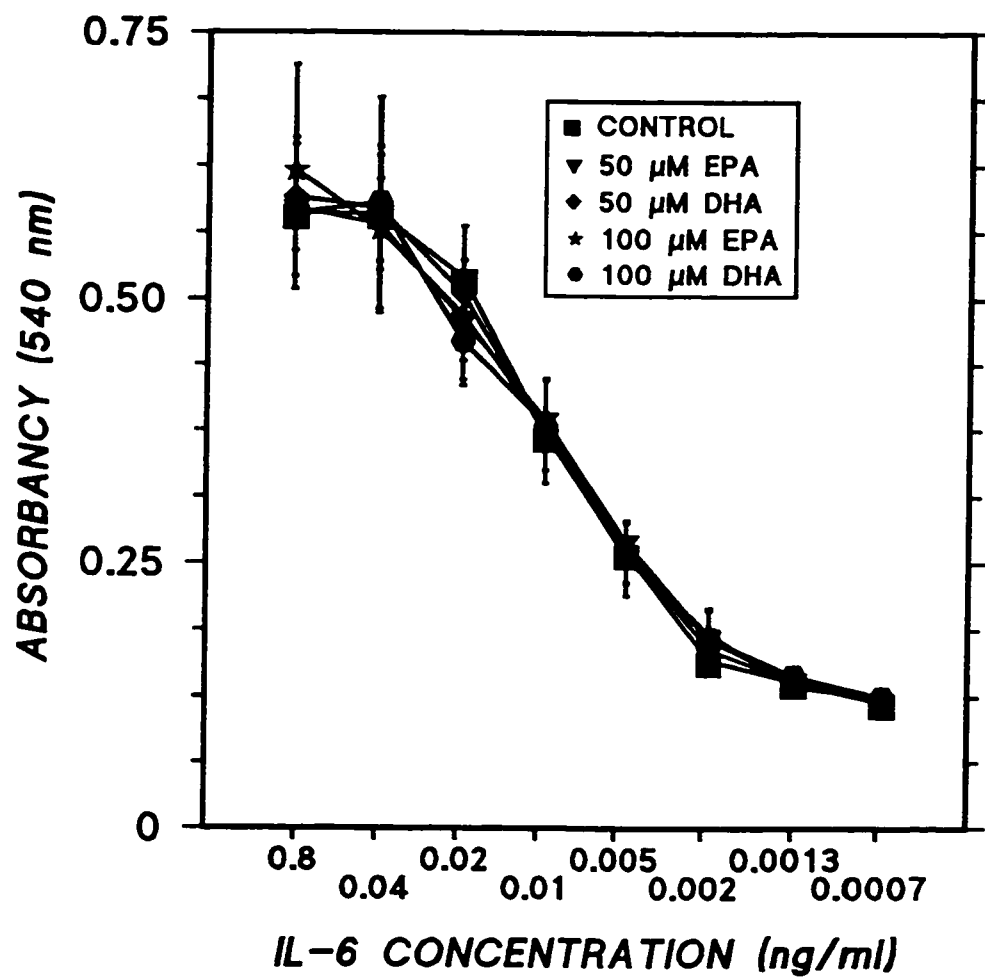
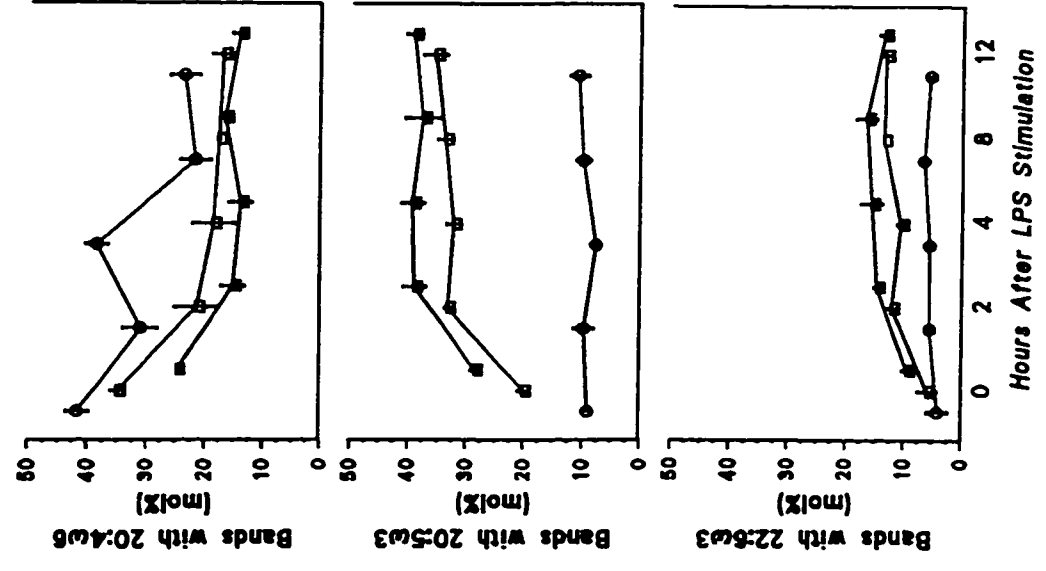
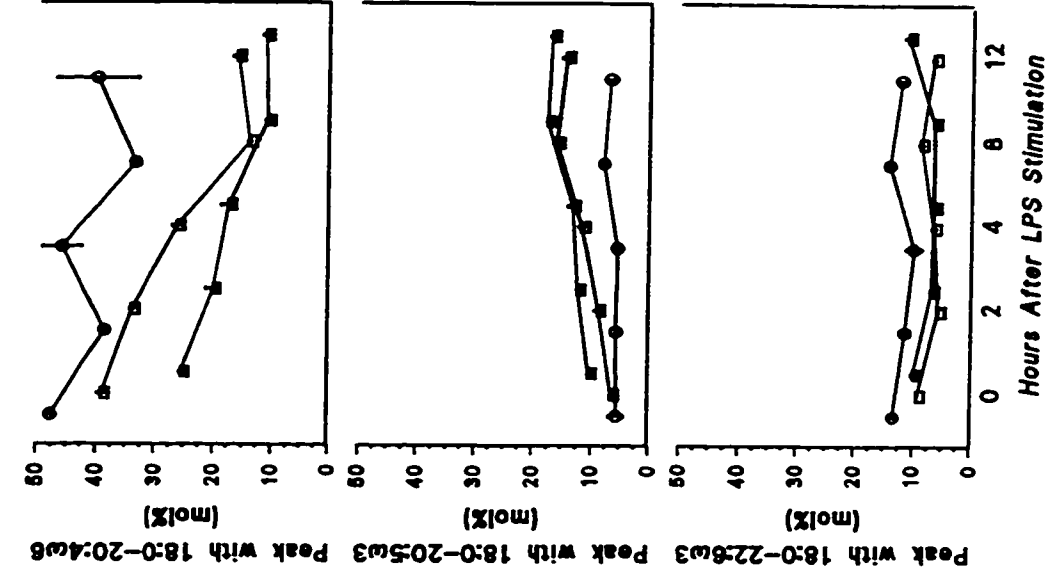


FIGURE 6. Diradylglycerol Molecular Species Analyses For LPS-Stimulated Murine Peritoneal Macrophages Incubated In EPA-Supplemented Media. Macrophages were incubated for 14 h in 0, 50 or 100 μ M EPA, prepared and left unstimulated or stimulated for an additional 0, 2, 4, 8 and 12 h with LPS (10 ng/ml). Results are shown for DG molecular species analyses for basal and LPS-stimulated macrophages. Open circles (control), open squares (50 μ M EPA) and closed squares (100 μ M EPA). (A) Argentation TLC for basal and LPS-stimulated macrophages are shown for DG molecular species fractions with (a) 20:4 ω 6 (b) 20:5 ω 3 (c) and 22:6 ω 3 in the *sn*-2 position. (B) HPLC analyses for basal and LPS-stimulated macrophages are shown for fractions containing the DG molecular species (a) 18:0-20:4 ω 6 (b) 18:0-20:5 ω 3 (c) 18:0-22:6 ω 3 (d) 16:0-20:4 ω 6 (e) 16:0-20:5 ω 3 and (f) 16:0-22:6 ω 3. Results are expressed as mol% of total DG, mean values $\pm$ SEM (n=6).

A



B



C

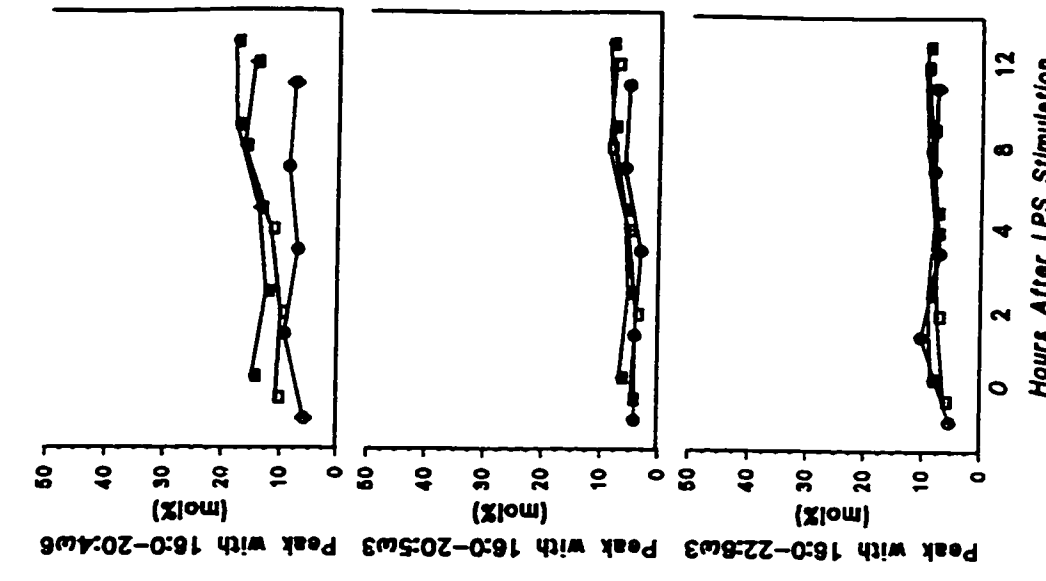
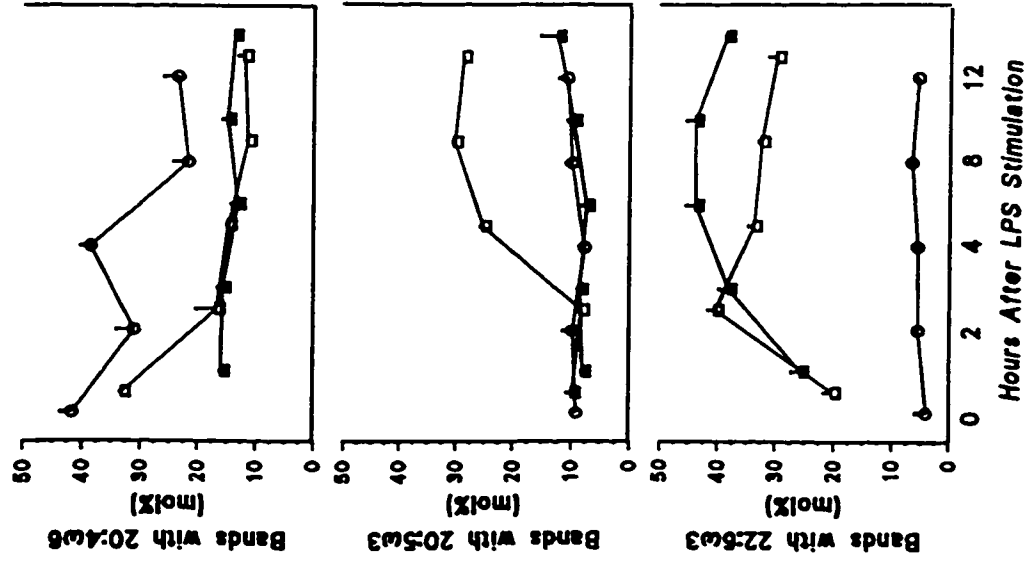
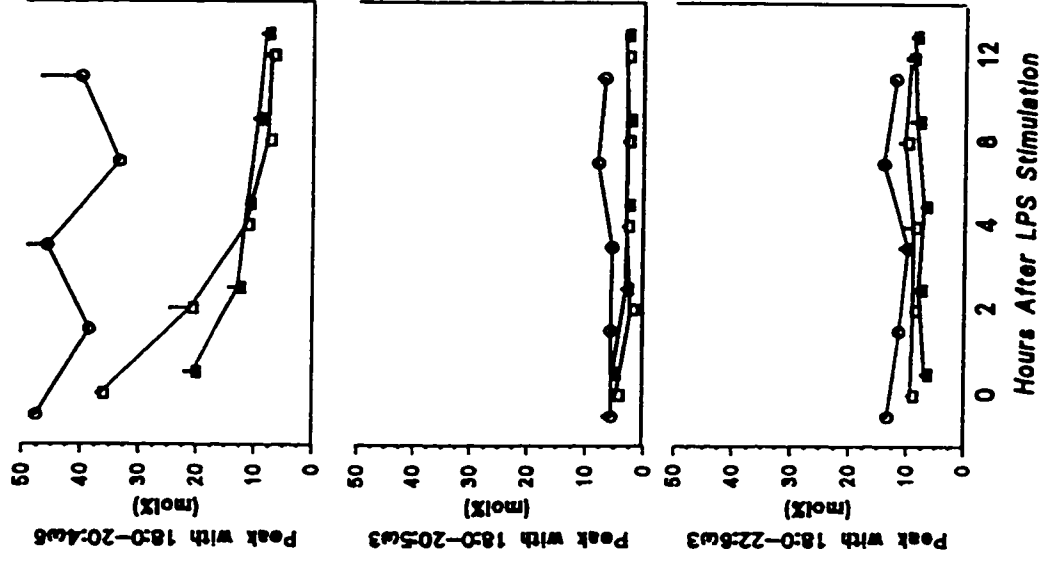


FIGURE 7. Diradylglycerol Molecular Species Analyses For LPS-Stimulated Murine Peritoneal Macrophages Incubated in DHA-Supplemented Media. Macrophages were incubated for 14 h in 0, 50 or 100 μ M DHA, prepared and left unstimulated or stimulated for an additional 0, 2, 4, 8 and 12 h with LPS (10 ng/ml). Results are shown for DG molecular species analyses for basal and LPS-stimulated macrophages. Open circles (control), open squares (50 μ M DHA) and closed squares (100 μ M DHA). (A) Argentation TLC for basal and LPS-stimulated macrophages are shown for DG molecular species fractions with (a) 20:4 ω 6 (b) 20:5 ω 3 (c) and 22: ω 3 in the *sn*-2 position. (B) HPLC analyses for basal and LPS-stimulated macrophages are shown for fractions containing the DG molecular species (a) 18:0-20:4 ω 6 (b) 18:0-20:5 ω 3 (c) 18:0-22:6 ω 3 (d) 16:0-20:4 ω 6 (e) 16:0-20:5 ω 3 and (f) 16:0-22:6 ω 3. Results are expressed as mol% of total DG, mean values $\pm$ SEM (n=6).

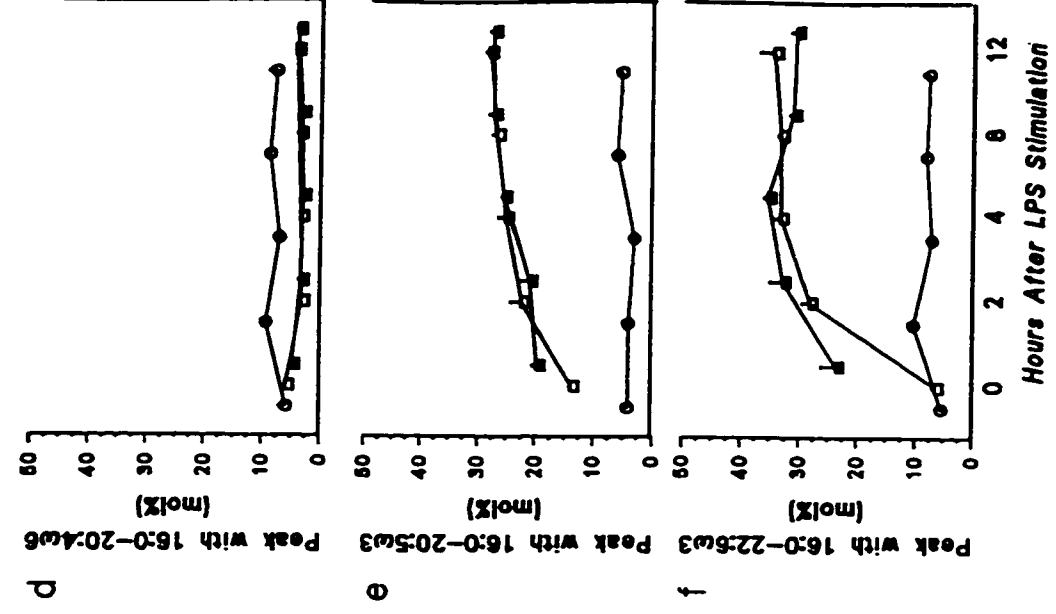
A



B



B



7.4.4 *Determination of Viability*

ω 3PUFA-supplemented media collected at different time intervals after LPS stimulation were analysed to investigate the effect of ω 3PUFA on macrophage production of IL-6. Viability determinations showed that after 14 h incubation followed by LPS stimulation for 8 h, 95-97% of the macrophages were viable under all media conditions.

7.5 DISCUSSION

Our results demonstrate for the first time that the production of the multipotent cytokine, IL-6, is suppressed in a dose-dependent manner in murine peritoneal macrophages incubated *ex vivo* in ω 3PUFA-supplemented culture media. Moreover, ω 3PUFA differentially alter IL-6 secretion in LPS-stimulated macrophages, with DHA being more potent than EPA in inhibiting IL-6 secretion. In order to determine whether IL-6 secretion in our experimental system may be dependent on the PKC signalling pathway, we characterized the incorporation of EPA and DHA into DG, in the same macrophages. Interestingly, the incorporation and retention of DHA in molecular species of DG was distinctly different from the incorporation and retention of EPA, as determined by reverse-phase HPLC. Furthermore, regression analyses shows that the suppression of IL-6 secretion is highly correlated with the incorporation and retention of DHA and/or EPA in DG. Our results suggest that at a functional level, DG molecular species with incorporated DHA is distinctly different from DG molecular species with incorporated EPA. Regression analyses confirmed the functional relationship between IL-6 secretion and differential incorporation and retention of ω 3PUFA. We conclude that the suppression of IL-6 secretion from LPS-stimulated macrophages may be attributable to alterations in protein kinase C (PKC) activity by in DG molecular species with DHA or EPA at the *sn*-2 position (Marignani et al. 1996).

The retroconversion of incorporated DHA to EPA in DG molecular species with 16:0 in the *sn*-1 position correlated with the suppression of IL-6 secretion from macrophages incubated in media containing DHA. Interestingly, retroconversion of incorporated DHA to EPA was not observed in DG molecular species with 18:0 at the *sn*-1 position. These findings suggest that the substituent fatty acids of DG molecular species *sn*-1 16:0 and *sn*-2 DHA is more apt to be retroconverted to 16:0-20:5 ω 3 as well as differentially modify the activation of PKC to a greater extent than DG molecular species with 18:0 at the *sn*-1 position and EPA at the *sn*-2 position.

The ω 3PUFA are different in that DHA is composed of 22 carbons and 6 double bonds while EPA is composed of 20 carbons and 5 double bonds. As a result of these structural differences, spatial modifications in membrane phospholipids and DG occur. We suggest that increasing the fluidity of membrane phospholipids through increasing the fatty acid chain length and unsaturation, together with the changes in DG molecular species formation after stimulation with LPS, contribute to the observed suppression of IL-6 secretion. Furthermore, incorporation of ω 3PUFA increases the fluidity of the macrophage membrane which may interfere with the release of IL-6. However, this reason may not be exclusive in explaining why we observed suppression of measurable IL-6 from supernatants. ω 3PUFA could also be inhibiting IL-6 production at either the transcriptional or at the translational level. Both changes in membrane fluidity and expression of IL-6 mRNA may contribute to our findings, however, these

mechanisms were not investigated in this paper.

An additional explanation for our results may be found in the AA-derived eicosanoid, PGE_2 , which may be involved in the regulation of IL-6 production in our system. The complex association between PGE_2 -mediated regulation of macrophage production and secretion of IL-6 is altered when ω 3PUFA are introduced into the system. The proportion of AA-derived eicosanoids are diminished as a result of increased proportions of 20:5 ω 3-derived eicosanoids (Croft et al. 1987; Hardardottir et al. 1992; Lokesh et al. 1988), thus the regulatory control of PGE_2 over the production of IL-6 is negated. PGE_2 regulation over IL-6 production has recently been described in rat peritoneal mast cells (Leal-Berumen et al. 1995) where it was found that IL-6 production could be induced by the addition of PGE_2 to mast cells and inhibited by the introduction of the cyclooxygenase downregulator, corticosteroid dexamethasone. Tumor necrosis factor- α ($\text{TNF}\alpha$), has also been found to be under PGE_2 regulatory control. Hardardottir and Kinsella (1992) showed that dietary enrichment with ω 3PUFA resulted in an increase production of $\text{TNF}\alpha$ from murine peritoneal macrophages. They attribute this increase to the reduction of PGE_2 production. Moreover, PGE_2 also suppresses $\text{TNF}\alpha$ mRNA accumulation (Scales et al. 1989) and secretion from LPS-stimulated macrophages (Hardardottir and Kinsella 1991, Kunkel et al. 1987). The enhancement of $\text{TNF}\alpha$ production by ω 3PUFA may contribute, in part, to the antitumorigenic effects of ω 3PUFA described in the literature (Anti et al. 1992 and Deschner et al. 1990).

The ramifications of cytokine suppression from macrophages incubated in ω 3PUFA and the incorporation of EPA and DHA into second messenger, DG, and the subsequent activation of signalling pathways have not been addressed. For instance, modifications in the synthesis and secretion of cytokines such as IL-6, TNF α and interleukin-1 (IL-1), attributable to ω 3PUFA, may directly or indirectly contribute to the amelioration of such diseases as arthritis, various cancers, lupus and coronary heart disease. However, the modulation of cytokine synthesis by ω 3PUFA and how this alters signalling in proinflammatory cells is less well understood.

Understanding disease processes at the level of signal transduction could potentially contribute to the clinical management of atherogenic syndromes, inflammatory diseases and neoplasia by developing ω 3PUFA-based therapies that target signalling pathways as an adjunct to the current management strategies of these diseases.

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CHAPTER EIGHT

PUBLISHED MANUSCRIPT

Marignani, P. A., Epand R. and Sebaldt, R. J. (1996) Acyl Chain Dependence Of Diacylglycerol Activation Of Protein Kinase C Activity *In Vitro*. *Biochemical and Biophysical Research Communications* 225: 469-473.

PREAMBLE

The purpose of these experiments was to address the hypothesis that DG molecular species with EPA or DHA at the *sn*-2 position activate PKC to a different extent than do other species of DG. I did this by comparing the activation of PKC by different pure DG molecular species in a mixed lipid vesicle system.



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10 August 1996.

Lipids

Dr. Howard R. Knapp
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Dear Dr. Howard Knapp

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Marignani PA and Sebaldt RJ. (1996) ω -3 Polyunsaturated Fatty Acid-Induced Changes In The Molecular Species Composition of Diacylglycerol In Murine Peritoneal Macrophages Remain Stable During Incubation Ex Vivo. *Lipids* 31: 771-776.

Please note that I am co-author of this work.

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Mary C. Lane

8.1 ABSTRACT

Stimulation of protein kinase C (PKC) activity in lipid vesicles *in vitro* was achieved by pure molecular species of diacylglycerol (DAG), specifically 1-stearoyl-2-acyl-*sn*-glycerol substituted with 2-arachidonoyl, 2-eicosapentaenoyl or 2-docosahexaenoyl (SAG, SEG and SDG, respectively). PKC activity was measured in lipid vesicles containing 30 mol% 1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phospho-L-serine (POPS), 68-70 mol% 1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphocholine (POPC) and 0-2 mol% DAG in the presence of 20 μ M calcium. Our results demonstrate that amplification of PKC activity differs significantly among these molecular species of DAG. In particular, SDG at 0.5 mol% is more potent in increasing PKC activity than is dioleoylglycerol (DOG), SEG or SAG, and SAG and SDG at 1.0 and 2.0 mol% have similar potencies which are greater than those of DOG or SEG. These findings demonstrate that *sn*-2 substitutions in DAG by specific n-3 and n-6 polyunsaturated fatty acids increase the potency of DAG to stimulate PKC activity *in vitro*.

8.2 INTRODUCTION

The consequences of cell membrane phospholipid incorporation of specific ω 3-polyunsaturated fatty acids (ω 3-PUFA) into diacylglycerol (DAG) (1) and the subsequent alterations in protein kinase C (PKC) activity by structurally modified DAG have not been explored.

PKC is a family of serine/threonine-specific protein kinases currently known to consist of twelve members that have been subdivided into three major groups based on their biochemical and structural properties. The activation of all isoforms of PKC is dependent upon the presence of anionic L-phosphatidylserine (2). The conventional PKC isoforms (cPKC) α , β I, β II and γ , are calcium-dependent and also require anionic lipids, DAG or phorbol esters for activation (3,4). The novel PKC isoforms (nPKC) δ , ϵ , η , θ and μ , are calcium-independent and are activated by DAG or phorbol esters (5-10). The atypical PKC isoforms (aPKC) ζ , λ and ι , are calcium-independent, insensitive to DAG or phorbol esters and can be activated by free fatty acids (10).

The single known function of DAG is to activate PKC by increasing the affinity of PKC for the membrane as well as by increasing the activity of the enzyme once it has partitioned into the membrane (11,12). The activation process is stereospecific in that only *sn*-1,2-DAG but not *sn*-1,3-DAG or *sn*-2,3-DAG is capable of activating PKC (13). Structural modification in *sn*-1,2-DAG could potentially alter the activation process of PKC and eventually affect cell growth

and differentiation. The work described above as well as work by others has shown that free PUFA enhance PKC activity, but the effects of arachidonic acid (AA, 20:4 ω 6), eicosapentaenoic acid (EPA, 20:5 ω 3) or docosahexaenoic acid (DHA, 22:6 ω 3) at the *sn*-2 position of DAG on PKC activation have not been studied to date. In this paper, we report the effects of the pure molecular species SAG (18:0/20:4), SEG (18:0/20:5) and SDG (18:0/22:6) on PKC activation *in vitro*.

8.3 MATERIALS AND METHODS

8.3.1 *Materials*

1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine, (PC-AA) 1-stearoyl-2-docosa-hexaenoyl-*sn*-glycerol-3-phosphocholine(PC-DHA), 1-stearoyl-2-eicosapentaenoyl-*sn*-glycerol (SEG), 1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phospho-L-serine (POPS), 1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphocholine (POPC) and 1,2-dioleoyl-*sn*-glycerol (DOG) were from Avanti Polar Lipids (Alabaster, AL). Histone H1 was from Gibco BRL (Grand Island, NY). Protein kinase C was from Molecular Probes, Inc (Eugene, OR). ATP sodium salt and bovine serum albumin fraction V were from Sigma (St. Louis, MO). [γ - 32 P]ATP was from NEN (Montreal, QC).

8.3.2 *Synthesis of Diacylglycerol*

1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine and 1-stearoyl-2-docosa-hexaenoyl-*sn*-glycerol-3-phosphocholine were ultrasonically dispersed in 0.1 ml of 50 mM potassium phosphate buffer, pH 7.0, and hydrolyzed to SAG or SDG with 96 units phospholipase C (Sigma, St. Louis, MO) in a final volume of 0.6 ml (25°C for 5 h). Polar reaction products were removed by passage through small chloroform-equilibrated silica columns (230-400 mesh). DAG was extracted into chloroform by the Bligh-Dyer (14) method. Quantification of DAG mass was performed as described (1,15).

8.3.3 PKC Activity Assay

The construction of lipid vesicles was modified from Mosior and Epand (12). Lipid mixtures of POPS (30 mol%), POPC (68-69.5 mol%) and DAG (0.5, 1.0, 2.0 mol%) were combined in chloroform/methanol (2:1, v/v) and dried under a stream of argon. Control lipid vesicles were similarly constructed with 30 mol% POPS and 70 mol% POPC. Lipid vesicles, at a final lipid concentration of 100 μ M, were resuspended in a modified standard buffer (100 mM KCl, 5 mM MgCl₂, and 20 mM Tris-HCl to pH 7.0) containing 20 μ M CaCl₂, 3 mg/ml BSA, 0.2 mg/ml histone and 50 ng PKC. The addition of 20 μ M [γ -³²P]ATP (0.2 μ Ci/ml) initiated the 10 min reaction at 25°C, and the addition of 2 ml 25% ice-cold TCA terminated the reaction. Samples were vortexed, placed on ice for 15 min, then filtered through GF/C Whatman filters. Filters were washed four times with 2 ml 25% TCA, dried and counted using efficiency-corrected Cerenkov counting. In order to minimize the likelihood of oxidation, argon was used extensively throughout the preparation of the lipid films and throughout sample preparation and analysis. Data represent three to four independent experiments, each performed in triplicate and expressed as mean $\pm$ SD. Student's t-tests were performed using the Fig.P computer program (Biosoft, Cambridge UK). One-way analyses of variance followed by post hoc comparisons with Duncan's multiple range test were performed at a significance level of $p < 0.05$ were used to test for significant differences within and among the DAG molecular species.

8.4 RESULTS

The phosphorylating activity of PKC in vesicles was determined by quantifying the incorporation of ^{32}P into histone from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ at a physiologically relevant calcium concentration in the micromolar range. Our results are novel in that the incorporation of SAG, SDG and SEG into lipid vesicles caused a marked concentration dependent increase in PKC activity compared to control (Fig.1). Lipid vesicles constructed with SAG (Fig.1, panel 2) and SDG (Fig.1, panel 4) increased PKC activity to a similar degree, 1.8 to 4.3 fold and 2.8 to 4.7 fold, respectively, compared to control. PKC activity was significantly greater in lipid vesicles constructed with SAG or SDG at 1.0 and 2.0 mol% than at 0.5 mol% ($p < 0.0001$). It was also significantly different between SAG at 1.0 and 2.0 mol% SAG ($p < 0.002$) and between SDG at 1.0 and 2.0 mol% SDG ($p < 0.05$). Lipid vesicles constructed with dioleoylglycerol (DOG) or SEG both increased PKC activity approximately 1.4 to 2.5 fold compared to control (Fig.1 panel 1 and 3). PKC activity was significantly greater ($p < 0.05$) with DOG at 2.0 mol% than at 0.5 mol% (Fig.1 panel 1) and with SEG at both 1.0 and 2.0 mol% than at 0.5 mol% ($p < 0.02$ and $p < 0.0001$, respectively) (Fig. 1 panel 3).

Comparison among the DAG molecular species show that SDG at 0.5 mol% was significantly more potent (1.8 times, $p < 0.05$, one-way analyses of variance) at promoting PKC activity than all other DAG molecular species at 0.5 mol% and both SAG and SDG were significantly better activators of PKC than were SEG or

DOG at all three concentrations ($p < 0.05$, one-way analyses of variance).

The concentration of POPS used in the above experiments was determined from studies that examined the effects of increasing POPS on the relative PKC activity towards histone (Fig.2). The relative PKC activity was 0.23 at 10 mol% POPS and increased to 0.73, 1.0 and 0.94 in vesicles constructed with 30, 50 and 70 mol% POPS, respectively. We chose 30 mol% POPS because PKC has not reached its maximum activity (Fig. 2) and because PKC is completely bound to membrane (data not shown, 3). We used a calcium concentration of 20 μM , which corresponds to a physiologically relevant range and which we have shown does not cause maximal activation of PKC in control vesicles (30 mol% POPS and 70 mol% POPC, data not shown).

FIGURE 1. PKC activity in vesicles constructed with oleoyl, arachidonoyl, eicosapentaenoyl or docosahexaenoyl at the sn-2 position of DAG.

PKC activity towards histone was determined in vesicles constructed with increasing concentrations (0.5, 1.0, 2.0 mol%) of the indicated DAG molecular species DOG, SAG, SEG and SDG. Activity was compared to control vesicles constructed with POPS (30 mol%), POPC (70 mol%) and no other additives. Data represent three to four independent experiments, each performed in triplicate and expressed as mean $\pm$ SD. Levels of statistical significance *: $p < 0.05$, **: $p < 0.02$, *** $p < 0.0001$.

PKC ACTIVITY TOWARDS HISTONES
(fold increase from control)

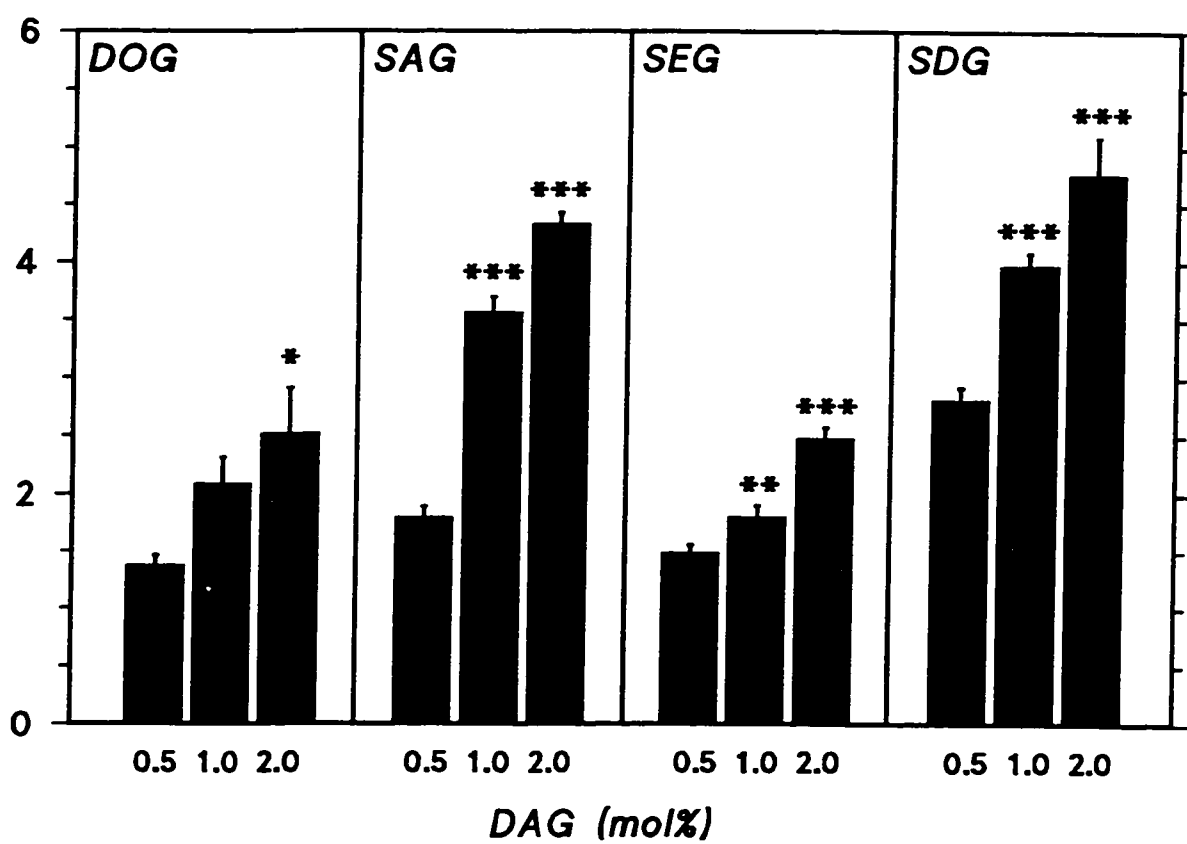
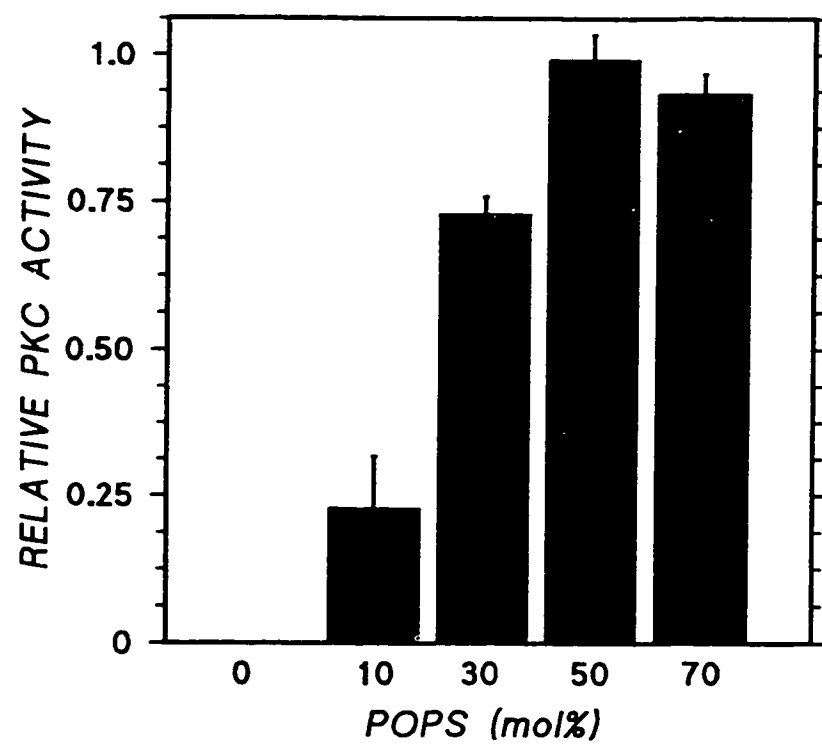


FIGURE 2. The effect of phosphatidylserine concentration on PKC activity.

Vesicles were constructed with 0, 10, 30, 50, or 70 mol% POPS, 1.0 mol% DOG and the remainder POPC. PKC activity is expressed relative to maximum activity determined with 50 mol% POPS. Data represent three independent experiments, each performed in triplicate and expressed as mean $\pm$ SD.



8.5 DISCUSSION

We have determined the individual effects of pure SAG, SEG and SDG, added to lipid vesicles containing 1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phospho-L-serine (POPS) and 1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphocholine (POPC), on changes in PKC activity towards ^{32}P incorporation from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ into histone. Bell and Sargent (20) describe changes in PKC activity towards histone in the presence of phosphatidylserine (PS) plus (ω 6)-rich DAG or (ω 3)-rich DAG. In these experiments, the (ω 3)-rich DAG was composed of a mixture of fatty acids prepared from cod roe (ω 3-type, 43 wt%; ω 6-type, 2.6 wt%; ω 9-type, 16.2 wt% and saturated, 32.3 wt%) and the PS was prepared from bovine brain and trout liver rich in stearic acid and DHA, respectively. The experimental design of the current investigation was different in that we were interested in determining the individual effects of pure SEG, SAG and SDG, added to lipid vesicles containing POPS and POPC, on changes in PKC activity towards histone. Others have shown that free fatty acid inclusion into vesicle systems enhances PKC activity (16-19, 21). More recently, Giorgione et al. (22) have shown that PKC activity towards histone increases in vesicles constructed with (18:1/22:6) PE, an abundant species of membrane phospholipid in cold-adapted fish brain.

Besides binding at a separate site from that of phorbol esters on several of the known PKC isoforms (23), DAG is also a promoter of the transition of phospholipids from bilayer to inverted hexagonal phase (24). Increasing the chain

length of the fatty acid at the *sn*-2 position of DAG could facilitate the conversion to hexagonal phase and thereby increase the activity of PKC. Increased unsaturation of the fatty acid chain reduce the temperature requirement for such a phase transition to occur. Recently, the dimensions of phospholipid bilayers, as measured from nuclear magnetic resonance (NMR) deuterium order parameter profiles, with DHA, AA and EPA at the *sn*-2 position, were determined to be 71.6, 70.6 and 69.1 Å², respectively (25). The AA and DHA acyl chains were able to increase the cross-sectional area of the membrane to a similar extent and to an extent greater than found with the EPA chain. These differences in cross-sectional area are likely related to differences in curvature stress in the membrane. Changes in membrane curvature resulting from increasing fatty acid unsaturation have been linked to enhanced activation of PKC (23). It is therefore especially interesting to note that our results showing a greater activation of PKC by SDG or SAG than by SEG correlate exactly with the observed differences in corresponding cross-sectional area (25).

Several researchers have studied the effects of free-fatty acids on PKC activation *in vitro*. Shinomura et al. (16) and others (17,18) have shown that saturated and *trans*-unsaturated free fatty acids are incapable of activating cPKC while *cis*-unsaturated fatty acids such as oleic (18:1 ω 9), linoleic (18:2 ω 6), AA and linolenic (18:3 ω 3) greatly enhance the phosphorylating capacity of cPKC *in vitro*. Hardy et al. (19) showed that PKC from purified rat brain could be activated by the addition of free fatty acids AA as well as by the ω 3PUFA, EPA, DHA and

dotriacontatetraenoic acids (34:6 ω 3) to the PKC activity assay. Speizer et al. (21) showed that EPA and DHA enhanced the catalytic activity of PKC purified from S49 lymphoma cells. In all experiments, the ability of highly unsaturated fatty acids to increase the activity of PKC towards histone may be attributable to the higher degree of unsaturation associated with the increase in fatty acid chain length. Although these studies were conducted using free fatty acids and not DAG with ω 3- or ω 6-type PUFA at the *sn*-2 position as in the current study, they provide an activity profile for PKC that may be of particular relevance for future *in vivo* studies.

Our current results provide evidence that the *sn*-2 substitution into DAG of PUFA, particularly DHA, in vesicle systems not only changes the structural characteristics of the membrane but also modifies PKC activity. We speculate that similar modulation of PKC activity may occur in cell membranes *in vivo*, which in turn may lead to modulation of PKC-dependent intracellular signalling pathways.

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UMI

CHAPTER NINE

9.1 DISCUSSION

Overall, my research investigated the changes in diacylglycerol (DG) and phospholipid (PL) molecular species that result from the incorporation of ω 3-polyunsaturated fatty acids (ω 3PUFA) into murine peritoneal macrophages. The implications of such incorporation on the secretion of interleukin-6 (IL-6) in macrophages were investigated and correlated to changes in DG molecular species composition. Furthermore, protein kinase C (PKC) activation by pure species of diacylglycerol with specific PUFA at the *sn*-2 position was investigated.

9.1.1 The Effects of ω 3PUFA on Diacylglycerol Formation *In Vivo*

ω 3PUFA have been observed to exert beneficial clinical effects in a number of medical disorders. However, the mechanisms by which ω 3PUFA exert their biological actions are not fully known. Over 40 years ago, Sinclair (1953) first reported a detailed inventory of the diet and an almost total absence of cardiovascular diseases in the Eskimo population. Subsequently, Bang and Dyerberg (Bang et al 1971, Dyerberg and Bang 1979) were inspired to search for an explanation of this epidemiological observation. Their early investigations

suggested a possible role for specific polyunsaturated fatty acids in the Inuit diet, namely ω 3PUFA, in the protection against coronary heart disease in this population. Currently, the best known explanation for the protective actions of ω 3PUFA involves the replacement of the membrane PL fatty acid AA by EPA and a resulting shift in the ratio of cellular AA:EPA-derived eicosanoids (Lokesh and Kinsella 1987, 1988b, Lokesh et al 1986, 1988, Croft et al 1987, Chapkin et al 1991, Hardardottir et al 1992, Endres et al 1993, Lawrence and Sorrell 1994).

The partial replacement of fatty acids of membrane PL in murine peritoneal macrophages by ω 3PUFA is well-documented in the literature (Huang et al 1986, Calder et al 1990, Chapkin and Carmichael 1990, Chilton et al 1993). Modifications in the fatty acid composition of membrane PL potentially predispose the cell to altered cellular function. My primary thesis hypothesis is that ω 3PUFA have other biological functions in addition to the known suppression of formation of AA-derived eicosanoids. The initial objective of the thesis work was to determine whether incorporation of ω 3PUFA into murine peritoneal macrophages alters the fatty acid composition of the second messenger, DG, a central cellular regulator that is derived from phospholipase activity on membrane PL. This seemed plausible on the basis of existing observations of the incorporation of ω 3PUFA into cellular PL (Huang et al 1986, Calder et al 1990, Chapkin and Carmichael 1990, Chilton et al 1993).

Initially, we used an *in vivo* dietary mouse model and demonstrated for the first time that when mice were fed a diet rich in ω 3PUFA (9% ω 3PUFA: 1%

COEE) for four weeks, EPA and DHA were significantly incorporated into DG as well as into PL, as determined by molecular species analysis (Chapter Three; Marignani and Sebaldt 1995). This was true both in the basal state as well as after cell stimulation with PAF, PMA or ionomycin. Of particular interest, membrane PI incorporated significant amounts of ω 3PUFA, largely at the expense of AA, although to a somewhat lesser extent than did membrane PC, PS and PE. This was an unexpected result since poor incorporation of ω 3PUFA into macrophage PI has been reported (Chapkin and Carmichael 1990, Chapkin et al 1988, 1991). Our demonstration of ω 3PUFA incorporation into PI may be due to the duration of our dietary regimens. However, since we also observed similar incorporation in our *in vitro* studies (see below), the details of our rapid processing of samples may also have contributed to our different result. We also demonstrated that PC is the primary parent PL from which late phase ω 3PUFA-enriched DG is derived after stimulation with PAF, PMA and ionomycin. This is consistent with published observations that early phase DG is predominantly derived from PI whereas late phase DG is primarily derived from PC, albeit indirectly via phosphatidic acid (Billah 1993, Exton 1990, Sebaldt et al 1992). The total mass of DG measured in ionomycin-stimulated macrophages, a measure of total cell capacity to form DG, was significantly reduced in the ω 3PUFA diet group relative to control. This reduction may be attributable to ω 3PUFA-induced changes in calcium-dependent signalling pathways that may be involved in the regulation of PLC and/or PLD (Dennis et al 1991, Mullmann et al 1993). In summary, these results provided

initial evidence that the biological effects of ω 3PUFA may be associated with cellular changes other than altered eicosanoid formation.

Having established that ω 3PUFA, at a fixed dietary intake ratio of EPA:DHA=2:1, are incorporated into DG, largely at the expense of AA, we next addressed the question whether there is a difference between the capacities of dietary EPA and DHA to be incorporated into DG. We hypothesized that the physicochemical differences between these two principal components of fish oil-derived ω 3PUFA might result in preferential incorporation of one over the other. The objective was to determine the effect of purified diets containing either EPEE or DHEE or a mixture of both on the incorporation of EPA and DHA into DG.

Total DG mass and DG molecular species in peritoneal macrophages were analyzed after mice were fed diets for three weeks with either 3% COEE or 2% EPEE/DHEE plus 1% COEE (Chapter Four: Marignani and Sebaldt 1996a). We found that incorporation of either EPA or DHA from an EPEE- or DHEE-containing diet was associated with a significantly lower total DG mass compared to the absence of their incorporation from a COEE-only control diet. Furthermore, EPA was associated with a greater suppression of total DG mass than was DHA. The incorporation of both EPA and DHA into DG, measured by molecular species analysis, occurred in a dietary concentration-dependent manner under both basal and stimulated conditions. Furthermore, we also showed that their incorporation into PL classes varied with dietary concentration. Because we were primarily interested in a direct comparison between EPA and DHA (rather than a possible

competition between them), our experimental conditions also included a diet with an equimolar ratio of EPA:DHA in which the total ω 3PUFA content was the same as in the other experimental conditions (2.0%). Our results clearly established that the degree of incorporation/retention per unit dietary concentration was greater for EPA than for DHA. Robinson and colleagues (1993) describe similar differences between EPA and DHA incorporation into splenocyte PL in mice fed 3% and 10% EPEE and DHEE diets. Recent studies on gastrointestinal absorption of EPA and DHA in both ethyl ester and triglyceride form showed no differences in absorption between the two ω 3PUFA (Nordøy et al 1991), making it unlikely that the observed differences in EPA and DHA incorporation in our study were attributable to this process. However, differences in the proportions of incorporated EPA and DHA could be a function of differences in the kinetics of their deacylation and reacylation reactions (Uhing et al 1987, Takayama et al 1987, Lokesh and Kinsella 1994). Our experiments do not exclude possible competition between EPA and DHA for incorporation into membrane lipids. However, because the extents of incorporation of both EPA and DHA in the 1.0%:1.0% diet were very close to half-way between their incorporation in the 2.0%:0% and 0%:2.0% diets, these data show no evidence that any competition is occurring.

Using a descriptive modelling approach, we fitted the data to polynomial equations and found that a linear fit was statistically significantly poorer than a quadratic fit in the case of results for EPA but not for DHA incorporation. The underlying kinetics are unknown and likely complex, so that we did not undertake

mechanism-based modelling. However, these descriptive differences may well reflect additional differences between EPA and DHA in their kinetics of uptake and remodelling, resulting in differences in their steady-state incorporation.

A possible problem with our results in the *in vivo* experimental design was a potential loss of ω 3PUFA that were previously incorporated as a result of diet, once macrophages were placed into culture for periods up to 16 h. I hypothesized, however, that ω 3PUFA behave similarly to other highly unsaturated fatty acids and have a relatively low rate of basal turnover. Furthermore, I hypothesized that a reduction of the protein content in the culture media would further minimize the loss *ex vivo* of previously incorporated ω 3PUFA, and my objective was to validate the culture conditions.

Macrophages from mice maintained for four weeks on diets containing either ω 3PUFA (9% ω 3PUFA: 1% COEE) or COEE were analyzed for DG and PL molecular species after either abbreviated (2 h) or standard (14h) incubation times and in either absence or presence of fetal calf serum (Chapter Five; Marignani and Sebaldt 1996b). When culture media included dfcs, the proportions of ω 3PUFA, particularly of EPA, incorporated into DG were noticeably diminished after standard incubation time compared to abbreviated incubation time, but this loss of initially incorporated ω 3PUFA was abrogated when dfcs was omitted from culture media. Moreover, the molecular species composition of PL classes also remained stable between abbreviated and standard incubation time when dfcs was omitted from the culture media. As a result of these observations, acceptable

culture conditions were established, which were those that we used in our *in vivo* experiments. Furthermore, we gained confidence in the validity of our experimental methods to establish the effects of ω 3PUFA on DG and PL metabolism.

9.1.2 An *In Vitro* Model To Analyze The Effects Of ω 3PUFA on Diacylglycerol Formation

To investigate further the implications of the results of the *in vivo* studies, I wished to develop an *in vitro* model. I hypothesized that the observed *in vivo* modifications in DG molecular species can be replicated *in vitro* in normal cultured murine peritoneal macrophages by the addition of DHA and EPA to culture media. The objective was to create an *in vitro* system that would be less cumbersome and time-consuming than the *in vivo* model, which requires meticulous diet preparation and handling and lengthy animal feeding regimens. I required that the *in vitro* model could reproduce the changes in DG and PL molecular species composition observed *in vivo*.

In the model that was developed, normal murine peritoneal macrophages were incubated for 14 h in culture media supplemented with 10% dfcs and increasing concentrations of EPA or DHA (0 - 100 μ M) (Chaper Six; Sebaldt and Marignani 1995). In contrast to the *in vivo* studies, dfcs was included in this *in vitro*

model to dissolve the hydrophobic ω 3PUFA in the aqueous culture media and to mimic a physiological state in which free fatty acids bind to albumin, from which they are taken up by cells and incorporated into membrane PL (Lokesh and Kinsella 1988b, Calder et al 1990). Both EPA and DHA increased total DG mass in a dose-dependent manner over a concentration range in the culture media of 25 - 100 μ M, compared to 0 μ M control medium, but at comparable concentrations, EPA was associated with a lesser increase than DHA. However, when compared to AA-supplemented control media at comparable concentrations, both EPA and DHA were associated with lower total DG mass (Sebaldt and Marignani 1993). We also found that both EPA and DHA were incorporated into DG and PC molecular species in a dose-dependent manner, reaching 27 - 31 mol% of total DG at 100 μ M of either EPA or DHA (Chapter Six; Sebaldt and Marignani 1995). Furthermore, EPA and DHA incorporation into DG occurred in both basal and PAF-stimulated macrophages (Chapter Six; Sebaldt and Marignani 1995). These results extend the findings of others. Lokesh and colleagues (1988b) analyzed fatty acid composition of PC, PE and neutral lipids in murine peritoneal macrophages incubated in culture media supplemented with increasing concentrations of EPA (5 - 100 μ M) and DHA (1 - 20 μ M) and reported concentration-dependent incorporation of the ω 3PUFA into membrane PL. At concentrations of 5 - 15 μ M, EPA was more extensively incorporated into PC and neutral lipids than was DHA, but incorporation into PE was similar. Interestingly, however, they found a functional difference between the ω 3PUFA in a direction

opposite to the extent of incorporation: DHA-enriched cells (15 μM) synthesized significantly less of the AA-derived eicosanoids LTC_4 and LTB_4 compared to EPA-enriched cells (25 μM). Hui and colleagues (1992) observed that incubation of rat mesenteric vascular smooth muscle cells in media supplemented with EPA (30 μM) significantly reduced AA at the *sn*-2 position of PC, PE, PS and PI, compared to cells incubated in AA or linoleic acid (18:2 ω 6). Furthermore, EPA attenuated arginine vasopressin-stimulated increases in the total mass of DG and PA, suggesting a suppressive role for EPA that may be relevant in vascular disease.

Thus, the *in vitro* model (Chapter Six; Sebaldt and Marignani 1995) is able to replicate key features of the *in vivo* model (Chapter Four; Marignani and Sebaldt 1996a). There is dose-dependent alteration of DG and PL molecular species by incorporation of ω 3PUFA. Furthermore, the effects of EPA and DHA on total DG mass are replicated in that EPA is associated with lower total DG mass than is DHA, and both are associated with lower total DG mass compared to appropriate controls (COEE-only diets *in vivo*, AA-supplemented medium *in vitro*). An approximate quantitative correspondence between the models also exists, as follows. The extent of incorporation into DG of EPA and DHA in the *in vivo* model using 9.0% dietary ω 3PUFA with EPA:DHA = 2:1 was 32 and 11 mol% of total DG, respectively. This was approximately reproduced in the *in vivo* studies when using 1.5 - 2.0% dietary EPEE (26 - 30 mol%) or 1.0% dietary DHEE (12 mol%). Corresponding results were obtained in the *in vitro* model when using 100 μM EPA (27 mol%) or 25 μM DHA (11 mol% of total DG). The higher

incorporation of DHA that was achieved when using 2.0% DHEE *in vivo* (18 mol%) was mimicked *in vitro* with 50 μ M DHA (21 mol%). The total DG mass determined in macrophages from mice fed diets of ω 3PUFA or COEE was found not to be different between the diet groups (Chapter Three; Marignani and Sebaldt 1995) and was approximately mimicked in the *in vitro* model with the higher concentration of ω 3PUFA used. However, in both the *in vivo* (Chapter Four; Marignani and Sebaldt 1996a) and the *in vitro* (Chapter Six; Sebaldt and Marignani 1995) models, EPA was associated with less total mass of DG than was DHA.

Our results are similar to those of Fowler and colleagues (1993b), who found that total DG mass was reduced more in unstimulated and concanavalin A (ConA)-stimulated splenocytes from mice fed purified EPA ethyl ester diet for three weeks compared to those given a purified DHA ethyl ester diet. At the same time, the analysis of individual molecular species of DG under both basal and stimulated conditions showed that the proportion of DG with incorporated EPA was significantly greater than that of DG with incorporated DHA in the *in vivo* model (Chapter Four; Marignani and Sebaldt 1996a). In contrast, no difference was observed between the incorporation of the two ω 3PUFA into molecular species of DG in the *in vitro* model (Chapter Six; Sebaldt and Marignani 1995). We have no proven explanation for these observed differences in ω 3PUFA incorporation other than the method by which the ω 3PUFA were introduced to macrophages. We believe that the *in vivo* dietary model provides a more prolonged mechanism by which to replace membrane fatty acid species in

macrophages to a steady-state, even in functional lipid pools with slower turnover (Marignani and Sebaldt 1995, 1996a, 1996b, Lokesh et al 1988b). In contrast, the supplementation of culture media with EPA and DHA *in vitro* may effectively replace membrane fatty acid species in more metabolically active pools that are fully labelled after 6 hr incubation *in vitro* (Lokesh et al 1988b) but may not be entirely representative of the long-term changes induced in the *in vivo* model (Lokesh et al 1988a).

The apparent metabolic origin of basal DG and of DG formed after cell stimulation was calculated to be primarily from PC in both our *in vivo* (Chapter Three; Marignani and Sebaldt 1995) and *in vitro* (Chapter Six; Sebaldt and Marignani 1995) models. Furthermore, in both studies, significant replacement of *sn*-2 AA by EPA and DHA was found to occur in PC. Physiologically, the replacement of AA by ω 3PUFA may have an impact on the production of PAF, an ether-linked bioactive PL involved in diverse processes in inflammation and in cardiovascular, renal and pulmonary diseases (Snyder 1995, review). Tanaka and colleagues (1994) showed that the production of PAF-like compounds from the oxidation of 1-*O*-alkyl-2-arachidonoyl-PC and 1-*O*-alkyl-2-docosahexaenoyl-PC were capable of inducing platelet aggregation *in vitro*. The oxidation products derived from 1-*O*-alkyl-2-arachidonoyl-PC and 1-*O*-alkyl-2-docosahexaenoyl-PC differed from PAF in that the *sn*-2 position contained propionate or butyrate residues. Platelet aggregation induced by the *sn*-1-*O*-alkyl PAF-like compound (derived from 1-*O*-alkyl-2-docosahexaenoyl-PC) was 5 times greater than platelet

aggregation induced by PAF-like compound (derived from 1-*O*-alkyl-2-arachidonoyl-PC) and 150 times greater than platelet aggregation induced by an *sn*-1-acyl PAF-like compound (derived from 1-acyl-2-docosaheptaenoyl-PC). As with PAF, platelet aggregation by PAF-like compounds was prevented by pretreatment of platelets with FR-900452, an antagonist of PAF. Further research should be conducted to determine the effect of PAF-like compounds on cells other than platelets, such as macrophages, and should include the effects of PAF-like compounds derived from *sn*-1-acyl subclasses of PL, since most cell membranes are rich in *sn*-1-acyl PL species. However, it still remains uncertain whether the formation of PAF-like compounds occurs physiologically. In our research, it is probable that PAF-like compounds derived from *sn*-1 ether-linked PL containing *sn*-2-linked EPA and/or DHA are produced. However, direct functional consequences of the formation of these PAF-like compounds in our macrophage model are not known.

9.1.3 The Effects of ω 3PUFA on Interleukin-6 Secretion From Macrophages

The experiments described above were conducted in murine peritoneal macrophages. Macrophages/monocytes are the primary source of the multipotent cytokine IL-6 that is released during an acute phase response, along with IL-1 and TNF- α (Kushner 1982, Heinrich et al 1990). These cytokines stimulate the synthesis

of acute phase proteins by hepatocytes in response to tissue injury. IL-6 is a widely distributed cytokine, for example, it prevents the migration of LPS-induced neutrophils into rat lungs (Ulich et al 1991), is secreted from epidermal cells and epidermoid carcinoma cell lines (Kirnbauer et al 1989, Gross et al 1993) and can be isolated from certain human carcinomas (Erroi et al 1989, Strassman et al 1992). Currently, there are a few studies that investigate the effects of ω 3PUFA replacement of *sn*-2-linked AA in membrane PL on cytokine secretion, including IL-6 (Billiar et al 1988, Endres et al 1989, 1993, Kremer et al 1990, Hardardottir and Kinsella 1991, Meydani et al 1991, Somers and Erickson 1994). I hypothesized that membrane incorporation of ω 3PUFA may alter IL-6 secretion in murine peritoneal macrophages, most likely by alterations in PL-derived second messengers. The specific objective was to demonstrate a correlation between IL-6 secretion in LPS-stimulated macrophages and ω 3PUFA incorporation into DG molecular species *in vitro*.

IL-6 secretion was measured in LPS-stimulated macrophages incubated in culture media supplemented with increasing concentrations of EPA and DHA (Chapter Seven; Marignani and Sebaldt 1996d, *submitted*). We found that IL-6 secretion is increasingly suppressed as the proportion of EPA and DHA in DG is increased. Incorporation of DHA into DG was associated with a greater attenuation of IL-6 secretion than was incorporation of EPA. Meydani and colleagues (1991) studied IL-6 production in mononuclear cells (MNC) from healthy volunteers given diets supplemented with ω 3PUFA-containing

triglycerides for 12 weeks and found that IL-6 production was suppressed. However, to the best of our knowledge, ours is the first report to describe differential effects of ω 3PUFA on IL-6 secretion.

The regulation of IL-6 production by PGE₂ has recently been described in rat peritoneal mast cells. IL-6 production was induced by the addition of PGE₂ to mast cells and inhibited by the introduction of the corticosteroid, dexamethasone, a cyclooxygenase-2 downregulator (Leal-Berumen et al 1995). Additional evidence for the regulation of cytokine production by PGE₂ has been obtained from dietary studies with ω 3PUFA. Hardardottir and Kinsella (1991) analyzed TNF- α production in LPS-stimulated peritoneal macrophages from mice maintained for 5 weeks on ω 3- or ω 6-PUFA-rich diets. TNF- α production, was greater in macrophages from mice in the ω 3-PUFA-rich diet group than in the ω 6-PUFA-rich diet group. Treatment of macrophages with indomethacin, a cyclooxygenase inhibitor, increased TNF- α production while the addition of exogenous PGE₂ decreased TNF- α production. These observations are consistent with the hypothesis that dietary ω 3PUFA reduce the inhibitory effect of PGE₂ on TNF- α production by reducing the production of AA-derived PGE₂. Although we did not measure PGE₂ production in these experiments, a decreased formation of AA-derived eicosanoids, including PGE₂, by EPA in macrophages is well-documented in the literature (see Chapter One: sections 1.0.2 and 1.0.3).

The reduction in IL-6 secretion in our system may be partially attributed to the replacement of the PGE₂ precursor, AA, by EPA and/or DHA, thus reducing

the extent of regulatory control of PGE₂ over IL-6 production. The antiaggregation and vasodilation effects of EPA-derived PGE₃ closely resemble those of AA-derived PGE₂. It is currently unknown whether PGE₃ possesses the same cytokine regulatory activity as PGE₂. However, in a number of models (Brouard and Pascaud 1990, Careaga-Houck and Sprecher 1990, Sebaldt et al. 1993), the reduction of AA-derived eicosanoids by EPA was greater than the increased formation of EPA-derived eicosanoids. Unlike EPA, DHA has not been found to be a substrate for production of E series prostaglandins, so that replacement of AA by DHA would reduce AA-derived PGE₂ without even a partially compensatory increase in the formation of another E series prostaglandin. Therefore, if indeed PGE₃ does exert at least some degree of regulatory control over cytokine production, then this may explain why incorporated DHA is associated with a more potent attenuation of IL-6 secretion than is incorporated EPA.

Other studies on the effects of ω 3PUFA on cytokine production have been reported. IL-1 β and/or TNF- α production were examined in either humans, mice or rats given diets containing various concentrations of ω 3PUFA. Endres and colleagues (1989, 1993) found cytokine production was suppressed in MNC from healthy human volunteers fed diets supplemented with ω 3PUFA-enriched triglycerides or ω 3PUFA ethyl esters for up to 6 weeks. Kremer and colleagues (1990) studied the effects of dietary ω 3PUFA ethyl esters on patients with rheumatoid arthritis and found that IL-1 β production in mononuclear cells was suppressed. Billiar and colleagues (1988) examined the effects of ω 3PUFA-

containing triglycerides on Kupffer cell production of IL-1 β and TNF- α and found them to be decreased. Somers and Erickson (1994) report the opposite effects of ω 3PUFA on cytokine production. Both TNF- α mRNA expression and production in LPS-stimulated macrophages from mice fed a ω 3PUFA-containing diet were increased compared to those in macrophages from mice fed a ω 6PUFA-containing diet. The reported effects of ω 3PUFA on cytokine production suggest that diverse mechanisms may be involved in the regulation of IL-6 production. Moreover, the pleiotropic actions of IL-6 suggest that additional unexplored regulatory mechanisms may be involved in its production. Any of these mechanisms may potentially be modified by ω 3PUFA.

9.1.4 Activation Of Protein Kinase C By ω 3PUFA-Modified Diacylglycerol

We have shown that ω 3PUFA modify the molecular species of the second messenger, DG, that are formed (Chapters Three, Four and Five; Marignani and Sebaldt 1995, 1996a, 1996b, Chapter Six; Sebaldt and Marignani 1995), and that such modifications correlate well with the attenuation of IL-6 secretion (Chapter Seven; Marignani and Sebaldt 1996d, *submitted*). The consequences of structural modification of DG by the incorporation of ω 3PUFA are interesting because DG is known to activate several isoforms of PKC. I hypothesized that DG molecular species with EPA or DHA at the *sn*-2 position might activate PKC to a different

extent than other species of DG. The specific objective was to compare the activation of PKC *in vitro* by different DG molecular species, including those with EPA or DHA at the *sn*-2 position. There are very few data in the literature concerning the effects of ω 3PUFA on PKC activity (Bell and Sargent 1987, see below). Hardy et al (1994) showed that the addition of the free fatty acids AA, EPA, DHA, dotriacontatetraenoic (32:4 ω 6) or tetratriacontahexaenoic acids (34:6 ω 3) to inside-out vesicles from human erythrocytes increased the activity of PKC towards histone *in vitro*. Speizer and colleagues (1991) demonstrated that the catalytic activity of PKC was enhanced in S49 lymphoma cells by the addition of free fatty acids EPA or DHA *in vitro*. Although these studies did not examine the effects of individual DG molecular species with EPA or DHA at the *sn*-2 position, they do provide evidence for the potential role of ω 3PUFA in modifying PKC activity *in vitro*.

We demonstrated for the first time that the activation of PKC *in vitro* differs significantly among PUFA-containing molecular species of DAG (Chapter Eight; Marignani et al 1996c). Since DOG is a DAG molecular species that is commonly used in *in vitro* systems and 1-stearoyl-2-arachidonoyl-*sn*-glycerol, which is abundant *in vivo*, enhances PKC activity to a greater extent than does DOG, studies of PKC activation conducted with DOG *in vitro* may not provide evidence directly applicable to the activation of PKC *in vivo*. Moreover, the activation of PKC by 1-stearoyl-2-arachidonoyl-*sn*-glycerol is the more appropriate control to which to compare the activation of PKC by 1-stearoyl-2-docosahexaenoyl-*sn*-

glycerol and 1-stearoyl-2-eicosapentaenoyl-*sn*-glycerol. Specifically, both 1-stearoyl-2-arachidonoyl-*sn*-glycerol and 1-stearoyl-2-docosahexaenoyl-*sn*-glycerol had similar potencies in activating PKC whereas 1-stearoyl-2-eicosapentaenoyl-*sn*-glycerol activated PKC to a lesser extent. Our results are consistent with the concept that increasing the chain length of the fatty acid at the *sn*-2 position of DAG reduces the temperature requirements for a phase transition that promotes the conversion to a hexagonal phase. These results correlate well with those described by Slater and colleagues (1994) and more recently, Separovic and Gawrisch (1996). Increases in fatty acid unsaturation are associated with increases in membrane curvature and enhanced activation of PKC (Slater et al 1994). The cross-sectional area of phospholipid bilayers with AA and DHA acyl chains increased to a similar extent (71.6 and 70.6 Å²) compared to a bilayer with an EPA acyl chain (69.1 Å²) (Separovic and Gawrisch 1996).

In contrast to the experimental design that determined the effects of pure individual DAG molecular species with *sn*-2 AA, *sn*-2 EPA or *sn*-2 DHA on PKC activity *in vitro* (Chapter Eight; Marignani et al 1996c), Bell and Sargent (1987) described the effects of a mixture of saturated fatty acids and PUFA-containing species of DAG on PKC activity *in vitro*. Their results show that ω3PUFA-rich DAG activated PKC the same as did ω6PUFA-rich DAG. However, the composition of ω3PUFA-rich DAG [derived from cod roe (ω3-type, 43 % by wt; ω6-type, 2.6 % by wt; ω9-type, 16.2 % by wt and saturated, 32.3 % by wt)] would make it very difficult to attribute the results solely to the ω3PUFA component in

the DAG. Furthermore, the PS used in their model was derived from bovine brain and trout liver and was rich in stearic acid and DHA.

Our findings suggest that PKC-dependent signal transduction pathways may be modified at an early stage, namely PKC activation, by varying the *sn*-2-substituted fatty acid in DAG to AA, EPA or DHA.

9.2 Interrelationship Amongst ω 3PUFA, Interleukin-6 and Protein Kinase C: An Hypothesis

To date, there have been no other studies that examine the simultaneous effects of individual ω 3PUFA on the formation of DG molecular species and IL-6 secretion in murine peritoneal macrophages *in vivo*, nor have the effects of DG with EPA or DHA at the *sn*-2 position on PKC activity been investigated *in vitro*.

Based on the above results and considerations, I propose that the reduced activation of PKC that results from the incorporation of EPA into DG indirectly results in the suppression of IL-6 secretion in peritoneal macrophages. Specifically, I have shown that PKC activation *in vitro* by 1-stearoyl-2-eicosapentaenoyl-*sn*-glycerol occurs but is less than that resulting from 1-stearoyl-2-docosahexaenoyl-*sn*-glycerol (Chapter Eight; Marignani et al 1996c), and this may very well occur *in vivo*. I hypothesize that this causes suppression of IL-6 secretion observed *in vitro* (Chapter Seven; Marignani and Sebaldt 1996d, *submitted*) by the mechanism

described below. Although the evidence in the literature described below supports this hypothesis in the case of activation of PKC by 1-stearoyl-2-eicosapentaenoyl-*sn*-glycerol, it does not support the case of PKC activation by 1-stearoyl-2-docosahexaenoyl-*sn*-glycerol. Furthermore, the literature does not address differences between EPA and DHA. I am led to conclude that the greater inhibition of IL-6 by DHA relative to that by EPA must be on the basis of a difference between these two ω 3PUFA that is not related to PKC activation. A precedent for a physiological role of DHA but not EPA is the substantial presence of DHA in the central nervous system (Salem et al 1986, Ward et al 1996).

9.2.1 The Effects of ω 3PUFA on Cytokine Synthesis

Studies analyzing the effects of ω 3PUFA on the production of some cytokines have been conducted. Renier and colleagues (1993), in an *in vivo* dietary model, demonstrated the suppressive effect of ω 3PUFA on TNF- α , IL-1 β and PGE₂ production in LPS-stimulated murine peritoneal macrophages from mice fed a diet containing 10% menhaden oil (ω 3PUFA-rich diet) compared to control diet group (ω 6PUFA-rich). Similar observations were made by Purasiri and colleagues (1994) in patients with advanced colorectal cancer given daily diets supplemented with a mixture containing EPA, DHA and γ -linolenic acid (132 mg, 20 mg, and 146 mg, respectively). Serum levels of IL-1 β , TNF- α and IL-6 were reduced by 61%, 73% and 83% of baseline values, respectively, after 6 months of dietary supplementation

and returned to pre-supplementation levels three months after cessation of the diet. Although these researchers report reductions in cytokine levels, the presence of γ -linolenic acid (18:3 ω 6) in the diet mixture prevents attribution of the reductions solely to dietary ω 3PUFA. Furthermore, analysis of serum cytokine levels may not reflect actual tissue levels or cellular production. Similar suppression of both IL-1 and TNF- α production have been observed in patients given diets supplemented with ω 3PUFA (Endres et al 1989, 1993, Billiar et al 1988, Kremer et al 1990, Meydani et al 1991, Purasiri et al 1994).

A key difference between my thesis research and the research presented in the literature regarding cytokine production is that my work investigated the individual effects of EPA and DHA whereas the work of others investigated the effects of mixed ω 3PUFA diets on cytokine production (Endres et al 1989, 1993, Billiar et al 1988, Kremer et al 1990, Meydani et al 1991, Renier et al 1993, Purasiri et al 1994). The composition of ω 3PUFA diets in these studies include both mixed ω 3- and ω 6 -type PUFA as well as ω 9- and ω 7-type fatty acids and saturated fatty acids. The observed reduction in cytokine synthesis described in these studies may not be solely attributable to the ω 3PUFA dietary component even though EPA and DHA are the predominant fatty acids in these diets and are in a ratio of 2:1 with respect to each other (13:2 in the case of Purasiri et al 1994). Aside from this difference, the literature supports my hypothesis in that the suppression of IL-6 secretion in macrophages correlates with the incorporation of ω 3PUFA into DG (Chapter Seven; Marignani and Sebaldt 1996d, *submitted*).

9.2.2 Regulatory Control of Interleukin-6 Synthesis by Interleukin-1 and Tumor Necrosis Factor- α : Protein Kinase C-Dependent Mechanism

Evidence that a PKC-dependent mechanism is involved in the synthesis of cytokines, including IL-6, has been obtained in various cell types (Sehgal et al 1987, Prabhakar et al 1993). Sehgal and colleagues (1987) demonstrated in human fibroblasts that enhanced expression of IL-6 is mediated by activation of PKC by the synthetic short-chain saturated DAG, 1,2-diocanoylglycerol, and the calcium ionophore, A23187. Furthermore, enhanced expression of IL-6 is inhibited by H7 (1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride), a non-specific inhibitor of PKC, by affecting ATP binding in the catalytic domain, but not by H8 (*N*-[2-methylamino)ethyl]-5-isoquinoline-sulfonamide dihydrochloride), an inhibitor of cAMP and cGMP-dependent protein kinases. Prabhakar and colleagues (1993) investigated the relationship between TNF- α production and PKC activity in human peripheral blood monocytes. LPS- and zymosan-induced production of TNF- α was inhibited by staurosporine, a non-specific inhibitor of PKC, by affecting ATP binding in the catalytic domain, and by calphostin C, a selective inhibitor of PKC that affects the phorbol ester binding in the C₁ region. Furthermore, long-term treatment of monocytes with PMA, which downregulates PKC, reduced LPS- and zymosan-induced TNF- α production. These results provide direct evidence that the initial steps involved in TNF- α production by human monocytes are PKC-dependent. However, interpretation of the above results must recognize that some

of the PKC inhibitors used in the studies have been shown to inhibit other serine/threonine kinases. For example, H7 has been shown to inhibit cyclic nucleotide dependent kinases and is more potent at inhibiting phosphorylation of histone *in vitro* than the phosphorylation of proteins *in vivo* (reviewed in Gordge and Ryves 1994), while staurosporine has been shown to also inhibit PKA (reviewed in Gordge and Ryves 1994) and induce apoptosis (Jacobson et al 1993). Therefore, the observed outcomes of these studies may not solely be due to alterations of a PKC-dependent mechanism.

IL-1 and TNF- α together have been shown to share regulatory control over IL-6 expression via a PKC-dependent mechanism (Evans et al 1992, Norris et al 1994, Grimaldi et al 1995). Evans and colleagues (1992) demonstrated that IL-1 α/β and TNF- α secreted by LPS-stimulated murine peritoneal macrophages *in vitro* induced the expression of IL-6 in co-incubated rhabdomyosarcoma MCA/76-9 cells. Induction of IL-6 expression in rat cortical astrocytes by IL-1 and PMA has been observed (Grimaldi et al 1995). Furthermore, the inhibition of PMA-activated PKC by calphostin C reduced IL-1-induced IL-6 production (Grimaldi et al 1995). The authors' data suggest a stimulatory role for PKC in IL-6 production. Similarly, Norris and colleagues (1994) showed that PKC inhibition with calphostin C prevented IL-1- and TNF- α -induced IL-6 expression in astrocytes, while stimulation of PKC with PMA and the calcium ionophore, A23187, induced IL-6 expression. The results suggest a stimulatory role for PKC in IL-1 and TNF- α -induced expression of IL-6 in astrocytes.

The studies described above can be reconciled with my hypothesis as follows. The reduction of IL-6 secretion (Chapter Seven; Marignani and Sebaldt 1996d, *submitted*) may be attributed to the EPA-induced suppression of IL-1 and TNF- α production (Endres et al 1989, 1993, Billiar et al 1988, Kremer et al 1990, Meydani et al 1991, Renier et al 1993, Purasiri et al 1994) via a PKC-dependent mechanism (Prabhakar et al 1993, Norris et al 1994, Grimaldi et al 1995), which in turn diminishes IL-1- and TNF- α -induced IL-6 production.

Future research using pure EPA and pure DHA in *in vitro* or *in vivo* models should be conducted to confirm the possibility of regulatory control of IL-6 production by other cytokines or by PKC, to directly substantiate my proposed hypothesis and begin to address the unexplained effect of DHA.

In summary, the research presented in this thesis has demonstrated novel biochemical effects of ω 3PUFA. The first phase of my research demonstrated that dietary manipulation of murine peritoneal macrophages results in the incorporation of EPA and DHA into DG molecular species (Chapters Three and Four; Marignani and Sebaldt 1995, 1996a). The validity of the experimental design was also established in this phase (Chapter Five; Marignani and Sebaldt 1996b). An *in vitro* model system was also developed and validated where key features of the *in vivo* work could be replicated (Chapter Six; Sebaldt and Marignani 1995). The second phase of our research investigated the functional implications of EPA and DHA, specifically the correlation between incorporation of EPA and DHA into DG and the secretion of IL-6 in murine peritoneal macrophages (Chapter Seven;

Marignani and Sebaldt 1996d, *submitted*). The final phase addressed the implications of these changes in DG molecular species on PKC activation, a pivotal enzyme involved in cell growth and differentiation (Chapter Eight; Marignani et al 1996c).

Our work together with that of others has begun to investigate the biochemical effects of ω 3PUFA at the level of signal transduction. This work may help to advance the understanding of some of the mechanisms underlying pathogenesis and treatment of atherogenic syndromes, inflammatory diseases and neoplasia.

9.3 Future Research

The results and discussions presented in this thesis provide substantial evidence for novel biochemical actions of ω 3PUFA in cellular signalling pathways. However, there is clearly a need for further investigation that would address the functional effects of EPA and DHA incorporated into DG on PKC activity *in vivo*. The biochemical significance of the incorporation of EPA or DHA into membrane PS and perhaps other membrane PL would be of additional interest with respect to how these modifications may alter the activation of PKC *in vitro*. Furthermore, it would be of interest to elucidate whether the effects of EPA and DHA incorporation into membrane PL and DG on cytokine synthesis are PKC-

dependent in our animal model. More specifically, the regulatory role of IL-1 and TNF- α expression and production on IL-6 expression and production in murine peritoneal macrophages should be investigated. Additional studies would verify the PKC dependency of cytokine synthesis in murine peritoneal macrophages modified by ω 3PUFA incorporation. To confirm that the outcomes of these studies are attributable to EPA and DHA incorporation into lipids, simultaneous analysis of DG molecular species and PL molecular species would be required.

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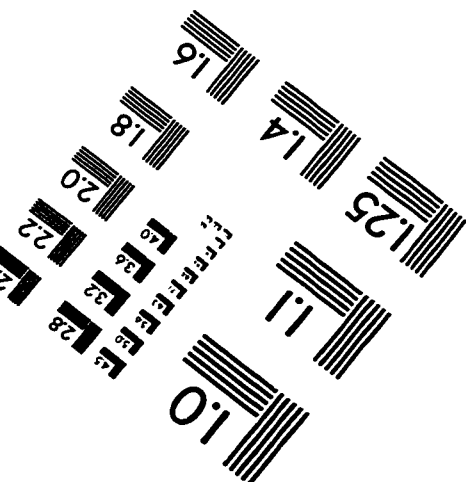
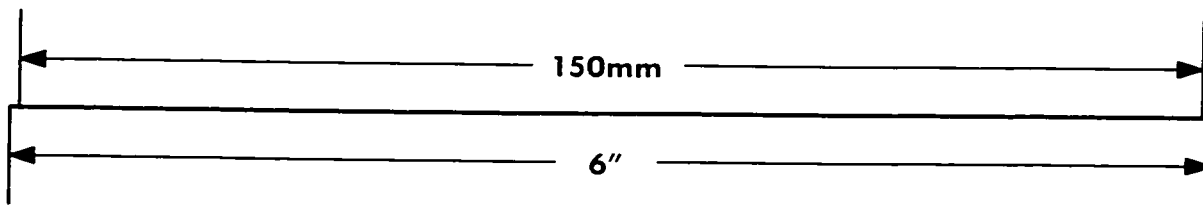
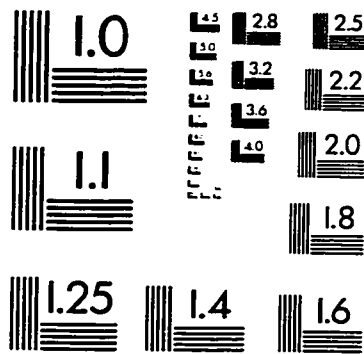
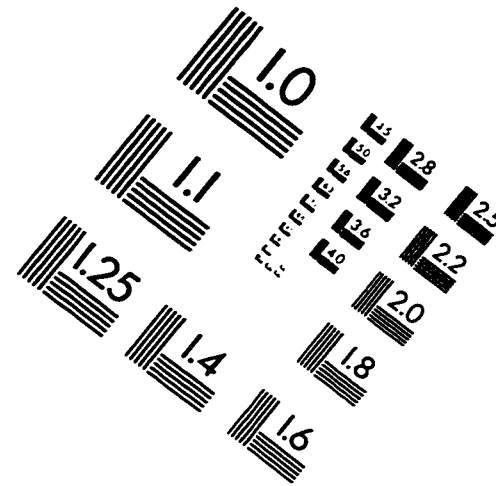
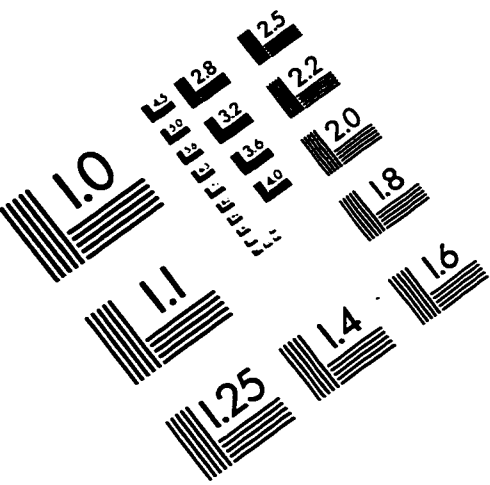
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IMAGE EVALUATION TEST TARGET (QA-3)



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